Journal of Chromatography, 391 (1987) 145–160
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 19 245

SYNTHESIS AND CHARACTERIZATION OF CHIRAL STATIONARY PHASES FROM AMINO ACIDS AND SMALL PEPTIDES FOR LIQUID CHROMATOGRAPHY FRACTIONATIONS OF A RACEMIC ALCOHOL

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(Received October 9th, 1986)

SUMMARY

A series of amino acid and dipeptide bonded phases have been prepared and the performances of these chiral stationary phases for separation of *R,S*-2,2,2-trifluoro-1-(9-anthryl)ethanol have been examined. The elution order in *n*-hexane, that contained a small percentage of a polar solvent, was shown to reverse when a D-configuration containing stationary phase was substituted for L-configuration on the surface of silica. Eluents, which contained different polar solvents (2-propanol and methylene chloride) in percentages that gave approximately the same retention times, gave different separation factors. Increases in the percentages of the polar solvents in the eluents decreased the retention times; however, for a given solvent, the separation factors remained constant. In addition, the effects of removing the butyloxycarbonyl (BOC) group from the immobilized valine and of substituting a benzyloxycarbonyl group resulted in a decrease with the separation factor. Bonding of second L-valine to the BOC-L-valine stationary phase gave an improved α value whereas additions of other amino acids usually decreased the separation factor, even when the acid had the same chirality.

INTRODUCTION

In the past decade there has been tremendous progress in the development of chromatographic separations of racemates[1–6]. Considerable interest has been focused on the resolution of stereoisomers by high-performance liquid chromatography (HPLC) using chiral stationary phases[3,4] or chiral mobile phases [7,8]. Pirkle and coworkers[9–17] introduced (*R*)-N-(3,5-dinitrobenzoyl)phenylglycine as a chiral stationary phase, and used it for HPLC either ionically or covalently bonded to γ-aminopropyl-silica. Its wide applicability for optical resolutions of various classes of organic compounds in nonaqueous solvents was demonstrated in a series of papers.

The present study follows up those studies and the related work by Fong and Grushka[18,19] and Howard *et al.*[20] in which a tripeptide-bonded stationary phase was

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0021-9673/87/$03.50

synthesized and then used to fractionate chiral dipeptides in aqueous solution. Our study was designed to evaluate the relative contributions of individual amino acids in the tripeptide-bonded stationary phase to separations of racemates. The first goal, therefore, was to prepare phases having a single optically active amino acid or a dipeptide on the surface of silica. Then, these phases were to be tested by using the same solute, the *R* and *S* forms of 2,2,2-trifluoro-1-(9-anthryl)ethanol (TFAE), so as to observe the effect of using different chiral phases in terms of the capacity ratio, k', and separation factor, α. Since completion of the present study, a similar, extensive study of the fractionation of dipeptide stereoisomers has been reported[7,8]. The results in our study are consistent with those. In addition, our NMR data[21] suggest how one might get additional useful information about their system.

The second goal was to study the effect on the elution order of reversing the configuration of the amino acid(s) in the stationary phase. It was also of interest to examine the effects on the retentions and the α values of removing the protecting group from the immobilized amino acid and of substituting a different protecting group.

In addition to single amino acid packings, dipeptide and tripeptide stationary phases were prepared so as to study the effects on the separation factor of the presence of more than one chiral center and of the location of an aromatic ring. Finally, it was of interest to observe the effects of increasing the percentages of polar solvents in the eluents on the k' and α values.

EXPERIMENTAL

Chemicals

All chemicals were J. T. Baker (Phillipsburg, NJ, U.S.A.) reagent-grade, HPLC-grade, or better unless otherwise stated. The following reagents for synthesizing the dipeptide and tripeptide using N-hydroxy-succinimide (HOSu), dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) were all obtained from Chemical Dynamics (South Plainfield, NJ, U.S.A.). The N-ethylmorpholine, 2-mercaptopyridine and triethylamine were obtained from Aldrich (Milwaukee, WI, U.S.A.).

The amino acid derivatives used in the syntheses were N-*tert.*-butyloxycarbonyl-L-valine (BOC-L-Val), L-alanine (L-Ala), D-Ala, L-proline (L-Pro), D-Pro, L-phenylalanine (L-Phe), D-Phe, L-valine methyl ester hydrochloride (L-Val-OMe · HCl), L-phenylalanine methyl ester hydrochloride (L-Phe-OMe · HCl), L-proline methyl ester hydrochloride (L-Pro-OMe · HCl) N-benzyloxycarbonyl-L-valine (CBZ-L-Val), L-tyrosine methyl ester hydrochloride (L-Tyr-OMe · HCl), CBZ-D-Val, L-alanine methyl ester hydrochloride (L-Ala-OMe · HCl), and D-Ala-OMe · HCl, all from Sigma (St. Louis, MO, U.S.A.).

The work-up of the coupling reactions employed house doubly-distilled, deionized water plus ligroine, *i.e.*, light petroleum b.p. 30–50°C and light petroleum b.p. 60–80°C, both from Eastman Kodak (Rochester, NY, U.S.A.); decolorizing carbon, decolorizing alkaline Norit-A, from Fisher Scientific (Norcross, GA, U.S.A.); isopropyl ether, methyl-cyclohexane, triethylamine from Aldrich; ethyl acetate, chloroform, diethyl ether, anhydrous magnesium sulfate, sodium chloride, citric acid monohydrate, sodium bicarbonate, 2-propanol (IPA), hydrochloric acid, sodium sul-

fate, methylene chloride (MCl), potassium hydroxide, methanol, sodium hydroxide, ethanol, hexane, and tetrahydrofuran (THF) from J. T. Baker. The THF was distilled over potassium prior to use.

LiChrospher Si 100 silica, (Alltech Assoc., Norcross, GA, U.S.A.) having a 10-μm particle diameter, was used for preparation of the bonded phases. The 4-aminobutyldimethylmethoxylsilane reagent used to bond the amino acid or peptide to silica was obtained from Petrarch Systems (Bristol, PA, U.S.A.).

The amino acid derivatives for bonding to silica were N-*tert.*-butyloxycarbonyl-L-alanine (BOC-L-Ala), Boc-L-Phe, BOC-L-isoleucine (BOC-L-Ile), BOC-L-leucine (BOC-L-Leu), Boc-D-Leu, BOC-L-Tyr, BOC-L-proline (BOC-L-Pro), all from Sigma.

