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COPPER(II) COMPLEXES OF DIAMINO-DIAMIDO-TYPE LIGANDS AS CHIRAL ELUENTS IN THE ENANTIOMERIC SEPARATION OF D,L-DANSYLAMINO ACIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

For purpose of studying the mechanism of chiral recognition in high-performance liquid chromatography (HPLC) (reversed phase), a model system is proposed, *i.e.*, copper(II) complexes of diamino-diamido-type ligands containing L-amino acids (AA-NN-*n*) which added to the eluent are able to perform the enantiomeric resolution of dansylamino acids on C_8 and C_{18} columns. Being potentially tetradentate, this system should be more liable to give an apical or outer-sphere interaction with the enantiomer rather than a simultaneous dechelation of two binding sites, as in the classical ligand-exchange mechanism. This model allows the variation of several parameters, such as the structural and electronic features of the initial complexes, their relative stabilities and lipophilicities and their correlation with enantioselectivity in HPLC.

INTRODUCTION

Chiral resolution of enantiomers via stereospecific and reversible interactions, involving the formation of labile diastereomeric complexes, is at present a very intensive and promising area of research. In gas chromatography (GC), high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) it is not only an analytical tool of great effectiveness, but also a rapid and valuable means for verifying the enantioselectivity of a system.

Chiral resolution of free and derivatized amino acids in HPLC (reversed phase) can be achieved on achiral phases by adding copper(II) complexes of L-amino acids[1–7] and amino acid derivatives to the mobile phase: L-prolylalkylamides[8], L-aspartylalkylamides[9–11], N-tosyl-L-phenylalanine[12], L-phenylglycine[13], N,N-dialkyl-L-amino acids[14–17], L-histidine methyl ester[18], aspartame and its methyl ester[19].

The mechanism of chiral resolution in these systems was mostly explained, by analogy with that obtained on a chiral phase incorporating L-amino acids, on the basis of the ligand-exchange theory (LEC = ligand-exchange chromatography)[20]. It

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is assumed that a ligand exchange occurs between the initial copper(II) complex and the incoming D- and L-amino acid, leading to formation of diastereomeric ternary complexes of different stabilities and/or affinities for the column. More recently, a new mechanism was proposed by Weinstein and Leiserowitz[21] mostly on the basis of crystallographic studies. An apical interaction or an outer sphere coordination of the enantiomers on the initial copper complex should be sufficient to give chiral resolution. Finally, it was stated[22] on the basis of thermodynamic studies that the difference in stability of the diasteromeric mixed complexes in water has a negligible influence on enantioselectivity, and that chiral recognition takes place on the organic phase where the initial complex is adsorbed.

For the purpose of studying the mechanism of chiral recognition, we recently[23] proposed a model system, *i.e.*, copper(II) complexes of diamino-diamido-type ligands containing L-amino acids, potentially tetradentate and, therefore, more liable to give an apical or outer-sphere coordination, rather than a simultaneous dechelation of two binding sites, as in the classical ligand-exchange mechanism.

Moreover, (*S,S*)-N,N′-bis(aminoacyl)ethane and propane diamines AA-NN-2 and AA-NN-3 (AA = alanine, phenylalanine, valine) (Fig. 1) allow the variation of several parameters, such as stability constants, geometry, lipophilicity of the complexes and their correlation with enantioselectivity in HPLC. The synthesis of the ligands AA-NN-*n* and potentiometric studies of their copper complexes have been published[24], as well as the enantiomeric separation of D,L-dansyl (Dns) amino acids in TLC using HPLC RP-18 plates saturated with the present ligands and copper acetate[25].

In the present paper we report the separation of D- and L-dansylamino acids on reversed-phase columns (C_8 and C_{18}) using mobile phases containing copper(II) complexes of AA-NN-*n* dissolved in water–acetonitrile mixtures at various pH values. The usual chromatographic parameters, *i.e.*, pH, eluent polarity, concentration, ligand-to-metal ratios, ionic strength and elution order have been examined. Particular attention has been paid to the structural and electronic features of the initial complexes, to their relative stabilities and affinities for the column. Also the lipophilicity and the bulk of each ligand were evaluated as parameters determining the enantioseparation process.

