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SYNTHESIS AND PROPERTIES OF A NOVEL CHIRAL STATIONARY PHASE FOR THE RESOLUTION OF AMINO ACID ENANTIOMERS

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SUMMARY

A novel chiral stationary phase has been synthesized by grafting L-valine–*tert.*-butylamide on to modified polycyanopropylmethyl phenylmethyl silicone (OV-225).

This chiral phase can be used for the separation of amino acid enantiomers at temperatures ranging from 60° to 230°. This allows the separation of the protein amino acid enantiomers in one run. The elution properties of six derivatives (O-isopropyl, O-*n*-butyl, N-trifluoroacetyl, N-pentafluoropropionyl, N-heptafluorobutyryl) on the new chiral phase are compared.

INTRODUCTION

The gas chromatographic (GC) separation of optically active compounds, particularly amino acids, has received much attention in recent years. This technique is especially interesting for the quantitative determination of amino acids by enantiomer labelling[1].

The separation of enantiomers can be achieved by two different methods: conversion of the enantiomers into diastereoisomeric derivatives followed by GC on conventional stationary phases[2–5] or by direct enantiomer separation on optically active stationary phases. To improve the resolution, both techniques can be combined in the analysis of the formed diastereoisomers on optically active phases[6].

Of these methods, direct separation on optically active phases has proved to be the most reliable. This method was first introduced by Gil-Av and coworkers[7–9], who synthesized the dipeptide phase, N-trifluoroacetyl (TFA)-L-valyl-L-valine cyclohexyl ester. Since this pioneering work, a number of different dipeptide and diamide phases have been prepared and evaluated[10–18]. The separation of D,L-isomers has been attributed to triple hydrogen bond formation of different strengths between the enantiomeric solutes and the chiral solvent[19]. Other molecular interactions (dipole–dipole interactions, dispersion forces) must also be considered, however[6].

The prerequisites for a chromatographic system that is capable of separating

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all naturally occurring amino acid enantiomers in one run are high selectivity, high efficiency and high temperature stability. The highest efficiency for amino acid enantiomer separations is obtained on diamide phases with the structure RCONHCH (*i*-Pr) CONHR', where R is unbranched and R' is a tertiary group[18]. The drawback to these phases is their low temperature stability. Therefore, the diamide part is better introduced in a polymer matrix with high temperature stability. Finally, high efficiency can be achieved with glass capillary column GC. The only optically active stationary phase reported so far that meets these requirements was described by Frank *et al.*[20]. The phase was synthesized by coupling L-valine–*tert.*-butylamide to a copolymer of dimethylsiloxane and carboxyalkylmethylsiloxane.

In this paper, a new approach for achieving a thermostable chiral phase is described. It is based on the incorporation of L-valine–*tert.*-butylamide in a modified form of the well known GC stationary phase polycyanopropylmethyl phenylmethyl silicone (OV-225). Some parts in this phase (diamide and the silicone matrix) are the same as in the phase described by Frank *et al.*[20]. There are, however, structural differences, *e.g.*, propyl link instead of ethyl link to the chiral centre and the presence of phenyl groups on the silicone matrix. The two phases are therefore chromatographically not identical as will be shown in the Discussion part of this paper.

EXPERIMENTAL

Synthesis of the chiral phase

The synthetic pathway is shown in Fig. 1.

