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RESOLUTION OF STEREOISOMERS OF DIPEPTIDES BY GAS CHROMATOGRAPHY ON CHIRASIL-VAL*

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SUMMARY

Several dipeptides have been separated into stereoisomers (enantiomers, diastereomers, sequential isomers) by gas chromatography on Chirasil-Val. Sufficiently volatile N-trifluoroacetyl dipeptide methyl esters were formed at ambient temperature, thus avoiding racemization (or epimerization, respectively), and cleavage of the peptide bond. This method is suitable for ascertaining the stereoisomeric composition of dipeptides in biological samples.

INTRODUCTION

Chromatographic separation of stereoisomers (enantiomers, diastereomers) is of increasing interest in most branches of chemistry and pharmacology. Successful applications of gas chromatography (GC)[1,4], liquid chromatography (LC)[5–8] or thin-layer chromatography (TLC)[9] have been described. Reflecting the increasing importance of life science, attention has been focused on amino acids[1–4,10] and dipeptides. Whereas oligopeptides have been analyzed by GC on achiral stationary phases for sequence determination[11,12], only few papers have dealt with diastereomer resolution[13].

In view of their commercial interest, synthetic dipeptides have recently been separated into the stereoisomers by means of TLC on the "Chiralplate" system[14,15]. The convenience of this direct method has been pointed out, whereas GC analysis requires derivatization that can be accompanied by racemization and side reactions. Indeed TLC, in conjunction with suitable detection devices (automatic spraying and scanning), is well suited for controlling industrial production of such compounds. Very recently, the resolution of dipeptides by LC on a "Pirkle-type" stationary phase has been reported[16].

However, when analyzing dipeptides, derived from degradation of peptides of either synthetic or natural origin, in order to establish racemization during and after

* Dedicated to Professor Ernst Bayer on the occasion of his 60th birthday.

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peptide synthesis, one is faced with complex mixtures of chemical compounds. Therefore, highly sensitive and selective techniques are required, such as GC–mass spectrometry (MS) with a chiral stationary phase, *e.g.*, Chirasil-Val[10] and single-ion monitoring (SIM). This technique has proven promising for the analysis of amino acids[17] and hydroxy acids[18]. For dipeptides, the thermostable polymeric stationary phase Chirasil-Val[4,10,19] is particularly useful[20]. A more detailed study of the derivatization and resolution of dipeptide stereoisomers is now outlined.

EXPERIMENTAL

Stereoisomers of dipetides were obtained from Bachem (Dubendorf, Switzerland). A large number of dipeptides was kindly provided by Dr. K. Günther (Degussa, Hanau, F.R.G.).

Derivatization of dipeptides

A sample of the dipeptide (1 mg) was dissolved in a solution of hydrogen chloride in methanol (prepared by mixing acetyl chloride and methanol, 1:10 (v/v) at 0°C). A 0.5-ml volume of the reagent was used per derivatization reaction, carried out in a 1-ml Reactivial (Macherey-Nagel, Düren, F.R.G.) and left to stand at ambient temperature for 3 h. The solvent was removed completely in a stream of dry nitrogen. The residue was allowed to react with trifluoroacetyl (TFA) anhydride (200 μl) for 15 min at ambient temperature. The reagent was carefully removed in a stream of dry nitrogen, in order completely to remove the by-product trifluoroacetic acid. The derivative should cover the surface of the Reactivial as a thin film. If a droplet is obtained, especially in the case of larger amounts of the sample, it is recommended to add 0.5 ml of dry toluene, which is subsequently removed, after vigorous mixing of the sample, in a gentle stream of dry nitrogen. Eventually, the derivative was dissolved in 0.1–0.5 ml of dichloromethane. In the case of polar derivatives, addition of 10% (v/v) methanol is necessary for the complete dissolution of the sample.

Gas chromatography

GC was performed with a Perkin-Elmer Sigma 1-Analyzer-System, equipped with an integrator and recorder. A fused-silica capillary column (H40, 25 m × 0.3 mm, Hewlett-Packard) was coated with D-Chirasil-Val[21], as described[22].