EXPERIMENTAL

Equipment

The chromatograph was a Waters Model 440 instrument equipped with a Model 420 fluorescence detector. The analytical columns were a LiChrosorb RP-8 (10 μm, 24 cm × 0.4 cm) and a C_{18} Novapak (4 μm, 15 cm × 0.4 cm).

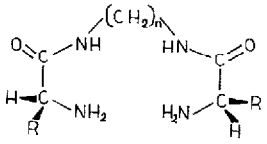

Fig. 1. Ligands AA-NN-*n*: *n* = 2, 3; R = $C_6H_5CH_2$ (Phe), $(CH_3)_2CH$ (Val), CH_3 (Ala).

Reagents

Twice distilled water was used. Acetonitrile (HPLC grade) and copper acetate (RPE-ACS grade) were obtained from Carlo Erba (Milan, Italy); L- and D,L-Dns-amino acids from Sigma. The ligands AA-NN-*n* were synthesized as described previously[24].

Mobile phase preparation

The ligand AA-NN-*n* and copper acetate were carefully weighed and added to a 0.3 *M* solution of sodium acetate. Then acetonitrile was added to the required percentage. The pH was adjusted with aqueous potassium hydroxide within the range 6.8–8.5. The mobile phase was then filtered and degassed under reduced pressure.

RESULTS AND DISCUSSION

Lipophilicity and bulk of the ligand

On the base of the Hansch coefficients[26], it is possible to estimate the relative lipophilicity of the ligands AA-NN-*n* and of the amino acid side-chain R of the amino acyl synthon (Table I).

The lipophilicity of the ligand side-chain appears to be determinant for enantioselectivity. Ligands containing L-phenylalanine (Phe-NN-2 and Phe-NN-3) give better separations than those with L-valine (Val-NN-2, Val-NN-3), whereas those with L-alanine (Ala-NN-2 and Ala-NN-3) are not selective. A full comparison of the data obtained with a C_{18} column is reported in Table II. However, by using a more efficient C_8 column, Val-NN-2 is able to separate some amino acids which were not resolved on the C_{18} column (Table III).

The overall lipophilicity of the ligand is less important since AA-NN-2 is more selective than AA-NN-3; other factors such as the different structural features and the relative stabilities of the complexes AA-NN-2/Cu(II) and AA-NN-3/Cu(II) must be more relevant.

Although the bulk of the R group of the ligands undoubtedly affects enantioselectivity, nevertheless it does not seem to be the discriminating factor. The best separations were obtained with ligands containing L-phenylalanine rather than the C_α-branched L-valine.

Structure and stability of the initial copper complexes: pH effect

Enantiomeric separation occurs in the range pH 6.8–8.5 with an optimum

TABLE I

LIPOPHILICITY OF LIGANDS AA-NN-*n*

Ligand	*R*	*log P*		
		R	*AA-NN-2*	*AA-NN-3*
Phe-NN-*n*	$C_6H_5CH_2$	2.62	0.74	1.24
Val-NN-*n*	$(CH_3)_2CH$	1.30	−2.04	−1.54
Ala-NN-*n*	CH_3	0.50	−3.64	−3.04

TABLE II

ENANTIOMERIC SEPARATION OF Dns-AMINO ACIDS BY AA-NN-*n*/Cu(II)

Conditions: 2 m*M* AA-NN-*n*/Cu(II); 0.3 *M* sodium acetate; pH 7.3; 25°C. Column: 15 cm × 0.4 cm, 4-μm C_{18} Novapak. Eluents: water–acetonitrile (a) 75:25; (b) 76:24; (c) 77:23. $\alpha = k'_L/k'_D$. αNBu = α-Aminobutyric acid.

