ELSEVIER

Journal of Chromatography A, 829 (1998) 101–113

JOURNAL OF CHROMATOGRAPHY A

Chiral discrimination of Dns- and unmodified D,L-amino acids by copper(II) complexes of terdentate ligands in high-performance liquid chromatography

Gianni Galaverna[a], Roberto Corradini[a], Arnaldo Dossena[a], Emma Chiavaro[a], Rosangela Marchelli[a,*], Francesco Dallavalle[b], Giuseppina Folesani[b]

[a]*Dipartimento di Chimica Organica e Industriale, Università di Parma, Viale delle Scienze, I-43100 Parma, Italy*

[b]*Dipartimento di Chimica Generale ed Inorganica, Chimica Analitica, Chimica Fisica, Università di Parma, Viale delle Scienze, I-43100 Parma, Italy*

Received 4 June 1998; received in revised form 15 September 1998; accepted 15 September 1998

Abstract

The enantiomeric separation of unmodified D,L-amino acids and Dns-amino acids in RP-HPLC by copper(II) complexes of terdentate diaminoamido ligands added to the eluent has been studied. The work is aimed at investigating whether a copper(II) complex with only one free equatorial position can still perform chiral discrimination of bidentate analytes such as unmodified and Dns-amino acids. The problem is approached by determining the nature and the stability of the initial copper(II) binary complexes and of the ternary complexes with amino acids in solution by potentiometry. Different chromatographic parameters were examined (pH, selector concentration, ionic strength, eluent polarity). All experimental data are consistent with a mechanism of chiral discrimination involving formation of diastereomeric complexes in which the amino acid coordinates at the free equatorial position and at the apical position of the initial complex which maintains its original chelate rings. It may still be considered a ligand exchange, implying that coordinated water molecules have been substituted by the analyte. The poor performance of the tricoordinated copper(II) complexes in the chiral discrimination of unmodified amino acids can be ascribed to the low stability of the mixed complexes and to their loose sterical requirements. With Dns-amino acids the most important feature seems to be the higher/lower affinity (match/mismatch) of the enantiomers for the selector adsorbed on the stationary phase.

Keywords: Enantiomer separation; Ligand-exchange chromatography; Stability constants; Terdentate ligands; Amino acids; Metal complexes

1. Introduction

Chiral ligand-exchange chromatography (CLEC), originally introduced by Rogozhin and Davankov [1], is one of the most powerful techniques for the enantiomeric separation of bidentate substrates in high-performance liquid chromatography (HPLC) [2,3]. It involves reversible formation of diastereomeric complexes between transition metal ions [copper(II) is the most common], chiral ligands (generally amino acids or derivatives) and enantiomers. A wide variety of systems has been proposed during the past years based on this principle, which is

*Corresponding author. Tel.: +39 521 905555, Fax: +39 521 905472, E-mail: marchell@ipruniv.cce.unipr.it

0021-9673/98/$ – see front matter
PII: S0021-9673(98)00783-3

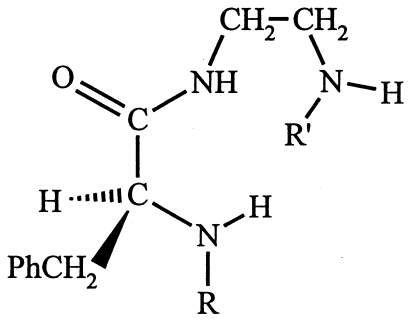
effective with both chiral stationary phases (CSPs) [4–9] or chiral mobile phases (CMPs) [10–15].

When the ligand is linked to a solid support, the stationary phase can form diastereomeric adsorbates with bidentate enantiomers. The relative stabilities of the adsorbates, if different, lead to the enantiomeric separation of the racemates [16]. When the chiral complex is added to the mobile phase (chiral eluent), the mechanism of chiral discrimination is, in general, quite complex, and it involves a series of complexation equilibria in the mobile and in the stationary phase as well as partition equilibria of the different species between the two phases [17,18].

Whether or not ligand exchange does occur in the chromatographic system has been discussed for several years. Special cases in which the analyte gives apical and outer sphere interactions with the selector, removing only molecules of water or solvent have been proposed [19]. We have already reported that, with bidentate ligands (namely, L-amino acid amides), ligand exchange does occur in the chromatographic system involving the displacement of one ligand from the initial binary complex, leading to formation of diastereomeric ternary complexes of different stability and/or affinity for the stationary phase [20].

We have also performed studies in solution of the equilibria of Cu^{II}/L-amino acid amide/D- or L-amino acid systems in order to define the species present at the various pH levels and the stability of the ternary complexes formed. These results indicate that the thermodynamic stereoselectivity in solution contributes but a minimum to the overall discrimination process and that the major factor involved in the recognition mechanism is the affinity of the complexes for the stationary phase and, therefore, the lipophilicity of the selectors is most important [21,22].

In order to investigate whether chiral discrimination with copper(II) complexes of terdentate ligands may occur only by coordination of an analyte at one free equatorial position, we considered aminoamido-type ligands containing as a third coordinating site either a poor (OH) or a strong (NH_2) donating group. Ligands of the former type actually behave as the analogous bidentate amino acid amides giving rise to L:M=2:1 complexes and perform chiral discrimination via ligand exchange, with the hydroxyl group eventually involved in the coordination at the copper(II) apical position [23,24].



R = R' = H : PheN-2 (**1a**)

R = CH_3, R' = H : MePheN-2 (**1b**)

R = R' = CH_3 : MePheNMe-2 (**1c**)

Fig. 1. Structures of the ligands.

In the present paper we report the enantiomeric separation of amino acids and Dns-amino acids by copper(II) complexes of terdentate ligands (Fig. 1) containing as a third donating site an amino or methylamino group. The mechanism of chiral discrimination is discussed on the base of the stability of the copper(II) binary complexes and of their ternary diastereomeric complexes with amino acids formed in solution and of the chromatographic parameters. The ligands are based on L-phenylalanine on account of its lipophilicity and for analogy with the corresponding amide which gave very good enantiomeric separation in HPLC [20,25]. Experiments were also performed with *N*-methylated ligands in order to vary the stability and the sterical arrangement of the copper(II) complexes.

2. Experimental

2.1. Materials and reagents

D,L-, D- and L-amino acids were obtained from Sigma (St. Louis, MO, USA); Dns-amino acids, OPA (*ortho*-phthalaldehyde), boric acid, EDTA, Pd/C (10%), benzyl chloroformiate and sodium hydride were obtained from Fluka (Buchs, Switzerland); 1,2-

diaminoethane, *N*,*N*′-dicyclohexylcarbodiimide and methyl iodide were purchased from Acros (NJ, USA); *N*-hydroxysuccinimide, 1-(*N*-methyl)-1,2-diaminoethane was from Aldrich (Steinheim, Germany); acetonitrile (LC-grade), methanol (LC-grade and RCS-grade), ethyl ether (RCS-grade), chloroform (RCS-grade), copper(II) acetate monohydrate and sodium acetate were obtained from Carlo Erba (Milan, Italy); 1,4-dioxane was from Lab-Scan (Dublin, Ireland); bidistilled water was produced in our laboratory utilizing an Alpha-Q system (Millipore).

