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RESOLUTION OF AMINO ACID DIASTEREOMERS BY MEANS OF PACKED COLUMN GAS CHROMATOGRAPHY*,**

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SUMMARY

The effect of an alcohol's structure upon the resolution of its N-alkylated amino acid diastereomeric ester was studied using several packed gas chromatographic columns. Using ± 3,3-dimethyl-2-butyl-N-trifluoroacetyl derivatives, fourteen protein amino acid diastereomers could be resolved to 93 % or better. Aspartic acid and proline derivatives could be resolved to 70 % and 82 %, respectively. Arginine, histidine and cystine derivatives were not studied.

INTRODUCTION

Almost simultaneously with the advent of the use of gas–liquid chromatography (GLC) for amino acid analysis, interest was generated for using the same technique to resolve the optical isomers of amino acids. Much of this interest was attributable to the possibility of using the technique to study optical isomerism in extraterrestrial life probes currently being planned by the United States National Aeronautics and Space Administration, but other interest has been shown in the areas of geochemistry, bacteriology and peptide synthesis.

To date most research in this area has been with capillary columns. While the results obtained with capillary columns often are quite good, the corresponding packed column technology would be beneficial in laboratories containing instrumentation not equipped for capillary columns. In some instances packed columns could also be used to resolve larger amounts of amino acids than could be done on capillary columns; AYERS *et al.*[1] showed that amino acid diastereomers could be resolved on a preparative or semipreparative scale.

Motivated by a desire to use GLC as a technique to study optical isomerism of amino acids in insect hemolymph, this study was initiated to select an alcohol which, when esterified with the proper N-alkylated amino acid derivatives, could be made to give suitable resolutions on conventionally packed GLC columns.

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MATERIALS AND METHODS

GLC supplies

All GLC supplies except the liquid phases OV-210 and OV-225 were purchased from the Anspec Co., Ann Arbor, Mich. The latter two phases were purchased from Regis Chemical Co., Chicago, Ill. Chemicals Procurement Laboratories, College Point, N.Y. was the supplier of (+)2-octanol; (—)2-methyl-1-butanol was purchased from Aldrich Chemical Co. Inc., Milwaukee, Wisc.; (+)2-butanol was prepared by the method of INGERSOLL[2]; and (+)3,3-dimethyl-2-butanol was prepared by the method of AYERS *et al.*[1].

Derivative formation

The amino acid esters were prepared by reacting 1–3 mg of the amino acid for 1 h at 100° with 1–1.5 ml of the appropriate alcohol which contained 1.2–1.5 mequiv. of HCl per ml. The alcohol was evaporated from the reaction mixture and the residue was dissolved in 1–1.5 ml methylene chloride–trifluoroacetic anhydride (4:1) and allowed to stand for 15–20 min at room temperature. This solution was then used for GLC analysis. In this manner the trifluoroacetyl derivatives of the 2-butyl, 2-pentyl, 2-octyl, 3-octyl, 4-octyl, 2-decyl, 3,3-dimethyl-2-butyl, 3-methyl-2-butyl, 2-methyl-1-butyl and 1,1,1-trifluoro-2-propyl esters of the various protein amino acids (except arginine, histidine and cystine) were prepared. These last three amino acids appear from our own studies as well as those of others[8] to be special cases and no attempt was made to work with them.

The 2-butyl and 2-octyl, N-acetyl, N-propyl, and N-trichloroacetyl derivatives of alanine, valine and phenylalanine were prepared in a similar manner except that the appropriate acid chlorides were used to form the alkyl amides.

Column packings

Column packings were prepared by weighing 15 g of solid support into a 300-ml round-bottom flask and covering it with a suitable solvent containing the liquid phase. The solvent was evaporated on a slowly turning rotating evaporator at *ca.* 60° until no more liquid was visible. During the next 15–30 min the support was maintained at the same temperature on the evaporator, and periodically rotated to expose a new surface to the vacuum. Final drying was accomplished in a shallow pan in an oven at 30–40°.

The packings were loaded and compacted with the aid of a vacuum pump and vibrator into 4-mm I.D. all-glass columns having all-glass injector ports as described elsewhere[1].