Amino acid	*Phe-NN-2 (a)*			*Phe-NN-3 (b)*			*Val-NN-2 (a)*			*Val-NN-3 (c)*			*Ala-NN-2 (c)*			*Ala-NN-3 (c)*		
	k'_D	k'_L	α	k'_D	k'_L	α	k'_D	k'_L	α	k'_D	k'_L	α	k'_D	k'_L	α	k'_D	k'_L	α
Glu	0.45	1.23	2.73	0.64	2.00	3.14	0.18	0.41	2.27	0.59	1.23	2.08	0.73	0.73	1.00	0.59	0.59	1.00
Asp	2.00	7.91	4.00	2.27	5.36	2.36	0.41	0.91	2.22	1.50	3.32	2.21	0.82	1.09	1.33	0.73	0.73	1.00
Ser	3.55	7.68	2.16	4.27	8.36	1.96	1.05	1.68	1.61	2.41	3.77	1.57	2.59	2.59	1.00	2.59	2.59	1.00
Thr	4.23	5.95	1.41	4.14	5.50	1.33	1.18	1.50	1.27	4.91	5.36	1.09	3.18	3.18	1.00	3.18	3.18	1.00
Ala	5.18	6.55	1.31	8.55	9.36	1.09	2.27	2.45	1.08	4.68	6.05	1.29	5.45	5.45	1.00	4.50	4.50	1.00
αNBu	7.68	8.50	1.11	8.91	12.09	1.36	3.00	3.00	1.00	6.95	6.95	1.00	8.09	8.09	1.00	6.77	6.77	1.00
Val	10.18	10.18	1.00	12.82	12.82	1.00	3.82	3.82	1.00	9.45	9.45	1.00	12.27	12.27	1.00	10.14	10.14	1.00
NVal	16.45	15.54	0.94	22.00	21.09	0.96	5.27	5.27	1.00	12.18	12.18	1.00	15.73	15.73	1.00	12.95	12.95	1.00
Met	21.36	21.36	1.00	26.82	29.27	1.09	6.23	6.23	1.00	12.64	13.77	1.09	15.27	15.27	1.00	13.18	13.18	1.00
Leu	23.00	21.00	0.91	28.64	26.82	0.94	7.95	7.95	1.00	18.55	18.55	1.00	25.91	25.91	1.00	20.86	20.86	1.00
Phe	49.63	58.63	1.18	60.27	66.45	1.10	13.64	13.64	1.00	37.64	37.64	1.00	33.55	33.55	1.00	32.73	32.73	1.00
Trp	80.91	109.09	1.35	79.27	118.18	1.49	13.27	22.36	1.45	38.55	58.09	1.51	42.45	42.45	1.00	33.64	33.64	1.00

TABLE III

ENANTIOMERIC SEPARATION OF Dns-AMINO ACIDS BY Val-NN-2/Cu(II)

Conditions: column, 24 cm × 0.4 cm, 10-μm LiChrosorb RP-8; 2 m*M* Val-NN-2/Cu(II), 0.3 *M* sodium acetate; pH 7.5; 25°C; water–acetonitrile (72:28).

Dns-amino acid	k'_D	k'_L	α
Glu	2.87	4.62	1.61
Asp	6.00	14.37	2.39
Ser	7.25	12.50	1.72
Thr	7.25	9.25	1.28
αNBu	9.25	10.75	1.16
Val	9.62	10.50	1.09
Met	15.87	19.12	1.20
Leu	14.63	14.63	1.00
Phe	25.62	35.50	1.38
Trp	39.12	89.00	2.27

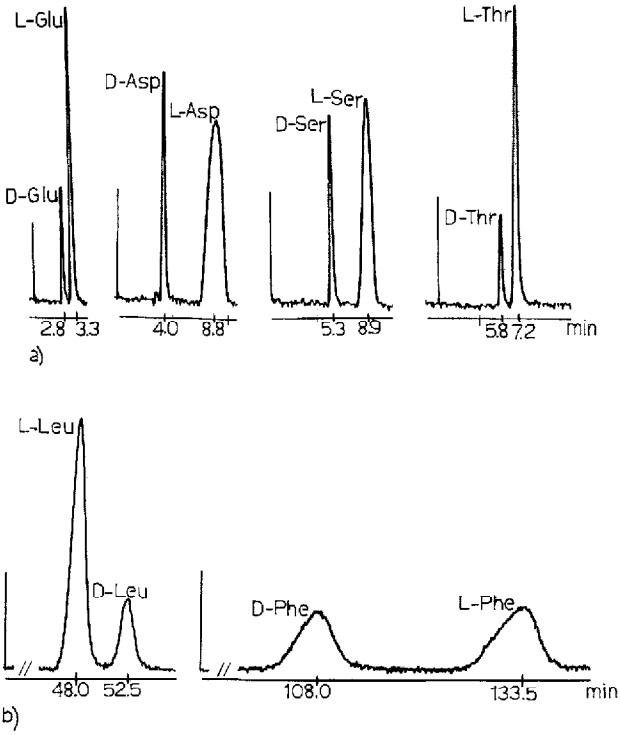

Fig. 2. Enantiomeric separation of Dns-amino acids by Phe-NN-2/Cu(II). Conditions: 2 m*M* Phe-NN-2/Cu(II); 0.3 *M* sodium acetate; pH 7.3; 25°C. Column: 15 cm × 0.4 cm, 4-μm C_{18} Novapak. Eluents: water–acetonitrile (a) 72:28; (b) 75:25.

