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Enantiomeric resolution of amino acid derivatives by high-performance liquid chromatography on chiral stationary phases derived from L-proline

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ABSTRACT

The enantiomeric separation of a series of N-3,5-dinitrobenzoyl amino acid esters was investigated by normal-phase high-performance liquid chromatography on four chiral stationary phases (CSPs) derived from L-proline as chiral selector. The chiral selector was covalently bonded to silica gel by the use of either a spacer with a three (CSP1 and 2) or eleven (CSP3 and 4) carbon chain length. The protective groups were benzyloxycarbonyl or *tert.*-butoxycarbonyl. The natures of the protective group and the spacer were important for chiral recognition. A spacer with eleven carbons allows a better resolution of a large number of N-3,5-dinitrobenzoyl amino acid esters. The mobile phase can induce major changes in the behaviour of solutes, thus leading to better enantioselectivity.

INTRODUCTION

The preparation of chiral stationary phases (CSPs) for high-performance liquid chromatography (HPLC) with a large field of application is of great current interest. Wainer [1] classified CSPs according to the type of interaction, and CSPs derived from amino acids are among the most important, especially the Pirkle-type phases.

The aim of this work was the preparation of new CSPs in which the chiral selector is an amino acid. The structures of these CSPs are shown in Fig. 1. The original feature of these four CSPs is the use of the L-proline, which is generally used in ligand-exchange chromatography (LEC) [2–6]. In general, enantioselective adsorption can be described by the model introduced by Dalgliesh [7], which reduces chiral recognition to the reversible formation of diastereoisomeric complexes between the chiral group of the CSP and the adsorbed derivative. To be resolved, three types of interaction must occur, of which at least one must be stereochemically dependent. Two types of interactions exist (hydrogen bonding and lipophilic interaction), and the third (π–π interaction occurs owing to the protective group, *i.e.*, benzyloxycarbonyl; the *tert.*-butoxycarbonyl group involves steric hindrance. The protected amino acid is

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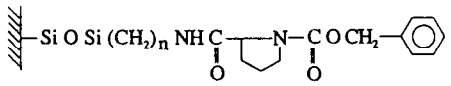
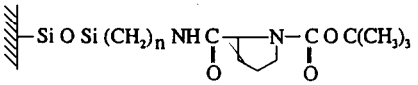


n=3	CBZ L PRO C_3	CSP_1
n=11	CBZ L PRO C_{11}	CSP_3



n=3	BOC L PRO C_3	CSP_2
n=11	BOC L PRO C_{11}	CSP_4

Fig. 1. Structures of chiral stationary phases CSP1, 2, 3 and 4.

bonded to silica gel by a linear alkyl chain three or eleven carbons long. The latter, not so widely used, should reduce the 'matrix–chiral selector' interactions. The solutes studied were N-3,5-dinitrobenzoyl amino acid esters, which have been used by several workers [8–13].

In this paper, the preparation of these CSPs and solutes is described, then the influence of the amino acid, the spacer and the protective group on the retention and the selectivity is discussed. The study of different mobile phases is also described.

EXPERIMENTAL

Preparation of chiral stationary phases

Before grafting, the silica gel is activated by the method proposed by Engelhardt *et al.* [14]. A 3-g portion of silica gel is hydrated with 1 *M* hydrochloric acid at room temperature for 1.5 h, then dried at 120°C for 12 h.

N-Benzyloxycarbonyl (CBZ) and N-*tert.*-butoxycarbonyl (BOC) amino acids were obtained by the method of Bodansky and Bodansky [15] and Wunsch *et al.* [16]. The activation of the N-benzyloxycarbonyl amino acids was carried out by the method of Lloyd [17].

Benzyloxycarbonyl-L-proline N-hydroxysuccinimide ester (1): yield 100%; m.p. 90°C; ^{1}H NMR (C^2HCl_3), d: 2.0 (m, 4H), 2.7 (s, 4H), 3.5 (t, 2H), 4.5 (t, 1H), 5.2 (s, 2H), 7.5 (s, 5H).

tert.-Butyloxycarbonyl-L-proline N-hydroxysuccinimide ester (2): yield 91%; m.p. 136°C; ^{1}H NMR (C^2HCl_3), d: 1.4 (s, 9H), 2.0 (m, 4H), 3.5 (t, 2H), 4.5 (t, 1H).

Preparation of CSP1 and CSP3

The preparation of the silane was effected by the method of Engelhardt *et al.* [14].

(*S*)-2-({[3-(Trimethoxysilyl)propyl]amino}carbonyl)-1-pyrrolidinecarboxylic

acid benzyl ester (3): yield 40%; ^{1}H NMR (C^2HCl), d: 1.2–1.5 (m, 8H), 3.05–3.45 (m, 4H), 3.52 (m, 9H), 4.2–4.4 (broad, 1H), 5.15 (s, 2H), 6.0–6.7 (m, 1H), 7.3 (s, 5H).