2.2. Instrumentation

Chromatographic analyses were performed with Waters Model 510 pumps equipped with a Model 470 fluorescence detector (λ_{ex}=330 nm and λ_{em}=440 nm for OPA derivatization; λ_{ex}=330 nm and λ_{em}=545 nm for dansyl-amino acids) or with a Model 440 UV detector set at 254 or 280 nm. The system was computer controlled by a MAXIMA 820 Chromatography Workstation for data handling. C_{18} Spherisorb ODS-2 columns (3 μm, 15×0.46 cm) and C_{18} PLRP-S 100 Å (15×0.46 cm) were used. ^{1}H nuclear magnetic resonance (NMR) and ^{13}C-NMR spectra were recorded on Bruker AC 300 MHz and AMX 400 MHz spectrometers. Infrared spectra were recorded on Perkin-Elmer Model 298 and Nicolet FT-IR 5PC spectrophotometers. Mass spectra were recorded on a Finnigan MAT SSQ 710 spectrometer using electron impact (70 eV, EI) or chemical ionization. Optical rotations were measured on an Autopol III Rudolph Research polarimeter, using a 10-cm cell.

2.3. Synthesis of the ligands

2.3.1. N-L-Phenylalanylethanediamine dihydrochloride (PheN-2·2HCl) (1a)

L-Phenylalanine (L-Phe) was *N*-protected to the *Z* (benzyloxycarbonyl) derivative [26], esterified to the corresponding hydroxysuccinimidyl ester (OSu) by reaction with *N*-hydroxysuccinimide and *N*,*N*-dicyclohexylcarbodiimide in dry dioxane and recrystallized (chloroform–light petroleum), according to the literature method [27]. *Z*-Phe-OSu (20 mmol, 7.92 g) dissolved in dioxane (20 ml) was added dropwise to a solution of 1,2-ethanediamine (200 mmol, 13.4 ml) in dry dioxane (200 ml) at 0°C. The mixture was stirred at 0°C for 1 h, then at room temperature for 14 h. After removal of the dicyclohexylurea formed by filtration, the solvent was removed under vacuo and the residue, dissolved in $CHCl_3$ (100 ml), was extracted with acidic water (3×50 ml). The combined aqueous layers, containing *Z*-Phe-*N*-2·HCl and 1,2-ethanediamine dihydrochloride, were adjusted to basic pH and reextracted with $CHCl_3$ (3×70 ml). The organic phase was washed with water (3×50 ml) to eliminate the residual 1,2-ethanediamine and dried under vacuum. The product was dissolved in HCl–MeOH to acid pH. After removal of the solvent, a white flocculent solid was obtained by crystallization from MeOH–Et_2O. The product was deprotected by hydrogenolysis (H_2 flux) in MeOH in the presence of Pd/C (10%) (20%, w/w to the substrate) as catalyst at 50°C for 20 h. The catalyst was then removed by filtration and, after addition of HCl–MeOH to acid pH, the solvent was evaporated under vacuo. The product was further purified by preparative HPLC by using a Spherisorb S_{10} nitrile column (25×1 cm I.D.). Total yield: ca. 50%. M.p. 73–76°C. $[\alpha]_D^{25°C}$=+5.68 (EtOH 95%, *c*=1). IR (KBr): ν 3450, 3220, 3000, 1680, 1580, 710 cm^{-1}. ^{1}H-NMR (400 MHz, $C^2H_3O^2H$): δ 3.01–3.16 (m, 2H, CH_2-NH_3^+), 3.19 (dd, 1 H, ^3J=7.8, ^2J=13.9, CH_2-Ph), 3.30 (dd, 1H, ^3J=7.0, ^2J=13.8 CH_2-Ph), 3.35–3.42 (m, 1H, CH_2-NHCO), 3.64 (m, 1H, CH_2-NHCO), 4.21 (t, 1H, ^3J=7.4, CH-CONH), 7.44–7.34 (m, 5H, H_{arom}), 8.09 (br. s, NH_3^+), 8.48 (br. s, NH_3^+), 8.69 (br. s, CONH) ppm. Mass spectrometry (MS) (*m*/*z*): 208 (M^+, 5), 178 (8), 165 (10), 131 (5), 120 (100), 99 (32), 91 (18), 77 (12).

2.3.2. 1-(N-Methyl-L-phenylalanyl)-1,2-ethanediamine dihydrochloride (MePheN-2·2HCl) (1b)

L-Phe was *N*-protected to the Boc (*tert*.-butoxycarbonyl) derivative [28] and then *N*-methylated according to the McDermott and Benoiton procedure [29]. It was subsequently esterified to the corresponding hydroxysuccinimidyl ester as for 1a and recrystallized. Boc-MePhe-OSu (20 mmol, 7.58 g) in dioxane (20 ml) was added dropwise to a solution of

1,2-ethanediamine (200 mmol, 13.4 ml) in dioxane (200 ml) at 0°C. The mixture was stirred at 0°C for 1 h and at room temperature for 14 h. Boc-MePheN-2 was deprotected by treatment with trifluoracetic acid (10 ml for 3 mmol of Boc-MePheN-2) for 20 min. The crude residue was then dissolved in water (100 ml), the solution was adjusted to basic pH and finally extracted with $CHCl_3$ (4×50 ml). The organic layer was evaporated under vacuo and the crude product was further purified by preparative HPLC by using a Spherisorb S_{10} nitrile column [eluent MeOH–CH_3CN (90:10)], obtaining a pale yellow waxy hygroscopic solid. Total yield 40%; $[\alpha]_D^{25°C}=+6.87$ ($c=1$, EtOH 95%); IR (KBr): $_2\nu$ 3400, 2990, 1670, 1550, 710 cm^{-1}; ^{1}H-NMR (C^2HCl_3, free amine): δ 1.50 (s, broad, 3H, $-NH_{2amine}+-NH_{amine}-$), 2.40 (s, 3H, N-$CH_3$), 2.80 (m, 3H, CH_2-NH_2 and CH_β), 3.30 (m, 4H, CH_2-NHCO+CH_β+CH_α), 7.30 (m, 6H, $H_{arom}+NH_{amide}$) ppm; ^{13}C-NMR ($C^2H_3O^2H$): δ 33.0, 37.2, 38.1, 40.3, 64.0, 128.8, 130.1, 130.8, 135.4, 169.5 ppm. MS (m/z): 285 (10), 257 (20), 222 (90), 204 (60), 162 (18), 134 (100), 130 (20), 111 (15).

2.3.3. 1-(N-Methyl-L-phenylalanyl)-2-(N-methyl)-1,2-ethanediamine dihydrochloride (MePheNMe-2·2HCl) (1c)

Boc-MePheMeN-2 was obtained as above starting from Boc-MePhe-OSu by using *N*-methylethanediamine. The same procedure applied as before afforded the product as a pale yellow oil, which could be conveniently stored as hydrochloride after treatment with HCl–MeOH. Total yield 30%; $[\alpha]_D^{25°C}=-34.14$ ($c=1$, CH_2Cl_2); IR (neat liquid): ν 3403, 2946, 1652, 1555, 749 cm^{-1}; ^{1}H-NMR (C^2HCl_3, free amine): δ 1.36 (s, broad, 2H, $-NH_{amine}+NH_{amine}$), 2.01 (s, 3H, N-CH_3), 2.08 (s, 3H, CH_2-N-CH_3), 2.34 (m, 2H, CH_2-N-CH_3), 2.50 (dd, 1H, CH_β), 2.82 (dd, 1H, CH_β), 2.94 (dd, 1H, CH_α), 3.04 (m, 2H, CH_2-NHCO), 7.00 (m, 5H, H_{arom}), 7.20 (s, broad, 1H, NH_{amide}) ppm; ^{13}C-NMR ($CDCl_3$, free amine): δ 35.2, 35.9, 38.2, 39.2, 50.8, 66.0, 126.6, 128.5, 129.0, 137.5, 173.5; MS (m/z): 157 (20), 134 (60), 111 (28), 99 (20), 85 (28), 59 (25), 51 (100), 48 (100).

