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A NEW METHOD FOR DETERMINATION OF INTACT OPTICAL PURITY OF PEPTIDES BY USE OF MASS FRAGMENTOGRAPHY[1)]

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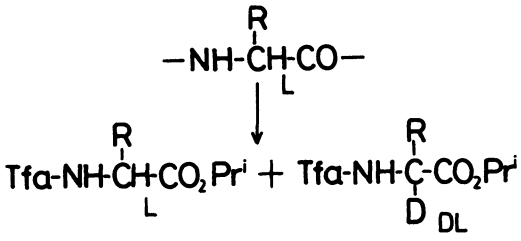
A novel method was established for the determination of true optical purity of amino acid residues in peptides. Hydrolysis in deuterium chloride followed by mass fragmentographic detection enables very accurate analysis without disturbance by racemization in the hydrolysis procedure.

Racemization is still one of the most important problems even in the modern peptide chemistry in spite of many efforts hitherto made to minimize it.[2)] The chiral purity of amino acid residues can not be completely retained in the coupling process in peptide synthesis, whatever method is employed. The diastereomeric peptides thus formed may contaminate the final product even after advanced purification procedures. Therefore, it is very important to test the optical purity of synthetic peptides prior to the precise discussion particularly on their biological activities. The most conventional method for this test is acid hydrolysis of the peptide followed by determination of optical purity of free amino acids by gas chromatography (GC)[3)] or amino acid analysis.[4)] However, new racemization occurs inevitably in the hydrolysis step to various extents, depending on the kind and the sequence of amino acids, and makes the exact determination of the intact chiral purity of the peptide impossible. An alternative procedure in which the purity of the peptides is examined by high performance liquid chromatography (HPLC) on a reverse phase column is now found to be very effective.[5)] In order to identify the racemized amino acid by this method, however, many kinds of adequate reference samples must be available for differentiation of each diastereomer. In this situation, we now established an accurate and convenient method for this purpose.[6)]

Although the racemization of component amino acids is unavoidable in acid hydrolysis, their true chiral purity may be able to be determined if the artificial racemates can be distinguished from the original ones present in the peptides. Manning proposed a tritium labeling method for the purpose.[7)] However, it has not been employed widely thereafter probably owing to the use of radioactive tritium chloride. In this study, deuterium was used in place of tritium for the labeling. When a peptide is hydrolyzed in deuterium chloride (DCl), the newly formed DL-amino acids are necessarily labeled since their α-hydrogen atoms are replaced with deuterium in the racemization process. This exchange reaction



Amino acid or peptide (0.5 mg)
↓
1) 6N DCl (99.5% deuterium, 1 ml)
110°C 24 hr in sealed tube
2) 6% dry HCl in Pr OH 90°C 1 hr
3) TFA anhydride in CH_2Cl_2
↓
Tfa-amino acid isopropyl ester(s)
↓
GC on OA-201 WCOT column (0.25mm x 50m)
↓
Monitor with JEOL JMS D300 mass spectrometer
JMA-2000 data analysis system

can be utilized for the racemization test by application of the modern technique of "mass fragmentography (MF)" in which a mass spectrometer is used as a GC detector specific to a definite mass peak.[8] Thus, the free amino acids in the DCl hydrolyzate are then converted into volatile Tfa isopropyl esters and subjected to GC on a chiral phase for separation of each D- and L-isomer. The column is directly connected to a mass spectrometer to monitor the intensity of adequate ion peaks. For example, ion peaks at m/e 140 and 168 are selected in order to test the optical purity of Ala and Val respectively. These ions correspond to the base peaks ($M-CO_2Pr^i$) of Tfa isopropyl esters of not deuterated species which were originally present in the peptide. On the other hand, the deuterated DL-isomers formed artificially can be excluded in this measurement since their corresponding peaks shift up by 1 mass unit to m/e 141 and 169.

The method was first applied to free amino acids. Thus, various samples of Ala, Phe and Asp with definite optical purities were analyzed according to the procedure shown in the scheme. After heating in DCl and derivatization, the enantiomers were separated on a glass capillary column of OA-201 (2,4-bis(L-Val-L-Val-OPr^i)-6-ethoxy-*s*-triazine).[9] Mass fragmentography was carried out with JEOL JMS-D300 mass spectrometer (in EI-mode at 70eV) equipped with JMA-2000 computer system. The observed optical purity is in excellent agreement with the true value in each case as shown in Table 1. In comparison with the conventional HCl-GC method,[10] the present method is evidently very accurate without being disturbed by the racemization during hydrolysis: the error range was 0.1 0.2%. It is noteworthy that even aspartic acid, which racemized considerably in hot acid, could be analyzed satisfactorily. For this measurement, the ion peak at m/e 214 corresponding to a main fragment peak ($M-OPr^i-C_3H_6$)[11] of β,β-bisdeuterated

Table 1. Determination of optical purity (D-isomer content) of free amino acids.