(*S*)-2-({[3-(trimethoxysilyl)propyl]amino}carbonyl)-1-pyrrolidinecarboxylic acid 1,1-dimethylethyl ester (4): yield 87%; ^{1}H NMR(C^2HCl_3), d: 0.5–1.0 (m, 4H), 1.5 (s, 9H), 2.0 (m, 6H), 3.0–3.5 (m, 2H), 3.6 (s, 9H), 4.2 (t, 1H), 7.0 (broad, 1H).

The reaction with the silane and silica gel was performed by suspending the silane in 50 ml of dry toluene, adding the silica (2 g) and refluxing for 16 h with stirring. The stationary phase was subsequently extensively washed with methanol and water.

Hydrolysis of methoxy groups and end-capping were performed by the method proposed by Engelhardt *et al.* [14].

CBZ-L-Pro-C_3 (CSP1): C10.50, H1.50, N1.50%, 0.53 mmol of chiral selector per gram of silica (based on N).

BOC-L-Pro-C_3 (CSP2): N1.65; 0.59 mmol of chiral selector per gram of silica (based on N).

Preparation of CSP2 and CSP4

10-Undecenamide and the 1-amino-10-undecene were obtained by the method of Dobashi and Hara [18].

To prepare the silane, a mixture of 19.5 mmol of 1 or 2 and 200 ml of dry acetonitrile was cooled to 0°C, followed by the addition of a mixture of 1-amino-10-undecene (21.4 mmol), and triethylamine (21.4 mmol) in 50 ml of acetonitrile. The mixture was stirred for 1.5 h at 0°C, then 500 ml of ethyl acetate and 300 ml of water were added. The layers were separated and the organic layer was washed with water, 1.5 *M* HCl and saturated $NaHCO_3$ and dried over Na_2SO_4. The solvent was evaporated and the residue was purified by flash chromatography on silica gel with hexane–acetone (75:25) as eluent.

(*S*)-2-{[(10-undecenyl)amino]carbonyl}-1-pyrrolidinecarboxylic acid benzyl ester (5): yield 99%; m.p. 64°C; ^{1}H NMR(C^2HCl_3), d: 1.35 (s, 14H), 1.8–2.4 (m, 6H), 3.03–3.3 (q, 2H), 3.3–3.6 (t, 2H), 4.2 (s, 2H), 5.5–6.1 (m, 1H), 6.1–6.9 (m, 1H, exchangeable with ^2H_2O), 7.3 (s, 5H).

(*S*)-2-{[(10-undecenyl)amino]carbonyl}-1-pyrrolidinecarboxylic acid 1,1-dimethylethyl ester (6): yield 58%; ^{1}H NMR (C^2HCl_3), d: 1.25 (s, 14H), 1.45 (s, 9H), 2.0 (m, 6H), 3.0–3.55 (m, 4H), 4.0–4.3 (m, 1H), 4.7–7.15 (m, 2H), 5.4–6.1 (m, 1H), 6.2–6.8 (m, 1H, exchangeable).

The chlorosilanes (7 and 8) were obtained by the method of Dobashi [18] and used directly, without purification, for the reaction with silica and end-capping.

CBZ-L-Pro-C_{11} (CSP3): C8.54, H1.45, N0.70%; 0.25 mmol of chiral selector per gram of silica (based on N).

BOC-L-Pro-C_{11} (CSP 4): C8.67, H1.60, N0.90%; 0.32 mmol of chiral selector per gram of silica (based on N).

Preparation of solutes

A mixture of 10 mmol of 3,5-dinitrobenzoyl chloride, 8 mmol of amino acid and 20 ml of 1 *M* NaOH was stirred for 1 h at room temperature, then acidified to pH 2 with 1 *M* HCl. The product was recrystallized from ethanol–water (1:1). Esterification was performed by the method of Boissonas [19]. All compounds showed the expected analytical and spectroscopic data (m.p., ^{1}H NMR).

Chromatographic conditions

Chromatography was carried out with a Jasco Trirotar VI equipped with a Jasco DG 3510 degasser (Prolabo, Paris, France). A Jasco Uvidec 100-VI variable wavelength UV detector (Prolabo) was used. The CSPs were packed into 150 × 4 mm I.D. stainless-steel columns by the classical slurry technique at 450 bar using acetone as the pumping solvent [20].

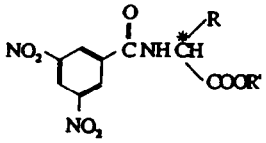
Solutes were injected at a concentration of 10^{-3} mol/l using a Rheodyne loop injector (20 μl) (Prolabo), at a flow-rate of 0.8 ml/min at room temperature.

The solvents 2,2,4-trimethylpentane (2,2,4-TMP, Rathburn, Walkerburn, U.K.), isopropanol and methylene chloride (Prolabo) were of an analytical-reagent grade.

The elution order of enantiomers was determined by the injection of the racemic mixture with an excess of L-enantiomer.