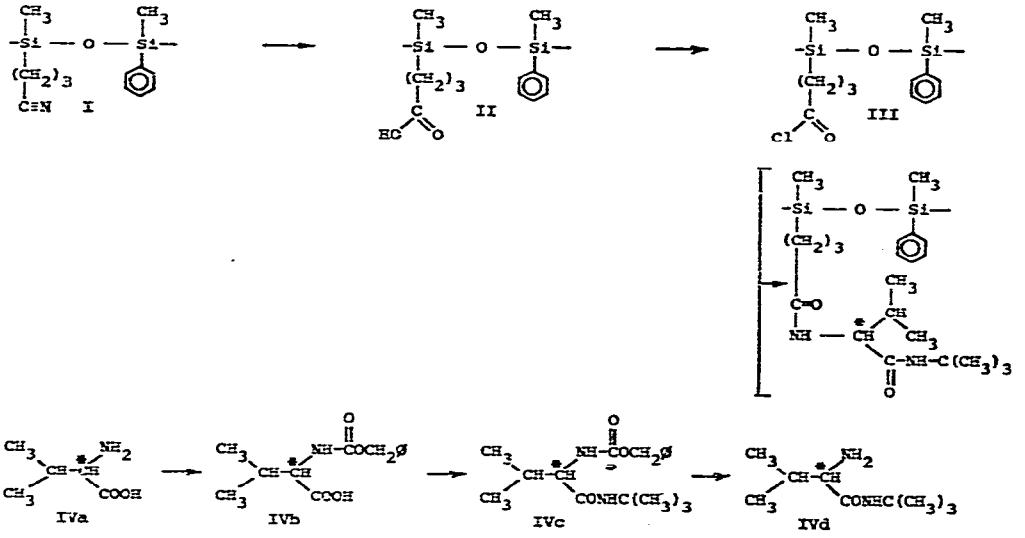

Fig. 1. Synthetic pathway for the chiral stationary phase.

The cyano group (I) of OV-225 is hydrolysed to the carboxylic function (II). By conversion to the acid chloride (III), a site is created for reaction with L-valine–*tert.*-butylamide. The latter compound (IVd) is synthesized by coupling carbobenzoxy-

L-valine (IVb) and *tert.*-butylamine (IVc). The protecting group is removed and the amide is linked to the polymer. Details of the synthesis are given below.

Synthesis of chloropropionylmethyl phenylmethyl silicone. OV-225 (1–10 g) is dissolved in diethyl ether and the cyano group is converted into the carboxylic function by acid hydrolysis. The reaction is followed by infrared (IR) spectroscopy. The total conversion of the cyano group (IR band 2270 cm^{-1}) to the carboxylic group (IR band 1730 cm^{-1}) marks the completion of the reaction. A 1-g amount of the acid thus obtained is dissolved in 50 ml of dry benzene. The mixture is cooled in an ice–salt bath and 3 ml of freshly distilled oxalyl chloride are added dropwise with constant stirring. After the evolution of hydrogen chloride gas, the mixture is refluxed for 1 h. The acid group is quantitatively converted to the acid chloride function as can be followed by IR spectroscopy (the 1730 cm^{-1} IR band shifts to 1815 cm^{-1}). Benzene and residual oxalyl chloride are removed on a rotary evaporator and the acid chloride is immediately dissolved in dry diethyl ether.

Synthesis of L-*valine–tert.-butylamide.* To a solution of 0.01 mole (1.17 g) of L-valine in 20 ml of water, 0.1 *N* sodium hydroxide solution is added until the pH of the solution is 10. The mixture is cooled in an ice–salt bath and 0.01 mole (1.7 g) of carbobenzoxy chloride, dissolved in 10 ml of diethyl ether, is added dropwise. During the reaction, the pH of the mixture is maintained at 10 by adding 0.1 *N* sodium hydroxide solution. Stirring is continued until the pH remains constant. Unreacted carbobenzoxy chloride is extracted with diethyl ether. The aqueous layer is acidified to pH 2 and extracted with diethyl ether. The ether extract is washed with water, dried over magnesium sulphate and the solvent removed. The dried derivatized or Z-amino acid is dissolved in dry tetrahydrofuran (THF) (20 ml). One equivalent (2.47 g) of N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) is added and the mixture is stirred for 1 h at room temperature. Thereafter one equivalent (0.73 g) of *tert.*-butylamine is added; the mixture is stirred for 30 min at room temperature and then refluxed for 2–3 h until the precipitate dissolves completely. The reaction mixture is cooled and washed three times with 0.1 *N* hydrochloric acid and with water to remove unreacted amine and quinoline. The solvent is evaporated and the compound crystallized from *n*-pentane–chloroform (9:1). The blocked L-valine–*tert.*-butylamide is dissolved in absolute methanol (30 ml). A few drops of glacial acetic acid are added and the mixture is stirred with 1 g of 10% Pd/C for 3 h at room temperature while hydrogen gas is blown just above the surface of the solution. After the reaction is completed, the mixture is filtered and methanol evaporated. The liberated amine is extracted with 0.1 *N* hydrochloric acid, regenerated with sodium hydroxide, extracted with chloroform and dried. L-Valine–*tert.*-butylamide is crystallized from isooctane, with the following properties. Yield, 60%, M.p., 48°. Spectroscopic values: 360 MHz. Proton nuclear magnetic resonance (NMR): γH 0.81δ; γ'H 0.97δ; βH 2.28δ; αH 3.10δ; *tert.*-Bu 1.36δ; NH_2 1.21δ; NH 7.13δ; $^3J_{\alpha,\beta}$ 3.1 Hz; $^3J_{\beta,\gamma}$ 7.0 Hz. Mass spectrum: $M^+ \cdot$ 172, *m/z* 72 (100%). IR spectrum: 3370, 1675, 1540 cm^{-1}.