Instrumentation

Two Model 600 Series Research Specialities gas chromatographs equipped with duel hydrogen flame detectors were used during the study. The carrier gas (either nitrogen or helium) flow rate was kept at 90 ml/min. Since both resolution and peak sharpness are dependent on retention time, the chromatograph oven temperature was adjusted to make retention times (injection to center between peaks) fall within a short time interval. In this manner it was possible to compare the abilities of several columns to resolve a given diastereomeric pair.

Selected derivatives from all classes prepared were chromatographed on the following columns: (1) 6 m 1½% cyclohexanedimethanol adipate on 60/80 a.w. Chromosorb W; (2) 3 m 10% OV-17 (phenylmethyl silicone) on 100/120 DMCS a.w. Chromosorb W; (3) 3 m 10% OV-1 (methyl silicone) on 100/120 DMCS a.w. Chromosorb W.

The 3,3-dimethyl-2-butyl-N-trifluoroacetyl derivatives were further tested on the following columns: (1) 3 m 5% Carbowax 20M on 100/120 DMCS a.w. Chromosorb W; (2) 3 m 10% GE-XE-60 (cyanoethylmethyl and dimethyl silicone) on 100/120 DMCS a.w. Chromosorb W; (3) 3 m 5% 1,2,3,4-tetrakis-(2-cyanoethoxy)-butane on 100/120 DMCS a.w. Chromosorb W; (4) 3 m 5% 1,2,3-tris-(2-cyanoethoxy)-propane on 100/120 DMCS a.w. Chromosorb W; (5) 3 m 10% OV-225 (phenylmethylcyanopropyl silicone) on 100/120 DMCS a.w. Chromosorb W; (6) 3 m 10% OV-210 (methyltrifluoropropyl silicone) on 100/120 DMCS a.w. Chromosorb W; (7) 3 m 5% cyclohexanedimethanol adipate on 100/120 DMCS a.w. Chromosorb W; (8) 6 m 1½% tetramethylcyclobutanediol adipate on 60/80 a.w. Chromosorb W.

In addition the 2-octyl and 3,3-dimethyl-2-butyl-N-trifluoroacetyl esters were tested on a 3 m 1% Carbowax 20M on 100/120 DMCS a.w. Chromosorb W column. In instances where the resolution of a particular diastereomer was in question, the problem was resolved either by mass spectroscopy and/or by preparing the derivative from resolved alcohols.

RESULTS

The degree of resolution obtained with the N-trifluoroacetyl derivatives was as good as and generally better than that obtained with the N-acetyl, N-trichloroacetyl or N-propyl derivatives. Less peak tailing usually occurred with these derivatives and the retention temperatures were generally lower than for the other derivatives. Because of their superiority the remainder of this paper is only concerned with N-trifluoroacetyl derivatives.

The effect of the structure of the alcohol upon the degree of resolution attainable with the different diastereomeric esters is quite pronounced. No resolution was achieved with 2-methyl-1-butyl esters. In the alkyl-2-ol series, 2-butyl through 2-decyl, the degree of resolution attainable increased with the size of the alcohol. In the larger

TABLE I

RESOLUTION OF OCTYL-N-TRIFLUOROACETYL ESTERS OF ALANINE AND VALINE ON TWO COLUMNS
Columns: (1) 3 m 10% OV-17 on 100/120 DMCS a.w. Chromosorb W and (2) 6 m 1½% cyclohexanedimethanol adipate on 60/80 a.w. Chromosorb W. Retention times between 15–20 min at a flow rate of 90 ml/min. Resolutions calculated by the method of BURCHFIELD AND STORRS[3] and (in parentheses) by the method of KAISER[4].

Column	*2-Octyl*		*3-Octyl*		*4-Octyl*	
	Alanine	*Valine*	*Alanine*	*Valine*	*Alanine*	*Valine*
1	1.14 (75.8)	0.80 (44.3)	Shoulder (Shoulder)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)
2	1.06 (79.9)	0.83 (41.7)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)

TABLE II

COMPARISON OF RESOLUTIONS ACHIEVED WITH 3-METHYL-2-BUTYL AND 3,3-DIMETHYL-2-BUTYL-N-TRIFLUOROACETYL DERIVATIVES OF THREE AMINO ACIDS ON TWO COLUMNS

Columns: (1) 3 m 10% OV-17 packed on 100/120 DMCS a.w. Chromosorb W and (2) 3 m 10% GE-XE-60 packed on 100/120 DMCS a.w. Chromosorb W. Retention times 20 ± 2 min at a flow rate of 90 ml/min. Resolutions calculated by the method of BURCHFIELD AND STORRS[3] and (in parentheses) by the method of KAISER[4].