2.4. Preparation of the chiral mobile phase

The mobile phase was prepared by dissolving the appropriate amount of the ligand and of copper(II) acetate in a 1:1 ratio, in a concentration range 0.5–2 m*M* in bidistilled water or in water–acetonitrile. Sodium acetate was added when required. The pH was adjusted with 0.1–1 *M* NaOH. The eluent was filtered and degassed under reduced pressure on HPLC filters (0.45 μm). In order to equilibrate the system, the mobile phase was allowed to flow through the column for about 30 min at a flow-rate of 0.5 ml/min until when a constant elution time of a standard was reached. The equilibrium conditions were checked by measuring the constance of the elution time. The dead volume (V_0) was determined from the elution time of an unretained solute (acetic acid).

2.5. Preparation of the derivatizing agent: OPA

Boric acid (18.55 g) was dissolved in bidistilled water (1 l) and the pH was adjusted to 10 by addition of solid KOH (pellets). EDTA (2.5 g) was added and the pH was readjusted to the previous value (pH 10). OPA (0.8 g) was dissolved in a mixture of mercaptoethanol (4 ml) and methanol (4 ml), then added to the former solution with a syringe under vigorous magnetic stirring. The resulting solution was filtered (0.45 μm) and degassed under reduced pressure.

2.6. Recovery of the chiral selector

The copper(II) complex adsorbed on the column was easily removed by washing with MeOH. Copper(II) was precipitated as CuS from the solution previously adjusted to pH 1–2 with HCl (6 *M*) by H_2S gas. After filtration of CuS and an extraction with $CHCl_3$ to eliminate impurities, the solution was evaporated to dryness and the resulting solid was washed with $CHCl_3$. The organic solvent was removed by vacuo and the ligand was recovered as free amine. Treatment with HCl–MeOH and recrystallization with MeOH–Et_2O afforded the original ligand as hydrochloride (85%).

2.7. Potentiometric determinations

All potentiometric measurements were carried out at $T=25.0\pm0.1$°C and $I=0.1$ *M* (KCl) under N_2 stream, using 50-ml samples. Computer-controlled titrations were performed using a 5-ml Metrohm 655 Dosimat motor-burette and a Radiometer PHM84

digital voltmeter equipped with B2905 glass and E7786 KCl-saturated calomel Ingold electrodes. The electrodic chain was calibrated in terms of $[H^+]$ by titrating HCl solutions (ca. 0.01 *M*) with standard KOH (ca. 0.2 *M* in 0.1 *M* KCl). The personal computer program BEATRIX [30], based on the Gran method, was used to calculate the equivalence volume, the electrode couple standard potential, and the ionic product of water [pK_w=13.76 (1)]. The ligands were dried over P_4O_{10} in vacuo and stock solutions (ca. 0.02 *M*) were prepared by weight and used within three to four days. PheN-2 and MePheN-2 dihydrochlorides were hygroscopic, so the titre of their stock solutions was determined by means of potentiometric titrations by a KOH solution. Cu^{II}, KOH and HCl solutions were prepared and standardized as already reported [21]. For the binary systems Cu^{2+}/L (L=PheN-2 or MePheN-2), four to five alkalimetric titrations of solutions with Cu/L ratios 1/1 and 1/2 (c_{Cu}=0.001–0.002 *M*) were carried out. For each of the ternary systems examined, five or six titrations were performed with Cu/L/A ratios 1:1:1 and 1:1:2 (c_{Cu}=0.001–0.0015 *M*; A=Val, Glu, Pro, Phe, Trp). The pH ranges explored varied between 3.0 and 11.0 ca.

The stability constants were calculated by the computer program HYPERQUAD [31], which employs the sum of the weighted squares of the residuals between observed and calculated electromotive force (e.m.f.) values as optimization function. The weighting of the experimental observations takes into account the errors of both e.m.f. and titrant volume that were estimated as 0.2 mV and 0.008 ml, respectively. During the refinement of the trial log β values for the ternary complexes, protonation and binary Cu^{II} complexation constants were fixed. For each system, the data from different titrations were treated in a unique batch.

2.8. Spectrophotometric determinations

The absorption spectra of the system Cu^{2+}/PheN-2 were recorded on a Uvikon 941 Plus Kontron spectrophotometer between 400 and 800 nm, at 2-nm intervals, against a 0.1 *M* (KCl) solution as reference. By a peristaltic pump the solution flowed from the potentiometric vessel through the spectrophotometric cell and the spectra were recorded at appropriate pH values. The experimental data were treated with the program SQUAD [32].

3. Results and discussion

3.1. Synthesis of the selectors

The synthesis of the ligands PheN-2 (1a), MePheN-2 (1b) and MePheNMe-2 (1c) was performed following the general rules of the peptide synthesis. The α-amino function of phenylalanine was protected with either the benzyloxycarbonyl (*Z*) or the Boc group and the carboxylic function activated as hydroxysuccinimidyl ester and reacted with ethanediamine (excess). By carefully controlling the reaction conditions and the stoichiometry, monoacylation of ethanediamine was obtained. When using 1-*N*-methyl-1,2-ethanediamine, the formation of the less hindered secondary amide was favoured. Final deprotection was easily accomplished by hydrogenolysis and acidic treatment. The ligands were purified by preparative HPLC. No racemization was observed during the synthesis. Full characterization of the ligands is reported in Section 2.3.

3.2. Enantiomeric separation of unmodified amino acids

Ligands 1a, 1b, 1c and copper(II) acetate in aqueous solution were used as chiral eluents in reversed-phase (RP) HPLC (C_{18} column) for the enantiomeric separation of unmodified amino acids.

With 1a and $Cu(CH_3COO)_2$ (1/1) only Val, Leu and Pro were resolved at pH=6.0, with a L<D elution order (Table 1 and Fig. 2).

The chromatographic pattern was often complicated by different system peaks which are known to

Table 1
Enantiomeric separation of D,L-amino acids with copper(II) complex of PheN-2 (1a)

Amino acid	k_L	k_D	α	R_s
Val	3.20	4.04	1.26	1.20
Leu	14.71	16.26	1.11	1.61
Pro	2.84	3.14	1.10	0.42

Conditions: 1a (0.5 m*M*), $Cu(CH_3COO)_2$ (0.5 m*M*), pH=6.0, room temperature, flow-rate=1 ml/min, column Spherisorb ODS-2 (3 μm, 15×0.46 cm I.D.), UV detection (λ=254 nm).

