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Note

Effect of the mobile phase composition and ligand structure on the separation of D- and L-dansylamino acids, as mixed metal complexes, by reversed-phase high-performance liquid chromatography

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It has been shown that the enantiomers of dansylated amino acids (Dns-Am) can be separated by reversed-phase high-performance liquid chromatography (RP-HPLC) when a chiral metal complex is added to the mobile phase[1–4]. Lam *et al.*[1] and Lam[2] used the L-proline–copper complex as a chiral additive and Karger and co-workers[3,4] applied the zinc–dodecyldiethyleneamine complex. In both instances a ternary metal complex was formed between the added chiral metal complex and the enantiomers of Dns-Am.

The difference in the stabilities of the L- and D-Dns-Am ternary complexes is the basis for their separation. This difference originates from steric effects in the interaction between the α-substituents of the Dns-Am and parts of the molecule of the chiral ligand in the metal complex. The stability of the ternary complex is dependent on the pH, the chiral metal complex and the organic modifier concentration[2]. Less is known about the effect of the nature of the organic modifier and the ring structure of the chiral ligand in the metal complex on the separation of Dns-Am enantiomers by RP-HPLC.

In this paper some results are presented on the effect of the type of modifier on the separation of Dns-Am enantiomers by RP-HPLC, using the L-proline–copper complex as a chiral additive in the mobile phase. Further, the effect of the ring structure of the chiral ligand in the copper complex was investigated using L-proline, with a five-membered ring, and its four- and six-membered ring analogues L-azetidine-2-carboxylic acid and L-pipecolic acid.

EXPERIMENTAL

Apparatus

The chromatograph consisted of a reciprocating pump (Orlita, Giessen, F.R.G.), a Bourdon-type pulse damper, a Model 7010 injection valve (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 20-μl loop, a 150 × 4.6 mm I.D. stainless-steel column and a fluorimeter (Shimadzu, Kyoto, Japan) set at 350 nm excitation and 530 nm emission.

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Materials

All solvents were of analytical-reagent grade (Merck, Darmstadt, F.R.G.). The amino acids were obtained from Nutritional Biochemical (Cleveland, OH, U.S.A.) and were converted into dansylated derivatives by the method described by Lawrence and Frei[5]. The reversed-phase column was slurry packed with Zorbax ODS, 7 μm (DuPont, Wilmington, DE, U.S.A.). L-Azitidine-2-carboxylic acid (L-Azi) and L-pipecolic acid (L-Pip) were obtained from Sigma (St. Louis, MO, U.S.A.). The following abbreviations are used: Dns = dansylated; AB = aminobutyrate; Met = methionine; Leu = leucine; Nleu = norleucine; Val = valine; Nval = norvaline; THF = tetrahydrofuran.

RESULTS AND DISCUSSION

Influence of the type of organic modifier

Table I gives the capacity ratios and selectivity factors of a number of enantiomers of Dns-Am, as measured with four different organic modifiers. The retention behaviour of the test solutes is similar with acetonitrile, methanol and 1,4-dioxane as modifier. The capacity ratios and selectivity factors (α values) decrease with increasing organic modifier content. The orders of elution of the test solutes and of the separate L- and D-isomers are the same with these three modifiers. However, the α values with acetonitrile as a modifier are significantly larger then those obtained with methanol and 1,4-dioxane.

A surprisingly different retention behaviour was observed with THF as the organic modifier. The capacity ratios decrease with increasing THF concentra-

TABLE I

CAPACITY RATIOS (k') AND SELECTIVITY FACTORS (α) OF DANSYLATED AMINO ACID ENANTIOMERS WITH VARIOUS ORGANIC MODIFIERS OBTAINED BY RP-HPLC

Mobile phase composition: water + organic modifier + 0.002 *M* copper sulphate + 0.004 *M* L-proline + 0.005 *M* ammonium acetate (pH 7).

Solute	*Acetonitrile*				*Methanol*				*Dioxane*	
	17%		*20%*		*35%*		*40%*		*20%*	
	k'	α	k'	α	k'	α	k'	α	k'	α
L-Dns-AB	8.18	1.20	3.66	1.14	7.00	1.18	3.58	1.11	5.74	1.11
D-Dns-AB	9.86		4.19		8.27		3.98		6.37	
L-Dns-Met	17.3	1.32	7.84	1.26	13.9	1.30	6.19	1.21	10.9	1.18
D-Dns-Met	22.8		9.86		18.1		7.48		12.9	
L-Dns-Val	12.6	1.30	6.38	1.27	11.9	1.27	6.05	1.22	9.20	1.20
D-Dns-Val	16.4		8.08		15.1		7.38		11.0	
L-Dns-Nval	18.3	1.33	8.03	1.27	16.6	1.23	7.70	1.13	12.1	1.18
D-Dns-Nval	24.3		10.2		20.4		8.71		14.3	
L-Dns-Leu	31.1	1.43	15.4	1.37	32.8	1.22	14.0	1.17	24.1	1.22
D-Dns-Leu	44.7		21.2		40.0		16.4		29.5	
L-Dns-Nleu	42.8	1.41	18.9	1.34	40.8	1.25	17.1	1.16	30.3	1.21
D-Dns-Nleu	60.6		25.3		51.2		19.9		36.7	

tion. However, at low THF concentrations the α values are much smaller than those obtained with the other modifiers, but increase with increasing THF concentration. Moreover, the D-isomers of the Dns-Am elute consistently before the L-isomers. This in contrast to the elution order of the L- and D-isomers found with the other modifiers, where the L-isomers always elute earlier than the D-isomers.