Coupling of chloropropionylmethyl phenylmethyl silicone and L-*valine–tert.-bytylamide.* The acid chloride solution is cooled in an ice–salt bath and an excess of L-valine–*tert.*-butylamide dissolved in diethyl ether is added dropwise. The mixture is stirred for 30 min. Excess valine reagent is extracted with 0.1 *N* hydrochloric acid. The ether layer is washed with water, dried over magnesium sulphate and the solvent

removed. The absorption bands of L-valine–*tert.*-butylamide are present in the infrared spectrum of the chiral phase. The NMR data indicate the presence of *tert.*-butyl ($\delta = 1.36$) and isopropyl protons (δ 0.92, overlap of γ,γ' Hs).

Capillary columns

Borosilicate glass capillaries, drawn on a Hupe Bush apparatus, were washed with methanol and dichloromethane and then heated overnight with dry nitrogen at 300°. The surface was modified by whisker formation[21,22] and deactivated with N,OH-containing compounds[23]. The columns were coated statically[24] with 0.2% solutions in dichloromethane or dynamically[25] with 4% solutions. In both instances a relatively thin film of *ca.* 0.2 μm is obtained.

Equipment

The GC separations were carried out on a Varian 3700 or a Carlo Erba 2900 gas chromatograph, both equipped with a flame-ionization detector.

Preparation of derivatives

N-TFA-D,L-amino acid isopropyl and *n*-butyl esters, N-pentafluoropropionyl (PFP)-D,L-amino acid isopropyl and *n*-butyl esters and N-heptafluorobutyryl (HFB)-D,L-amino acid isopropyl and *n*-butyl esters were prepared according to the methods described in the literature[1,20,26].

RESULTS AND DISCUSSION

The novel chiral stationary phase was evaluated with a mixture of sixteen pairs of amino acid enantiomers and L-lysine. D-Lysine, D,L-arginine and D,L-histidine were not included as they were not available at the time.

In Fig. 2, a chromatogram of the N-PFP-D,L-amino acid isopropyl esters on a 22 m × 0.25 mm I.D. column is shown. The thermal stability and low volatility of the chiral phase are demonstrated by the wide temperature range over which the columns can be used (90–190°) in temperature-programmed runs. This stability makes it possible to resolve both high- and low-boiling amino acid enantiomers in one run on 20–25-m columns in less than 50 min. During the initial conditioning period a decrease in capacity factor is noted. After this, further conditioning overnight at 230° does not reduce the capacity factors. As in other instances mentioned before[22], the film of stationary phase is stabilized effectively by the very rough structure of the whisker surface. On moderately roughened surfaces (sodium chloride deposit), temperature programming can only be extended to about 180°.

The temperature stability of the phase is illustrated by the fact that a column accidentally conditioned overnight at 230° without a carrier gas flow showed only very minor deterioration. The high selectivity of the stationary phase for the protein amino acid enantiomers can be deduced from Fig. 2. The first peak of the enantiomer pairs corresponds to the D-enantiomer.

