N-Pivaloyl Methyl Esters as Novel Derivatives of Amino Acid Enantiomers for Chiral-Phase Capillary Gas Chromatography

Iwao Abe,* Yoshifumi Nakao, and Taketoshi Nakahara
Department of Applied Chemistry, College of Engineering, Osaka Prefecture University, 1-1, Gakuen-cho, Sakai, Osaka 593

(Received March 27, 1997; CL-970227)

Amino acids were first acylated with pivaloyl chloride and then esterified with trimethylsilyldiazomethane in order to form volatile derivatives for enantiomer separation by gas chromatography using capillary column coated with chiral stationary phase. All the derivatives showed complete separation of their enantiomeric pair, especially in the case of Pro which is difficult to separate completely as in conventional N-perfluoroacyl alkyl ester derivatives.

Amino acid enantiomer separation by gas chromatography (GC) made remarkable progress in these two decades with the development of highly enantioselective and thermostable chiral stationary phases.[1] The method has been applied to the configurational analysis of peptide antibiotics,[2] the quantification of the racemization during peptide synthesis,[3] the age-dating of the fossils from the extent of amino acid racemization,[4] etc.

Of the many chiral stationary phases in GC, diamide type phase of Chirasil-Val is known as a suitable phase for the separation of amino acid enantiomers as in a form of N(O)-perfluoroacyl isopropyl esters in a single chromatographic run.[5] However, the derivative type is a laborious one to be prepared that spend over 2 h for treatment of one sample. Moreover, complete separation of the Pro enantiomeric pair is difficult, especially for an analysis of sample containing the Pro in much larger quantities.[6]

In previous papers,[7] we have described the N(O)-alkyloxy-carbonyl alkyl ester derivatives of amino acids in order to speed up the enantiomer analysis. However, the separation factors of the derivatives on Chirasil-Val capillary column were generally low, and it could not be separated the Pro enantiomer after all.

In this present study, we have prepared a novel derivative of N-pivaloyl methyl esters of amino acids according to the procedure first undergo acylation and then esterification, which have been separated on a capillary column coated with a chiral stationary phase similar to Chirasil-Val but with higher enantioselectivity.

Amino acid derivatives of N-pivaloyl methyl esters were prepared according to the following procedure:

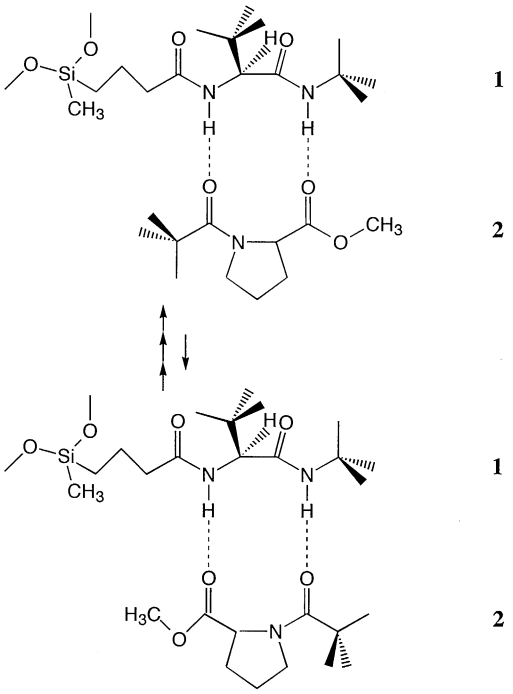
Standard amino acid solution of 100 μl aliquot containing each D,L-amino acid to a concentration of 5 μmol/ml was pipetted into a micro-reaction tube (8 x 50 mm) with polyethylene cap. To this solution, 100 μl of 10%-Na_2CO_3 was added and the total volume was made up to 300 μl with an addition of water. The solution was then adjusted to pH 10.8-11.3. After 10 μl of pivaloyl chloride was added, the tube was capped immediately, and the mixture was shaken vigorously by hand for 30 s in order to convert amino acids into N-pivaloyl derivatives. After addition of 200 μl of saturated NaCl solution, the mixture was acidified to pH 2-3 with an addition of 100 μl of 2.7 M-H_3PO_4. The acidified solution was extracted three times with 300 μl of diethyl ether with shaking for 3 min. The ethereal layers were transferred into another tube by means of Pasteur pipet, and the combined extract was dehydrated over 0.1 g of anhydrous-Na_2SO_4. The supernatant was transferred into a Reacti-Vial (1 ml volume) and once evaporated to dryness with nitrogen stream at room temperature. After the residue was redissolved in 300 μl of ether, 200 μl of methanol and 30 μl of trimethylsilyldiazomethane was added successively for esterification. After standing for about 5 min at room temperature, the solution was evaporated to dryness at 90 °C with gentle stream of nitrogen. The residue was dissolved in 30 μl of dichloromethane and 1-2 μl of the solution was injected into the GC. Dimethylpolysiloxane anchored with L-*t*-leucine-*t*-butylamide[8] was used as chiral stationary phase which has been synthesized according to the reference in order to coat on glass capillary by a static method.[9]

Table 1. Separation data for N-pivaloyl methyl ester derivatives of amino acid enantiomers