A possible explanation for this deviating behaviour with THF might be sought in the replacement of one of the coordinated water molecules in the metal complex. L-Proline and Dns-Am coordinate with copper in a *trans* conformation with two water molecules in the axial position[2] (see Fig. 1). The ring system of L-proline stands above the coordination plane and interacts with the α-substituent of the L-Dns-Am. This interaction (steric hindrance) results in the L-proline–copper–L-Dns-Am complex being less stable than the L-proline–copper–D-Dns-Am complex, with the result that the L-Dns-Am will elute earlier than the D-Dns-Am. However, when a water molecule can be replaced with a THF molecule, a different situation will occur. The replacement of a water molecule is possible because the oxygen atom in THF bears two lone electron pairs, like the oxygen atom in water, and hence can coordinate with the metal complex at the axial positions of the coordination plane. It is reasonable to assume that the water molecule below the coordination plane is easier to replace with a THF molecule than is the water molecule above the coordination plane, because the ring system of L-proline stands above this plane and hence will sterically hinder the coordination with a THF molecule in this orientation. If the water molecule below the coordination plane is replaced with a larger THF molecule, the stability of the L-proline–copper–D-Dns-Am complex will decrease owing to interaction of the α-substituent of the D-Dns-Am with this THF molecule. The stability

Dioxane		*Solute*	*THF*							
25%			*17.5%*		*15%*		*20%*		*22.5%*	
k'	α		*k'*	α	*k'*	α	*k'*	α	*k'*	α
2.23	1.00	D-Dns-AB	4.33	1.13	2.19	1.17	1.18	1.23	0.76	1.31
2.23		L-Dns-AB	4.89		2.57		1.46		0.92	
4.35	1.11	D-Dns-Met	9.19	1.10	4.77	1.13	2.65	1.19	1.71	1.22
4.83		L-Dns-Met	10.1		5.41		3.15		2.09	
3.53	1.12	D-Dns-Val	7.55	1.00	3.78	1.04	2.21	1.09	1.43	1.12
3.95		L-Dns-Val	7.55		3.93		2.39		1.59	
4.73	1.12	D-Dns-Nval	10.8	1.09	5.23	1.10	3.01	1.12	1.88	1.16
5.28		L-Dns-Nval	11.4		5.72		3.39		2.17	
9.13	1.16	D-Dns-Leu	18.1	1.00	8.81	1.00	5.52	1.04	3.26	1.06
10.3		L-Dns-Leu	18.1		8.81		5.75		3.49	
11.4	1.14	D-Dns-Nleu	26.0	1.00	12.3	1.06	7.03	1.11	4.40	1.12
13.0		L-Dns-Nleu	26.0		13.1		7.76		4.93	

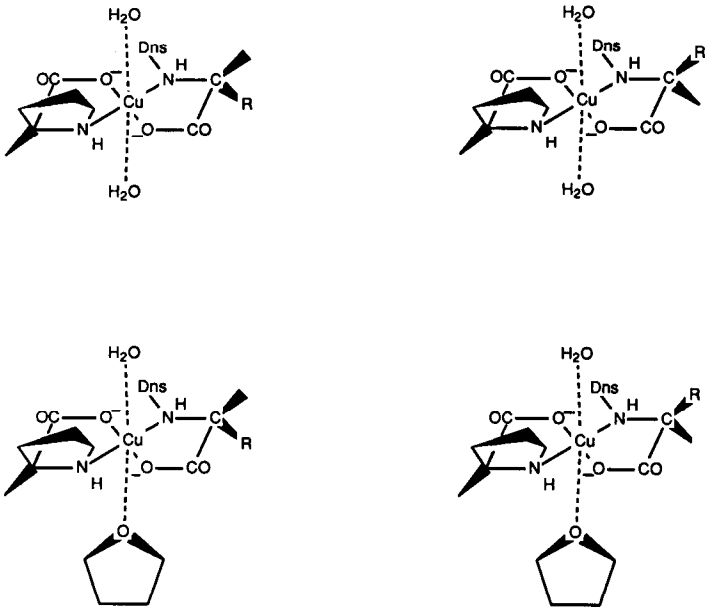

Fig. 1. Proposed structures of the ternary complexes of L-proline and D,L-isomers of dansylated amino acids with copper(II). (a) Coordination with water; (b) coordination with water and THF.

of the L-proline–copper–L-Dns-Am complex is hardly influenced by the presence of a THF molecule below the coordination plane. This means that when a water molecule is replaced with a THF molecule, the difference in stability between the L-proline–copper–L-Dns-Am and the L-proline–copper–D-Dns-Am complexes decreases and can even cause a reversal in the stability order of the two enantiomers, resulting in the D-isomer eluting before the L-isomer. The more water molecules are replaced with THF molecules (at higher THF concentrations in the mobile phase), the larger becomes the difference in stability between the two isomers and hence the α values will increase with increasing THF content in the mobile phase. However, there is a limit to the maximum THF content in the mobile phase, because the retention of the ternary metal complexes decreases with increasing THF concentration.