Good separation factors for all of the D,L-amino acid pairs were obtained except for D,L-proline. This separation proved to be difficult and a low resolution factor on dipeptide phases has also been reported by other groups[18,20]. Removal of the hydrogen-bonding NH group in proline by the derivatization can explain this

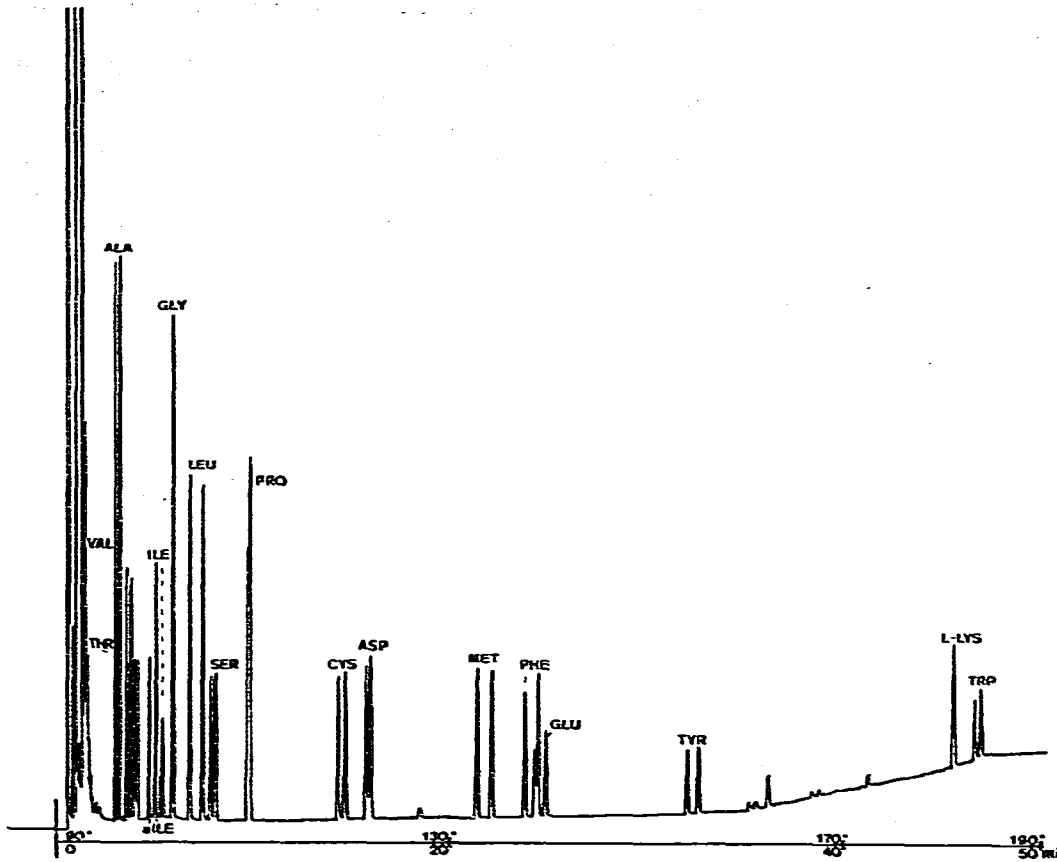

Fig. 2. Chromatogram of the N-PFP-amino acid isopropyl esters of sixteen pairs of amino acid enantiomers together with L-lysine. Conditions: 22 m × 0.25 mm I.D. whiskered capillary column; temperature-programmed at 2°/min from 90° to 190°; injector and detector temperature, 250°; carrier gas, hydrogen at a linear velocity 50 cm/sec.

behaviour. In the conditions used to obtain Fig. 2, incomplete separation is observed for D,L-threonine and D,L-aspartic acid; overlapping is found in the case of L-*allo*-isoleucine and D-isoleucine and for D-glutamic acid and L-phenylalanine. Baseline separation of D- and L-threonine is achieved by modifying the initial temperature (70°) and temperature programming rate or by chromatographing isothermally over a short period. The resolution of D,L-aspartic acid can be improved by the preparation of other derivatives. The problem of peak overlap with L-phenylalanine and D-glutamic acid can also be overcome in the same way (see below). The separation of the leucine isomers can be achieved by using a thicker film of stationary phase (0.5 μm).