TABLE I

STRUCTURES OF THE N-3,5-DINITROBENZOYL AMINO ACID DERIVATIVES



No.	Abbreviation[a]	R	R′
1a	3,5-DNB-Phe-Ala-OMe	$CH_2C_6C_5$	CH_3
1b	3,5-DNB-Phe-Ala-OEt		C_2H_5
1c	3,5-DNB-Phe-Ale-OiPr		$CH(CH_3)_2$
2a	3,5-DNB-Phe-Gly-OMe	C_6H_5	CH_3
2b	3,5-DNB-Phe-Gly-OEt		C_2H_5
2c	3,5-DNB-Phe-Gly-OiPr		$CH(CH_3)_2$
3a	3,5-DNB-Val-OMe	$CH(CH_3)_2$	CH_3
3b	3,5-DNB-Val-OEt		C_2H_5
3c	3,5-DNB-Val-OiPr		$CH(CH_3)_2$
4a	3,5-DNB-Nor-Val-OMe	$(CH_2)_2CH_3$	CH_3
4b	3,5-DNB-Nor-Val-OEt		C_2H_5
4c	3,5-DNB-Nor-Val-OiPr		$CH(CH_3)_2$
5a	3,5-DNB-Leu-OMe	$CH_2CH(CH_3)_2$	CH_3
5b	3,5-DNB-Leu-OEt		C_2H_5
5c	3,5-DNB-Leu-OiPr		$CH(CH_3)_2$
6a	3,5-DNB-Iso-Leu-OMe	$CH(C_2H_5)CH_3$	CH_3
6b	3,5-DNB-Iso-Leu-OEt		C_2H_5
6c	3,5-DNB-Iso-Leu-OiPr		$CH(CH_3)_2$
7a	3,5-DNB-Nor-Leu-OMe	$(CH_2)_3CH_3$	CH_3
7b	3,5-DNB-Nor-Leu-OEt		C_2H_5
7c	3,5-DNB-Nor-Leu-OiPr		$CH(CH_3)_2$
8a	3,5-DNB-Pro-OMe	$(CH_2)_3$	CH_3
8b	3,5-DNB-Pro-OEt		C_2H_5
8c	3,5-DNB-Pro-OiPr		$CH(CH_3)_2$
9a	3,5-DNB-Ala-OMe	CH_3	CH_3
9b	3,5-DNB-Ala-OEt		C_2H_5
9c	3,5-DNB-Ala-OiPr		$CH(CH_3)_2$

[a] Me = methyl; Et = ethyl; iPr = isopropyl.

RESULTS AND DISCUSSION

The structures of the N-3,5-DNB racemates are shown in Table I. All these CSPs permit the separation of a large number of solutes. A comparison of the retentions and selectivities for the solutes obtained on the different CPSs with different mobile phases was made, and the influence of the mobile phase and of the polar modifier was studied.

First, some general statement on the retention and selectivity for all of the solutes can be made regardless of the CSPs and the mobile phase used, namely, an increase in the size of the ester group (methyl, ethyl) or in the branching (isopropyl) leads to a decrease in the capacity factor, k' (Fig. 2). Also, an increase in the linear chain length (Ala, NVal, NLeu) of the amino acid leads to a decrease in k' (Figs. 3 and 4), as expected in normal-phase HPLC. Further, solutes which have the same configuration as the CSPs are more retained.

The 'three-point' attractive interactions model [7] has been widely used as the basis of a chiral recognition model. Several workers have shown that the interactions between the solute and the CSP do not have to be all attractive. A model based on two attractive interactions [21,22] or even one [23] could be sufficient to achieve resolution.

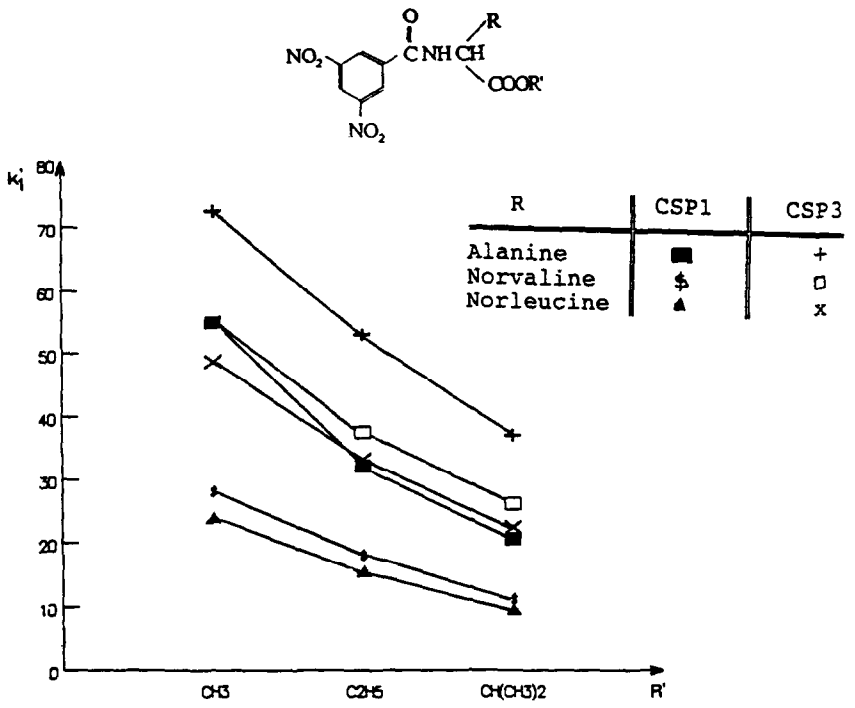


Fig. 2. Influence of the nature of the R' substituent group of N-3,5-DNB-α-amino acid esters on the capacity factor, k'_1, of the first enantiomer eluted, on CSP1 and CSP3. Mobile phase, isooctane–methylene chloride (90:10); flow-rate 0.8 ml/min; UV detection at 254 nm.