performance (enantioselectivity, resolution, peak sharpness at pH 7.3–7.5 for all ligands) (Fig. 2).

Species distribution diagrams[24], obtained from potentiometric titration of the free ligands and of the copper complexes, are shown in Fig. 3. In the pH range considered, two species $Cu_2L_2H_{-2}^{2+}$ and $CuLH_{-2}$ are observed for the Cu(II)/AA-NN-2 systems, which may be responsible for enantiomeric separation. In contrast, for the system Cu(II)/AA-NN-3 only the species $CuLH_{-2}$ is present. The structures of the complexes, as derived from visible[24] and ESR (electron spin resonance)[27] data, are reported in Fig. 4.

Both complexes were shown to be enantioselective. Since $CuLH_{-2}$ is the only species present for AA-NN-3 and performs enantioresolution, it is reasonable to assume that the same species formed by AA-NN-2 is enantioselective. $CuLH_{-2}$ is a square-planar neutral complex and may be adsorbed on the column to a greater degree than the charged species $Cu_2L_2H_{-2}^{2+}$. This fact might be significant if the

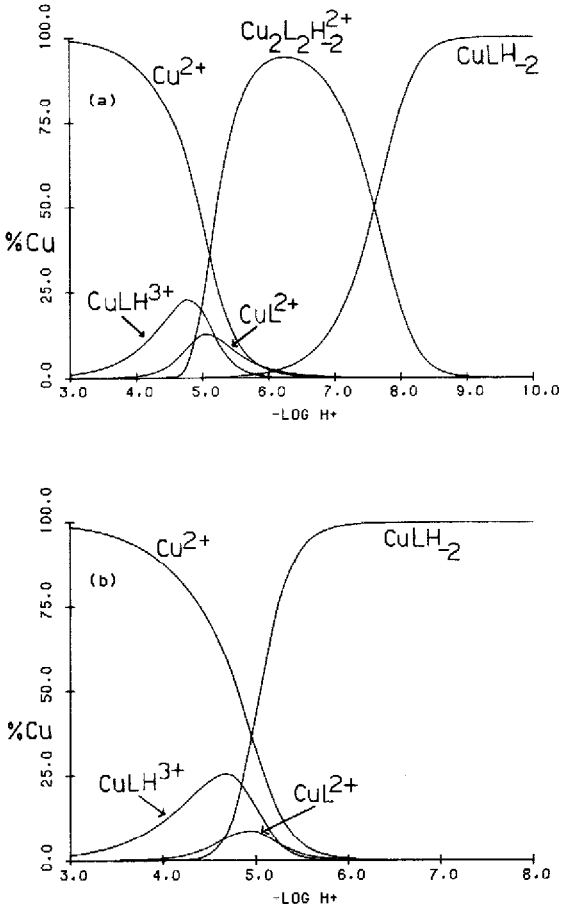

Fig. 3. Species distribution for Phe-NN-*n*/Cu(II) as a function of pH, obtained by potentiometric titration: (top) $n = 2$; (bottom) $n = 3$.

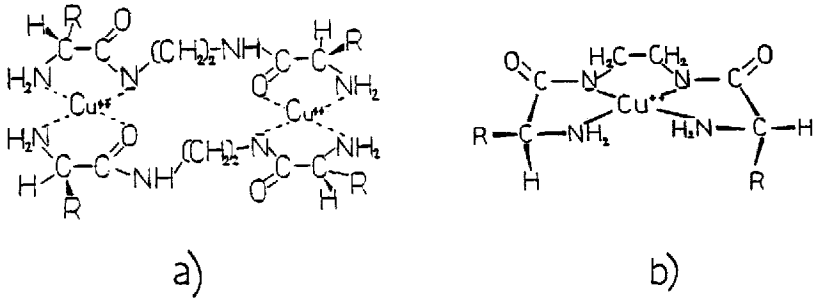

Fig. 4. Structures of copper complexes: (a) $Cu_2L_2H_{-2}^{2+}$; (b) $CuLH_{-2}$.