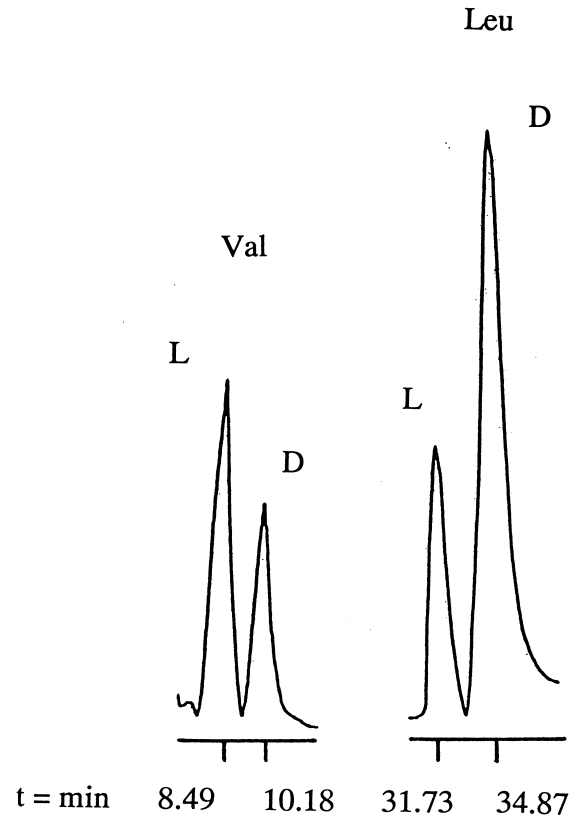


Fig. 2. Enantiomeric separation of D,L-amino acids with the system 1a/copper(II). Conditions as in Table 1.

occur when copper containing eluents are used in RP-HPLC in connection with UV detection [33]. With the analogous monomethylated ligand 1b and dimethylated 1c the results were even worse and no separation was observed. With the dimethylated ligand (1c), it was possible to use post-column derivatization with OPA and fluorescence detection, thus improving the chromatographic performance: however, no enantiomeric separation was obtained. The performance did not improve by increasing the pH to 9 by using a polymeric column in order to prevent deterioration of the silica-based stationary phase.

3.3. Enantiomeric separation of Dns-amino acids

Good enantiomeric separations (Fig. 3 and Table 2) of D,L-Dns-amino acids were achieved by using

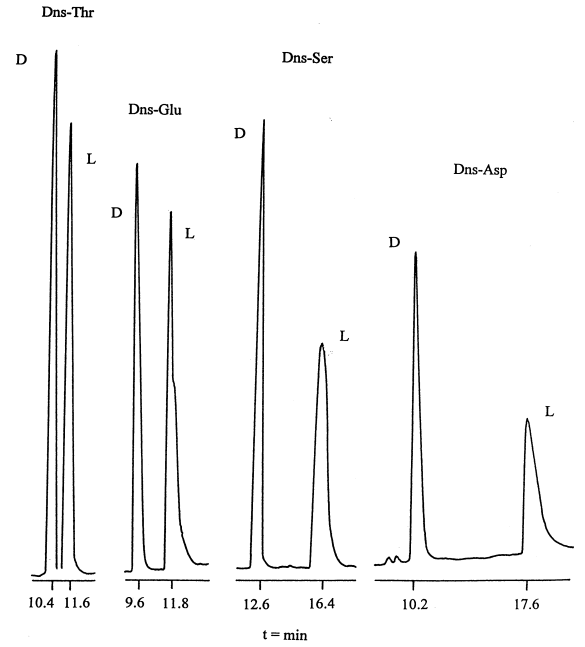


Fig. 3. Enantiomeric separation of D,L-Dns-amino acids. HPLC conditions: column Spherisorb 3 ODS-2 (3 μm, 15×0.46 cm I.D.); eluent, 2 m*M* 1a (for Dns-Ser and Dns-Glu) or 2 m*M* 1b (for Dns-Thr and Dns-Asp), 2 m*M* copper(II) acetate, 0.3 *M* sodium acetate, pH 5.5; column temperature, 25°C; flow-rate, 1 ml/min; fluorescence detection, (λ_{ex}=330, λ_{em}=545).

copper(II) complexes of 1a, 1b and 1c added to the eluent in RP-HPLC.

In particular, very high enantioselectivity factors (α) were obtained for polar Dns-amino acids (α= 5.23 for Dns-Asp). Generally, the enantioselectivity increased with the mono-methylated ligand (1b), while the introduction of a second methyl group on the ethanediamine terminal group had different effects. The elution order was always D<L for all Dns-amino acids.

The chromatographic performance was influenced by the complex concentration in the eluent: in fact, when the complex concentration was increased to 2 m*M* all polar and apolar Dns-amino acids were separated. The *N*-methylated ligands showed good results already at a 1 m*M* concentration. When using a ligand:copper(II) ratio 2:1 the chromatographic results did not change, as shown in Table 3 for 1b.

With all ligands, by increasing the percentage of acetonitrile in the eluent, both the enantioselectivity and capacity factors decreased (Table 4 for 1a).

Table 2
Enantiomeric separation of D,L-Dns-amino acids with copper(II) complexes of 1a, 1b and 1c

Dns-amino acid	1a[a]			1b[b]			1c[b]		
	k_D	k_L	α	k_D	k_L	α	k_D	k_L	α
Dns-Ser	0.85	1.31	1.55[c]	1.16	2.32	2.00	0.83	1.97	2.37
Dns-Thr	1.15	1.38	1.20[c]	1.32	2.56	1.94	0.94	1.62	1.72
Dns-Glu	2.45	5.18	2.11[d]	0.32	0.81	2.52	0.78	1.82	2.33
Dns-Asp	0.35	0.85	2.44[c]	0.46	2.41	5.23	1.50	3.84	2.56
Dns-α-NBu	18.63	22.36	1.20[d]	3.76	5.38	1.42	2.15	3.10	1.44
Dns-Val	27.36	31.00	1.13[d]	5.28	6.81	1.29	2.78	4.30	1.55
Dns-NVal	8.09	12.40	1.53	13.09	18.81	1.44	4.21	5.84	1.39
Dns-Leu	7.64	11.36	1.48	9.86	12.30	1.25	7.30	11.64	1.59
Dns-NLeu	–	–	–	13.09	10.09	1.46	9.13	12.89	1.41
Dns-Met	5.73	9.91	1.73	7.19	8.81	1.23	3.95	7.14	1.81
Dns-Phe	16.54	35.54	2.15	19.00	31.95	1.68	3.84	7.31	1.90
Dns-Trp	–	–	–	22.81	47.19	2.07	5.03	11.28	2.24

Conditions: column, Spherisorb 3 ODS-2 (3 μm, 15×0.46 cm I.D.).
[a] Eluent, 2 m*M* 1a, 2 m*M* copper(II) acetate, 0.3 *M* sodium acetate in water–acetonitrile (75:25, v/v), pH 7.0; column temperature, 25°C; flow-rate, 1.2 ml/min; fluorescence detection, (λ_{ex}=330, λ_{em}=545); t_0=1.05 min.
[b] Eluent, 1 m*M* 1b or 1c, 1 m*M* copper(II) acetate, pH 7.5; flow-rate, 1.5 ml/min for 1b and 0.7 ml/min for 1c; other conditions as in (a).
[c] One m*M* 1a, 1 m*M* copper(II) acetate, other conditions as in (a).
[d] Water–acetonitrile (82:18, v/v).