The reason why 1,4-dioxane does not show the same behaviour as THF must be attributed to the lack of a dipole moment in 1,4-dioxane and the fact that its oxygen atom is significantly less electronegative than that in THF. For these reasons 1,4-dioxane is not able to replace water in the metal complexes.

The results obtained with THF indicate that it is worth investigating further whether the replacement of coordinated water molecules in chiral metal complexes by organic molecules is a good means of "tuning" the chiral selectivity for Dns-Am.

Influence of the ring structure of the chiral ligand

In the ternary L-proline–copper–Dns-Am complex, the proline ring is situated above the coordination plane. This ring interacts with the α-substituent of the L-Dns-Am, which decreases the stability of this complex compared with that of the L-proline–copper–D-Dns-Am complex. The extent of interaction (and thus the sta-

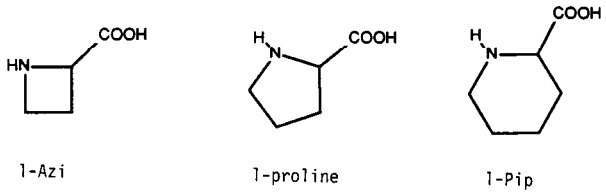

Fig. 2. Structures of chiral ligands for the separation of the D,L-isomers of dansylated amino acids with copper(II).

bility of the L-Dns-Am metal complex) depends on the size of the ring of the chiral ligand and the α-substituent in the Dns-Am.

In order to investigate the effect of the size of the ring in the chiral ligand on the selectivity, three chiral ligands with different ring sizes were tested (see Fig. 2): L-azitidine-2-carboxylic acid (L-Azi), L-proline and L-pipecolinic acid (L-Pip). The capacity ratios and selectivity factors, as measured with the chiral ligands, are given in Table II. All three ligands show selectivity towards the Dns-Am enantiomers. The elution order of the Dns-Am and of the L- and D-isomers is the same, but the capacity ratios increase in the order L-Azi < L-proline < L-Pip, which corresponds to the order of hydrophobicities of the rings and hence of the overall metal complex. As expected, the α values obtained with L-Azi are smaller then those obtained with L-proline, because the extent of interaction of the α-substituent of the Dns-Am is smaller with a four- than with a five-membered ring. However, the α values obtained with the six-membered ring (L-Pip) were smaller then those obtained with L-proline, but larger than those found with L-Azi. This does not fit well with the proposed interaction model, where one would expect the largest α values with L-Pip. The found

TABLE II

CAPACITY RATIOS (k') AND SELECTIVITY FACTORS (α) OF ENANTIOMERIC DANSYLATED AMINO ACIDS OBTAINED BY RP-HPLC USING CHIRAL LIGANDS WITH DIFFERENT RING SIZES

Mobile phase composition: water–acetonitrile (4:1, v/v) + 0.002 *M* copper sulphate + 0.004 *M* chiral ligand + 0.005 *M* ammonium acetate (pH 7).

Chiral ligand	L-*Azi*		L-*Proline*		L-*Pip*	
	k'	α	k'	α	k'	α
L-Dns-AB	2.83	1.07	3.66	1.14	5.29	1.14
D-Dns-AB	3.03		4.19		6.04	
L-Dns-Met	5.37	1.09	7.84	1.26	11.3	1.14
D-Dns-Met	5.84		9.86		12.8	
L-Dns-Val	4.77	1.12	6.38	1.27	8.91	1.21
D-Dns-Val	5.33		8.08		10.8	
L-Dns-Nval	5.76	1.13	8.03	1.27	12.8	1.16
D-Dns-Nval	6.52		10.2		14.9	
L-Dns-Leu	11.2	1.17	15.4	1.37	24.3	1.20
D-Dns-Leu	13.1		21.2		29.2	
L-Dns-Nleu	13.9	1.17	19.0	1.34	30.1	1.19
D-Dns-Nleu	16.2		25.3		35.9	

selectivity order L-Azi < L-Pip < L-proline is different to that found by Lefebre *et al.*[6] and Gübitz *et al.*[7], who bound the same chiral ligands to a solid support for the separation of underivatized amino acids. They found the order L-Azi < L-proline < L-Pip, in accordance with the proposed interaction model. An explanation for the unexpected behaviour of L-Pip has not yet been found. It might be that the pH has a significant effect, because L-Pip has a different protolysis constant to L-proline. This aspect is now under investigation.

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