enantioresolution occurs on the stationary phase of the column or at the interphase, while it is less relevant if enantiomeric discrimination occurs in the mobile phase. The elution order of D- and L-enantiomers of Dns-amino acids is the same at pH 7.0, where the concentration of $CuLH_{-2}$ is very small in comparison with that of $Cu_2L_2H_{-2}^{2+}$, and at pH 8.4 where $CuLH_{-2}$ is the only species present. Moreover, the elution order is the same for AA-NN-2 and AA-NN-3 ($k'_D < k'_L$) (Table I). Thus, both complexes are enantioselective. In every case the elution order was checked by injecting a solution of the D,L-Dns-amino acid enriched with the L-enantiomer.

On the other hand, it is not easy to establish which complex is more enantioselective, since the pH of the mobile phase in a chromatographic analysis affects not only the species distribution of the enantioselective system, but also the chelating abilities of the Dns-amino acids. Dns-amino acids may behave towards Cu(II) as monodentate or bidentate ligands depending on the pH, as well as mono- or dianions. The sulphonamidic nitrogen in the presence of Cu(II) undergoes deprotonation, $pK_a = 8.0$, in water[28].

Now, the retention times and the selectivity coefficients α, with Phe-NN-2/-Cu(II) (2 m*M*) increase with increasing pH for Glu and Asp, but only slightly for

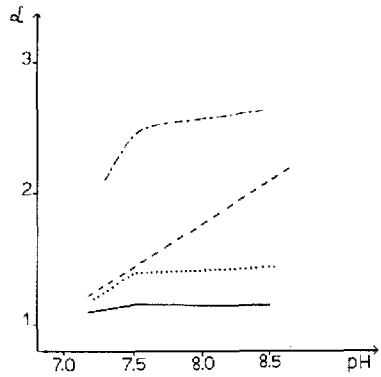

Fig. 5. Variation of the enantioselectivity coefficient, α, as a function of pH with Phe-NN-2/Cu(II). Conditions: 2 m*M* Phe-NN-2/Cu(II); 0.3 *M* sodium acetate; water–acetonitrile (72:28); column, 15 cm × 0.4 cm, 4-μm C_{18} Novapak. – – –, Glu; — · — · , Asp; · · · · , Thr; ———, αNBu.

the other amino acids after pH 7.5. Apparently, the former, which can become tridentate at higher pH, may give further electrostatic interactions with the initial complex, whereas the latter reach the final equilibrium at such a pH (Fig. 5).

Comparing AA-NN-2/Cu(II) and AA-NN-3/Cu(II), the better enantioselectivity in general exhibited by the former may be ascribed either to the lower stability of the relative complexes $CuLH_{-2}$ [log $\beta(CuLH_{-2}) = -6$ for L = AA-NN-2, log $\beta(CuLH_{-2}) = -3$ for L = AA-NN-3][24] or to a more favourable geometry of the complex, which allows enantioselection.

Concentration and ligand/Cu(II) ratio

The ratio ligand/Cu(II) = 1/1 was found to be the best according to the stoichiometry of the species $Cu_2L_2H_{-2}^{2+}$ and $CuLH_{-2}$.

For the system Phe-NN-2/Cu(II), on increasing the concentration from 1 to 2 m*M* the capacities increase remarkably, improving the separation coefficients, while at 3 m*M* the capacities still increase while the separation coefficients slightly increase or remain constant (Table IV). This behaviour is in accord with the resolution occurring on the stationary phase, where the initial complex is most probably adsorbed.

Stationary phase and mobile phase

LiChrosorb RP-8 (10 μm, 24 cm × 0.4 cm) and C_{18} Novapak (4 μm, 15 cm × 0.4 cm) columns were used. Both gave good results, but with surprising differences: with Phe-NN-2, D,L-Dns-valine is separated on the C_8 (Table IV) and not on the C_{18} column (Table II). The C_{18} chains of the stationary phase might solvate the valine side-chain, levelling off the discriminating effect. The same deduction can be applied to the ligand Val-NN-2, which is more enantioselective on the shorter C_8 phase.