Table 3
Effect of the ligand/Cu^{2+} ratio on the enantioselectivity in the enantiomeric separation of D,L-Dns-amino acids with MePheN-2

Dns-amino acid	L:Cu^{2+}=2:1			L:Cu^{2+}=1:1		
	k_D	k_L	α	k_D	k_L	α
Dns-Ser	3.06	3.06	1.00	5.68	5.94	1.04
Dns-Thr	4.31	4.68	1.08	8.56	9.19	1.07
Dns-Glu	5.75	5.94	1.03	11.50	11.81	1.03
Dns-Asp	3.44	5.62	1.63	6.62	10.56	1.59

Conditions: column, Spherisorb 3 ODS-2 (3 μm, 15×0.46 cm I.D.); eluent, 2 or 1 m*M* 1b, 1 m*M* copper(II) acetate, 0.3 *M* sodium acetate, pH 5.5, water–acetonitrile (75:25, v/v); column temperature, 25°C; flow-rate, 0.5 ml/min; fluorescence detection, (λ_{ex}=330, λ_{em}=545).

3.4. Binary Cu^{II} complex formation equilibria of PheN-2 and MePheN-2 in aqueous solution

In order to investigate which copper(II) complexes of the ligands 1a and 1b are present in the eluent within the pH range used in HPLC, a potentiometric study of the two systems was carried out at T=25°C and I=0.1 *M* (KCl). The stability constants obtained are reported in Table 5. The log *K* values concerning the protonation of the amino group of the ethanediamine residue are 8.86 for 1a and 8.70 for 1b, respectively, whereas those referring to the amino groups of the amino acid are 6.93 (1a) and 7.01 (1b), respectively.

Table 4
Effect of the eluent polarity on the enantioselectivity in the enantiomeric separation of D,L-Dns-amino acids with PheN-2 (1a)

Dns-amino acid	Water–acetonitrile (65:35, v/v)			Water–acetonitrile (60:40, v/v)		
	k_D	k_L	α	k_D	k_L	α
Dns-Ser	13.68	21.68	1.58	6.69	9.25	1.38
Dns-Thr	17.12	19.75	1.15	9.25	10.12	1.09
Dns-Glu	8.00	11.75	1.46	4.94	6.35	1.26

Conditions: column, Spherisorb 3 ODS-2 (3 μm, 15×0.46 cm I.D.); eluent, 2 m*M* 1a, 2 m*M* copper(II) acetate, 0.3 *M* sodium acetate, pH 5.5; column temperature, 25°C; flow-rate, 1 ml/min; fluorescence detection, (λ_{ex}=330, λ_{em}=545).

Table 5
Logarithms of protonation and Cu^{II} complex-formation contants ($\beta_{pqr}=[Cu_pL_qH_r]/[Cu]^p[L]^q[H]^r$) of PheN-2 and MePheN-2, (L)

Ligand	$[HL]^+$	$[H_2L]^{2+}$	$[CuLH]^{3+}$	$[CuLH_{-1}]^+$	$[CuLH_{-1}(OH)]$	$[CuL_2H_{-1}]^+$	s^{2a}	n^a
PheN-2	8.86 (1)	15.79 (1)	–	–	–	–	0.16	291
	–	–	12.61 (3)	2.98 (1)	−6.21 (1)	5.45 (3)	0.71	257
MePheN-2	8.70 (1)	15.71 (1)	–	–	–	–	2.31	191
	–	–	12.32 (1)	2.46 (1)	−6.58 (1)	4.69 (3)	2.10	404
PicN-2[b]	9.17	10.92	–	–	–	–	–	–
	–	–	12.26	3.27	−6.21	5.84	–	–

T=25°C, I=0.1 M (KCl). Standard deviations are given in parentheses.
[a] $s^2=\Sigma w_i(E_i^{obs}-E_i^{calc})^2/(n-m)$=sample variance; $w_i=1/\sigma_i^2$, where σ_i is the expected error on each observed e.m.f. value (E_i^{obs}); n=number of observations; m=number of parameters refined.
[b] Ref. [34]; other species, $[CuL_2H_2]^{4+}$, log β=23.25.

Both ligands form the same copper(II) complexes, namely $[CuLH]^{3+}$, $[CuLH_{-1}]^+$, $[CuLH_{-1}(OH)]$ and $[CuL_2H_{-1}]^+$. The model reported in the literature [34] for a similar terdentate ligand, *N*-picolinoylethanediamine, (PicN-2), is substantially the same, although a minor species $[Cu(LH)_2]^{4+}$ was found in addition with a L/Cu ratio >4. The two most important species formed by PheN-2 and MePheN-2 are $[CuLH_{-1}]^+$ and $[CuLH_{-1}(OH)]$, whereas the other two are minor species, as it appears in the distribution diagram for the system Cu^{2+}/PheN-2 reported in Fig. 4. In the conditions utilized for the chromatographic separations (Cu:L= 1:1) $[CuLH]^{3+}$ never exceeds the 10%.

In the formation of $[CuLH]^{3+}$ from: $Cu^{2+}+LH^+=[CuLH]^{3+}$, the ligands can be considered as bidentate (N,O) with the amino group and the carbonyl amide acting as coordination sites. The log *K* values of 1a (3.75) and 1b (3.62) are similar to those previously observed [35] for the corresponding amides, phenylalaninamide (4.42) and *N*-methylphenylalaninamide (3.79), respectively. The $[CuLH_{-1}]^+$ species is the only complex present in the pH range 5.5–8.0, where the chromatographic experiments were performed. The log β of these species (2.98 for 1a and 2.46 for 1b) are similar to that obtained for the tricoordinated complex of PicN-2 (3.27) [34] and are much higher than that observed for the ligand N^2-[(*S*)-2-hydroxypropyl]-(*S*)-phenylalaninamide (−2.19) [24], which behaves only as bidentate. The presence of the methyl on the phenylalanine amino group slightly decreases the stability of 1b, in agreement with what previously observed for the corresponding amides [35].

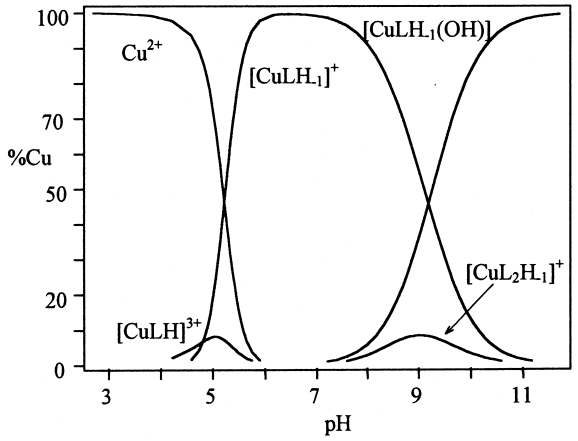


Fig. 4. Species distribution for Cu^{II}/PheN-2 (1:2) system. c_{Cu}=1 m*M*.

The ionization of the equatorial water molecule occurs above pH 8, giving rise to a square planar hydroxo-complex, $[CuLH_{-1}(OH)]$. The log *K* values of the ''hydrolytic'' equilibrium $[CuLH_{-1}(OH_2)]^+=[CuLH_{-1}(OH)]+H^+$ are −9.19 for 1a and −9.04 for 1b, in agreement with the data reported in the literature for other terdentate ligands [diethylenetriamine (dien), dipeptides, log *K* range, −9.0/−9.6] [36,37].

