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LIQUID CHROMATOGRAPHIC RESOLUTION OF ENANTIOMERS ON NORMAL-PHASE CHIRAL AMIDE-BONDED SILICA GEL

RETENTIONS OF OPTICALLY ACTIVE α-AMINO ACID DERIVATIVES ON N-ACYL HOMOLOGUES OF L-VALYLAMINOPROPYLSILANIZED SILICA PHASES

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SUMMARY

In order to develop a chiral stationary phase for the direct resolution of optical isomers in liquid chromatography, new chemically bonded phases, involving less specific and milder diastereomeric association with the solute molecule than with previously known phases, were prepared. N-Acyl homologues of L-valylaminopropylsilanized packing materials containing chiral amide bonds were synthesized from microparticulate silica gel. Racemic N-acetyl-α-amino acid and benzyloxycarbonyldipeptide esters were resolved into their antipodes in a normal-phase system using high-efficiency columns packed with a slurry of the new chiral materials. The retention mechanism was confirmed from the relationship between the capacity ratio and the binary solvent composition. The conformations and the steric fitting of the graft moiety and the solute molecule were discussed by examining space-filling models.

INTRODUCTION

There have been a series of recent advances in the design and preparation of chiral stationary phases for the resolution of optical isomers[1]. The highly selective diastereomeric interactions in solution have been incorporated into the liquid chromatographic system. Host–guest complexation using optically active crown ether-bonded silica has been applied to chiral recognition[2]. Ligand-exchange resolution of racemic α-amino acids has been developed by preparing L-proline-bonded resins which were coupled with transition metal ions[3,4]. Charge-transfer complexation has also been applied to the separation of helicene isomers by coating or bonding chiral electron acceptors on silica gel[5,6]. Such procedures have depended on a high degree of selective interaction between the solute molecule and the configuration of the stationary surface. As a result, applicable solutes are limited to the particular set of compounds specifically favoured by the chiral stationary matrix. As yet, there have been only a few isolated cases of work on systems involving less stereospecific and weaker diastereomeric association than in earlier work, which can be expected in

widening the applicable range of the solute compounds. One such study consisted in the preparation of optically active polymeric amide supports by the polymerization of olefinic amides as the chiral monomers[7].

With the advent of highly efficient column technology employing microparticulate packings, resolution of enantiomers involving less selective processes has become feasible. Hence, interest was focused on the design of less specific and milder active sites than the system previously mentioned, made possible by employing columns with a large number of theoretical plates. The chiral amides were grafted to the microparticulate support and direct resolution of racemic α-amino acid derivatives was examined. The resolution of the racemates was successfully accomplished. Preliminary communications describing the results obtained by employing N-formyl- (FVA-silica) and N-acetyl-L-valylaminopropylsilanized silica gel (AVA-silica) normal phases have been reported recently[8,9]. For the characterization and exploitation of the chiral amide-bonded phase, higher homologues of N-acyl-L-valine-bonded phases were additionally synthesized and the retention behaviours of α-amino acid derivatives were compared. The mechanism of the retention process was also investigated.

EXPERIMENTAL

Preparation of 3-aminopropylsilanized silica (APS-silica)[10,11]

APS-silica was prepared from microparticulate spherical shaped silica gel (Kusano Scientific Co., Tokyo, Japan; average particle size 10 μm, average pore diameter 95 Å, specific surface area 380 m^2/g). Toluene for silanization was dried by distillation over calcium hydride under argon and 3-aminopropyltriethoxysilane (APS) was distilled twice *in vacuo* (boiling point 108°/15 torr). Before reaction, the physically adsorbed water was removed from the silica and glassware by heating at 200° *in vacuo* for 12 h.

The bonding reaction was carried out in a dry argon atmosphere. About 20 g of dried silica gel were suspended in 400 ml of absolute toluene and 20 ml of APS were added to the suspension. The mixture was heated at 100° for 12 h with gentle stirring and the ethanol formed was removed by azeotropic distillation. After the reaction, the suspension was cooled to room temperature, filtered off and washed successively with toluene, acetone, absolute methanol and diethyl ether. Finally, APS-silica was dried over phosphorus pentoxide *in vacuo* for 12 h at room temperature. The results of the elemental analysis (C, N) yielded 1.59% of nitrogen of APS-silica and a surface concentration of 3.0 μmole/m^2 of APS monomers.

