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SEPARATION OF DIPEPTIDE DIASTEREOMERS BY HIGH-RESOLUTION GAS CHROMATOGRAPHY

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SUMMARY

Separation of trimethylsilylated diastereomers of dipeptides was achieved by high-resolution gas chromatography on a fused silica capillary column coated with an achiral (conventional) stationary phase. L,L and D,D forms (enantiomers) were separated from their diastereomers, L,D and D,L with excellent resolution. Enantiomers were not separable from each other (L,L from D,D and L,D from D,L) on this column. The nominal structures of the separated stereoisomers were confirmed by gas chromatography–mass spectrometry.

INTRODUCTION

In the past, dipeptide diastereomers have been separated by a variety of chromatographic procedures, in conjunction with investigations of racemization during peptide synthesis. Weygand *et al.*[1] first succeeded in separating two diastereomeric dipeptides by gas chromatography (GC) as their N-trifluoroacetyl (N-TFA) methyl esters. Later, the same group was able to separate many more N-TFA methyl esters of diastereomeric dipeptides by GC on steel capillary columns[2]. Despite strongly tailing peaks, good resolution of the diastereomers has been obtained. The same derivatives have also been used by other workers for the GC separation of some dipeptide diastereomers[3,4]. Moreover, GC has been applied to the separation of diastereomeric cyclic dipeptides (diketopiperazines) using their trimethylsilyl derivatives[5].

Paper, thin-layer and classical column chromatographic procedures have also been successfully used for the same purpose[4,6–13].

In addition to the techniques mentioned above, high-performance liquid chromatography is a powerful tool for peptide separations in general. Several workers have successfully utilized this methodology to separate dipeptide diastereomers[14–18].

Recently, we described the separation of a large number of trimethylsilylated dipeptides with excellent resolution by GC on a fused silica capillary column[19]. In this paper, we report the separation of trimethylsilylated diastereomers of dipeptides by the same method. This method has been developed to study the racemization of peptides by ionizing radiation.

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EXPERIMENTAL*

Apparatus

A Hewlett-Packard Model 5880A microprocessor-controlled gas chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a flame-ionization detector was used. The injection port and detector were maintained at 250°C. Separations were carried out on a fused silica capillary column (12 m × 0.2 mm I.D.) coated with SE-54 (5% phenyl-, 1% vinylmethylsilicone gum; siloxane deactivated) (Hewlett-Packard). The measured efficiency was *ca.* 5400 theoretical plates per meter based on the pentadecane peak at 120°C ($k' = 6.16$; linear velocity = 39.9 cm/sec). Helium was used as the carrier gas at an inlet pressure of 100 kPa. The splitting ratio was 1:60. The ratio of 2,6-dimethylphenol to 2,6-dimethylaniline[20] was 1.0.

Materials

Dipeptides were purchased from Sigma (St. Louis, MO, U.S.A.). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and acetonitrile were obtained from Pierce (Rockford, IL, U.S.A.).

Trimethylsilylation

A sample of 0.5 mg of each dipeptide was placed in a Teflon-capped Hypo-Vial (Pierce) and trimethylsilylated with 0.4 ml of BSTFA–acetonitrile (1:1) by heating for 15 min at 140°C in a sand-bath.

Gas chromatography–mass spectrometry (GC–MS)

Mass spectra were measured in the laboratory of Dr. H. C. Krutzsch of the National Institutes of Health (Bethesda, MD, U.S.A.) using an LKB (Rockville, MD, U.S.A.) Model 2091 gas chromatograph–mass spectrometer at 70 eV with an ionizing current of 50 μA. Separations were performed on a wide-bore fused silica capillary column (25 m × 0.3 mm I.D.; Hewlett-Packard) coated with OV-1 (dimethylsilicone gum; siloxane deactivated) and programmed from 100 to 250°C at 4°C/min.

RESULTS**

There are four possible stereoisomers (L,L, D,D, L,D and D,L) of a peptide, due to the presence of two asymmetric centers. However, not all of the possible stereoisomers for each dipeptide examined here were commercially available. In most instances, only DL,DL-dipeptides were available, which should contain all four possible stereoisomers. For this reason, the separated compounds were also analyzed by GC–MS to ensure their authenticity. Mass spectra in this particular instance were interpreted on the basis of the data published by Krutzsch and Pisano[21].