Because of the phenyl groups on the polymer back-bone, the polarity of the new phase differs from the polarity of the phase described by Frank *et al.*[20]. Comparison of the elution order of the N-PFP isopropyl derivatives of amino acids reflects this difference; on the modified OV-225 phase, glycine elutes after the isoleucines, proline

after serine and cysteine before aspartic acid. The sequence of the amino acids eluting after methionine is the same on both phases.

In order to compare their chromatographic behaviours, N-TFA, N-PFP and N-HFB derivatives of the isopropyl and *n*-butyl esters of some amino acids were analysed. The elution times in a programmed run (from 90° to 190° at 2°/min) are listed in Table I, and show that N-PFP-amino acid isopropyl esters and N-HFB-amino acid isopropyl esters are more volatile than the corresponding N-TFA derivatives. Table I reveals, however, that the highest selectivity of enantiomer separation is obtained with the N-TFA-isopropyl derivatives. This is illustrated in Fig. 3, which compares the analysis of the N-TFA- and the N-PFP-isopropyl derivatives of six amino acids. Note the baseline separation of the D,L-enantiomers of the N-TFA-isopropyl derivatives of aspartic acid.

TABLE I

ADJUSTED RETENTION TIMES OF DERIVATIVES OF SOME PROTEIN D,L-AMINO ACIDS ON A 22 m × 0.25 mm I.D. WHISKERED CAPILLARY COLUMN TEMPERATURE PROGRAMMED FROM 90° TO 190° AT 2°/min

Amino acid	*Derivative*					
	TFA-i-Pr	*TFA-n-Bu*	*PFP-i-Pr*	*PFP-n-Bu*	*HFB-i-Pr*	**HFB-n-Bu*
D-Val	4.00	8.96	3.00	7.02	2.90	6.40
L-Val	4.40	9.42	3.28	7.40	3.10	6.72
Gly	6.24	13.00	5.40	11.36	5.26	10.80
D-Leu	7.80	14.70	6.24	12.16	5.90	11.30
L-Leu	8.70	15.52	6.90	12.90	6.60	12.04
D-Pro	10.90	18.70	9.14	16.10	8.30	14.60
L-Pro	11.00	18.80	9.22	16.22	8.40	14.68
D-Asp	17.48	33.10	15.22	29.96	14.16	28.20
L-Asp	17.80	33.22	15.44	30.12	14.40	28.40
D-Phe	25.70	33.60	22.90	30.50	21.80	28.74
L-Phe	26.40	34.08	23.56	31.00	22.44	29.22
D-Glu	26.26	41.64	23.30	38.26	22.00	36.00
L-Glu	26.80	42.10	23.90	38.62	22.60	36.40

* The carrier gas flow-rate was slightly higher; normally retention times of PFP and HFB derivatives are identical.

The acidic amino acids, aspartic and glutamic acids, are more strongly retarded than the neutral amino acids if the *n*-butyl esters are formed. This peak shift is illustrated in Fig. 4, which shows the analysis of the N-PFP-*n*-butyl esters of aspartic acid, phenylalanine and glutamic acid. The GC conditions used to obtain Fig. 4 are the same as for Fig. 2 and the formation of *n*-butyl esters can be used to differentiate L-phenylalanine and D-glutamic acid.

The properties of the chiral phase for the separation of other optically active compounds have not yet been studied sufficiently to permit conclusions to be drawn. The enantiomers of amino alcohols, however, can be separated, as illustrated in Fig. 5, which shows the separation of the D,L-enantiomers of the pentafluoropropionyl derivative of norephedrine.