Preparation of N-n-valeryl-L-valyl-aminopropylsilanized silica (VVA-silica) and other N-acyl homologues

Condensation of acyl chloride (*n*-valeryl, *n*-butyryl, propionyl and pivaloyl chloride) with L-valine to give the desired N-acyl-L-valine was carried out by the usual Schotten-Baumann procedure. A typical procedure is as follows.

L-Valine (1.5 g, 12.8 mmole) was dissolved in 7.1 ml of ice-cold 2 *N* sodium hydroxide solution and five additions of 9.6 ml of 2 *N* sodium hydroxide solution and 1.86 ml of *n*-valeryl chloride (15.4 mmole) were made at intervals of 10 min with vigorous stirring and cooling in an ice-bath. After the addition of reagents, the mixture was stirred for a further 2 h at room temperature. It was then washed once

with diethyl ether, acidified (pH 2) by the careful addition of 5 *N* hydrochloric acid and extracted three times with ethyl acetate. The ethyl acetate fraction was dried over anhydrous sodium sulphate and evaporated *in vacuo*. The residue was recrystallized from ethyl acetate (melting point 139.5–140.5°). Other N-acyl-L-valine derivatives prepared by the procedure described above, and identified by NMR and IR spectrometry and melting point determinations, were N-*n*-butyryl-L-valine (m.p. 149–150.5°), N-propionyl-L-valine (m.p. 131–132°) and N-pivaloyl-L-valine (m.p 144.5–145°).

To a suspension of APS-silica (2.2 g) in 8 ml of dimethylformamide (DMF) degassed *in vacuo* for 5 min were added a solution of 1-hydroxybenzotriazole (1.02 g, 7.53 mmole) in 3 ml of DMF and a solution of N-acyl-L-valine (5.02 mmole; N-*n*-valeryl-L-valine 1.01 g, N-*n*-butyryl-L-valine 940 mg, N-propionyl-L-valine 869 mg) in 3 ml of DMF. The resulting mixture was treated with 3 ml of DMF containing 1.14 g of dicyclohexylcarbodiimide at 0° for 1 h with stirring, and then stirred at room temperature for 48 h. The grafted silica was separated from dicyclohexylurea by centrifugation and washed successively with chloroform, acetone, absolute methanol and diethyl ether, and then dried over phosphorus pentoxide *in vacuo* for 6 h. BVA-, PVA- and PivVA-silica were obtained by the same procedure.

As formyl-L-valine, when used in conjunction with the carbodiimide procedure, does not racemize, there is no necessity to add 1-hydroxybenzotriazole. However, this reagent was, in fact, added in order to keep grafting conditions constant.

IR spectra were obtained using a Hitachi Model-215 spectrometer. As the IR spectra taken in potassium bromide were too weak, tablets were obtained from direct pressurization of the grafted silica at 600 bar for 35 min. IR spectra of the grafted silica showed absorption peak at 1660 cm^{-1} due to C=O in the amide group.

Microelemental analysis was performed by the Microanalytical Centre of this college. The results are given in Table I.

Column packing procedure

Columns were packing using a high-pressure slurry packing apparatus equipped with a constant-pressure pump (Model DSTV-122G, Haskel Eng. and Supply Co., Burbank, Calif., U.S.A.) shown schematically Fig. 1. This pump can deliver a maximum pressure of 20,000 p.s.i. The slurry reservoir was constructed of stainless-steel tubing and was 50 cm in length with I.D. 10 mm (Chemco Co., Osaka, Japan). To 2.1 g of grafted silica dried over phosphorus pentoxide was added a solution that contained 5 ml of tetrachloromethane, 10 ml of chloroform and 10 ml of dioxan and which had been degassed in an ultrasonic bath for 3 min. Slurry Solvent B conc (Macherey, Nagel & Co., Düren G.F.R.) was added for stabilization and the slurry was further homogenized in an ultrasonic bath for 3 min. A solution consisting of chloroform and methanol (1:1, 200 ml) was used to pressurize the slurry. This solution was introduced to the bottom of the slurry reservoir. The slurry was transferred to a reservoir. The chromatographic column (precision-bore stainless-steel tubing of length 20 cm and I.D. 4 mm) was attached to the slurry reservoir. The slurry packing apparatus was initially pressurized to 6000 p.s.i. with the valve closed. The valve was opened, and the slurry was forced from the reservoir into the column. After half of the slurry solvent had been eluted, the pressure was increased to 8000 p.s.i. Just before the solvent reservoir became empty, *n*-hexane was added and 200