The solvents used for bonding the silane reagent to silica and then the peptide to the silane-derivatized silica were toluene and THF. The toluene was either distilled or dried over calcium chloride before being stored over sodium. N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) from Aldrich was used to accelerate the bonding reaction. Formic acid (98%) and 5% palladium-on-charcoal from Aldrich were used to remove the BOC and CBZ protecting groups, respectively. Dimethoxyethane (DME) from Eastman Kodak, THF, acetonitrile and dioxane were used in the syntheses of the peptides. The DME was dried over sodium or molecular sieve 5A prior to use.

Isooctane, cyclohexanol, carbon tetrachloride, and IPA were used to pack the columns. Hexane, MCl, and IPA were used to wash the columns after packing.

The *R*(−) and *S*(+) forms of 2,2,2-trifluoro-1-(9-anthryl)-ethanol (TFAE) were used as test solutes to evaluate the peptide-bonded column packings. All solvents were filtered using a 0.5-μm FH filter for organics (Millipore, Bedford, MA, U.S.A.) and were thoroughly degassed with helium before use. Nitrogen, helium and hydrogen were obtained from Selox (Atlanta, GA, U.S.A.).

Apparatus

Two chromatographic systems were employed in this study, a microbore system and a conventional system. The microbore system consisted of an isocratic Varian 8500 syringe pump (Varian; Sunnyvale, CA, U.S.A.) with mixing devices which had been modified for a microbore column,[22] an electronically-actuated injector valve (Model EC14W; Valco, Houston, TX, U.S.A.) with an internal 0.5-μl sample loop, a variable-wavelength UV–VIS detector (Model SF769Z, Kratos Instruments, Ramsey, NJ, U.S.A.) set at 254 nm and 0.02 a.u.f.s., and a strip-chart recorder (Model 4523, Houston Instrument; Austin, TX, U.S.A.).

The conventional system consisted of a solvent metering pump (Model 110A, Altex; Berkeley, CA, U.S.A.), a Rheodyne 7125 injection valve having a 10-μl loop, a variable-wavelength detector (Model SF770p, Kratos) set at 254 nm and a chart recorder (Model 385, Linear Instruments Co.; Reno, NV, U.S.A.) or a PRO 350 microcomputer (Model PC-350-D2, Digital Equipment Co.; Maynard, MA, U.S.A.). The latter recorded directly through a real-time module (Model ADMPC-A2) equipped with letter printer (Model LA-100-PC). All programming was accomplished using the UGA Scientific Applications Package developed for the PRO 350 by Dr. L. A. Carreira.

Procedures

Preparations of dipeptides and tripeptides. These were prepared using a variety

of reactions which are described below. The preparation of an active ester and its coupling will be described first, following by coupling with DCC and, finally, removal of the protecting groups.

N-Hydroxysuccinimide ester[23]. The N-*tert.*-butyloxycarbonyl amino acid (BOC-AA) or (BOC-AA-AA) (50 mmol) and HOSu (50 mmol.) were dissolved together in anhydrous dimethoxyethane (40 ml) at 0°C. The DCC (50 mmol and 20% excess), also dissolved in DME, was added to the mixture slowly while stirring, and the solution kept at 0–5°C for 48 h. The precipitate of dicyclohexylurea which formed was removed using a sintered glass funnel and the filtrate evaporated to dryness in a rotating evaporator, leaving a residue of crude product. Two successive recrystallizations from methylcyclohexane–methylene chloride (MCH–MCl) or from IPA gave the pure product, N-hydroxysuccimide ester of N-*tert.*-butyloxycarbonyl amino acid (BOC-AA- OSu or BOC-AA-AA-OSu).

The synthetic results are summarized below:

N-Hydroxysuccinimide ester of butyloxycarbonyl-L-valine (BOC-L-Val-Osu), yield 86%,m.p. 127–129°C. Calculated for $C_{14}H_{22}N_2O_6$: C 53.49%; H 7.05%; N 8.91%. Found: C 53.56%; H 7.07%; N 8.90%.

BOC-D-Val-OSu, yield 83%, m.p. 126–128°C. Calculated for $C_{14}H_{22}N_2O_6$: C 53.4%; H 7.05%; N 8.91%. Found: C 53.5%; H 7.07%; N 8.86%.

BOC-L-Val-L-Ala-OSu, yield 57%; m.p. 178–181°C. Calculated for $C_{17}H_{27}N_3O_7$: C 53.01%; H 7.00%; N 11.02%. Found: C 53.08%; H 7.07%; N 10.90%.

BOC-L-Val-D-Ala-OSu, yield 20%, m.p. 168–170°C. Calculated for $C_{17}H_{27}N_3O_7$: C 53.01%; H 7.00%; N 11.02%. Found: C 52.89%; H 7.07%; N 10.81%.

BOC-L-Val.-L-Phe-OSu, yield 70%; m.p. 162–164°C. Calculated for $C_{23}H_{31}N_3O_7$: C 59.87%; H 6.72%; N 9.11%. Found: C 59.89%; H 6.77%; N 9.09%.

BOC-L-Val-D-Phe-OSu, yield 63%; m.p. 159–161°C. Calculated for $C_{23}H_{31}N_3O_7$: C 59.87%; H 6.72%; N 9.11%. Found: C 59.88%; H 6.77%; N 9.03%.

BOC-L-Val-L-Val-OSu, yield 17%, m.p. 165–167°C. Calculated for $C_{19}H_{31}N_3O_7$: C 55.21%; H 7.51%; N 10.17%. Found: C 55.29%; H 7.57%; N 10.01%.

BOC-D-Val-D-Val-OSu, yield 21%, m.p.172–175°C. Calculated for $C_{19}H_{31}N_3O_7$; C 55.2%; H 7.51%; N 10.17%. Found: C 55.10%; H 7.11%; N 10.87%.

BOC-L-Val-L-Ile-OSu, yield 27%, m.p. 133–136°C. Calculated for $C_{19}H_{31}N_3O_7$: C 56.21%; H 7.73%; N 9.84%. Found: C 56.33%; H 7.81%; N 9.82%.