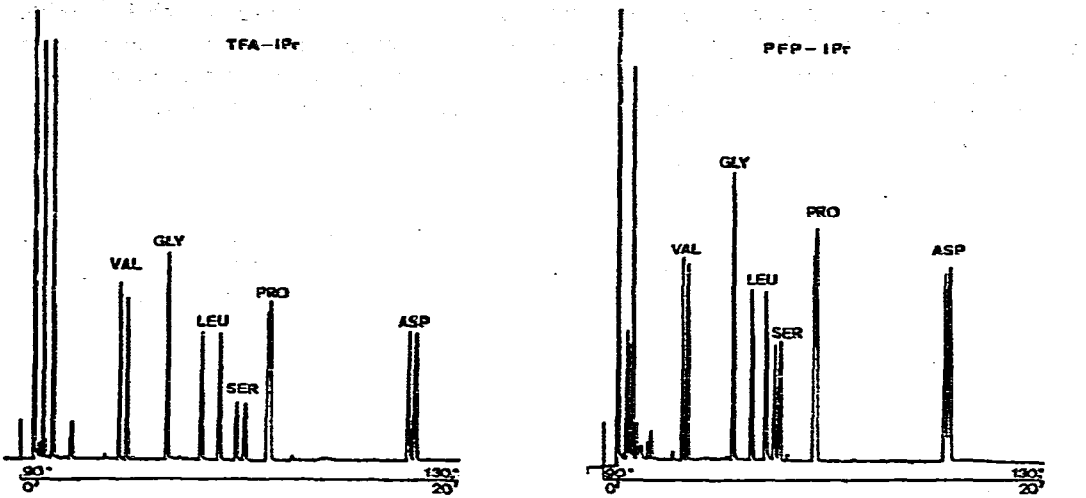

Fig. 3. Chromatogram of the N-TFA-D,L-amino acid isopropyl esters (TFA-*i*-Pr) and N-PFP-D,L-amino acid isopropyl esters (PFP-*i*-Pr) of valine, glycine, leucine, serine, proline and aspartic acid. Conditions as in Fig. 2.

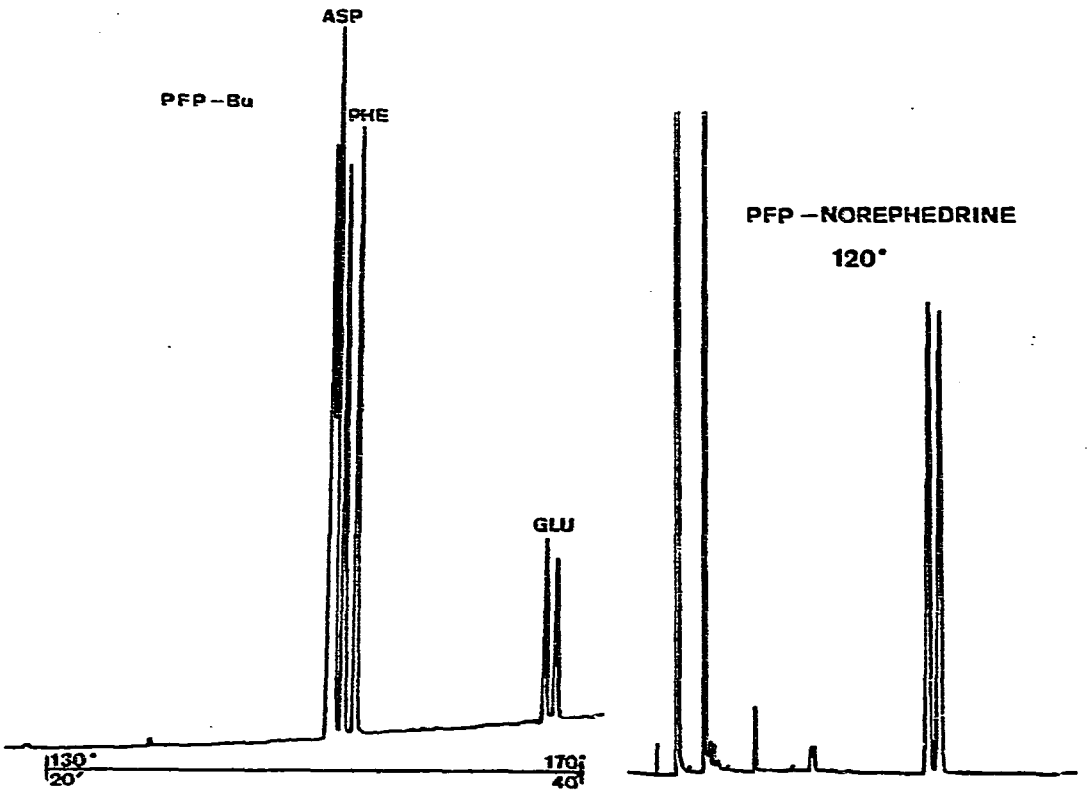

Fig. 4. Chromatogram illustrating the peak shift of the N-PFP-D,L-amino acid *n*-butyl esters (PFP-Bu) of aspartic acid, phenylalanine and glutamic acid. Conditions as in Fig. 2.