ml of *n*-hexane were passed through the column at 8000 p.s.i. in order to remove the slurry solvent from the packed column. Then the pump was shut off and column pressure relieved.

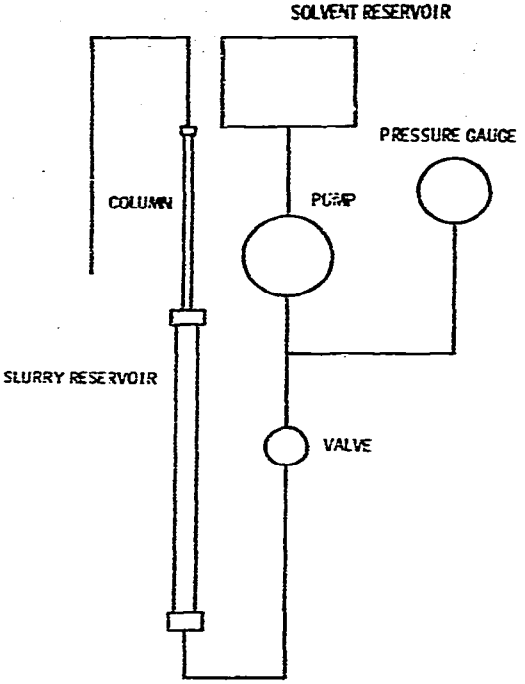

Fig. 1. High-pressure slurry packing apparatus.

Samples

α-Amino acid and dipeptide derivatives were prepared in our laboratory by employing reagent-grade chemicals.

Apparatus and chromatographic procedure

The apparatus was a Spectra-Physics (Stenderway, Santa Clara, Calif., U.S.A.) Model SP 8000 chromatograph equipped with a Jasco UVIDEC-100-II (Japan Spectroscopic Co., Hachioji, Tokyo, Japan) variable-wavelength UV detector operated at 230 and 254 nm.

The chromatographic solvents, *n*-hexane and 2-propanol were of analytical-reagent grade and were distilled once over calcium hydride.

The amount of N-acetylamino acid methyl esters injected was 40 μg, dissolved in 4 μl of chloroform. The amount of dipeptide derivatives was 80 μg in 8 μl of chloroform.

RESULTS AND DISCUSSION

It has been suggested that the main factors necessary for chiral recognition are strong specific binding interactions and extensive spacial recognition between the solute molecule and the stationary surface[5]. In spite of such suggestions, in the

present study the chiral amide function was selected to be a less specific and milder group with less stereoselectivity than those previously utilized for resolving enantiomeric isomers. The amide group is a very common polar function which has the ability to serve as either a donor or acceptor in hydrogen bonding and rotation around the amide bond is restricted, providing a fixed direction for association with the solute molecule. Therefore, the chiral amide-bonded phase seemed to be a suitable stationary phase for enantiomer resolution. Optically active N-acyl-L-valine as a chiral element was thus activated and grafted on to 3-aminopropylsilanized silica gel. Another chiral amide bond was introduced simultaneously on to the surface of the support. As a result, the chiral surface structure containing the two amide functions supported by the isopropyl group bound to the asymmetric carbon can differentiate enantiomeric sample molecules.

For preparing high-efficiency columns, a slurry of the chiral packing materials prepared from spherical microparticulate silica gel was packed into stainless-steel tubes. The numbers of theoretical plates given by the columns of N-acyl homologous phases are shown in Table I.