2-Pyridyl thiolester of valine[24]. A solution of dicyclohexylcarbodiimide (45 mmole) in ethyl acetate (60 ml) was added dropwise to a stirred solution of the N-*tert.*-butyloxycarbonyl-L-valine (45 mmol) and 2-mercaptopyridine (45 mmol) in ethyl acetate (100 ml) at −18°C. After stirring for another 30 min at −18°C, the temperature was allowed to rise. The next day the solution was filtered and the filtrate washed successively with saturated sodium chloride (brine), saturated sodium carbonate, and brine. The organic layer was dried over magnesium sulfate, and evap-

orated *in vacuo*. The residue was recrystallized from ethyl acetate–light petroleum b.p. 60–80°C to yield the pure product 2-pyridyl thiolester of N-*tert.*-butyloxycarbonyl-L-valine (BOC-L-Val-SPy). Yield 89%, m.p. 119–120°C. Calculated for $C_{15}H_{22}N_2O_3S$: C 58.1%; H 7.2%; N 9.0%; S 10.3%. Found: C 57.9%; H 7.1%; N 9.1%; S 10.2%.

Acylation with N-hydroxysuccinimide ester[23]. A solution of BOC-AA-OSu or BOC-AA-AA-OSu (20 mmol) in DME (40 ml) was slowly added to a solution of amino acid (20 mmol) dissolved in water (20 ml) and adjusted at room temperature to pH = pK_2 of the amino acid using sodium bicarbonate and 1 *M* sodium hydroxide. After three days, 20 ml of water was added and the solution evaporated in a rotating evaporator for 20 min below 40°C. After cooling to room temperature the solution was acidified with citric acid to pH 2 and then saturated with ammonium chloride before being extracted three times with 50 ml of choroform. The chloroform extracts were combined, washed with 50 ml of water, dried over magnesium sulfate and concentrated in vacuo to yield the crude product. Two successive recrystallizations from ethyl acetate or ethyl acetate–hexane or ethyl acetate–light petroleum b.p. 60–80°C gave the pure dipeptide product or tripeptide, the BOC drivative of the free acid [BOC-AA-AA or BOC-AA-AA-AA].

N-*Tert.*-Butyloxycarbonyl-L-valyl-L-alanine (BOC-L-Val-L-Ala), yield 72%, m.p. 154–156°C. Calculated for $C_{13}H_{24}N_2O_5$: C 54.17%; H 8.33%; N 9.72%. Found: C 54.30%; H 8.40%; N 9.69%.

BOC-L-Val-D-Ala, yield 62%, m.p. 162–164°C. Calculated for $C_{13}H_{24}N_2O_5$: C 54.17%; H 8.33%; N 9.72%. Found: C 54.28%; H 8.37%; N 9.70%.

BOC-L-Val-L-Phe, yield 65%, m.p. 136–138°C. Calculated for $C_{19}H_{28}N_2O_5$: C 62.64%; H 7.69%; N 7.69%. Found: C 62.44%; H 7.33%; N 7.64%.

BOC-L-Val-D-Phe, yield 68%, m.p. 134°C. Calculated for $C_{19}H_{28}N_2O_5$: C 62.64%; H 7.69%; N 7.69%. Found: C 62.76%; H 7.75%; N 7.66%.

BOC-L-Val-L-Val, yield 43%, m.p. 159–161°C. Calculated for $C_{15}H_{28}N_2O_5$: C 56.96%; H 8.86%; N 8.86%. Found: C 57.23%; H 9.01%; N 8.61%.

BOC-L-Val-L-Ile, yield 60%, m.p. 146–148°C. Calculated for $C_{16}H_{30}N_2O_5$: C 58.18%; H 9.09%; N 8.48%. Found: C 58.29%; H 9.17%; N 8.44%.

BOC-L-Val-L-Pro, yield 50%, m.p. 154–156°C. Calculated for $C_{15}H_{26}N_2O_5$: C 57.33%; H 8.28%; N 8.92%. Found: C 57.18%; H 8.37: N 8.87%.

BOC-D-Val-L-Ala, yield 65%, m.p. 164–166°C. Calculated for $C_{13}H_{24}N_2O_5$: C 54.17%; H 8.33%; N 9.72%. Found: C 54.21%; H 8.42%; N 9.72%.

BOC-D-Val-D-Ala, yield 62%, m.p. 154–156°C. Calculated for $C_{13}H_{24}N_2O_5$: C 54.17%; H 8.33%; N 9.72%. Found: C 54.27%; H 8.42%; N 9.68%.

BOC-D-Val-D-Val, yield 76%, m.p. 160–162°C. Calculated for $C_{15}H_{28}N_2O_5$: C 56.96%; H 8.86%; N 8.86%. Found: C 56.83%; H 8.95%; N 8.57%.

BOC-D-Val-L-Ile, yield 62%, m.p. 150–152°C. Calculated for $C_{16}H_{30}N_2O_5$: C 58.18%; H 9.09%; N 8.48%. Found: C 58.13%; H 9.16%; N 8.38%.

BOC-L-Val-D-Phe-L-Pro, yield 34%, m.p. 113–125°C. Calculated for $C_{24}H_{35}N_3O_6$: C 62.47%; H 7.59%; N 9.11%. Found: C 62.01%; H 7.88%; N 9.45%.

BOC-L-Val-L-Ala-L-Pro, yield 40%, m.p. 189–191°C. Calculated for $C_{18}H_{31}N_3O_6$: C 56.10%; H 8.05%; N 10.91%. Found: C 56.10%; H 8.18%; N 10.46%.

BOC-L-Val-D-Ala-L-Pro, yield 25%, m.p. 189–192°C. Calculated for $C_{18}H_{31}N_3O_6$: C 56.10%; H 8.05%; N 10.91%. Found: C 56.30%; H 7.98%; N 10.80%.

BOC-D-Val-D-Ala-D-Pro, yield 25%, m.p. 188–192°C. Calculated for $C_{18}H_{31}N_3O_6$: C 56.10%; H 8.05%; N 10.91%. Found: C 56.02%; H 8.20%; N 10.50%.

Acylation with 2-pyridyl thiolester (SPy)[24]. The BOC-L-Val-SPy (25 mmol) was added to the solution of L-isoleucine methyl ester hydrochloride (30.5 mmol) with triethylamine (30.5 mmol) in dioxane (40 ml). After 1 h, the mixture was poured into the brine and the product extracted with ethyl acetate. The combined extracts were washed successively with 1 *N* hydrochloride, aqueous sodium bicarbonate and brine before being dried over magnesium sulfate. After decolorization with carbon and evaporation of the solvent, the crude product was recrystallized from ethyl acetate to yield the pure product, N-*tert.*-butyloxycarbonyl-L-valyl-L-isoleucine methyl ester (BOC-L-Val-L-Ile-OMe), yield 76%, m.p. 149–151°C.