TABLE IV

ENANTIOMERIC SEPARATION OF Dns-AMINO ACIDS BY Phe-NN-2/Cu(II): INFLUENCE OF THE CONCENTRATION OF THE ADDITIVE

Conditions: column, 24 cm × 0.4 cm, 10-μm LiChrosorb RP-8; 0.3 *M* sodium acetate, pH 7.5, 25°C; water–acetonitrile (72:28, v:v); V_0 = 1 ml.

Dns-amino acid	*1 mM*			*2 mM*			*3 mM*		
	k'_D	k'_L	α	k'_D	k'_L	α	k'_D	k'_L	α
Glu	2.87	4.62	1.61	3.50	7.50	2.14	4.25	9.37	2.20
Asp	6.00	14.37	2.39	8.37	20.75	2.48	9.12	22.75	2.49
Ser	7.25	12.50	1.72	10.00	20.00	2.00	11.75	23.25	1.98
Thr	7.25	9.25	1.28	10.37	15.75	1.52	12.12	18.50	1.53
αNBu	9.25	10.75	1.16	14.25	17.50	1.23	16.50	21.00	1.27
Val	9.62	10.50	1.09	14.25	16.63	1.17	16.87	20.00	1.18
Met	15.87	19.12	1.20	25.62	32.62	1.27	30.62	39.62	1.29
Leu	14.63	14.63	1.00	23.25	23.25	1.00	27.12	27.12	1.00
NVal	–	–	–	25.00	25.00	1.00	28.87	28.87	1.00
Phe	25.62	35.50	1.38	44.12	71.50	1.62	52.87	77.25	1.46
Trp	39.12	89.00	2.27	–	–	–	–	–	–

The opposite occurs with D,L-Dns-leucine and norvaline on the C_{18} phase, which gives a reversed elution order: the β- or unbranched side-chain of the D-enantiomers, in this case, may have a lipophilic interaction with the aromatic ring of the ligand rather than a steric repulsion, as in every other case.

The mobile phase plays an important rôle in the separation since the chromatographic process involves the partition of the solutes between the mobile and the stationary phase. The eluent polarity influences both the capacity factor, k', and the enantioselectivity coefficient, α.

Various water–acetonitrile mixtures were used in order to dissolve the solutes [the complexes AA-NN-*n*/Cu(II) and the Dns-amino acids] and to allow the interaction of the copper complexes with the organic phase of the column. According to the lipophilic character of each complex AA-NN-*n*/Cu(II) and of the particular Dns-amino acid, the optimum percentage of acetonitrile was found. The best mixture on the C_{18} column is 25–28% for Phe-NN-2, 24% for Phe-NN-3, 25% for Val-NN-2 and Val-NN-3 and 23% acetonitrile for Ala-NN-2. For the aromatic Dns-amino acids, however, it is more convenient to use an higher percentage of acetonitrile, resulting in less enantioselectivity but shorter retention times and better resolution.

It is known[29] that the ionic strength affects the retention times of solutes and that the strongly lipophilic Dns-amino acids require a relatively high ionic strength in order to reduce the retention times and to favour the partition in the mobile phase. 0.3 *M* sodium acetate was added to the eluent also in order to buffer the solution since deprotonation of ligands and solutes occurs.

The mechanism of recognition

The following considerations on the mechanism of enantioselectivity are based on the sound assumption that the main complex involved in resolution is $CuLH_{-2}$, whose structure is clearly known[24], and which is present in the pH range where optimum enantiomeric recognition occurs.

The fundamental questions concern where and how the enantioselective interactions take place. There are two possibilities: (1) the initial complex $CuLH_{-2}$ and the enantiomers (L,D) form in the mobile phase two diastereomeric ternary complexes of different stabilities which give different interactions with the column; (2) the initial complex is adsorbed on the organic phase of the column, where it performs the enantiomeric recognition.