Fig. 5. Enantiomer separation of the pentafluoropropionyl derivative of D,L-norephedrine. Conditions: 18 m × 0.25 mm I.D. whiskered capillary column; temperature, 120°; carrier gas, hydrogen at a linear velocity of 40 cm/sec.

The reaction scheme for the synthesis of the chiral phase for amino acids provides possibilities for preparing "tailor-made" stationary phases for specific problems. Other optically active compounds can be coupled to the polymer matrix via the acid chlorides. Such work is the subject of current investigations.

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REFERENCES

1 H. Frank, G. J. Nicholson and E. Bayer, *J. Chromatogr.*, 167 (1978) 187.
2 R. Charles, G. Fischer and E. Gil-Av, *Israel J. Chem.*, 1 (1963) 234.
3 G. Pollack, V. Oyama and R. Johnson, *J. Gas Chromatogr.*, 3 (1965) 174.
4 E. Gil-Av., R. Charles and G. Fischer, *J. Chromatogr.*, 17 (1965) 408.
5 B. Halpern and J. Westley, *Bicchem. Biophys. Res. Commun.*, 19 (1965) 361.
6 W. A. Koenig, K. Stoelting and K. Kruse, *Chromatographia*, 10 (1977) 444.
7 E. Gil-Av, B. Feibush and R. Charles, *Tetrahedron Lett.*, (1966) 1009.
8 B. Feibush and E. Gil-Av, *Tetrahedron Lett.*, (1967) 3345.
9 S. Nakaparksin, P. Birrell, E. Gil-Av and J. Oró, *J. Chromatogr. Sci.*, 8 (1970) 177.
10 W. A. Koenig, W. Parr, H. A. Lichtenstein, E. Bayer and J. Oró, *J. Chromatogr. Sci.*, 8 (1970) 183.
11 W. Parr and P. J. Howard, *Angew. Chem.*, 84 (1972) 586.
12 W. A. Koenig and G. J. Nicholson, *Anal. Chem.*, 47 (1975) 951.
13 F. Andrawes, R. Brazell, W. Parr and A. Zlatkis, *J. Chromatogr.*, 112 (1975) 197.
14 R. Brazell, W. Parr, F. Andrawes and A. Zlatkis, *Chromatographia*, 9 (1976) 57.
15 B. Feibush, *Chem. Commun.*, (1971) 544.
16 I. Abe, T. Kohno and S. Musha, *Chromatographia*, 11 (1978) 393.
17 K. Stoelting and W. A. Koenig, *Chromatographia*, 9 (1976) 331.
18 R. Charles, U. Beitler, B. Feibush and E. Gil-Av, *J. Chromatogr.*, 112 (1975) 121.
19 B. Feibush and E. Gil-Av, *Tetrahedron*, 26 (1970) 1361.
20 H. Frank, G. J. Nicholson and E. Bayer, *J. Chromatogr. Sci.*, 15 (1977) 174.
21 J. D. Schieke, N. R. Comins and V. Pretorius, *J. Chromatogr.*, 112 (1975) 97.
22 P. Sandra and M. Verzele, *Chromatographia*, 10 (1977) 419.
23 P. Sandra, M. Verstappe and M. Verzele, *J. High Resol. Chromatogr. Chromatogr. Commun.*, 1 (1978) 28.
24 J. Bouche and M. Verzele, *J. Gas Chromatogr.*, 5 (1968) 501.
25 G. Dykstra and J. De Goey, in D. H. Desty (Editor), *Gas Chromatography 1958*, Butterworths, London, 1958, p. 56.
26 C. F. Poole and M. Verzele, *J. Chromatogr.*, 150 (1978) 439.