Coupling using DCC[25]. The L-tyrosine methyl ester was prepared from L-tyrosine methyl ester hydrochloride by treatment with alcoholic potassium hydroxide. L-Tyrosine methyl ester (10 mmol) was dissolved in 100 ml of acetonitrile by warming. N-Protected-valine (10 mmol) was added and the mixture cooled to 0°C. DCC (12 mmol) was added and the reaction mixture was stirred for 4 h at 0°C followed by stirring overnight at room temperature. Dicyclohexylurea was filtered off and washed with 50 ml of hot acetone. The filtrate and washings were concentrated *in vacuo* and the residue crystallized from ethyl acetate to give the protected dipeptide methyl ester.

N-Benzyloxycarbonyl-L-valyl-L-tyrosine methyl ester (CBZ-L-Val-L-Tyr-OMe), yield: 79.3%, m.p. 155–156°C. Calculated for $C_{23}H_{28}N_2O_6$: C 64.49%; H 6.54%; N 6.54%. Found: C 64.32%; H 6.62%; N 6.51%.

N-Butyloxycarbonyl-L-valyl-L-tyrosine methyl ester (BOC-L-Val-L-Tyr-OMe), yield 67%, m.p. 165°C. Calculated for $C_{20}H_{30}N_2O_6$: C 60.91%; H 7.61%; N 7.11%. Found: C 60.99%; H 7.63%; N 7.10%.

Coupling with DCC in the presence of HOBt[26]. N-Protected-amino acid (or BOC-AA-AA) (10 mmol), amino acid methyl ester hydrochloride (or dipeptide methyl ester hydrochloride) (10 mmol) and 1-hydroxybenzotriazole hydrate (20 mmol) were dissolved in freshly distilled THF following which N-ethylmorpholine (10 mmol) was added while stirring. The solution was then cooled to 0°C before adding DCC (10.6 mmol) dissolved in THF. The mixture was stirred for at least 4 h at 0°C and then overnight at room temperature. (In the case of BOC-(L-Val)$_3$-OMe, the solvent MCl was used and the reaction time extended to 4 days at 0°C). The precipitate was filtered off using suction and the filtrate concentrated. The residue was dissolved in a two-phase 2:1 mixture of ethyl acetate and saturated sodium bicarbonate solution. After removing the aqueous phase, the organic phase was washed successively with 2 *M* hydrochloric acid or 10% citric acid followed by water, saturated sodium bicarbonate solution, and then water before being dried over sodium sulfate. The ethyl acetate was then removed by evaporation. The residue was triturated with light petroleum, b.p. 30–50°C, to remove impurities before the remaining product was recrystallized from ethyl acetate–light petroleum b.p. 60–80°C.

N-Benzyloxycarbonyl-L-valyl-L-valine methyl ester (CBZ-L-Val-L-Val-OMe), yield 97%, m.p. 104–106°C. Calculated for $C_{19}H_{28}N_2O_5$: C 62.64%; H 7.69%; N 7.69%. Found: C 63.04%; H 7.94%; N 7.81%.

BOC-L-Val-OMe, yield 70%; m.p. 167°C. Calculated for $C_{16}H_{30}N_2O_5$: C 58.18%; H 9.09%; N 8.48%. Found: C 58.22%; H 9.16%; N 8.43%.

BOC-L-Phe-L-Val-OMe, yield 86%, m.p. 120–122°C. Calculated for $C_{20}H_{30}N_2O_5$: C 63.50%; H 7.94%; N 7.41%. Found: C 63.57%; H 8.10%; N 7.49%.

BOC-L-Val-L-Phe-OMe, yield 91%, m.p. 112–113°C. Calculated for $C_{20}H_{30}N_2O_5$: C 63.50%; H 7.94%; N 7.41%. Found: C 63.64%; H 7.99%; N 7.44%.

BOC-L-Val-L-Val-L-Val-OMe, yield 68%, m.p. 167–172°C. Calculated for $C_{21}H_{39}N_3O_6$: C 58.74%; H 9.09%; N 9.79%. Found: C 58.84%; H 9.19%; N 9.75%.

BOC-L-Val-D-Phe-L-Pro-OMe, yield 72.7%, oily.

BOC-L-Val-NH-$(CH_2)_4$-Si$(CH_3)_2$-OMe (BOC-L-Val-silane), yield 72%. Calculated for $C_{17}H_{36}N_2O_4Si$: C 56.67%; H 10.00%; N 7.78%. Found: C 55.97%; H 9.90%; N 8.26%.

Hydrogenation[27]. For hydrogenolysis of the N-benzyloxycarbonyl group, a solution of N-CBZ-L-Val-L-Val-OMe (8 mmol) in methanol (50 ml) was prepared in a three-necked round-bottom flask (100 ml) provided with a magnetic stirrer, a gas inlet-outlet tube, and surrounded by a large evaporating dish. The air was displaced by a slow stream of nitrogen before a 5% palladium-on-charcoal catalyst (1 g) and hydrochloric acid (8 mmol) were added. A slow stream of hydrogen was bubbled through the liquid for 3 h at room temperature. The remaining hydrogen was then displaced by nitrogen and the catalyst removed by filtration through diatomaceous earth (celite). The filtrate was concentrated to dryness in vacuum and the residue dried in air before finally being dried over P_2O_5 *in vacuo*.

Val-Val-OMe · HCl, yield 77.6%, m.p. 95–105°C. Calculated for $C_{11}H_{23}N_2O_3Cl$: C 49.35%; H 8.63%; N 10.51%. Found: C 50.91%; H 8.60%; N 9.41%.