The other minor species $[CuL_2H_{-1}]^+$ does not exceed the 10% of the total copper (Fig. 4), the second ligand probably acting only as monodentate through the amine residue of ethanediamine with the eventual coordination of the carbonyl oxygen at the apical position. In fact, the stepwise equilibrium constants observed for the reaction $[CuLH_{-1}]^++L=$

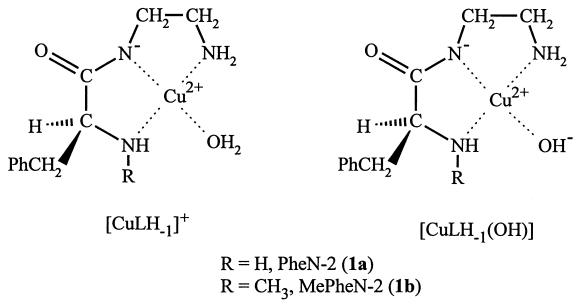


Fig. 5. Proposed structures of binary complexes $[CuLH_{-1}]^{+}$ and $[CuLH_{-1}(OH)]$ for PheN-2 and MePheN-2.

$[CuL_2H_{-1}]^{+}$ (log K=2.47 for 1a, 2.23 for 1b) are consistent with that of PicN-2 (2.57) [34].

In order to characterize the two main species of the Cu^{2+}/PheN-2 system, a spectrophotometric study was carried out in the range 400–800 nm. Spectral data were processed by the program SQUAD [32] and the molar absorptivities (ϵ) as a function of λ were calculated for $[CuLH_{-1}]^{+}$ and $[CuLH_{-1}(OH)]$, using the formation constants obtained by potentiometry. The λ_{max} (nm) and the corresponding ϵ (mol^{-1} dm^3 cm^{-1}) values, obtained for $[CuLH_{-1}]^{+}$ (589/86) and for $[CuLH_{-1}(OH)]$ (588/79), were consistent with those reported in the literature for other terdentate ligands such as PicN-2, $[CuLH_{-1}]^{+}$ (617/104), $[CuLH_{-1}(OH)]$ (602/89) [34] and dien, $[CuL]^{2+}$ (615/82), $[CuL(OH)]^{+}$ (605/73) [36], for which a square planar coordination has been proposed.

Thus, the structure of $[CuLH_{-1}]^{+}$ may be considered as square planar, in which the ligand forms two coplanar five-membered chelate rings with two amino and a deprotonated amide nitrogens. The fourth position is occupied by a water molecule, with the possibility of two more distant apical water molecules.

The proposed structures for $[CuLH_{-1}]^{+}$ and $[CuLH_{-1}(OH)]$ are reported in Fig. 5.

3.5. Ternary Cu^{II} complex formation equilibria of PheN-2 with D- or L-amino acids in aqueous solution

Formation of the ternary complexes in solution by the Cu^{II} complexes of 1a and some D- and L-amino acids (Val, Glu, Phe, Pro, Trp) was investigated by potentiometry. Protonation and Cu^{2+} complexation constants of the amino acids, used for the calculation of the stability constants of the ternary complexes, were taken from the literature [38] (Table 6).

With the reasonable assumption that Cu^{II} can bind one terdentate (L=1a) and one bidentate ligand (A^{-}=aminoacidate), only species with Cu:L:A= 1:1:1 stoichiometry were considered. The model initially tested for the calculation of the log β of the ternary species included $[Cu(LH)A]^{2+}$, $[CuLA]^{+}$ and $[CuLH_{-1}A]$: however, the first species was rejected. The refined formation constants obtained for the various systems are reported in Table 7. An example of species distribution, as a function of pH, for the system Cu^{2+}/1a/L-Phe is reported in Fig. 6.

Two ternary complexes $[CuLA]^{+}$ and $[CuLH_{-1}A]$ are formed. The former is present in the pH range 5.5-9.0 and reaches the 20% of the total copper concentration around pH 7. Its molecular structure may be considered as square planar, with the aminoacidate anion acting as bidentate chelate and the ligand PheN-2 coordinated via the phenylalanine amino group and the carbonyl oxygen of the amide.

Table 6
Literature values (from Ref. [38]) for the protonation and Cu^{II} complex-formation constants (log β) of Val, Glu, Pro, Phe, Trp (A), used in the calculations

Species	L-Val	L-Glu	L-Pro	L-Phe	L-Trp
[HA]	9.49 (3)	9.59 (9)	10.41 (10)	9.09 (4)	9.32 (5)
$[H_2A]^{+}$	11.75 (2)	13.79 (8)	12.30 (10)	11.26 (4)	11.67 (5)
$[H_3A]^{2+}$	–	15.97 (10)	–	–	–
$[CuA]^{+}$	8.09 (4)	8.33 (6)	8.84 (2)	7.90 (4)	8.25 (3)
$[CuA_2]$	14.90 (10)	14.84 (10)	16.36 (8)	14.80 (10)	15.40 (10)
$[CuHA_2]^{+}$	–	12.48 (5)	–	–	–

T=25°C, I=0.1 M (KCl). Standard deviations are given in parentheses.

Table 7
Formation constants (log β_{pqrs}; $\beta_{pqrs}=[Cu_pL_qA_rH_s]/[Cu]^p[L]^q[A]^r[H]^s$) of the ternary Cu^{II} complexes of PheN-2 (L) with L- or D-amino acids (A)

Species	Val		Phe		Trp		Pro		Glu	
	D	L	D	L	D	L	D	L	D	L
$[CuLA]^+$	14.70 (9)	14.64 (3)	14.79 (7)	14.77 (3)	15.64 (2)	15.72 (2)	15.63 (4)	15.66 (3)	14.98 (7)	14.98 (5)
$[CuLH_{-1}A]$	6.36 (3)	6.42 (1)	6.51 (3)	6.69 (2)	6.90 (2)	7.00 (2)	7.18 (2)	7.18 (1)	6.58 (3)	6.66 (2)
s^2	1.37	0.40	1.61	0.77	1.90	1.35	1.69	0.86	3.88	3.42
n	366	297	290	233	345	410	297	293	308	454

T=25°C and I=0.1 M (KCl). Standard deviations are given in parentheses (s^2, n as in Table 5).

The diaminoethane amino group does not seem to be involved in complexation (Fig. 7a).

The main ternary species present $[CuLH_{-1}A]$ (40% of the total copper concentration) starts at pH 7, reaches a maximum at pH 9.5 and vanishes at pH>11.0. It is probably tetragonal pyramidal with the ligand coordinated by the two amino groups and the deprotonated amide, as in the binary complex $[CuLH_{-1}]^+$, and the amino acid binding at the equatorial ($-NH_2$) and apical ($-COO^-$) positions (Fig. 7b). Indeed, the stability constant of the equilibrium $[CuLH_{-1}]^+ + A^- = [CuLH_{-1}A]$ for 1a (log K=3.71, A=L-Phe) is much lower than that formed by phenylalaninamide (Phe-NH_2) and L-Phe [21] (log K=7.09), where the aminoacidate is coordinated to two equatorial positions and it is consistent with that observed for $[Cu(dien)(Phe)]^+$ (log K=3.91) [39].

The data reported in Table 7 show that stereoselectivity is never observed for the species $[CuLA]^+$, whereas for the species $[CuLH_{-1}A]$ it may be appreciated only for the system PheN-2/Phe [$\Delta\log\beta=-0.18$ (4)], and it is almost negligible for Trp [−0.10 (3)]. With both Phe and Trp the diastereoisomeric complexes with the L-enantiomers are more stable than those with the D-enantiomers on account of the steric hindrance of the selector and amino acid side groups. The poor enantioselectivity may be due to the fact that the mixed complexes are less stable than those formed with the amino acid amides since the aminoacidate is coordinated at only one equatorial position and the carboxyl group can be accommodated at either apical positions (Fig. 7b), thus compensating for the eventual destabilizing interactions.