RESULTS AND DISCUSSION

In order to resolve dipeptides into stereoisomers (enantiomers, diastereoisomers) by GC, a number of problems must be solved. The thermostability of the stationary phase, Chirasil-Val, is guaranteed by the polymeric backbone[23]. Racemization of the valine diamide residues at elevated temperature is largely suppressed by the very careful purification of the stationary phase[24]. Accordingly, no alkaline or nitrogen basic material should be injected in substantial amount. Therefore, the use of reagents like imidazolides, though carried out under mild conditions, must be avoided. Derivatization of substrates under acidic conditions can be used, provided the reagents are removed completely prior to chromatography.

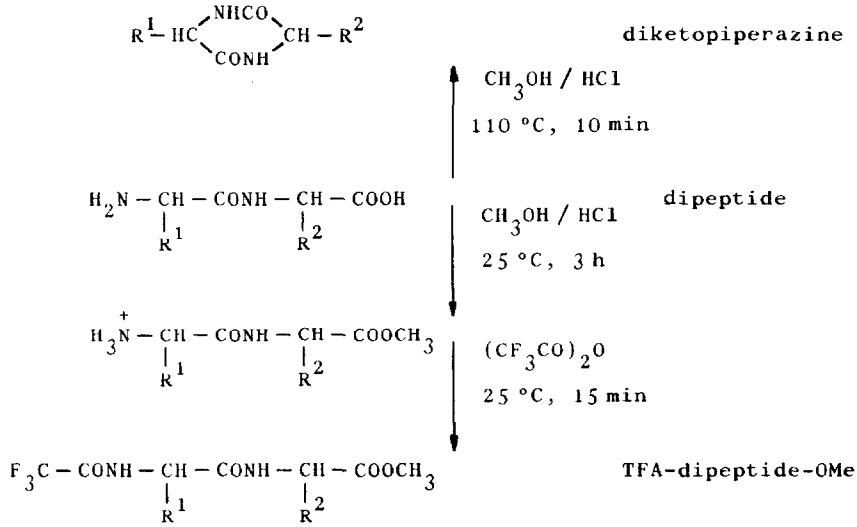
Thus, we decided to transform the dipeptides to the alkyl esters (catalyzed by

dry hydrogen chloride), and thereafter to the corresponding perfluoroacyl derivatives. Preliminary experiments revealed that the conditions usually applied to amino acids (hydrogen chloride in the appropriate alcohol, 1:10 (v/v) of acetyl chloride in alcohol is used instead of dry hydrogen chloride gas) at 110°C for 10 min (for the most reactive alcohol, methanol) when applied to dipeptides lead to the almost exclusive formation of the diketopiperazine. At lower concentrations of hydrogen chloride (the solution is prepared by mixing 2% (v/v) acetyl chloride and methanol at 0°C), ring formation is largely supressed. Even for the most reactive dipeptide investigated, *i.e.*, Ala–Gly, only 5% of diketopiperazine was formed, and methanolysis of the peptide bond appeared to be negligible under these conditions. Further studies revealed that esterification is best carried out at ambient temperature, but at the higher hydrogen chloride concentration (as described in the Experimental). For methanol, a reaction time of 3 h was sufficient. For the less reactive alcohols, ethanol, 1-propanol and 2-propanol, a reaction time of 17 h was chosen in order to ensure almost complete conversion.

The formation of the trifluoroacetyl derivative proceeds smoothly at ambient temperature within a few minutes. Standard conditions are shown in Scheme 1. After removal of the reagents, the derivatives were dissolved in dichloromethane and injected via a split inlet, at an injector temperature of 270°C.

The proper choice of suitable derivatives was guided by the demand for optimum resolution factors, α, for the stereoisomers within reasonable retention times. Volatility is important, since the resolution factors usually decrease at higher temperatures, due to the unfavourable entropy contribution to diastereoselective interaction of the substrate with the chiral stationary phase[25].

Throughout this investigation, TFA has been used for derivatization of the terminal amino group, for various reasons. First, the N-TFA-amino acid esters often exhibit larger resolution factors than the corresponding perfluoropropanoic (PFP)



Scheme 1. Derivatization procedure.