Hydrolysis[28]. For base-catalyzed hydrolysis of alkyl esters, a solution of N-protected dipeptide methyl ester (10 mmol) in methanol (10 ml) was surrounded by a water bath at room temperature. Then 1 *N* sodium hydroxide (10 mmol) was added while stirring. The mixture was kept at room temperature for 2 h before 10% citric acid (10 ml) was added. Methanol was removed by a rotating evaporator. The aqueous solution was stirred during adjustment to pH 2 using citric acid following which sodium chloride was added to salt out the product when the flask was cooled in an ice-water bath for 2 h. The mixture was extracted with three 50-ml portions of ethyl acetate. The combined ethyl acetate extracts were washed with water and dried over magnesium sulfate before concentration *in vacuo*. Two successive recrystallizations from ethyl acetate–diethyl ether or ethyl acetate–hexane or ethanol–water yielded the N-protected amino acid.

BOC-L-Val-L-Val-L-Val, yield 70%, m.p. 155–160°C. Calculated for $C_{20}H_{37}O_6N_3$: C 57.83%; H 8.92%; N 10.12%. Found: C 57.97%; H 9.02%; N 9.89%.

N-*tert.*-Butyloxycarbonyl-L-valyl-L-phenylalanine ((BOC-L-Val-L-Phe), yield 75%, m.p. 136–139°C.

BOC-L-Val-D-Phe-L-Pro, yield 29.4%, m.p. 115–120°C. Calculated for $C_{24}H_{35}N_3O_6$: C 62.47%; H 7.59%; N 9.11%. Found: C 61.84%; H 7.77%; N 8.87%.

BOC-L-Val-L-Ala, yield 87.9%, m.p. 154–156°C. BOC-L-Val-D-Ala, yield 70%, m.p. 165–166°C.

Acidolysis[29]. The N-*tert.*-butyloxycarbonyl dipeptide methyl ester (6 mmol) was dissolved in 3 *M* hydrochloric acid in THF (16 ml) and kept for 30 min at room temperature. The mixture was washed twice with diethyl ether (20 ml), and evaporated to dryness and then dried over potassium hydroxide pellets in a vacuum oven. The dipeptide methyl ester hydrochloride was crystallized from methanol–diethyl ether.

L-Val-L-Val-OMe · HCl, yield 68.4%, m.p. 145–150°C. Calculated for $C_{11}H_{22}N_2O_3 \cdot HCl$: C 49.53%; H 8.63%; N 10.51%. Found: C 49.18%; H 8.78%; N 10.14%.

Preparation of derivatized silica. The 4-aminobutyl-derivatized silica for bonding the amino acid or peptide to the silica was prepared by suspending silica gel Si 100 (2 g) in dry toluene (50 ml) and then adding the silane reagent, 4-aminobutyldimethylmethyloxysilane (1 ml). The reaction was refluxed at 110°C for 48 h with removal of water by azeotropic distillation. The derivatized silica was isolated by filtration and washed successively with 100 ml each of toluene, methanol, diethyl ether and finally pentane before being dried at 110°C overnight *in vacuo*. Elemental analysis: Found 6.5% carbon, 1.75% hydrogen, 1.12% nitrogen (Atlantic Microlab, Inc., Atlanta, GA, U.S.A.). Calculated: 4.23 μmol/m^2 (based on carbon), 3.67 μmol/m^2 (based on nitrogen). Calculations of surface coverage were made by using the formulas of Berendsen and De Galan[30], assuming a surface area of 250 m^2/g for LiChrospher Si 100.

The N-protected amino acid or peptide was bonded to the 4-amino butyl-derivatized silica (spacer-silica) by suspending the latter (2 g) in dry THF (100 ml) followed by adding the N-protected amino acid or peptide (8 mmol) and N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) (8 mmol) while swirling[9]. After 24 h at room temperature, the silica was collected by filtration and washed successively with 100-ml portions of the following solvents: THF, methanol, acetone and diethyl ether. Fines were removed by adding the chiral bonded-phase silica to a 250-ml graduated cylinder that contained methanol. The mixture was stirred and the fines were decanted after the larger particles had fallen to the bottom of the cylinder. After being dried at 110°C *in vacuo* overnight, the silica was ready for packing into the column.

The BOC-L-Val-spacer packings were synthesized using 4 mmol of BOC-L-Val-silane and 1 g of LiChrosphere Si 100 and 50 ml of toluene. The BOC-D-Val (dilute) packing was synthesized using 100 ml of dry THF, 2 g of the spacer-silica, 0.8 mmol of BOC-D-Val and 0.8 mmol of EEDQ.

The results from the elemental analyses and the surface coverage calculations of bonded phases are shown in Table I. The surface coverages of the chiral groups were calculated from the difference of carbon percentage for the chiral bonded-phase silica and the silane-derivatized silica except for the BOC-L-Val-spacer packing[30].

Packing the HPLC columns. The microbore columns were packed using a slurry procedure similar to that reported by Powley *et al.*[22]. The tubing [1.59 mm O.D., 1 mm I.D. (Alltech Assoc.)] was cut into 25-cm pieces. The inner bore of this tubing was polished by pumping a slurry of Carborundum 600 polishing compound in water using a recirculating peristaltic pump for 24 h. The viscosity technique was used to

TABLE I

ELEMENTAL ANALYSES AND SURFACE COVERAGES OF HPLC PACKINGS

HPLC packing	*C%*	*H%*	*N%*	*μmol/m²*
Amino acid-derivatized silica				
BOC-L-Val	11.49	2.18	1.49	2.14
BOC-L-Ile	10.52	2.16	1.22	1.48
BOC-L-Leu	11.98	2.47	1.36	2.10
BOC-L-Phe	12.66	2.18	1.29	1.77
BOC-L-Tyr	11.39	2.10	1.29	1.41
BOC-L-Ala	10.29	2.19	1.49	1.87
CBZ-Val	13.13	2.11	1.54	2.05
BOC-D-Val	11.14	2.26	1.41	1.89
CBZ-Val	9.95	1.77	1.12	1.50
BOC-L-Val-spacer	8.09	1.69	1.16	1.85
BOC-D-Val (dilute)	5.15	1.42	0.75	0.19
BOC-L-Pro	10.08	2.10	1.42	1.47
Dipeptide-derivatized silica				
BOC-L-Val-L-Ala	11.07	2.35	1.98	1.36
BOC-L-Val-L-Val	10.62	2.23	1.68	1.17
BOC-L-Val-D-Ala	11.93	2.30	1.98	1.72
BOC-D-Val-L-Ala	11.26	2.28	1.87	1.51
BOC-L-Val-L-Phe	13.36	2.24	1.76	1.67
BOC-D-Val-L-Ile	10.08	2.14	1.33	0.73
BOC-D-Val-D-Val	11.06	2.25	1.56	1.29
Trieptide-derivatized silica				
BOC-L-Val-L-Ala-L-Pro	13.45	2.54	2.55	1.55
BOC-L-Val-D-Ala-L-Pro	12.28	2.30	1.97	1.28
BOC-L-Val-D-Phe-L-Pro	14.54	2.32	2.00	1.35
BOC-D-Val-D-Ala-D-Pro	13.42	2.50	2.50	1.55
BOC-L-Val-L-Val-L-Val	10.97	2.17	1.85	0.92