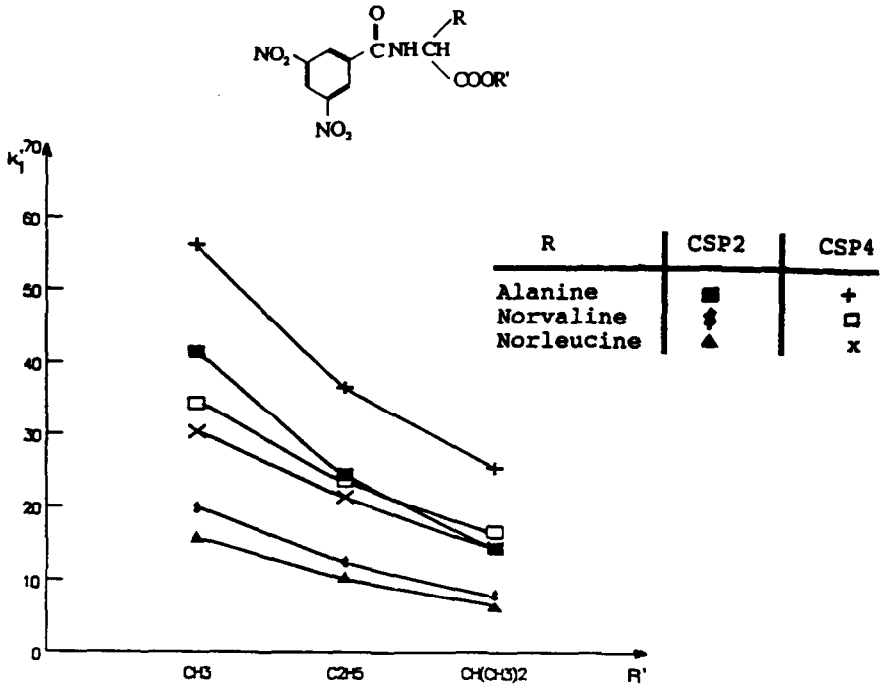


Fig. 3. Influence of the nature of the R′ substituent group of N-3,5-DNB-α-amino acid esters on the capacity factor, k'_1, of the first enantiomer eluted, on CSP2 and CSP4. Conditions as in Fig. 1.

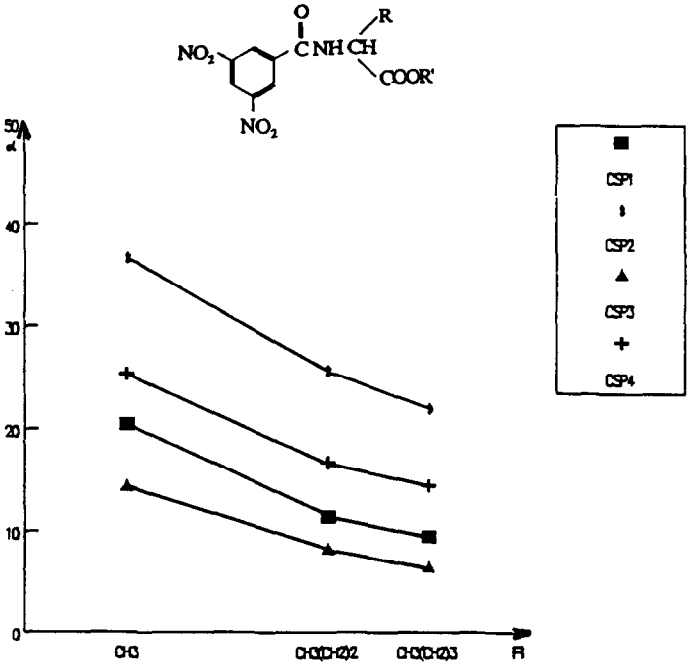


Fig. 4. Influence of the nature of the R substituent group of N-3,5-DNB-α-amino acid esters on the capacity factor, k'_1, of the first enantiomer eluted on the CSPs 1–4. Conditions as in Fig. 1.

TABLE II

ENANTIOMERIC RESOLUTION OF N-3,5-DINITROBENZOYL PHENYLALANINE ESTERS ON CSP3 AND CSP4 USING METHYLENE CHLORIDE AS POLAR MODIFIER

Mobile phase, isooctane–methylene chloride (90:10); flow-rate, 0.8 ml/min; room temperature; UV detection at 254 nm. k'_2 is the capacity factor of the second-eluted enantiomer, $k'_2 = (t_{r_2} - t_0/t_0)$, where t_{r_2} is the retention of the last-eluted enantiomer and t_0 the retention of a non-retained solute. The selectivity, α, between two enantiomers is the ratio of their respective capacity factors (k'_1/k'_2).