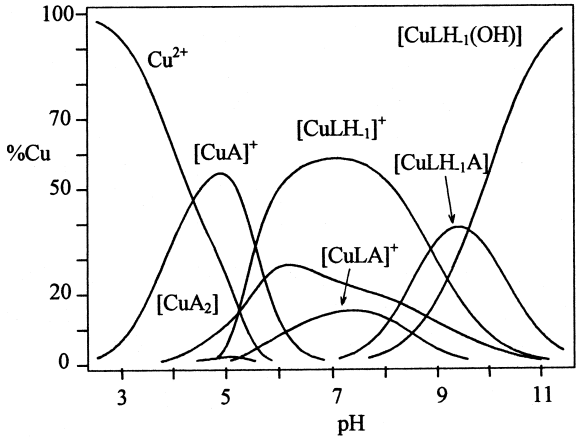


Fig. 6. Species distribution for Cu^{II}/PheN-2/L-Phe (1:1:1) system. c_{Cu}=1.5 mM.

3.6. Mechanism of chiral separation

The species predominant in the pH range utilized for the chromatographic experiments (5.5–9) is the complex $[CuLH_{-1}]^+$, which reaches a 100% of copper complexation. This species gives rise to ternary species $[CuLH_{-1}A]$, without modifying its chelate rings, but rather accepting the amino group of the amino acid at the free equatorial position and the carboxylate at the apical position. Thus, it appears that one free equatorial position is sufficient for the amino acid complexation, although with a stepwise formation constant much lower (log K= 3.71, L-Phe) than that previously observed for the bidentate ligand L-Phe-NH_2 (log K=7.09) [21]. Since the stereoselectivity in solution is low and a fairly good separation in HPLC was observed only for Val, Leu and Pro, it is feasible that chiral discrimination does not occur in solution, but rather by interaction with the selector $[CuLH_{-1}]^+$ adsorbed

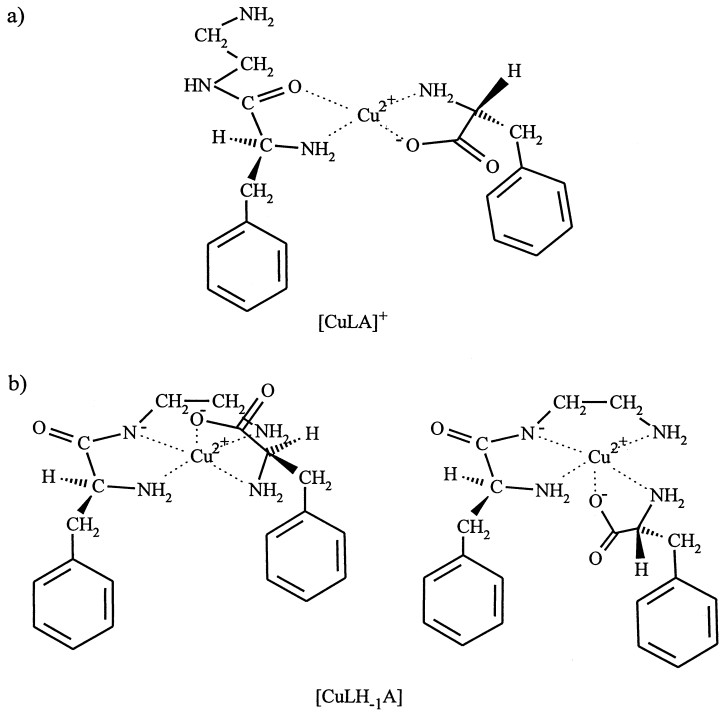


Fig. 7. Proposed structures of the ternary complexes of Cu^{II} with PheN-2 and D-Phe. (a) $[CuLA]^+$; (b) $[CuLH_{-1}A]$: the two different coordination modes at the apical positions are outlined.

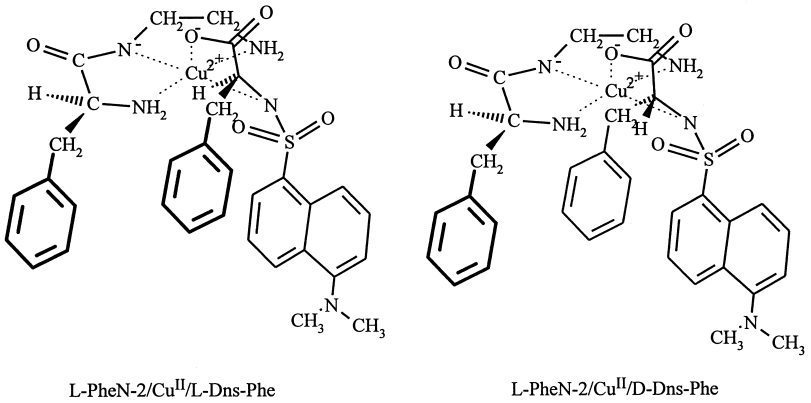
on the column stationary phase to form the neutral species $[CuLH_{-1}A]$. In this way, D-Leu, D-Val and D-Pro are more retained, being able to establish a lipophilic interaction between their side-chain and the stationary phase.

The other potential selector $[CuLH]^{3+}$ is not present at pH 6 and the charged mixed species $[CuLA]^+$ should not be relevant in the conditions utilized in HPLC, being much less adsorbed on the column. Indeed, in the chromatographic system the amino acid is added only after the selector has been adsorbed on the column. Thus, we are inclined to conclude that the equilibria eventually occurring in solution are not relevant to the overall discrimination process.

In conclusion, the poor performance of the diaminoamido 1a, 1b and 1c copper(II) complexes in the chiral discrimination of unmodified amino acids can be ascribed to the fact that $[CuLH_{-1}]^+$ does not displace any of its original binding groups and, having only one free equatorial position, it gives rise to mixed complexes of a too low stability to compete with the formation of the binary complexes of the aminoacidate. Thus, when the amino acid is injected in the mobile phase, it strongly competes with the selector for copper complexation. Only lipophilic amino acids which can undergo favourable partition equilibria between the mobile and the stationary phase can be separated. Tryptophan and phenylalanine are too strongly retained on the column and are not eluted.

In contrast, the copper(II) complexes of the terdentate ligands 1a, 1b and 1c gave very good separation of Dns-amino acids in HPLC. Also in this case the Dns-amino acid may coordinate at the equatorial position with the deprotonated dansylamino group and at the apical position with the carboxylate. The dansyl moiety, on account of its bulkiness and lipophilicity, gives a strong lipophilic interaction with the C_{18} stationary phase, which can be modulated by adding an organic modifier (acetonitrile) to the mobile phase.

The elution order is the same for all the Dns-amino acids (D<L), as observed with the analogous



L-PheN-2/Cu^{II}/L-Dns-Phe L-PheN-2/Cu^{II}/D-Dns-Phe

Fig. 8. Proposed recognition model for Dns-Phe by L-PheN-2.

diaminodiamido tetradentate ligands [40]. In contrast, for the bidentate ligands amino acid amides an opposite elution order for polar (D<L) and apolar Dns-amino acids (L<D) was observed [25]. In the latter case, the data were consistent with the formation of mixed complexes in solution and subsequent partition between the two phases, with the amino acid side chains playing a definite role in the adsorption mode on the column.