TABLE I

PHYSICAL PROPERTIES OF CHIRAL AMIDE-BONDED SILICA PHASES AND COLUMNS

Property	*FVA-silica*	*AVA-silica*	*PVA-silica*	*BVA-silica*	*VVA-silica*	*PivVA-silica*
Elemental analysis, N (%)	2.11	2.04	1.65	1.95	1.85	1.93
Surface coverage (%)*	37	42	53	59	51	39
Number of theoretical plates per 20 cm**	1300	1300	1100	1200	1000	900

* The ratio of N-acyl-L-valine grafted on APS-silica is shown.

** Number of theoretical plates per 20 cm was obtained by using N-Ac-L-Leu-OMe as the solute employing a linear velocity of *ca.* 0.18 cm/sec, a temperature of 40° and a mobile phase consisting of 8% (v/v) 2-propanol in *n*-hexane ($k' \approx 2.9$).

Separation of racemic N-acyl-α-amino acid derivatives

The new columns are suitable for both normal- and reversed-phase systems, but in the present study the characteristics of the column were checked by utilizing only the normal-phase adsorption mode. Racemic N-acetyl-α-amino acid methyl esters were selected as the solute compounds because they contain amide and ester functions as active sites, bound to the asymmetric carbon atom. It was assumed that the racemic solutes would associate in a different manner with the chiral graft bonded covalently to the stationary phase. A binary solvent system consisting of *n*-hexane and 2-propanol was prepared as the mobile phase. When the solvent strength was adjusted to afford a medium with a capacity ratio of 6–9, pairs of enantiomers were resolved into the corresponding antipodes using FVA-silica[8] and AVA-silica[9] columns. A typical chromatogram is shown in Fig. 2. As a further effort in determining the features of the stationary phase, columns prepared from silica grafted with higher homologues of N-acyl-L-valine were examined. These homologues include the N-propionyl-(PVA-), N-*n*-butyryl-(BVA-), N-*n*-valeryl-(VVA) and N-pivaloyl-L-valyl-aminopropylsilanized silica (PivVA-silica) derivatives.

TABLE II

RESOLUTION OF DL-α-AMINO ACID AND DIPEPTIDE DERIVATIVES USING N-ACYL HOMOLOGUES OF L-VALYLAMINOPROPYL-SILANIZED SILICA

DL-α-Amino acid and dipeptide derivatives were analysed on six chiral columns under the following conditions: column dimensions, 20 × 0.4 cm I.D.; temperature, 40°; linear velocity, (1) FVA-silica 0.080 cm/sec, (2) AVA-silica 0.093 cm/sec, (3) PVA-silica 0.090 cm/sec, (4) BVA-silica 0.104 cm/sec, (5) VVA-silica 0.095 cm/sec, (6) PivVA silica 0.096 cm/sec.

Racemate	*FVA-silica*[a]	*AVA-silica*[a]	*PVA-silica*				*BVA-silica*				*VVA-silica*				*PivVA-silica*			*Mobile phase: concentration of 2-propanol in n-hexane (%)*
	R_s[b]	R_s	k_1'	k_2'	α	R_s	k_1'	k_2'	α	R_s	k_1'	k_2'	α	R_s	k_1'	k_2'	α	
N-Ac-Leu-OMe	0.79	0.71	5.63	6.39	1.13	0.92	6.90	7.78	1.13	0.99	6.83	7.61	1.11	0.76	7.55	**		4
N-Ac-Nle-OMe	0.70	0.63	5.89	6.60	1.12	0.86	6.69	7.51	1.12	0.91	6.61	7.38	1.12	0.83	—	—		4
N-Ac-Ile-OMe	0.62	0.54	5.37	6.00	1.12	0.85	5.98	6.67	1.12	0.90	6.01	6.63	1.10	0.68	—	—		4
N-Ac-Val-OMe	0.63	0.55	5.23	5.78	1.11	0.77	6.86	7.55	1.10	0.76	7.23	7.91	1.09	0.63	7.84	1.00		4
N-Ac-Phe-OMe	0.56	0.57	8.15	8.94	1.10	0.74	8.59	9.36	1.09	0.70	9.13	9.93	1.09	0.64	10.26	1.00		4
N-Ac-S-Bz-Cys-OMe	0.40	0.24	8.98	9.41	1.05	0.37	9.37	9.76	1.04	0.31	10.01	10.33	1.03	0.22	—	—		4
N-Ac-Met-OMe	0.39	0.31	7.36	7.73	1.05	0.37	6.90	7.24	1.05	0.38	6.50	6.89	1.06	0.41	—	—		8
N-Ac-Ala-OMe	0.31	**	6.28	6.68	1.06	0.43	5.92	6.32	1.07	0.52	7.20	7.49	1.04	0.28	—	—		8
Z-Leu-Leu-OMe	0.84	0.46	5.48	5.89	1.07	0.49	5.51	5.98	1.09	0.66	5.47	5.88	1.07	0.47	6.42	1.00		2
Z-Phe-Phe-OMe	0.70	**	6.23	6.54	1.05	0.36	6.51	6.85	1.05	0.38	6.29	6.46	1.03	0.20	—	—		3