pack the columns[31]. The column was filled with tetrachloromethane before packing was started, and a slurry of the packing material (10%, w/v) in IPA–cyclohexanol (1:3, v/v) was poured into the slurry reservoir which was 25 cm × 4.6 mm I.D. tubing. A constant-pressure air-driven reciprocating pump (Model 10-600-50, SC Hydraulics; Los Angeles, CA, U.S.A.) was used to force isooctane through the column at 22000 p.s.i. Pure isoctane was allowed to pass through the column for 1 h, after which the "slamming" technique[32], in which the column is allowed to depressurize and then is rapidly repressurized, was used several times to consolidate the packed bed. The column was connected directly to the injector port, which contained a 2-μm porosity, 1.59 mm diameter screen (Valco). A 1.59 mm diameter, 0.79 mm thick, 0.5-μm porosity frit (Valco) was used in the detector flow-cell inlet port.

The conventional HPLC columns used in this study were 25 cm × 3.2 mm I.D. columns which were packed in our laboratory using the viscosity technique. A 10% slurry of the bonded-phase silica in cyclohexanol–2-propanol (3:1, v/v) was placed in the slurry packer and displaced into the column using isooctane at a pressure of 10000 p.s.i. Carbon tetrachloride, placed in the column prior to packing,

prevented the slurry from settling into the column. Hexane, 20% methylene chloride in hexane, and 2% 2-propanol in hexane were used successively to wash the column prior to evaluation.

Analytical procedure. The flow-rate of the mobile phase was 3 ml/h for the microbore columns and 1 ml/min for the conventional columns. The microbore column was equilibrated with the appropriate mobile phase for 16 h prior to a chromatographic run whereas the conventional column was equilibrated for 3 h. The *R,S*-TFAE mixture was injected at concentrations of 100 μg/ml solution in hexane using a 0.5-μg sample loop for microbore columns and at concentrations of 20 μg/ml solution using 10-μl sample loop for conventional columns. Columns were usually flushed with hexane before being stored overnight.

The time (volume) for a nonretained substance, used in calculating the capacity factor, k', was determined by adding toluene to the sample solution. Because peaks for the sample solution. Because peaks for the solutes were quite symmetrical, the retention times (volumes) were taken at the peak maximum. The capacity factor was calculated using $k' = (t_R - t_0)/t_0$ where t_R is the retention time of the component of interest and t_0 is the value for an unretained peak. The separation factor, α, was calculated as k'_2/k'_1 where $k'_2 > k'_1$. If the *R,S*-TFAE mixture failed to resolve separate peaks in a single chromatographic run, the individual components of the mixture were injected separately at a 5-min interval.

RESULTS AND DISCUSSION

Elution order

Using either pure *R*- or *S*-TFAE and an eluent of hexane, which contained either 2-propanol or methylene chloride, the *R*-TFAE was the first to elute from all the columns in which an L-configuration of the amino acid or dipeptide was on the stationary phase. The reverse order was found for a D-configuration amino acid on the surface. Fig. 1 shows a typical separation using 20% methylene chloride in hexane. Table II summarizes the chromatographic results obtained with different chiral stationary phases. Separation factors for all of these stationary phases were clearly different from 1.00. The order of elution, in general, agreed with that reported by Pirkle and co-workers[9,10]. Note that BOC-L-Ile had the same α value as the BOC-L-Val packing but the corresponding Phe, Ala and Leu were significantly smaller. The effects of using di- and tri-peptides will be discussed later.

Effect of eluent composition

The average k' and α values of *R,S*-TFAE separations from five replicate runs are given in Table III. In general, an increase in the percentage of a polar solvent in the eluent decreased the retention time, but for a given eluent, the α value remained essentially constant. The eluent compositions that gave approximately the same retention times for two different polar solvents, *e.g.*, 25% methylene chloride or 1% 2-propanol in hexane, gave different α values for the BOC-D-Val-D-Val column. A similar effect was also found on the BOC-D-Val column. Unlike methylene chloride, 2-propanol may be more effective as a mobile phase additive with respect to TFAE because it, too, is an alcohol. As the 2-propanol content in the mobile phase increased, its displacement by TFAE became more and more difficult. In contrast,

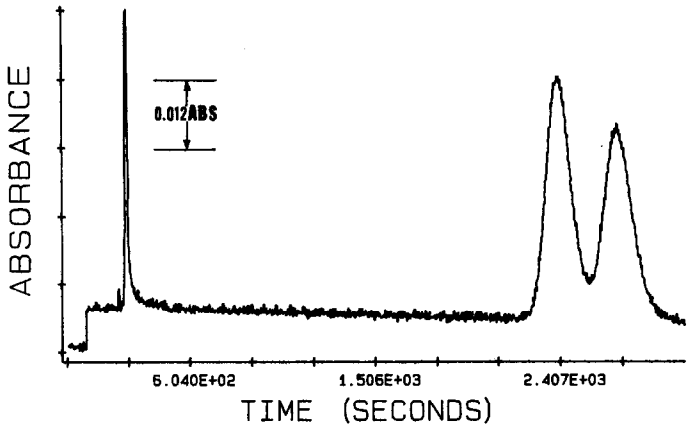

Fig. 1. Chromatogram of the *R,S*-TFAE separation on a BOC-L-Val-L-Val column using 20% methylene chloride in hexane. Column dimensions: 25 cm × 1 mm I.D. Flow-rate: 3 ml/h. Detection: at 254 nm.

TABLE II

SEPARATION OF *R,S*-TFAE ON DIFFERENT CHIRAL STATIONARY PHASES

Mobile phases: A, 1% 2-propanol in hexane; B, 20% methylene chloride in hexane; C, 2% 2-propanol in hexane; D, 30% methylene chloride in hexane; E, 40% methylene chloride in hexane.