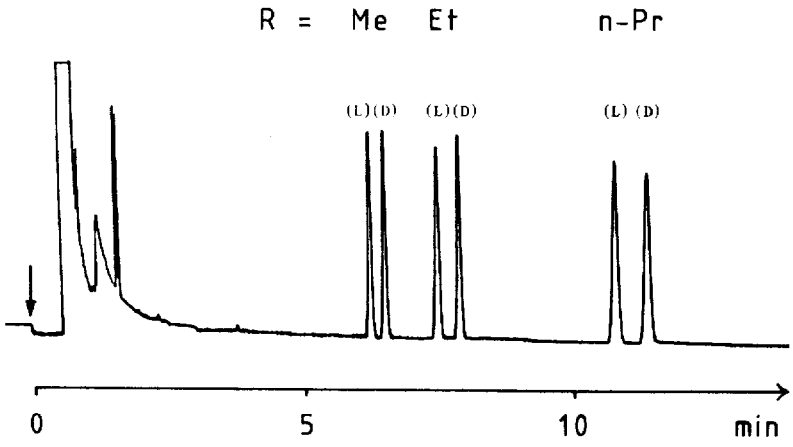


Fig. 1. Enantiomer resolution of N-TFA–Gly–Val–OR by GC on D-Chirasil-Val (H40); 160°C isothermal; 0.5 bar hydrogen; dead-time 0.69 min. Me = methyl; Et = ethyl; n-Pr = *n*-propyl.

derivatives[24,26]. Secondly, PFP anhydride is more expensive, though its chemical purity is not always acceptable[27].

As for the ester part, a brief study of four different alcohols was performed with Gly–Val as the test compound. At a column temperature of 160°C, a clean separation of the enantiomers was observed for all esters investigated (see Fig. 1). The results are compiled in Table I. As expected, the methyl ester is the most volatile. In view of the upper temperature limit set by the thermal stability of the less volatile dipeptides, the slightly increased resolution factors for the ethyl, 1-propyl and 2-propyl esters are outweighed by the disadvantage of decreased volatility. Since a standard procedure for derivatization should be applicable to all dipeptides as far as possible, the N-TFA-dipeptide methyl esters were chosen, the more so as these derivatives are most conveniently prepared.

Dipeptides containing one achiral glycine unit were resolved into enantiomers. The net retention times, t_R', and the resolution factors, α, are summarized in Table II. The enantiomers were usually resolved completely on typical Chirasil-Val columns within an acceptable analysis time. Dipeptides bearing the glycine at the NH_2-terminal position emerged in the order expected from the amino acids (L stronger retained on L-Chirasil-Val). In contrast, samples having a glycine residue at the

TABLE I

COMPARISON OF DIFFERENT ESTERS OF THE TYPE TFA–Gly–Val–OR

Fused-silica capillary, coated with D-Chirasil-Val (H40); 160°C; 0.5 bar hydrogen, dead-time 0.69 min.

R	t_R' (L) (min)	t_R' (D) (min)	α (160°C)
Methyl	5.84	6.11	1.047
Ethyl	7.09	7.50	1.058
Isopropyl	7.31	7.81	1.068
n-Propyl	10.80	11.45	1.060

TABLE II

ENANTIOMER RESOLUTION OF TFA-DIPEPTIDE METHYL ESTERS CONTAINING THE ACHIRAL GLYCINE UNIT

Fused-silica capillary, coated with D-Chirasil-Val (H40); 0.5 bar hydrogen. All retention data are given in minutes.

Compound	*140°C* t_M = 0.65		*160°C* t_M = 0.69		*180°C* t_M = 0.73		*200°C* t_M = 0.77		*Configuration on D-Chirasil-Val*
	t_R'	α	t_R'	α	t_R'	α	t_R'	α	
Ala–Gly	9.51	1.043	2.83	1.039					D
	9.92		2.94						L
Leu–Gly	24.15	1.084	6.53	1.080	2.48	1.080			D
	26.19		7.05		2.68				L
Gly–Val*	18.46	1.063	5.73	1.047					
	19.64		6.00						
Gly–Leu	32.82	1.061	9.20	1.045	3.70	1.032			L
	34.82		9.61		3.82				D
Gly–Phe			42.20	1.021	14.25	1.019			L
			43.10		14.52				D
Gly–Trp (di-TFA)							24.45	1.024	L
							25.03		D
Gly–Trp (mono-TFA)							37.68	1.010	L
							38.06		D

* Configuration not assigned.