	Ala*1				Phe*2	Asp*3
True content of D-amino acid	0.01%	0.14%	1.07%	9.13%	0.00%	0.00%
This method (DCl - MF)	0.06	0.19	1.07	9.24	0.18	0.26
Conventional method (HCl - GC)	1.5	1.6	2.7	10.2	1.5	4.2

*1 monitored in MF at m/e 140, *2 at m/e 216, *3 at m/e 214

Tfa-Asp-$(OPr^i)_2$ was used since its two β-hydrogens were completely exchanged with deuterium under the hydrolysis condition. This indicates that such a deuterium exchange at other than α-position is not necessarily severe interference in this method, provided that an adequate ion peak is monitored.

This DCl-MF method was then actually used to analyze the optical purity of peptides. Model peptides, L-Ala-L-Val and L-Phe-L-Ala-L-Val, containing D-Ala peptides in various ratios[12] were hydrolyzed in DCl and analyzed according to the standard procedure. The mass fragmentogram obtained for the pure L-L-L-tripeptide is shown in Fig. 1 as an example. Almost no peaks of D-amino acids are observed in monitoring the adequate peaks even after acid hydrolysis. As summarized in Table 2 and 3, the observed values are in very good agreement with the original optical purities for all amino acid residues.

For evaluation of the present method, racemization degrees in "fragment condensation" products were determined in the coupling reaction of Boc-L-Phe-L-Ala and L-Val-OBu^t. After condensation with various reagents, the tripeptide fraction was directly hydrolyzed in DCl and analyzed as above. For comparison, the content of L-D-L-peptide was determined by HPLC after deprotection with trifluoroacetic acid. The observed values in both methods coincide very well each other as shown in Table 4, indicating the high accuracy and sensitivity of the present method.

From the results described above, this DCl-MF method offers a very versatile and reliable way for determination of the true chiral purity of amino acid residues in a relatively simple procedure. The remarkable advantages of this method could be summarized as follows: 1) any amino acid in any position in the peptide sequence can be analayzed if its enantiomers can be separated on GC, 2) no reference samples of diastereomeric peptides are needed for the analysis, and 3) several (up to 8) amino acids can be analyzed simultaneously in one GC measurement with less than 1 μg of a sample using the present D300 MF system.

Table 2. Determination of D-amino acid content in Ala-Val.

True content of D-amino acid	Ala Val	0 % 0	0.10% 0	0.99% 0	8.95% 0
This method (DCl - MF)	Ala*1 Val*2	0.18 0.00	0.25 0.00	1.08 0.00	8.16 0.00
Conventional method (HCl - GC)	Ala Val	1.8 0.5	1.8 0.5	2.6 0.5	9.4 0.4

*1 monitored in MF at m/e 140, *2 at m/e 168

Table 3. Determination of D-amino acid content in Phe-Ala-Val.

True content of D-amino acid	Ala Val Phe	0.00% 0 0	0.10% 0 0	0.96% 0 0
This method (DCl - MF)	Ala*1 Val*2 Phe*3	0.15 0.00 0.23	0.26 0.00 0.26	1.16 0.00 0.24
Conventional method (HCl - GC)	Ala	2.4		

*1 monitored in MF at m/e 140, *2 at m/e 168, *3 at m/e 216

Fig. 1. Mass fragmentogram of Tfa isopropyl ester mixture obtained after DCl-hydrolysis of L-Phe-L-Ala-L-Val.

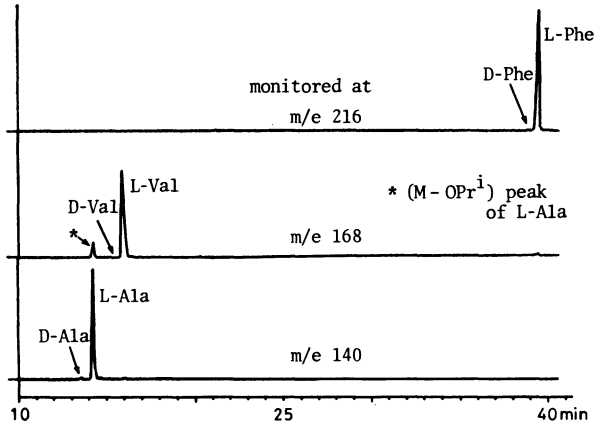


Table 4. Racemization test in coupling of Boc-L-Phe-L-Ala with L-Val-OBu^t.

Coupling reagent	D-Ala content in tripeptide determined by DCl - MF	HPLC
DCC	14.9 %	13.6 %
DCC - HONSu	0.41	0.17
DCC - HONb	0.42	0.18
DCC - HOBt	0.16	+
EEDQ	0.41	0.19
Azide (Rudinger)	0.21	+

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References and Notes

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11) E. Gelpi, W. A. Koenig, J. Gilbert, and J. Oro, J. Chromatogr. Sci., 7, 604 (1969).
12) These model peptides were prepared by mixing definite amounts of pure L-Ala-L-Val and its D-L isomer, or L-Phe-L-Ala-L-Val and its L-D-L isomer, which had been synthesized separately by DCC-HONSu method and purified extensively.

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