Stationary phase	*Separation factor, α*	*Capacity factor of 1st eluted enantiomer*	*Mobile phase*	*Absolute configuration of 1st eluted enantiomer*
(a) BOC-L-Val	1.08	6.89	A	*R*
(b) BOC-L-Ile	1.10	5.92	A	*R*
(c) CBZ-L-Val	1.03	7.01	A	*R*
(d) BOC-L-Phe	1.05	9.41	A	*R*
(e) BOC-L-Val-L-Val	1.10	7.63	A	*R*
(f) BOC-L-Val-L-Phe	1.04	2.19	C	*R*
(g) BOC-L-Val-L-Ala	1.05	5.01	A	*R*
(h) BOC-D-Val	1.10	4.41	A	*S*
(i) CBZ-D-Val	1.04	5.34	A	*S*
(j) BOC-D-Val-D-Val	1.09	4.13	A	*S*
(k) BOC-L-Val	1.10	4.65	B	*R*
(l) CBZ-L-Val	1.05	5.80	B	*R*
(m) BOC-L-Ala	1.02	6.10	B	*R*
(n) BOC-L-Ile	1.09	5.88	B	*R*
(o) BOC-L-Phe	1.03	4.66	B	*R*
(p) BOC-L-Leu	1.05	5.36	B	*R*
(q) BOC-L-Val-L-Val	1.18	7.65	B	*R*
(r) BOC-L-Val-L-Phe	1.06	7.54	B	*R*
(s) BOC-L-Val-L-Ala	1.07	5.45	B	*R*
(t) BOC-L-Val-L-Ala-L-Pro	1.05	5.14	D	*R*
(u) BOC-D-Val	1.11	5.66	B	*S*
(v) CBZ-D-Val	1.07	5.35	B	*S*
(w) BOC-D-Leu	1.03	4.44	B	*S*
(x) BOC-D-Val-D-Val	1.16	6.61	B	*S*
(y) BOC-D-Val-D-Ala-D-Pro	1.07	3.20	E	*S*
(z) BOC-L-Val-L-Val-L-Val	1.20	3.60	D	*R*

TABLE III

EFFECT OF ELUENT COMPOSITION ON THREE CHIRAL STATIONARY PHASES FOR SEPARATION OF *R,S*-TFAE

Eluent composition	*Stationary phase*			
	BOC-D-*Val*-D-*Val*		*BOC*-L-*Val*-L-*Val*	*BOC*-D-*Val*
	k'*	α	α	α
Hexane–methylene chloride				
80:20	6.61 ± 0.18	1.158 ± 0.035	1.184 ± 0.014	1.082 ± 0.008
75:25	4.34 ± 0.29	1.142 ± 0.025	–	–
60:40	2.60 ± 0.05	1.174 ± 0.064	1.159 ± 0.037	1.097 ± 0.009
Hexane–2-propanol				
99:1	4.13 ± 0.15	1.090 ± 0.006	1.097 ± 0.009	1.094 ± 0.012
98.5:1.5	2.36 ± 0.02	1.085 ± 0.007	–	1.085 ± 0.006
98:2	1.97 ± 0.04	1.099 ± 0.011	1.082 ± 0.010	1.090 ± 0.007

* *S*-TFAE is eluted first.

when methylene chloride of the same percentage was substituted for 2-propanol in the mobile phase, the TFAE apparently displaced the adsorbed solvent more readily. Thus, approximately the same k' value resulted when one used 1% of 2-propanol having a polarity of 0.14 and 25% methylene chloride having a polarity of 0.85 (ref. 32).

Effect of an aromatic ring and the BOC group

To evalute the contributions of these groups to the fractionations and to reveal

TABLE IV

CHROMATOGRAPHIC DATA FOR *R*- AND *S*-TFAE USING DIFFERENT CHIRAL STATIONARY PHASES

Stationary phase	*Mobile phase*			
	1% IPA in hexane		*20% MCl in hexane*	
	k'*	α	k'*	α
BOC-L-Val	6.89 ± 0.39	1.095 ± 0.022	4.65 ± 0.12	1.105 ± 0.017
CBZ-L-Val	7.01 ± 0.20	1.025 ± 0.006	5.80 ± 0.05	1.050 ± 0.014
L-Val**	5.64 ± 0.03	1.045 ± 0.009	5.92 ± 0.12	1.061 ± 0.008
BOC-D-Val	5.31 ± 0.18	1.094 ± 0.011	5.66 ± 0.13	1.112 ± 0.013
CBZ-D-Val	5.34 ± 0.09	1.040 ± 0.006	5.35 ± 0.16	1.071 ± 0.018
BOC-L-Ala	5.41 ± 0.11	1.003 ± 0.003	6.35 ± 0.09	1.016 ± 0.013
BOC-L-Phe	9.41 ± 0.28	1.049 ± 0.007	4.66 ± 0.04	1.034 ± 0.015
BOC-L-Val-spacer***	6.40 ± 0.20	1.039 ± 0.004	8.44 ± 0.29	1.077 ± 0.016
BOC-D-Val (dilute)§	3.45 ± 0.02	1.018 ± 0.001	–	–

* Capacity factor for first eluted enantiomer.

** BOC group removed from BOC-L-Val packing using formic acid.

*** Spacer combined with BOC-L-Val before bonding to the silica.

§ Less concentrated "regular" BOC-D-Val bonded on the silica surface.

some details about the chiral recognition mechanism, two sets of experiments were run. First, an aromatic ring was placed either in the protecting group or in the amino acid itself. Second, the BOC group was removed by formic acid[20]. Table IV shows the chromatographic data, which are averages of five replicates, that were obtained for different chiral stationary phases using two different eluents. In general, the α values on the BOC-L-Val stationary phase were larger than those on CBZ-L-Val stationary phase in both eluents, while the k' values on the latter were either close to those on the former or somewhat larger. The same trend was found on the BOC-D-Val and CBZ-D-Val columns as well as on the BOC-L-Val and BOC-L-Phe columns. These data suggest that adding the aromatic ring either in the protecting group or in the amino acid gives a smaller α value. On the other hand, while the α values of the BOC-L-Phe column are larger than those of BOC-L-Ala column, the k' value of the latter is larger using 20% methylene chloride in hexane than that of the former. Adding an aromatic ring to the BOC-L-Ala stationary phase in either place resulted in an improved α value, probably because the aromatic ring may act as a π-donor to the anthryl group of the solute. This kind of interaction was also observed by other authors in comparable cases[7–10].