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* Certain commercial equipment, instruments or materials are identified in this paper in order to specify the experimental procedure adequately. Such identification does not imply recommendation or endorsement by the National Bureau of Standards, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

** Abbreviations for amino acids follow IUPAC–IUB recommendations [see *Biochem. J.*, 126 (1972) 773].

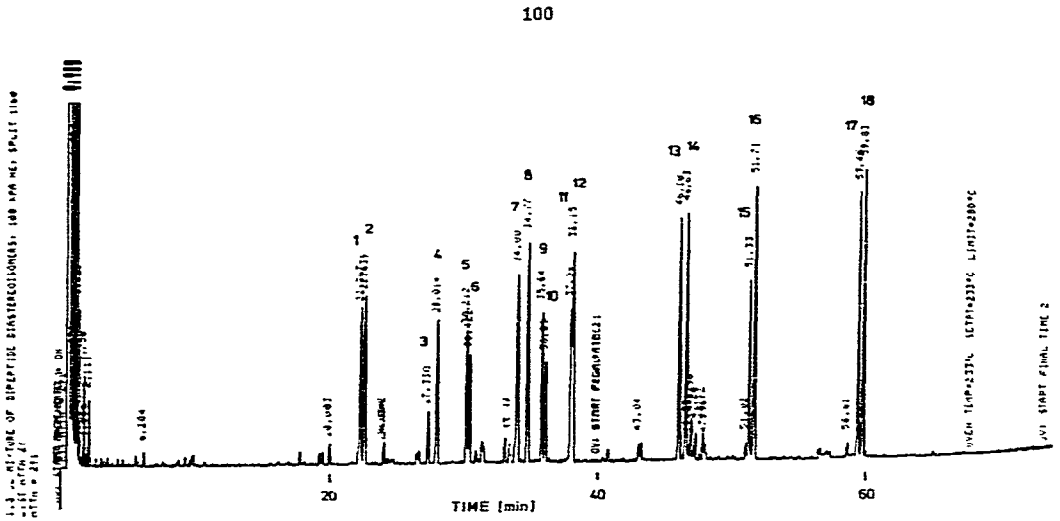

Fig. 1. Separation of trimethylsilylated diastereomers of dipeptides by high-resolution GC. Column, fused silica SE-54 (12 m × 0.2 mm I.D.) programmed at 2°C/min from 70 to 150°C then at 3°C/min from 150 to 250°C. For other column details see Experimental. Peak identifications are given in Table I. Identification was achieved by comparison of retention times of individually injected compounds and by GC–MS. Each peak corresponds to about 0.5–3 pmol of dipeptide.

TABLE I

IDENTIFICATION OF PEAKS IN FIG. 1

Peak No.	*Dipeptide*	*Peak No.*	*Dipeptide*
1	L-Ala–L-Ala	10	L-Leu–D-Val*
	D-Ala–D-Ala		D-Leu–L-Val*
2	L-Ala–D-Ala	11	L-Leu–L-Leu
	D-Ala–L-Ala		D-Leu–D-Leu
3	L-Ala–L-Val	12	L-Leu–D-Leu
	D-Ala–D-Val		D-Leu–L-Leu
4	L-Ala–D-Val*	13	L-Ala–L-Phe
	D-Ala–L-Val*		D-Ala–D-Phe
5	L-Ala–L-Leu	14	D-Aia–L-Phe
	D-Ala–D-Leu*		
6	L-Ala–D-Leu*	15	L-Leu–L-Phe
	D-Ala–L-Leu*		D-Leu–D-Phe*
7	L-Ala–L-Ser	16	L-Leu–D-Phe*
	D-Ala–D-Ser*		D-Leu–L-Phe*
8	L-Ala–D-Ser*	17	L-Leu–L-Tyr
	D-Ala–L-Ser*		
9	L-Leu–L-Val	18	D-Leu–L-Tyr
	D-Leu–D-Val*		

* The assignment of the peaks corresponding to these compounds was based on the order of elution of the other dipeptides in this table. For more details see the text.

Fig. 1 shows the separation of trimethylsilylated diastereomers of nine dipeptides on a fused silica capillary column coated with SE-54, an achiral stationary phase. Peak identifications are given in Table I.

With Ala–Ala, all the four stereoisomers were available. L-Ala–L-Ala and D-Ala–D-Ala gave peak 1, while L-Ala–D-Ala and D-Ala–L-Ala are represented by peak 2. Excellent resolution of L,L and D,D forms from their diastereomers, L,D and D,L forms, were obtained, but the enantiomers (L,L and D,D; L,D and D,L) were not separable from each other on this column.