Solute	CSP3			CSP4		
	k'_1	k'_2	α	k'_1	k'_2	α
Phe-Ala:						
1a	69.00	77.17	1.12	38.72	40.80	1.05
1b	48.14	54.43	1.13	28.03	29.55	1.05
1c	33.79	38.42	1.14	20.20	21.32	1.05

The results indicate that the chiral recognition mechanism is based on both attractive and steric interactions. The elution order of the enantiomers is not affected by changes in the structure of the CSP or the solute or in the composition of the mobile phase. These results indicate that the chiral recognition mechanism is similar for all four CSPs.

It is not necessary to have interactions to obtain resolution of the racemates but, in general, their presence increases the enantioselectivity by stabilizing the solute–CSP complex (Table II), as stressed by Wainer and Alembik [24]. In the present instance, the blocking agent of the CSP plays only a secondary role in obtaining resolution. Fig. 5 shows the hydrogen bonding, dipole–dipole and steric interactions involved in the chiral recognition mechanism.

Tables IV–IX show that the ester moiety does not play a significant role in the chiral recognition mechanism. The predominant steric interactions seem to occur at the chiral centre of the CSP.

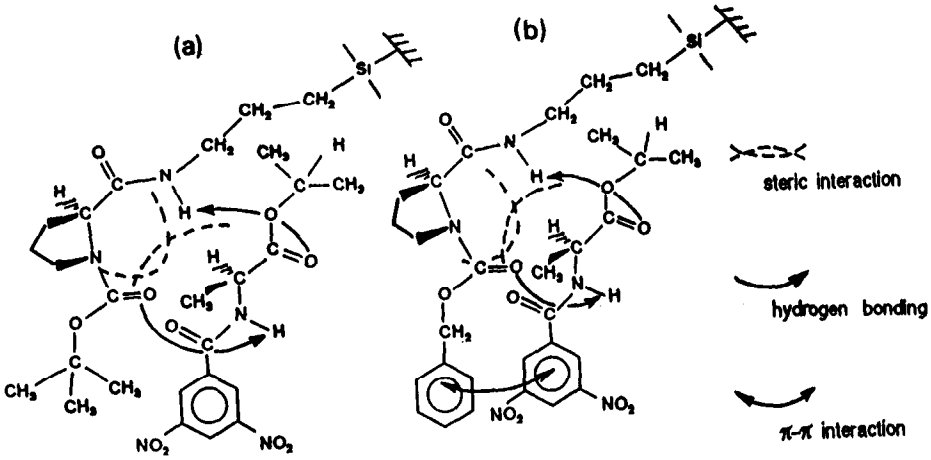


Fig. 5. Proposal chiral recognition model for the isopropyl ester of N-3,5-dinitrobenzoylalanine (**9c**) on (a) CSP2 and (b) CSP1. The most strongly retained enantiomer is represented.

Influence of the mobile phase

The use of methylene chloride or isopropanol, which are defined as a strong dipole and proton acceptor, respectively, (Table III) by Snyder [25] according to the Rohrschneider solubility data (26), leads to different results: the capacity factors are greater when methylene chloride is used although it has a larger polarity ($P'_{2,2,4\text{-TMP/2-PrOH}} = 17.6$, $P'_{2,2,4\text{-TMP/CH}_2\text{Cl}_2} = 40$). This increase in retention generally leads to an increase in the separation factor.

TABLE III

SELECTIVITY PARAMETERS AS DEFINED AND CALCULATED BY SNYDER [25] ACCORDING TO ROHRSCHNEIDER'S DATA [26]

The values in italics indicate the dominant character of the polar modifier; X_e (proton acceptor), X_d (proton donor), X_n (strong dipole), P' (polarity).

Polar modifier	X_e	X_d	X_n	P'
Isopropanol	*0.55*	0.19	0.27	3.90
Methylene chloride	0.29	0.18	*0.53*	3.10

It seems possible to relate the dominant character of each modifier with its ability to favour hydrogen bonding or dipole–dipole interactions. However, solvation or the conformation of both the solute and the CSP are affected by a change in the polar modifier.

The chiral recognition mechanism involves the establishment of dipole–dipole interactions and hydrogen bonding. These interactions do not seem to play an equivalent role in the chiral recognition mechanism.

The use of methylene chloride instead of isopropanol leads to an increase in the separation factor. Isopropanol, because of its proton acceptor character, could interact preferentially with the NH moieties of the CSP and the solute. Hence the solute–CSP interactions are less important, which leads to a decrease in the separation factor.

On the other hand, methylene chloride tends to reduce dipole–dipole interactions between the solute and the CSP but as already mentioned, an increase in the separation factor occurred. This leads to the conclusion that the predominant interaction is hydrogen bonding in the chiral recognition mechanism. Nevertheless, dipole–dipole interactions are fairly important.

It has been demonstrated that the use of 1,2-ethylene chloride [which is a weaker dipole ($X_n = 0.49$) than methylene chloride] instead of methylene chloride leads to an increase in the separation factor [27]. Similar results have been observed by other workers with different CSPs and solutes and an inversion of the elution order of enantiomers has been reported [28].