COOH-terminal position emerged in an order (the D-enantiomer more strongly retained on L-Chirasil-Val) that is the reverse of that of the N-TFA amino acid esters. These findings deserve further comment (see below). Sequential isomers, *e.g.*, Leu–Gly and Gly–Leu were also completely separated from each other. The dipeptide Gly–Trp, having an appreciable molecular weight, yielded two derivatives of different but sufficient volatility. Both derivatives were eluted at 200°C within a fairly short analysis time. Due to the notorious instability of TFA-amides of the indol nitrogen[28], one of the two derivatives had a free N–H group in the tryptophan side chain.

Apart from additional chirality in the side chain, dipeptides formed from the two chiral amino acids have four different stereoisomers. Usually, all four isomers were completely resolved on typical Chirasil-Val columns. The results are compiled in Table III. For the examples investigated, enantiomers (as indicated by equal peak areas) showed rather high resolution factors, comparable to those of simple amino acid derivatives. The retention times, too, were as expected from those of the corresponding amino acids. The most volatile representative, TFA–Ala–Ala–OCH_3, was eluted in the form of four isomers in *ca.* 20 min at 120°C. On aged columns, the second and the third peak showed partial overlap. Even the less volatile derivative of Ala-Phe could be analyzed within less than 4 min at 200°C, as shown in Fig 2. Even under these conditions, the four stereoisomers are baseline separated. The peaks have been assigned to the absolute configurations at the two chiral centres by using synthetic reference compounds, as indicated in Table III. The separation of a mixture of dipeptides is shown in Fig. 3.

A detailed analysis of the diastereoselectivity observed on L-Chirasil-Val re-

TABLE III

RESOLUTION OF ALL FOUR STEREOISOMERS (ENANTIOMERS, DIASTEREOMERS) OF TFA-DIPEPTIDE METHYL ESTERS CONTAINING TWO ASYMMETRIC CENTRES

Fused-silica capillary, coated with D-Chirasil-Val (H40); 0.5 bar hydrogen. All retention data are given in minutes.

Compound	*120°C* t_M = 0.62		*140°C* t_M = 0.65		*160°C* t_M = 0.69		*180°C* t_M = 0.73		*200°C* t_M = 0.77		*Configuration on D-Chirasil-Val*
	t_R'	α	t_R'	α	t_R'	α	t_R'	α	t_R'	α'	
Ala–Ala I	18.53	1.148	6.27	1.132							DL
	21.28		7.10								LD
Ala–Ala II	20.85	1.111	7.04	1.060							LL
	23.17		7.46								DD
Ala–Leu I*			16.08	1.070	5.04	1.065					
			17.21		5.37						
Ala–Leu II*			16.67	1.127	5.18	1.116					
			18.80		5.78						
Ala–Phe					19.42	1.080	7.32	1.077	2.97	1.074	DD
					20.97		7.88		3.19		LL
					23.43	1.068	8.62	1.067	3.45	1.061	DL
					25.03		9.20		3.66		LD

* Configuration not assigned; I, II are the enantiomeric pairs, indicated by equal peak areas.