In our case, by assuming that the selector is adsorbed on the stationary phase and that it is the dansyl group which gives an aspecific lipophilic interaction with the column, the nature of the amino acid side-chain becomes irrelevant, and all the L-enantiomers, which have the same configuration as the selector, are more retained on account of a better ''fitting'' on the phase: L-selector/L-Dns-amino acid (match), L-selector/D-Dns-amino acid (mismatch), (Fig. 8).

The presence of methyl groups on the amino groups increases the lipophilicity of the ligand and the steric hindrance in the complexation, leading to a higher enantioselectivity.

4. Conclusions

In conclusion, copper(II) complexes of terdentate diaminoamido ligands perform good enantiomeric discrimination of Dns-amino acids and poor enantioseparation of unmodified amino acids. The chiral recognition mechanism takes place by coordination of the amino (or deprotonated sulphonamide for the dansyl) group of the amino acid at the free equatorial position and the carboxylate at the apical position. None of the three strongly binding groups of the terdentate ligand are displaced from the Cu(II) coordination sphere, but only water molecules.

Thus, chiral discrimination can occur also when only one equatorial position is available in the initial copper complex, but it is less efficient. The interaction of the analyte with the selector adsorbed on the column is fundamental. Indeed, in solution the formation of the mixed ternary complexes $[CuLH_{-1}A]$ strongly competes with the more stable aminoacidate binary species. Moreover, the structures of the mixed complexes do not have strict steric requirements, being able to accommodate the carboxylate at either apical positions.

Acknowledgements

This paper was supported by a grant from CNR (Consiglio Nazionale delle Ricerche, Rome), Progetto Finalizzato Chimica Fine e Secondaria II, and by MURST (Ministero dell'Università e della Ricerca Scientifica e Tecnologica). NMR and mass spectra were recorded at CIM (Centro Interdipartimentale di Misure), University of Parma. Professor A. Vacca (University of Florence) is kindly thanked for providing us with the latest version of the HYPERQUAD program.

References

[1] S.V. Rogozhin, V.A. Davankov, Chem. Commun. (1971) 490.
[2] V.A. Davankov, J.D. Navratil, H.F. Walton, Ligand Exchange Chromatography, CRC Press, Boca Raton, FL, 1988.
[3] V.A. Davankov, Adv. Chromatogr. 18 (1980) 139.
[4] V.A. Davankov, Pure Appl. Chem. 54 (1982) 2159.
[5] G. Gübitz, W. Jellenz, W. Santi, J. Chromatogr. 203 (1981) 377.
[6] V.A. Davankov, S.V. Rogozhin, A.V. Semetchin, B.A. Baranov, J. Chromatogr. 93 (1974) 363.
[7] G. Gübitz, W. Jellenz, G. Löffler, W. Santi, J. High Resolut. Chromatogr. Chromatogr. Commun. 2 (1979) 145.
[8] H. Brückner, Chromatographia 24 (1987) 725.
[9] P. Roumeliotis, K.K. Unger, A.A. Kurganov, V.A. Davankov, J. Chromatogr. 255 (1983) 51.
[10] B. Galli, F. Gasparrini, D. Misiti, C. Villani, R. Corradini, A. Dossena, R. Marchelli, J. Chromatogr. A 666 (1994) 77.
[11] W. Lindner, J.N. Le Page, G. Davies, D.E. Seitz, B.L. Karger, J. Chromatogr. 185 (1979) 323.
[12] S. Weinstein, M.H. Engel, P.E. Hare, Anal. Biochem. 121 (1982) 370.
[13] N. Nimura, T. Suzuki, Y. Kasahara, T. Kinoshita, Anal. Chem. 53 (1981) 1380.
[14] E. Gruskha, R. Leshem, C. Gilon, J. Chromatogr. 255 (1983) 41.
[15] E. Gil-Av, A. Tishbee, P.E. Hare, J. Am. Chem. Soc. 102 (1980) 5115.
[16] V.A. Davankov, Yu.A. Zolotarev, A.A. Kurganov, J. Liq. Chromatogr. 2 (1979) 1191.
[17] V.A. Davankov, A.A. Kurganov, Chromatographia 17 (1983) 686.
[18] V.A. Davankov, A.A. Kurganov, T.M. Ponomareva, J. Chromatogr. 452 (1988) 309.
[19] S. Weinstein, L. Leiserowitz, Isr. J. Chem. 25 (1985) 334.
[20] G. Galaverna, R. Corradini, E. de Munari, A. Dossena, R. Marchelli, J. Chromatogr. A 657 (1993) 43.
[21] F. Dallavalle, G. Folesani, R. Marchelli, G. Galaverna, Helv. Chim. Acta 77 (1994) 1623.
[22] F. Dallavalle, G. Folesani, E. Leporati, G. Galaverna, Helv. Chim. Acta 79 (1996) 1818.
[23] R. Marchelli, R. Corradini, T. Bertuzzi, G. Galaverna, A. Dossena, F. Gasparrini, B. Galli, C. Villani, D. Misiti, Chirality 8 (1996) 452.
[24] F. Dallavalle, G. Folesani, T. Bertuzzi, R. Corradini, R. Marchelli, Helv. Chim. Acta 78 (1995) 1785.
[25] E. Armani, L. Barazzoni, A. Dossena, R. Marchelli, J. Chromatogr. 441 (1988) 287.
[26] J.P. Greenstein, M. Winitz, Chemistry of the Amino Acids, Vol. 2, Wiley, New York, 1961, p. 887.
[27] G.W. Anderson, J.E. Zimmerman, F.M. Callahan, J. Am. Chem. Soc. 86 (1964) 1839.
[28] O. Keller, W.E. Keller, G. Van Look, G. Wersin, Org. Synth. Coll. VII (1990) 70.
[29] J.R. McDermott, N.L. Benoiton, Can. J. Chem. 51 (1973) 1915.
[30] A. Braibanti, C. Bruschi, E. Fisicaro, M. Pasquali, Talanta 33 (1986) 471.
[31] P. Gans, A. Sabatini, A. Vacca, Talanta 43 (1996) 1739.
[32] D.J. Legget, W.A.E. McBryde, Anal. Chem. 47 (1975) 1065.
[33] N. Mizrotsky, L. Kristol, E. Grushka, J. Chromatogr. A 691 (1995) 21.
[34] T. Kaden, A.D. Zuberbuhler, Helv. Chim. Acta 54 (1971) 1361.
[35] F. Dallavalle, E. Fisicaro, R. Corradini, R. Marchelli, Helv. Chim. Acta 72 (1989) 1479.
[36] H. Gampp, H. Sigel, A.D. Zuberbuhler, Inorg. Chem. 21 (1982) 1190.
[37] T. Kiss, Z. Szucs, J. Chem. Soc. Dalton Trans. (1986) 2443.
[38] A.E. Martell, R.M. Smith, Critical Stability Constants, Vol. 5, First Supplement, Plenum Press, New York, 1982.
[39] T. Murakami, K. Murata, Y. Ishikawa, Inorg. Chim. Acta 244 (1996) 51.
[40] E. Armani, A. Dossena, R. Marchelli, R. Virgili, J. Chromatogr. 441 (1988) 275.