Influence of the structure of the solutes

The ester group and the alkyl chain of the amino acid are the two main parameters of the structure of the solutes. It is difficult to interpret the variations in the separation factor, α, in terms of the size of the ester group. In general, α is constant or increases slightly when a methyl group is replaced with an ethyl or an isopropyl

TABLE IV

ENANTIOMERIC RESOLUTION OF N-3,5-DINITROBENZOYL AMINO ACID ESTERS ON DIFFERENT CHIRAL STATIONARY PHASES USING ISOPROPANOL AS POLAR MODIFIER

Mobile phase: isooctane–isopropanol (98:2); other conditions as in Table II.

Solute	CSP1			CSP3			CSP2			CSP4		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
Ala:												
9a	36.96	42.49	1.15	27.63	29.95	1.08	14.20	15.54	1.09	21.33	22.17	1.04
9b	20.53	23.25	1.13	20.43	22.40	1.09	8.85	9.55	1.08	14.75	15.25	1.03
9c	16.24	18.28	1.13	17.71	19.57	1.10	6.14	6.50	1.06	11.33	11.83	1.04
Nor-Val:												
4a	20.01	20.68	1.03	19.45	20.84	1.07	7.66	8.15	1.06	16.16	–	1.00
4b	12.33	12.89	1.04	14.33	15.39	1.07	5.17	5.43	1.06	10.37	–	1.00
4c	9.25	9.69	1.05	11.81	12.81	1.08	3.81	–	1.00	7.73	–	1.00
Nor-Leu:												
7a	17.15	–	1.00	19.74	20.90	1.06	6.55	6.99	1.07	14.77	–	1.00
7b	11.62	–	1.00	13.77	14.71	1.07	4.43	4.66	1.05	10.95	–	1.00
7c	7.88	–	1.00	10.47	11.24	1.07	3.33	–	1.00	6.95	–	1.00

group. Isopropyl is a bulkier group than ethyl and is significantly larger than methyl, and exerts a greater degree of conformational control. An exception can be noted on CSP2 (Tables IV and V). The diastereoisomeric complexes are less stable; this difference in stability could be due to the steric hindrance of the protective group.

The esters of N-3,5-DNB-leucine have higher k' values than the esters of N-3,5-DNB-valine, but the valine derivatives are better separated. The k' value measures the overall affinity of a compound for the stationary phase. If the compound has a

TABLE V

ENANTIOMERIC RESOLUTION OF N-3,5-DINITROBENZOYL AMINO ACID ESTERS ON DIFFERENT CHIRAL STATIONARY PHASES USING METHYLENE CHLORIDE AS POLAR MODIFIER

Mobile phase: isooctane–methylene chloride (90:10); other conditions as in Table II.

Solute	CSP1			CSP3			CSP2			CSP4		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
Ala:												
9a	54.94	63.95	1.16	72.54	81.11	1.12	41.25	45.83	1.11	56.03	59.26	1.06
9b	31.96	37.17	1.16	52.89	59.65	1.13	24.43	26.95	1.10	36.50	39.14	1.07
9c	20.43	23.62	1.16	36.91	41.95	1.13	14.42	15.71	1.09	25.39	27.33	1.08
Nor-Val:												
4a	28.48	31.72	1.08	54.90	61.06	1.11	20.15	21.98	1.09	34.19	35.97	1.05
4b	18.33	19.93	1.09	37.30	41.91	1.12	12.79	13.93	1.09	23.68	25.13	1.06
4c	11.45	12.43	1.08	25.93	29.04	1.12	8.29	8.85	1.07	16.72	17.90	1.07
Nor-Leu:												
7a	23.88	24.94	1.04	48.50	53.72	1.11	15.68	17.18	1.09	30.28	31.77	1.05
7b	15.33	16.18	1.05	33.04	36.82	1.11	10.41	11.34	1.09	21.41	22.70	1.06
7c	9.49	10.09	1.06	22.26	24.99	1.12	6.51	6.99	1.07	14.54	15.57	1.07

TABLE VI

ENANTIOMERIC RESOLUTION OF N-3,5-DINITROBENZOYL AMINO ACID ESTERS ON DIFFERENT CHIRAL STATIONARY PHASES USING ISOPROPANOL AS POLAR MODIFIER

Mobile phase: isooctane–isopropanol (98:2); other conditions as in Table II.

Solute	CSP1			CSP3			CSP2			CSP4		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
Val:												
3a	15.37	16.96	1.10	14.59	16.40	1.12	6.50	7.16	1.09	10.78	11.41	1.06
3b	10.32	11.37	1.10	10.54	11.86	1.12	4.29	4.60	1.07	7.55	8.00	1.06
3c	7.55	8.29	1.10	7.86	8.86	1.13	3.19	–	1.00	5.67	6.00	1.06
Leu:												
5a	16.48	–	1.00	18.10	18.98	1.05	6.86	7.08	1.03	14.40	–	1.00
5b	11.25	–	1.00	13.37	14.09	1.05	4.88	–	1.00	10.49	–	1.00
5c	8.47	–	1.00	10.14	10.77	1.06	3.59	–	1.00	7.28	–	1.00
Pro:												
8a	11.62	–	1.00	11.39	–	1.00	5.12	–	1.00	10.32	–	1.00
8b	8.41	–	1.00	8.34	–	1.00	3.23	–	1.00	6.78	–	1.00
8c	6.10	–	1.00	6.21	–	1.00	2.24	–	1.00	4.87	–	1.00