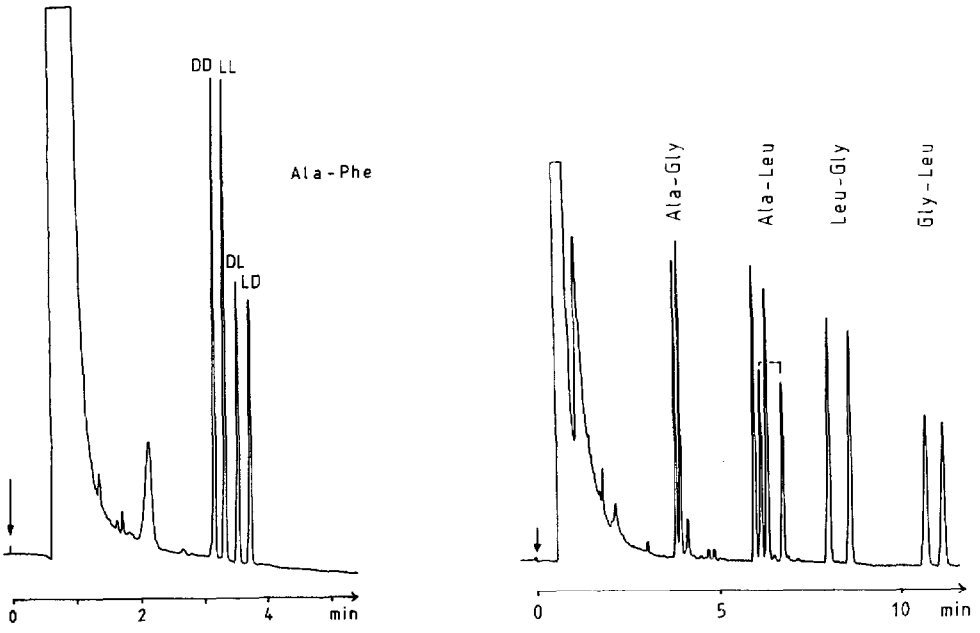
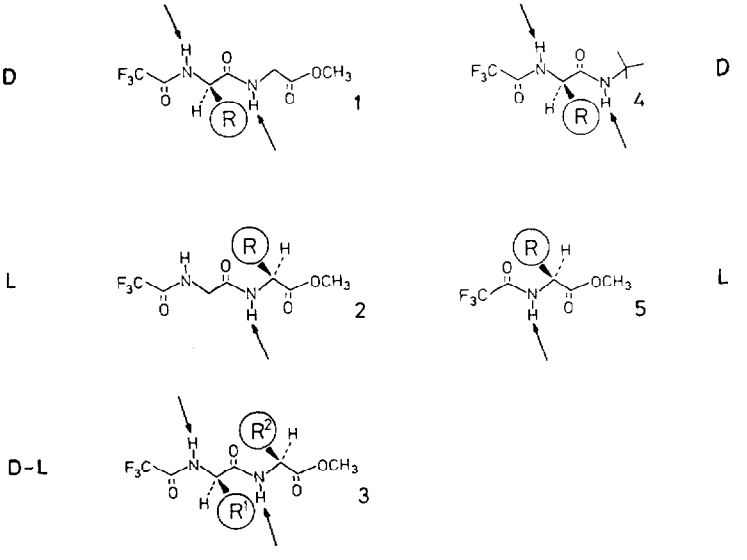


Fig. 2. Fast resolution of N-TFA–Ala–Phe–OCH_3 stereoisomers by GC on D-Chirasil-Val; 200°C isothermal; 0.6 bar hydrogen; dead-time 0.65 min.

Fig. 3. Resolution of a mixture of N-TFA-dipeptide methyl esters by GC on D-Chirasil-Val; 160°C isothermal; 0.5 bar hydrogen.



Scheme 2. Comparison of absolute configurations of stereoisomers strongly interacting with L-Chirasil-Val (as compared to the antipode).

veals some intriguing findings. The stereoisomers most strongly retained are depicted in Scheme 2. TFA– Leu–Gly–OCH_3 (1, R = isobutyl) can be compared to a N-acyl amino acid amide. In fact, TFA–valine–*tert.*-butylamide (4, R = isopropyl) shows a similar order of emergence, the D-enantiomer being more strongly retained on L-Chirasil-Val[29]. This order is the reverse of that of the well known N-acyl amino acid esters (5), where the order of emergence (L more strongly retained on L-Chirasil-Val) is strictly conserved for all 2-amino acids studied[1]. The order of emergence for a particular class of compound cannot be predicted *a priori*, though the behaviour is usually very regular within a certain class of compound. Rules and mechanisms established in the past on a fairly narrow basis of experience may become invalid when extended to a broad variety of classes of compounds. The value of such theories must be judged on the basis of their ability to predict the behaviour of new classes of compounds.