Amino acid	*Column 1*		*Column 2*	
	3-Methyl-2-butyl	*3,3-Dimethyl-2-butyl*	*3-Methyl-2-butyl*	*3,3-Dimethyl-2-butyl*
Alanine	1.42 (85.0)	2.10 (95.3)	0.94 (50.7)	1.41 (91.0)
Valine	1.29 (80.2)	1.64 (95.0)	1.30 (83.9)	1.95 (98.9)
Phenylalanine	0.74 (13.9)	1.20 (73.1)	Shoulder (Shoulder)	1.33 (72.5)

alcohols of this series, when the hydroxy group occupied a more median position on the alkyl chain the degree of resolution decreased markedly (Table I). The difference between 2-decanol and 3-decanol was similar to that found between 2-octanol and 3-octanol.

The alcohols, 3-methyl-2-butanol and 3,3-dimethyl-2-butanol, were found to be significantly superior to any of the alcohols tested. Of these two alcohols, 3,3-dimethyl-2-butanol was the best, as can be seen from Table II. While complete resolution of only one or two amino acids and partial resolution of several more were obtainable with the larger 2-alkyl derivatives, resolutions of 93% or better could be obtained with 3,3-dimethyl-2-butyl derivatives for all the amino acids tested except aspartic acid and proline. Resolutions of about 70% and 82%, respectively, were obtained for these amino acids. The notable exception to this general situation occurred with proline. The larger straight-chained 2-alkyl derivatives gave resolutions superior to that of the 3,3-dimethyl-2-butyl derivatives.

The 1,1,1-trifluoro-2-propyl-N-trifluoroacetyl derivatives of alanine, valine, and phenylalanine gave excellent resolutions but esterification was found to be extremely difficult to achieve and 1,1,1-trifluoro-2-propanol was not tested further.

While the straight-chained alkyl derivatives gave essentially equal areas for the two peaks of a given diastereomeric pair, the L+ and D— peaks were greater in area than the L— and D+ pairs for 3,3-dimethyl-2-butyl derivatives. The ratio of the smaller peak to the larger peak varied from amino acid to amino acid and ranged from *ca.* 0.303 to 0.714. The differences of the peak areas were found to be slightly less for the 3-methyl-2-butyl derivatives than for the 3,3-dimethyl-2-butyl derivatives. Proline formed the exception to the general situation with both peaks of a given diastereomeric pair being essentially equal in area.

The liquid phases 1,2,3,4-tetrakis-(2-cyanoethoxy)-butane, 1,2,3-tris-(2-cyanoethoxy)-propane and OV-210 were generally found to be unsuitable for the resolution of the various derivatives because of lack of resolving power and/or thermal stability. The two phases tetramethylcyclobutanediol adipate and OV-1 gave at least partial

resolution of some of the derivatives but were judged inferior to the remainder of the phases tested.

Table III contains data pertinent to the resolution achieved by the 3,3-dimethyl-2-butyl-N-trifluoroacetyl derivatives on the remainder of the phases. When retention times were kept to between 15 and 20 min the degree of resolution was not markedly affected by the percent liquid phase on the solid support within a range of 1–5 % for the non-silicone phases (1–10 % for the silicone phases). The resolutions tended to be slightly better, however, at the higher phase concentrations.

Often the temperature needed to achieve a given retention time was considerably less at the lower phase levels (Table IV). At times the degree of degradation increased significantly with the higher liquid phase concentrations. This was especially significant for the di-trifluoroacetyl derivatives of the hydroxy and sulfhydryl amino acids on polar columns.