high affinity for the stationary phase, its enantiomers are more likely to be separated [29,30]; an example is given in Tables IV and V with the retention and resolution of linear alkyl chain derivatives. However, this is not always true, as can be seen with the compounds valine and leucine, mentioned at the beginning of this paragraph (Tables VI and VII). A longer retention leads to a better enantiomeric resolution if, and only if, the enantiomeric retention mechanisms are dominant. If, on the other hand, the dominant retention mechanisms are not enantioselective, then an increase in the

TABLE VII

ENANTIOMERIC RESOLUTION OF N-3,5-DINITROBENZOYL AMINO ACID ESTERS ON DIFFERENT CHIRAL STATIONARY PHASES USING ISOPROPANOL AS POLAR MODIFIER

Mobile phase: isooctane–methylene chloride (90:10); other conditions as in Table II.

Solute	CSP1			CSP3			CSP2			CSP4		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
Val:												
3a	16.13	18.37	1.14	29.63	34.57	1.17	10.87	12.23	1.12	18.74	20.30	1.08
3b	10.33	11.79	1.14	18.81	23.20	1.17	6.52	7.22	1.11	12.73	13.86	1.09
3c	6.70	7.60	1.13	14.26	16.65	1.17	4.44	4.81	1.08	9.55	10.66	1.12
Leu:												
5a	30.79	–	1.00	68.14	75.46	1.11	23.35	24.79	1.06	–	–	–
5b	20.71	21.48	1.04	44.92	49.81	1.11	14.65	15.46	1.05	27.89	29.55	1.06
5c	13.36	13.97	1.05	32.27	36.16	1.12	9.92	10.33	1.04	20.33	21.40	1.05
Pro:												
8a	4.90	–	1.00	7.62	–	1.00	4.38	–	1.00	4.82	–	1.00
8b	3.51	–	1.00	5.71	–	1.00	2.58	–	1.00	3.11	–	1.00
8c	2.56	–	1.00	4.05	–	1.00	1.76	–	1.00	2.34	–	1.00

retention will not affect the enantiomeric resolution [31]. In this event, the valine derivative is better resolved.

As has been mentioned previously, the steric hindrance at the chiral centre seems to play an important role in the chiral recognition mechanism. The relative steric hindrance for the α-position of the chiral centre is more pronounced for the valine than for the leucine derivatives, and this could explain the increase in the separation factor. An example of a separation is given in Fig. 6.

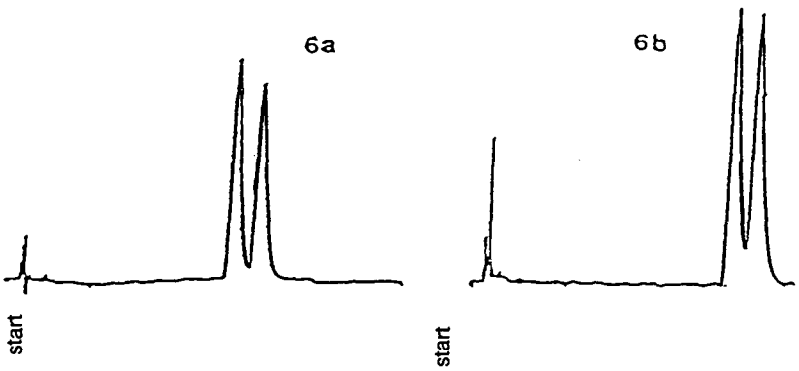


Fig. 6. Examples of separations of (a) the methyl ester of N-3,5-dinitrobenzoylvaline and (b) the isopropyl ester of N-3,5-dinitrobenzoylalanine.

The phenylalanine derivatives are well resolved with either CSP1, 3 or 4 when methylene chloride is used, whereas the phenylglycine derivatives are never resolved (Tables VI and VII). These results are in a good agreement with the literature [9] for the separation of these derivatives on different types of phases. If this difference in the separation factor for phenylalanine and phenylglycine derivatives is due to steric hindrance at the chiral centre, this would indicate that the relative steric bulk at the chiral centre is more important with a benzyl ring than a phenyl ring.

The behaviour of the derivatives of proline is unusual (Tables VIII and IX). It is

TABLE VIII

ENANTIOMERIC RESOLUTION OF N-3,5-DINITROBENZOYL AMINO ACID ESTERS ON DIFFERENT CHIRAL STATIONARY PHASES USING ISOPROPANOL AS POLAR MODIFIER

Mobile phase: isooctane–isopropanol (98:2); other conditions as in Table II.