Amino acid	Retention time		α^c	Rs[d]	Column temp(°C)
	t_D[a]	t_L[b]			
Ala	3.80	4.03	1.105	3.85	140
Val	4.98	5.24	1.078	3.46	140
allo-Ile	6.40	6.76	1.075	3.41	140
Ile	6.76	7.22	1.090	4.29	140
Leu	7.26	8.08	1.146	7.70	140
Pro	12.34	13.38	1.097	5.87	140
Asp	14.97	15.48	1.038	2.48	140
Glu	14.30	15.57	1.100	5.74	160
Thr	8.48	9.04	1.082	2.83	170
Ser	8.78	9.97	1.059	3.39	170
Phe	7.20	7.53	1.060	3.22	190
Orn	11.63	12.42	1.080	3.45	220
Tyr	15.22	15.79	1.043	2.14	220
Lys	17.11	17.98	1.057	2.54	220
Trp	26.81	27.91	1.044	2.00	240

[a]Retention time of the D-enantiomer; [b]Retention time of the L-enantiomer; [c]Separation factor(corrected); [d]Resolution.

Table 1 represents the retention times of D and L enantiomers, separation factors, and resolutions for 15 amino acids as in a form of N-pivaloyl methyl ester derivative. All the enantiomeric pairs are separated completely with the resolution of above 2.00. The separation factors are not very high as in the case of N-perfluoroacyl alkyl ester derivatives. Nevertheless, Pro and Asp enantiomeric pairs have shown complete baseline separation. Especially, the resolution of Pro is the highest on this type of chiral stationary phase, hydrogen bonding interaction is the major basis of chiral recognition. The separation mechanism is ascribed to the formation of association complexes as depicted in Eq. 1 between the stationary phase **1** and the enantiomers **2** to be separated primarily to the action of two-point hydrogen bonding. As the two carbonyl groups on Pro act as hydrogen acceptor, two interaction models can be postulated. In the Eq. 1, the steric hindrance between the two *t*-butyl groups on Pro and the chiral stationary phase of amide nitrogen is considered to shift the



(1)

association equilibrium greatly to the upper side. The shifted association probably plays an important role in enhancement of the separation factor of the Pro enantiomers. As the size of the alkyl substituent of acyl group on Pro becomes smaller and the alkyl substituent of the ester group becomes larger, the equilibrium would likely to shift to the lower side. Consequently, the separation factors of the Pro enantiomers might be decreased.

N-Acylated amino acids are said to have a risk of racemization arises from activation of the carbonyl group. In a side reaction, an oxazolinone may form, which racemizes rapidly via a resonance stabilized tautomer.[10] The ease of formation of an oxazolinone depends primarily upon the nature of the N-acyl group, presence of bases, temperature, and the side α-alkyl group of each individual amino acid. Figure 1 shows two GCs of N-pivaloyl methyl ester derivatives of amino acids prepared from (a) L-amino acids and (b) D,L-amino acids, respectively. As can be seen easily from the Figure 1 by comparing (a) and (b), racemization was not observed in all amino acids including Asp, the amino acid with fastest racemization rate due to its electron-withdrawing carboxymethyl side chain and the resultant stabilization of the incipient carbanion. The electron-releasing nature of the *t*-butyl group on N-acyl moiety is considered to help preventing the formation of the oxazolinone.

Although the derivative type of N-pivaloyl methyl has already been reported for a quantitative analysis of iodoamino acids,[12] all the derivatives were prepared in the order of esterification and acylation which can not apply directly to the aqueous amino acid samples.

The authors thank the Grant-in-Aid for Scientific Research from the Ministry of Education of Japan, Science and Culture for partial support of this work.

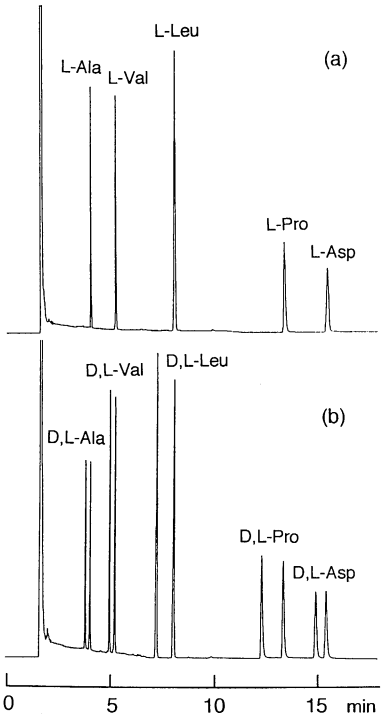


Figure 1. Gas chromatograms of N-pivaloyl methyl ester derivatives of amino acid enantiomers prepared from (a) L-amino acids and (b) D,L-amino acids. Column: Glass capillary column (20m x 0.25 mm) coated with 3-carboxypropyl-substituted dimethylpolysiloxane coupled with L-*t*-leucine-*t*-butylamide;[11] Column temp.: 140 °C, isothermal; Carrier gas: He, 1.5 kg/cm^2; Detector: FID; Split ratio: 1:35. For each amino acid enantiomeric pair, the D-enantiomer eluted faster.

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