TABLE III

RESOLUTION OF 3,3-DIMETHYL-2-BUTYL-N-TRIFLUOROACETYL AMINO ACID DERIVATIVES ON VARIOUS COLUMNS WITH A CONSTANT CARRIER GAS FLOW RATE OF 90 ml/min

Columns: (1) 3 m 10% OV-17 on 100/120 DMCS a.w. Chromosorb W; carrier gas, nitrogen. (2) 3 m 10% OV-225 on 100/120 DMCS a.w. Chromosorb W; carrier gas, nitrogen. (3) 3 m 10% GE-XE-60 on 100/120 DCMS a.w. Chromosorb W; carrier gas, nitrogen. (4) 6 m 1½% cyclohexanedimethanol adipate on 60/80 a.w. Chromosorb W; carrier gas, nitrogen. (5) 3 m 5% cyclohexanedimethanol adipate on 100/120 DMCS a.w. Chromosorb W; carrier gas, nitrogen. (6) 3 m 1% Carbowax 20M on 100/120 DMCS a.w. Chromosorb W; carrier gas, nitrogen or helium. Retention times between 15–20 min. Resolutions calculated by the method of BURCHFIELD AND STORRS[3] and (in parentheses) by the method of KAISER[4].

Amino acid	*Column 1*	*Column 2*	*Column 3*	*Column 4*	*Column 5*	*Column 6*	
						N_2	*He*
Alanine	1.82 (95.5)	1.69 (96.8)	1.61 (96.6)	1.48 (89.3)	1.75 (93.3)	3.39 (98.3)	3.50 (94.1)
Valine	1.39 (83.6)	1.73 (97.0)	2.00 (99.5)	1.57 (94.0)	1.70 (88.3)	1.91 (93.1)	2.23 (97.5)
Leucine	1.58 (91.1)	1.79 (90.8)	1.93 (98.3)	1.70 (92.0)	1.61 (94.2)	2.85 (98.1)	3.11 (98.5)
Isoleucine	1.43 (92.3)	1.88 (90.1)	2.21 (98.6)	1.92 (98.0)	1.94 (99.4)	2.50 (93.9)	2.78 (96.6)
Threonine	0.76 (40.0)	1.00 (64.0)	1.50 (93.3)	1.60 (90.8)	0.62 (29.2)	1.35 (92.3)	2.26 (95.9)
Proline	Sh [a] (Sh)	0.97 (55.6)	1.03 (61.8)	0.62 (36.7)	0.36 (4.5)	1.09 (80.5)	1.07 (82.1)
Serine	2.00 (99.9)	1.16 (73.5)	1.81 (97.3)	1.65 (95.4)	1.39 (89.8)	2.56 (97.6)	2.96 (99.1)
Cysteine	1.93 (99.9)	1.60 (95.5)	2.53 (95.8)	2.35 (94.6)	2.05 (95.0)	[b]	[b]
Methionine	0.00 (0.0)	0.51 (26.7)	1.48 (87.1)	1.42 (85.8)	1.39 (84.4)	2.38 (97.7)	2.60 (98.4)
Aspartic acid	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)	Sh (Sh)	0.00 (0.0)	0.92 (62.5)	1.04 (69.8)
Phenylalanine	0.88 (61.5)	1.06 (60.6)	1.86 (99.6)	1.70 (97.0)	1.83 (97.0)	2.44 (95.8)	2.67 (99.3)
Glutamic acid	0.90 (51.0)	0.91 (51.5)	[c]	1.17 (92.5)	1.41 (84.1)	1.77 (94.7)	1.67 (95.5)
Tyrosine	1.02 (74.0)	1.03 (62.4)	[c]	[d]	1.78 (92.6)	2.23 (93.5)	2.84 (96.7)
Lysine	0.54 (27.3)	0.36 (15.8)	[c]	1.04 (77.7)	1.24 (62.8)	1.58 (96.4)	1.73 (93.5)
Tryptophan	0.89 (60.8)	1.05 (70.0)	[c]	1.65 (91.6)	1.72 (93.4)	2.32 (97.8)	2.24 (97.5)

[a] Sh = shoulder.
[b] Multiple peaks apparently do to degradation on column.
[c] Not found; excessive column bleed at these temperatures.
[d] Apparently totally degraded on column.