Most results reported in the literature favour the latter hypothesis[22,30], mainly on account of the fact that the slight differences in the stability constants of the mixed

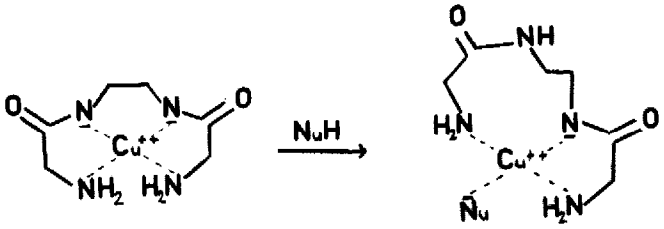

Fig. 6. Decomplexation mechanism of a ligand AA-NN-2 in the presence of a nucleophile[31].

complexes, determined spectroscopically and potentiometrically in water, do not explain the observed enantioselectivities. However, studies of the mixed complexes have been performed exclusively with free amino acids since the coordination mode of the dansyl derivatives has not been studied so far.

Despite this situation, which deserves experimental investigation, we think that our data clearly support the hypothesis that recognition occurs on the column. The best enantioselectivity is displayed by the more lipophilic ligands (Tables I and II), which mainly operate in a pH range where a neutral species, $CuLH_{-2}$, is predominant (Figs. 3 and 5). To get enantioseparation it is necessary to "saturate" the column with the complex (Table IV) by allowing the eluent to flow at an appropriate concentration for several minutes (20–25 min). Enantioselectivity is maintained by eluting without the additive for about 30–45 min. Moreover, RP_{18} TLC plates saturated with the initial complex and eluted with an eluent not containing the complex gave enantioseparation[25].

Although these data are not conclusive, we believe that a sequence of successive adsorption–desorption equilibria of the initial and mixed species occurs along the column, leading to enantiodifferentiation.

As regards the mechanism of chiral recognition, a "classical" ligand exchange with simultaneous dechelation of two binding sites does not seem feasible with these systems. The kinetics of decomplexation of tetradentate ligands is slower than that of the analogous binary complexes and has been reported[31] to occur by reprotonation of a deprotonated amidic nitrogen, and a shift of an amino group from its equatorial position to that set free by the amidic nitrogen, thus leaving a site free for a nucleophilic attack (Fig. 6). Moreover, the elution order with the system AA-NN-*n*/Cu(II), with few exceptions, is the same for all amino acids: the D-enantiomer is eluted before the L-enantiomer (Table II). It appears more reasonable that the complex $CuLH_{-2}$, embedded on the column, interacts with the carboxylic group of the Dns-amino acids at the apical position and with the sulphonamidic nitrogen on a free equatorial position. Granted that the major interaction with the organic phase is provided by the naphthyl group of both dansyl enantiomers, the D-enantiomer (polar or apolar) must give a repulsive interaction with the side-chain of the ligand in order to be less strongly retained (Fig. 7). On the contrary, D-Dns-Leu and D-

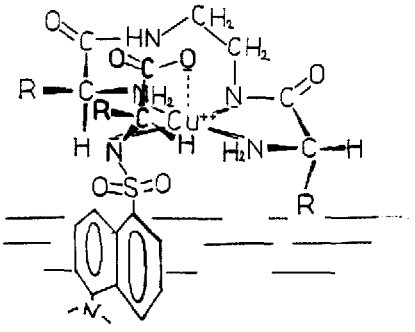

Fig. 7. Stereoselective interactions of the species $CuLH_{-2}$ with a D-Dns-amino acid.

Dns-NVal, which have a lipophilic chain not branched at C_α, can establish lipophilic interactions with the aromatic group of the ligands Phe-NN-2 and Phe-NN-3, giving a reversed elution order.

CONCLUSIONS

We have devised a model system which provides some clues as to the mechanism of the enantiomeric recognition of Dns-amino acids in HPLC (reversed phase).

It has been shown that the square-planar, neutral copper(II) complexes are enantioselective and that the less stable species $CuLH_{-2}$ gives better performance.

The enantioselective complex seems to be initially adsorbed on the stationary phase where it performs chiral recognition. A sequence of adsorption–desorption equilibria of the initial and mixed species occurs along the column leading to enantioseparation.

It is suggested that the enantiomer interacts with the initial complex either in the inner coordination sphere of the metal ion, with partial substitution of the ligand, or by more complex outer-sphere interactions.

If a ligand exchange were to occur, a different elution order would be observed. This assumption has been checked with the analogous bis(L-amino acid amidato) copper(II) complexes[32].

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