[a] Resolution (R_s) was calculated according to the equation $R_s = 1/4\ (\alpha-1)\sqrt{N[k'/(1+k')]}$, where k' is the capacity factor and α is the separation factor, calculated according to the equations $k' = (k_1' + k_2')/2$ and $\alpha = k_1'/k_2'$.

** Shoulders could be definitely detected in all instances.

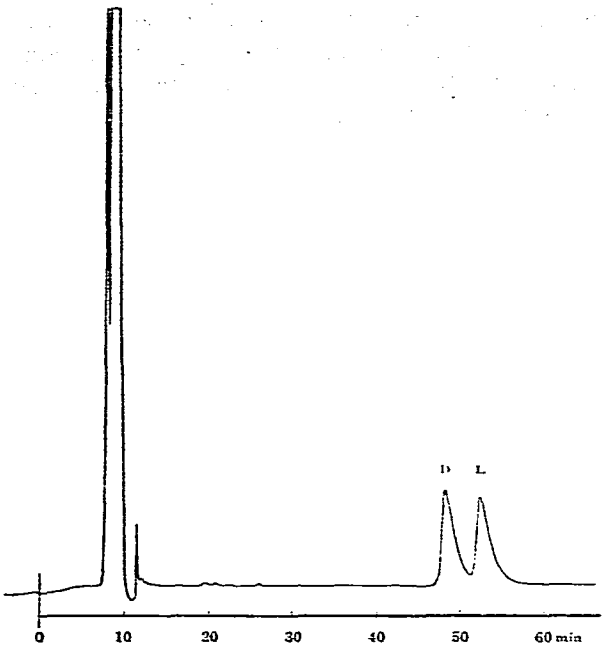

Fig. 2. Resolution of N-Ac-DL-Leu-OMe. Column, FVA-silica ×2; mobile phase 4% (v/v) 2-propanol in *n*-hexane; temperature, 40°; linear velocity, 0.086 cm/sec; detection, UV at 230 nm, 0.16 a.u.f.s.

It was found that PVA-, BVA- and VVA-silica columns gave good chiral recognition. The results are summarized in Table II. All stationary phases constructed from homologues of N-acyl-L-valine afforded very similar capacity ratios. Whereas the PivVA-silica column was found to be "blind" concerning chiral recognition, other homologues having hydrogen or a straight alkyl chain in the N-acyl function gave very similar separation factors.

It was observed that D-α-amino acid derivatives eluted faster than the corresponding L-isomers in every instance. Separation factors for pairs of D-,L-isomers varied from 1.03 to 1.13. The average value of approximately 1.08 was smaller than in the work mentioned above[2–4], indicating that the chiral differentiation of this system was weaker than earlier ones. The values for leucine homologues were relatively large, whereas the values for alanine derivatives were rather small.

Benzyloxycarbonylpeptide esters have been known as useful intermediates for peptide synthesis. Therefore, two pairs of enantiomeric benzyloxycarbonyldipeptide methyl esters were synthesized and their retention behaviours were examined on chiral columns. The resolution of the protected dipeptides was also successful. The retention data are given in Table II. These enantiomers were eluted faster than the corresponding N-acetyl-α-amino acid esters. L,L-Dipeptide derivatives eluted faster than D,D-isomers. It was known that the retention sequences of the dipeptide and α-amino acid derivatives were optically reversed.