With respect to the function of the BOC group, the α values on the BOC-L-Val column were a little larger than those on the L-Val column in both eluents while the k' value of the latter was not necessarily smaller than that of the former. These data indicate that the BOC group attached to the nitrogen atom assists the resolution of the enantiomers while not necessarily resulting in longer retention. It is reasonable to suggest that hydrogen bonding between the carbinyl hydrogen of the solute and NH group of the stationary phase also contributes to enantioselective absorption. The increase or decrease in the k' value probably reflects solvent competition for the silanols which may, in turn, play a minor role in the differentiation of the racemates compared to the role of the alkyl chain reported by Roumeliotis *et al.*[4].

Changes in derivatization procedure

The α values on both the BOC-L-Val-spacer column and the BOC-D-Val (dilute) column are smaller than those on the "regular" BOC-L-Val and BOC-D-Val columns. The decreases may be due to two different causes. First, although the concentration of BOC-L-Val was nearly the same for the packing derivatized first with spacer alone and for the one to which BOC-L-Val was first added to the spacer, the latter had more unreacted silanols on it. (In the regular procedure, most of those silanols reacted with spacer but did not, according to calculations of carbon content, react later with the BOC-L-Val.) In the second case, the lower concentration of the BOC-D-Val in the regular but more dilute, preparation probably accounts for the lower α.

Interesting differences were also seen in the retentions, although care must be taken in attaching too much significance to absolute values of k' for different columns. The k' values of the "spacer" and "dilute" phases were smaller when using 1% 2-propanol in hexane. However, the k' value of the BOC-L-Val-spacer column was larger than that of the BOC-L-Val column using 20% methylene chloride in hexane. These data, especially the reversal with solvent change of the order of k' value for the regular and "spacer" preparations must reflect the presence of a retention mechanism independent of chiral recognition. The presence of residual silanol

groups and spacer groups on the silica surface are possible sources of the solvent-dependent adsorption. Further experiments should give more information about the mechanism of adsorption.

Effects of additional chiral centers

BOC-Dipeptide and BOC-tripeptide stationary phases that have been synthesized were reported in Table II. The performances of these dipeptide stationary phases were examined in more detail in two different mobile phases using *R,S*-TFAE enantiomers in Table V. Table II indicated that the k' value of dipeptide stationary phase was, in general, larger than that of a single amino acid stationary phase in the same solvent. A similar trend was also found on the tripeptide column. One should note in Table V that adding a second amino acid, even when it was of the same chirality had no influence on the α value except for the BOC-L-Val-L-Val (bb) and BOC-D-Val-D-Val (gg) columns in the 20% methylene chloride of hexane where α value had a singificant increase. For example, there was a decrease in the α value upon adding a second amino acid that had the same chirality as valine but was less active, *e.g.*, phenylalanine (cc) or alanine (ee). Part of the decrease in α value may have been due to the smaller BOC-L-Val concentration on the silica surface. However, when the chirality was different as in BOC-L-Val-D-Ala (dd), BOC-D-Val-L-Ala (hh), BOC-D-Val-L-Ile (ii) columns, the α value was reduced as expected, and the decrease was a larger one for the more effective Ile than for the Ala. Note in Table II that adding a third amino acid Pro (t,y) did not change the α value and neither did the Val (z).

TABLE V

THE SEPARATION FACTORS, α, AND CAPACITY FACTORS, k', OF TFAE ENANTIOMERS ON CHIRAL STATIONARY PHASES

Stationary phase	*Mobile phase*			
	20% MCl in hexane		*1% IPA in hexane*	
	k'_1*	α	k'_1*	α
(aa) BOC-L-Val**	4.03 ± 0.11	1.099 ± 0.011	2.84 ± 0.03***	1.076 ± 0.010***
(bb) BOC-L-Val-L-Val	4.61 ± 0.03	1.184 ± 0.014	7.63 ± 0.14	1.097 ± 0.009
(cc) BOC-L-Val-L-Ala	5.44 ± 0.07	1.069 ± 0.011	4.91 ± 0.08	1.065 ± 0.013
(dd) BOC-L-Val-D-Ala	4.62 ± 0.09	1.040 ± 0.009	3.67 ± 0.07	1.038 ± 0.015
(ee) BOC-L-Val-L-Phe	7.38 ± 0.12	1.055 ± 0.015	2.18 ± 0.03§	1.038 ± 0.015
(ff) BOC-D-Val**	5.87 ± 0.10	1.082 ± 0.008	4.41 ± 0.07	1.094 ± 0.012
(gg) BOC-D-Val-D-Val	6.61 ± 0.18	1.158 ± 0.035	4.13 ± 0.15	1.090 ± 0.006
(hh) BOC-D-Val-L-Ala	4.04 ± 0.28	1.062 ± 0.015	3.27 ± 0.06	1.034 ± 0.013
(ii) BOC-D-Val-L-Ile	8.06 ± 0.09	1.010 ± 0.006	3.75 ± 0.04***	1.011 ± 0.007***

* Capacity factor for the first eluted enantiomer.
** Microbore column.
*** 1.5% 2-propanol in hexane.
§ 2% 2-propanol in hexane.

CONCLUSIONS

We have confirmed the expected reversal of elution orders of an enantiomeric test solute (*R,S*-TFAE) on changing the chirality of the amino acid derivative on the surface of the silica column packing. In addition, the presence of the BOC group on the immobilized valine was found to result in an increase of the separation factor. The use of a dipeptide on the surface showed that the inner amino acid had approximately the same contribution to chiral separation as when it was alone, though the contribution usually appeared to be slightly smaller. That is best shown by D-valine and L-isoleucine where the BOC-derivatives of the individual amino acids had approximately the same α values, but the D-, L-dipeptide had a small α change in the direction governed by the D-valine.

Increases in the percentage of the polar (strong) solvent in the eluents decreased the retention times. However, the separation factors remained constant.

ACKNOWLEDGEMENTS

We acknowledge the support of this work by the Department of Basic Energies, U.S. Department of Energy, Contract Number DE-AS09-76ER00854. We also thank Dr. L. A. Carreira for helping with the acquisition of data using a microcomputer and Dr. M. R. Rayco for help in packing the columns.

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