Solute	CSP1			CSP3			CSP2			CSP4		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
Phe-Ala:												
1a	42.95	43.92	1.01	39.34	42.77	1.09	11.60	–	1.00	24.74	–	1.00
1b	27.26	28.51	1.05	27.41	29.96	1.09	7.43	–	1.00	16.37	–	1.00
1c	18.92	19.92	1.05	19.29	21.21	1.10	5.05	–	1.00	11.68	–	1.00
Phe-Gly:												
2a	34.84	–	1.00	27.72	–	1.00	10.88	–	1.00	20.68	–	1.00
2b	24.32	–	1.00	20.19	–	1.00	7.21	–	1.00	18.00	–	1.00
2c	15.24	–	1.00	17.39	–	1.00	5.05	–	1.00	11.53	–	1.00

TABLE IX

ENANTIOMERIC RESOLUTION OF N-3,5-DINITROBENZOYL AMINO ACID ESTERS ON DIFFERENT CHIRAL STATIONARY PHASES USING METHYLENE CHLORIDE AS POLAR MODIFIER

Mobile phase: isooctane–methylene chloride (90:10); other conditions as in Table II.

Solute	CSP1			CSP3			CSP2			CSP4		
	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α	k'_1	k'_2	α
Phe-Ala:												
1a	39.46	42.21	1.07	69.00	77.17	1.12	19.83	21.08	1.06	38.72	40.81	1.05
1b	24.25	26.22	1.08	48.14	54.43	1.13	12.95	13.65	1.05	28.03	29.55	1.05
1c	17.18	18.61	1.08	33.79	38.72	1.14	9.29	–	1.00	20.20	21.32	1.05
Phe-Gly:												
2a	36.57	–	1.00	60.14	–	1.00	18.42	–	1.00	33.17	–	1.00
2b	23.21	–	1.00	44.71	–	1.00	12.96	–	1.00	23.17	–	1.00
2c	15.41	–	1.00	29.57	–	1.00	8.35	–	1.00	16.84	–	1.00

the only case where the capacity factor decreases when methylene chloride is used instead of isopropanol. The non-resolution of these derivatives (**8a, 8b** and **8c**) could be due to the absence of a hydrogen atom on the nitrogen. Several workers have given the same explanation. Thus, Lloyd [17], who studied a phase derived from (*R*)-phenylglycyl-(*S*)-α-naphthylethylamide, reported the lack of resolution for the methyl ester of N-3,5-dinitrobenzoylproline whatever the mobile phase used [hexane–isopropanol (90:10), hexane–methylene chloride (80:20 or 60:40)].

Hara and Dobashi [32] observed the same phenomena by testing the isopropyl ester of N-4-nitrobenzoylproline in normal-phase HPLC on silica gel with a chiral selector (N-acetyl-L-valine-*tert.*-butylamide) in the mobile phase.

Dobashi *et al.* [33] also reported the lack of resolution of the isopropyl ester of N-4-nitrobenzoylproline on a diamide phase. Berndt and Krüger [34] observed the same results for the isopropyl ester of N-3,5-dinitrobenzoylproline on CBZ-D-phenylglycine and BOC-D-phenylglycine. More recently, Kuropka *et al.* [35] reported the lack of resolution of these derivatives on a phase grafted with acryloyl-D-phenylglycylpropylamide.

The results in Tables II–IX demonstrate that the performance of a given CSP is determined not only by the structure of the resolving agent or the blocking agent, but also by the connecting arm. An example is given by the resolution of the derivatives of isoleucine (**6a, 6b** and **6c**), which are resolved into four diastereoisomers on CSP3, whereas only one peak is observed on CSP1 (Table X). Nevertheless, no calculation of α was made as the four pure diastereoisomers were not available.

The use of the connecting arm has not been elucidated; depending on the blocking group employed, the use of a spacer with a C_{11} alkyl chain usually involves an increase in the separation factor in comparison with its C_3 homologue for phases with a benzyloxycarbonyl group (CSP3 and CSP1), whereas a slight decrease is observed when using CSP4 instead of CSP2. Hence it seems that the spacer interferes with the chiral recognition mechanism by altering the relative importance of phenomena that occur during the process of the separation.

The influence of the connecting arm has been studied by other workers and it is

TABLE X

ENANTIOMERIC RESOLUTION OF N-3,5-DINITROBENZOYL ISOLEUCINE ESTERS ON CSP1 AND CSP3 USING ISOPROPANOL AS POLAR MODIFIER

Mobile phase: isooctane–isopropanol (98:2) other conditions as in Table II.

Solute	CSP1:	CSP3			
	k'_1	k'_1	k'_2	k'_3	k'_4
6a	11.62	12.81	13.81	14.09	14.91
6b	8.41	9.26	9.82	Shoulder	10.66
6c	6.10	6.98	7.37	7.76	7.97

clear that the connecting arm plays a role in the chiral recognition mechanism; the spacer could induce a change in the dominant character of the chiral recognition mechanism, leading eventually to an inversion of the elution order of enantiomers [9].

CONCLUSIONS

CSPs derived from L-proline permit the separation of π-acid dinitrobenzoyl racemates. The use of a spacer with a long alkyl chain allows a better enantioselectivity. The organic modifiers contained in the mobile phase have a great influence on the resolution. NMR studies of bimolecular solute–CSP and solute–solvent complexes would be useful means of elucidating the chiral recognition process, as they would also take into account the contribution of the solvent.

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