It is not surprising that the behaviour of TFA–Gly–Leu–OCH_3 (2, R = isobutyl) parallels that of compound 5. Obviously, the order of emergence is dictated by the amide groups adjacent to the chiral centre (arrows in Scheme 2), and most probably the configurations of homologous compounds may be assigned according to this rule. As expected, the most strongly retained stereoisomer of TFA–Ala–Phe–OCH_3 (3, R^1 = methyl, R^2 = benzyl), *i.e.*, D-L on L-Chirasil-Val, combines both principles in its molecular structure. D-Ala takes the place of D-Leu in 2.

For analytical purposes, the virtual absence of racemization during derivatization is highly desirable. However, minimum racemization (or epimerization, respectively) must be accepted, even during storage of chemically pure chiral compounds. The acceptability of a certain degree of racemization depends on the analytical purpose itself. On the other hand, any determination of racemization is crucially affected by the original stereochemical integrity of the test sample. The term "maximum degree of racemization" for a given reaction is based on the assumption that the original compound was isomerically pure. Obviously, the error inherent in such an approach is effectively reduced if the stereoisomeric purity observed for the reaction product is exceedingly high. As for the dipeptides, we are ignorant of the original purity of the samples obtained commercially. Particular stereoisomers of Ala-Phe from Degussa contained the three minor stereoisomers at levels of 0.1–0.5%. Presumably, these isomeric impurities were at least partially present in the original samples, since racemization during derivatization of amino acids is suppressed almost completely (<0.01%), if the derivatization reaction is carried out at ambient temperature[30].

The technique described proved particularly useful for the stereochemical analysis of peptides[30]. Usually, control of racemization during peptide synthesis is achieved by total hydrolysis to the amino acids[31]. Alternatively, dipeptides could be checked without degradation, after suitable derivatization, on a chiral stationary phase, since the derivatives are formed without significant side-reactions and racemization (or epimerization, respectively). In peptide hydrolysis, a certain part of the D-amino acids obtained is formed artificially in the course of the analytical procedure. Though racemization of the free amino acids under these conditions is slight (usually less than 0.3%)[32], some amino acid residues in polypeptides may undergo an accelerated racemization due to catalytic effects of neighbouring groups[32]. A systematic study of 37 different dipeptides revealed the striking effect of neighbouring-group

participation in the stabilization of achiral charged intermediates[33]. However, since up to 40% racemization was observed in some samples after treatment in aqueous solution (pH 7.6, 122.5°C, 8 h)[33], the racemization observed following hydrolysis of the dipeptides prior to GC analysis of the amino acid derivatives is not easily distinguished from racemization during hydrolysis. It has been pointed out that dipeptides are even more resistant to hydrolysis than are polypeptide bonds[34], a fact that may also account for the marked resistance of the dipeptides to alcoholysis during the first step of the derivatization reaction (Scheme 1). Protein hydrolysis *in vivo* gives rise to a certain level of dipeptides in body fluids[35], that are readily investigated by the technique outlined, in combination with SIM in GC–MS[30]. *In vitro*, the use of dipeptidyl aminopeptidases has become a tool for the sequence analysis of proteins[36,37]. The significance of amino acid racemization and epimerization in geochronology and paleobiology has been recognized for many years, and several review articles have appeared[38–42]. The question of stereochemical integrity of proteins was posed as early as 1970[43]. The separation of stereoisomers of dipeptides and higher peptides may become a valuable tool to answer some of the questions addressed, thus complementing the analysis of amino acids.

CONCLUSIONS

The number of dipeptide stereoisomers formed by the 20 different protein amino acids is $19 \times 19 \times 4 + 19 \times 2 \times 2 + 1$. Thus, 1520 chiral dipeptide stereoisomers and the achiral dipeptide Gly–Gly are possible. Obviously, the present study can give only a tentative insight into the possibilities and limitations of dipeptide stereoisomer resolution. As indicated, such studies not only open up new analytical possibilities, but also shed some new light on the mechanism of enantiomer resolution.

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