In addition to the D,D- and L,L-enantiomers, D,L- and L-leucyl-D-leucine derivatives were prepared as another pair of antipodes. These four stereoisomers were resolved simultaneously using an FVA-silica column. The chromatogram is shown

in Fig. 3. The elution sequence of the four dipeptide derivatives was as follows: L,L- > D,D- > D,L- > L,D-. The results indicated that protected dipeptides containing a D-α-amino acid as a C-terminal constituent were more retained than the antipodes on a column having an L-α-amino acid moiety.

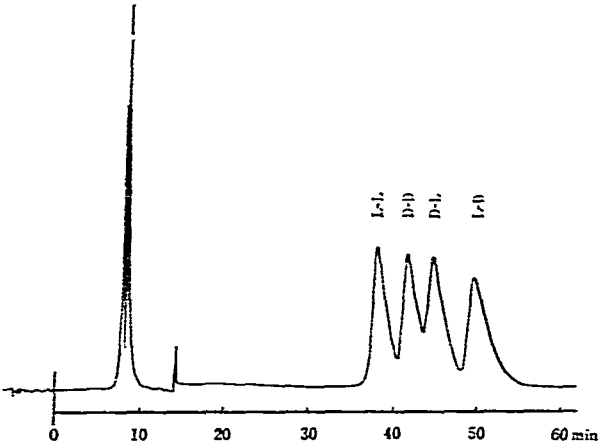

Fig. 3. Resolution of Z-Leu-Leu-OMe. (L-L, D-D, D-L, L-D). Column, FVA-silica ×2; mobile phase, 2% (v/v) 2-propanol in *n*-hexane; temperature, 40°; linear velocity, 0.086 cm/sec; detection, UV at 254 nm, 0.16 a.u.f.s.

Retention mechanism on the chiral amide-bonded stationary phase

In order to study the retention mechanism of α-amino acid derivatives on the chiral amide-bonded silica gel phase, the correlation between the capacity ratio and the binary solvent composition was examined, based on the recently established concept of the adsorption–desorption equilibrium on the adsorbent surface[12–17].

A number of studies concerning the adsorption mechanism on the silica gel surface in normal-phase liquid chromatography have been performed. An adsorption model dealing with the competition between the solute and solvent molecules for the active sites on the stationary surface has been proposed[12]. Based on a model for binary solvent systems, a relationship between the capacity ratio and the stronger solvent concentration has been derived as follows:

$$\log k' = c - n \log X_s$$

where X_s is the molar fraction of the stronger solvent in a diluent and c and n are constants[13,14]. The linear relationship was proved experimentally by applying systematically prepared binary solvent systems[15–17]. If such a relationship is confirmed, the retention mechanism of the system can be concluded to be liquid–solid adsorption.

Capacity ratios of N-acetyl-D,L-leucine methyl esters as model compounds on normal-phase chiral stationary phases were obtained by varying the concentration of the *n*-hexane–2-propanol system. Plotting of the logarithm of the retention *versus* the logarithm of the molar fraction for the binary solvent yielded a straight line (Fig. 4). When the least-squares procedure was applied, the correlation coefficient was

0.999 with a very small deviation from a linear relationship. Constants were obtained as follows: $c_D = 2.16$, $c_L = 2.26$; $n_D = 1.47$, $n_L = 1.53$. These data suggest that the system employed in this work involves a liquid–solid adsorption mechanism.