TABLE IV

COMPARISON OF THE RETENTION TEMPERATURES OF THE ± 3,3-DIMETHYL-2-BUTYL-N-TRIFLUOROACETYL AND DI-TRIFLUOROACETYL DERIVATIVES OF SEVERAL AMINO ACIDS ON 1% AND 5% CARBOWAX 20M COLUMNS

The columns were both packed on 100/120 DMCS a.w. Chromosorb W. Retention times 20 ± 1 min at a flow rate of 90 ml/min.

Amino acid	*Retention temperature (°C)*	
	1% Carbowax 20M column	*5% Carbowax 20M column*
Threonine	74	100
Serine	95	125
Aspartic acid	128	167
Tyrosine	148	177

Generally slightly better resolutions were obtained when helium instead of nitrogen was used as the carrier gas (see Table III).

The retention times and degree of resolution were found to be dependent upon several factors such as the tightness of packing and history of the column. The resolutions achieved on the 10% OV-17 column improved considerably with age (data in Table III are from an old OV-17 column with many hours of use). The Carbowax columns tended to have a short useful life but this was only very pronounced for selected amino acids, most notably aspartic acid. The resolution of aspartic acid deteriorated in many cases after only about a week's use, even though the column temperature was generally kept well under 180°.

DISCUSSION

The degree of resolution attainable by an N-trifluoroacetyl amino acid ester appears to be dependent not only upon closeness of the two asymmetric centers and the size of the alcohol as reported by several authors[5,6], but also upon the similarity of three non-hydroxy groups on the asymmetric carbon of the alcohol. This was alluded to by the esters of octanol and decanol with the hydroxy position more median than the 2-position. For example, in 3-octanol the largest group off of the 2-carbon ((pentyl group)) would be considerably larger than the largest group off of the 2-position in either 2-butanol or 2-pentanol (ethyl and propyl groups, respectively), yet the latter two alcohols achieved better resolutions than the larger 3-octanol. The superiority of 3-methyl-2-butanol, 3,3-dimethyl-2-butanol and 1,1,1-trifluoro-2-propanol tends to support this supposition; however, these alcohols when considered alone would not discredit the hypothesis that their superiority is based upon bulk alone.

Relevent to the work reported for capillary columns, relatively non-polar phases such as OV-17 achieve surprisingly good resolutions for the various 3,3-dimethyl-2-butyl-N-trifluoroacetyl derivatives. Such phases should find general utility in resolving the di-trifluoroacetyl derivatives of serine, threonine, cysteine and tyrosine. This would be an advantage over the additional steps suggested by POLLOCK *et al.*[7] to circumvent degradation problems encountered when trying to resolve them on polar columns. Phases of this type may also prove useful for the resolution of arginine, histidine and cystine since such phases are needed for chromato-

graphic studies of the *n*-butyl-N-trifluoroacetyl derivatives of these amino acids[8].

Often it was found that the di-trifluoroacetyl derivatives (with the probable exception of the cysteine derivative, which apparently underwent serious degradation) could be resolved on polar phases. While there is undoubtedly some deterioration during chromatography, it is of such an order on low liquid phase packings that analysis in the microgram range could conveniently be carried out on columns containing these packings. This lessened deterioration on columns of low phase concentrations may be a result of the lower retention temperatures needed on these columns. Column supports advertised to work best at low phase concentrations might prove very useful in solving this problem. It was noticed on several occasions using a 5 % Carbowax 20M column and successive analyses of one of the di-trifluoroacetyl derivatives that the peak areas for a given size injection increased from one injection to the next, and eventually reached an area equal to that given by the same sized injection on a 1 % Carbowax 20M column. The phenomenon appeared to be at least partly reversible if the column was not used for a period of several days. No explanation is offered for this pecularity.

In no instance was any one column capable of separating all the amino acids while at the same time resolving the optical isomers. One or more overlaps occurred on each column. The problem could often be circumvented by using several columns since the overlap pattern is often different on different columns.

For quantitative purposes 3,3-dimethyl-2-butyl derivatives often would not be as convenient as esters having identical formation rates for each member of a given diastereomeric pair. However, if an investigator were working with traces of one amino acid isomer in much larger quantities of the other isomer, advantage could be taken of the dissimilar formation rates by choosing the proper optical form of the alcohol utilized in the esterification.

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