DL-Ala–DL-Val, DL-Ala–DL-Leu, DL-Ala–DL-Ser and DL-Leu–DL-Val each gave two peaks which were completely separated (peaks 3 and 4; 5 and 6; 7 and 8; and 9 and 10, respectively). The retention times of their L,L-isomers, which were available, correspond to that of the first peak in each instance. Mass spectra taken from the two peaks of each compound were identical, proving that the two peaks correspond to isomers.

With Leu–Leu, all four possible stereoisomers were also available. L,L and D,D forms gave peak 11, while their diastereomers, L,D and D,L forms, are represented by peak 12. The resolution, however, is not as good as that of the diastereomers of other dipeptides examined here.

Peak 13 was obtained from L-Ala–L-Phe and D-Ala–D-Phe; D-Ala–L-Phe gave peak 14. L-Ala–D-Phe was not available.

DL-Leu–DL-Phe is represented by peaks 15 and 16. The retention time of the L,L-isomer of this dipeptide corresponds to that of peak 15. Mass spectra of both peaks were identical.

Peaks 17 and 18 represent L-Leu–L-Tyr and D-Leu–L-Tyr, respectively. Their mass spectra were also identical. The other two stereoisomers of this dipeptide were not available.

DISCUSSION

As the results show, two peaks from each DL,DL-dipeptide were obtained. It was concluded that the two peaks correspond to L,L- and D,D-isomers (shorter retention time), and to L,D- and D,L-isomers (longer retention time), respectively. This conclusion was based on the order of elution of all four possible stereoisomers of Ala–Ala and Leu–Leu, which were available. This was also supported by the fact that, in all instances, the L,L-isomers had the same retention time as the first peak of each DL,DL-dipeptide. In addition, three stereoisomers of Ala–Phe (L,L; D,D and D,L) and two stereoisomers of Leu–Tyr (L,L and D,L), which were available, followed the same elution order. Moreover, mass spectra of the two separated compounds from each DL,DL-dipeptide were identical, proving that these compounds were true isomers. The data obtained and assignment of the peaks show that L,L- and D,D-isomers (enantiomers) could be easily separated from their diastereomers, L,D and D,L forms, but the enantiomers were not separable (L,L from D,D and L,D from D,L). The lack of complete separation of all the four stereoisomers does not diminish the usefulness of this method in a study of racemization of natural peptides and proteins under the influence of diverse agents. For instance, radiation-induced L → D conversion will produce mainly the L,D- and D,L-dipeptides.

The method described here is also extremely sensitive. For example, the smal-

lest peak in Fig. 1 (except the impurities) corresponds to about 0.5 pmol of Ala–Val (peak 3). The detection of smaller amounts is also plausible by using the highest sensitivity setting of the detector. Moreover, this method yielded symmetrical peaks and the column efficiency was excellent (*ca.* 5400 theoretical plates per meter).

We also tested two other fused silica capillary columns for the separation of dipeptide diastereomers: a 12-m × 0.2 mm I.D. SP-2100 column (methylsilicone fluid, Carbowax 20M deactivated) (Hewlett-Packard) that we had used to separate a complex mixture of dipeptides in recent work[19] and a 25 m × 0.3 mm I.D. wide-bore OV-1 column (dimethylsilicone gum; siloxane deactivated) (Hewlett-Packard) which was used in GC–MS. These columns did not provide the same performance for resolution of the diastereomers examined here as the SE-54 column. Longer narrow bore columns (25 m × 0.2 mm I.D.) coated with SE-54 and SP-2100 also did not improve the resolution. In addition, trimethylsilylated dipeptides with long retention times tend to decompose on the longer narrow bore columns.

The superior performance of the SE-54 column in comparison with the SP-2100 column may be due to the type of column deactivation or simply to the higher polarity of SE-54 stationary phase (compare the McReynolds constants[22]). Other silicone stationary phases with different structures and higher polarities, such as OV-17 and Dexsil 410, might yield generally similar results to those with SE-54 for dipeptide separations. However, these other columns were not available to us.

Based on the results, we recommend the use of short (12 m) narrow-bore (0.2 mm I.D.) fused silica capillary columns coated with SE-54 for the separation of trimethylsilylated dipeptide diastereomers and of dipeptide mixtures in general. We believe that this is an important conclusion, as the sensitive and efficient separation of dipeptides by GC is also very important in sequencing of polypeptides using the dipeptidyl peptidase–GC–MS method[23].

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