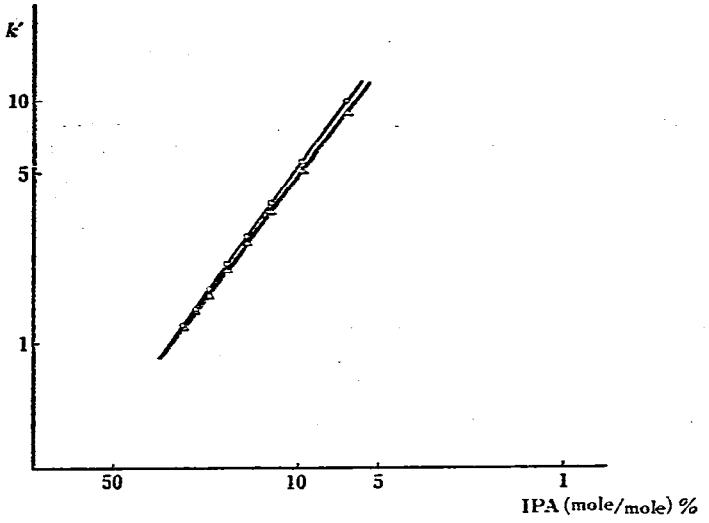

Fig. 4. Graph of the logarithm of the capacity ratio *versus* the logarithm of the molar fraction of 2-propanol (IPA) in *n*-hexane. ○, N-Ac-L-Leu-OMe; △, N-Ac-D-Leu-OMe. Capacity ratios were determined by using N-Ac-DL-Leu-OMe as the solute on a FVA-silica phase. Linear velocity, 0.090 cm/sec; temperature, 40°; mobile phases, 2-propanol in *n*-hexane.

Mobile phase composition mole % 2-propanol in n-hexane	*Capacity ratios*	
	N-Ac-L-Leu-OMe	*N-Ac-D-Leu-OMe*
6.64	9.77	8.92
9.82	5.54	5.10
12.91	3.70	3.43
15.94	2.72	2.53
18.87	2.10	1.97
21.82	1.61	1.53
24.61	1.32	Shoulder was detected
27.34	1.15	Shoulder was detected

As the adsorption sites in the stationary surface were assumed to be two amide bonds, retention and chiral differentiation must be associated with these two active functions. By constructing a space-filling CPK model, preferential conformation of the graft moiety was assumed. The conformation of the amide bonds in the graft is probably *s-trans*, and consequently complexation of the stationary surface with a solute molecule may involve an amide carbonyl group and an imino hydrogen. Two active sites on opposite sides of the graft moiety are thus able to associate with a solute molecule which has two active functions, such as amide and ester groups. An example of the probable configurational adaptation of an amide ester molecule as a solute is illustrated in Fig. 5. The chiral recognition is assumed to involve selective complexation between the graft and the solute amide group, and to some extent the steric effect of the chiral alkyl group in the localized solute molecule.

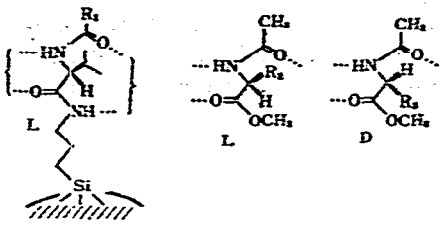

Fig. 5. Supposed favourable conformations of the grafted moiety on the stationary phase with N-acetyl-L-α-amino acid and D-α-amino acid methyl esters as solutes. Two amide groups of the graft moiety are able to associate with a solute molecule which has two active functions, *e.g.* amide and ester groups. R_1 = H–, CH_3–, CH_3CH_2–, $CH_3CH_2CH_2$–, $CH_3CH_2CH_2CH_2$–, $(CH_3)_3C$–. R = $CH_3CH_2(CH_3)CH$–, $CH_3(CH_2)_2CH_2$–, $(CH_3)_2CHCH_2$–, $(CH_3)_2CH$–, $C_6H_5CH_2$–, $C_6H_5CH_2SCH_2$–, $CH_3SCH_2CH_2$–, CH_3–.

Whereas a reduction in chiral differentiation was shown by the PivVA-silica column, having a tertiary alkyl group in the N-acyl moiety, recognition of sufficient and similar extent was afforded by *n*-alkyl homologues of N-acylamide-grafted phases such as FVA-, AVA-, PVA-, BVA- and VVA-silica. The difference can be attributed to the steric hindrance caused by the particular N-acyl group in the graft moiety. This suggests that the amide function located in N-terminal group of the valine graft makes a greater contribution than that located in the C-terminal group to the resolution of enantiomers.

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