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# New Peptide Models for Studying Racemization[1,2]

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A new test for racemization was developed and used to assess known coupling reagents and procedures. The procedure consists of coupling between BOC–Ala–Met–Leu–OH and *tert*-butyl ester of amino acid [Leu, Ile or Asp(OBu$^t$)], cleaving of the resulting peptide by cyanogen bromide to form dipeptide diastereomers, and analyzing both isomers with an amino acid analyzer.

The following results were obtained:

1) The DCC-additive (Eintopf) method, especially the HONB-DCC procedure, was somewhat superior to the standard azide method.

2) Even in the azide method, epimerization during peptide bond formation considerably increased in the presence of excess base.

3) Significant racemization was observed in some of the known coupling methods which have been shown, by the Young or Anderson test, to be free from racemization.

**Keywords**——racemization; peptide synthesis; coupling method; azide coupling; DCC-additive method; cyanogen bromide; dipeptide diastereomer

Partial epimerization of an optically active amino acid residue during assembly of peptide fragments has particularly become a serious problem in practical peptide synthesis, because the presence of enantiomeric residue in a biologically active polypeptide will dramatically affect the biological activity if the residue interferes with the correct conformation folded by the primary structure, as previously suggested by several investigators.[4]

Although peptide synthesis is usually carried out under conditions considered to be safe from racemization after checks by several evaluation methods, most of the available methods for detecting epimerization are too simple as models for practical peptide synthesis, *e.g.*, including the methods of Anderson, Young, Taschner, Izumiya and Weygand.[5] Recently, a highly sensitive method for detecting racemization based upon isotope dilution was developed by Kemp, *et al.*[6] This method, however, is still based on the Anderson and Young tests. When the amino acid residue penultimate to the C-terminus of the carboxy component is glycine, the extent of racemization during the coupling reaction decreases remarkably compared with a carboxy component having other amino acids at the position, according to Sieber

1) Presented at the 14th Symposium on Peptide Chemistry (Japan), Hiroshima City, Nov. 4, 1976; M. Fujino, C. Kitada, and I. Yoshida, "Peptide Chemistry, 1976," ed. by T. Nakajima, Protein Research Foundation, Osaka, 1977, p. 28.

2) Amino acids, peptides and their derivatives in this paper are of the L-configuration unless otherwise mentioned. The following abbreviations are used: Z=benzyloxycarbonyl, BOC=*tert*-butoxycarbonyl, OBu$^t$=*tert*-butyl ester, HONB=N-hydroxy-5-norbornene-2,3-dicarboximide, DCC=N,N′-dicyclohexylcarbodiimide, DCHA=dicyclohexylamine, TEA=triethylamine, TFA=trifluoroacetic acid, THF=tetrahydrofuran, DMF=N,N-dimethylformamide.

3) Location: *Juso-honmachi, Yodogawa-ku, Osaka, 532, Japan.*

4) M. Monahan, M. Amoss, H. Anderson, and W. Vale, *Biochemistry*, **12**, 4616 (1973); M. Fujino, I. Yamazaki, S. Kobayashi, T. Fukuda, S. Shinagawa, R. Nakayama, W.F. White, and R.H. Rippel, *Biochem. Biophys. Res. Commun.*, **57**, 1248 (1974), **60**, 406 (1974); J. Rivier, M. Brown, and W. Vale, *ibid*, **65**, 746 (1975).

5) For a review, see M. Bodanszky, Y.S. Klausner, and M.A. Ondetti, "Peptide Synthesis," John Wiley and Sons, New York, 1976, p. 137.

6) D.S. Kemp, S.W. Wang, G. Busby, and G. Hugel, *J. Am. Chem. Soc.*, **92**, 1043 (1970).

and Riniker.[7] Therefore, good methods are needed to determine ideal conditions and/or to compare the usefulness of coupling procedures.

We report here some racemization tests using an amino acid analyzer and also the results of the influence of several known coupling reagents or procedures (see Table I) on the extent of epimerization during activation of the carboxy group of the carboxy components.

One of the present tests is a modification of the Bodanszky and Conklin test,[8] which originally consisted of the coupling between acetyl-isoleucine and glycine ethyl ester and the

Table I. Extent of Racemization during Peptide Bond Formation with Various Coupling Methods

| Coupling method (in DMF) | Reaction temperature C° | Detection method (A) allo-Ile | (B) Leu-Ile | (C) Leu-Leu | (D) Leu-Asp |
|---|---|---|---|---|---|
| | | Free base[a] | TEA-HCl[b] | TEA-HCl[b] | Free base[a] |
| DCC[d] (1.5 eq) | (0) | 26.8± 1.1[c] | 8.7 (8.0)[a] | 9.3 (4.6)[a] | 14.8 (20.1)[b] |
| HONB-DCC[e] (2 eq/1.5 eq) | (0) | 0.8± 0.4 | 0.7 | <1.5 | 3.4 (6.0)[b] |
| HOSu-DCC[f] (2 eq/1.5 eq) | (0) | 2.9± 3.0 | 1.6 | <2.9 | 9.2 |
| HOBt-DCC[g] (2 eq/1.5 eq) | (0) | 7.5± 3.0 | 0.7 | <1.9 | 9.1 |
| EEDQ[h] (1.2 eq) | (0) | 12.2—27.6 | 0.7 | <1.2 | 5.1 |
| CCBT-N-methylmorphorine[i] (1 eq/1 eq) | (0) | 4.9± 2.1 | 12.4 | 19.2 | 6.7 |
| $(Ph)_3P$ in $CH_2Cl_2$[j] | (0) | 33.0 | 19.8 (9.6)[a] | 26.5 (13.5)[a] | 11.7 |
| $(Ph)_3P$+Pyrid-2-thione[j] | (−5) | 24.7±2.1 | | | |
| DPPA-TEA[k] (1.2 eq/1 eq) | (0) | 14.1± 2.1 | 1.8 | 3.9 | 6.7 |
| DEPC-TEA[l] (1.2 eq/1 eq) | (0) | 11.9 | 0.4 | | |
| Azide (extracted with AcOEt)[m] | (−10) | | 1.6 | 2.7 | 5.5 |
| Azide (Rudinger)[n] | (−30) | 1.4± 0.4 | 1.3 | 2.3 | 8.0 |
| Azide (in situ)[o] | (−15) | 11.2± 0.8 | 4.2 | 4.5 | 7.0 |
| MA (Anderson)[p] | (−20) | | 3.9 | | |
| Pepsin (in 30% aqueous MeOH)[q] | (37) | | 1.6 | | |
| Stepwise elongation | | | 0.8 | | |

(A): BOC–Leu–Ile–OH (or $-NHNH_2$)+H–Asn–Leu–$OBu^t$ —conpling, acid hydrolysis→ chromatographic anal.

(B): BOC–Ala–Met–Leu–OH (or $-NHNH_2$)+H–Ile–$OBu^t$ —coupling, TFA, BrCN→ chromatographic anal.

(C): BOC–Ala–Met–Leu–OH (or $-NHNH_2$)+H–Leu–$OBu^t$ —coupling, TFA, BrCN→ chromatographic anal.

(D): BOC–Ala–Met–Leu–OH (or $-NHNH_2$)+H–Asp$(OBu^t)_2$ —coupling, TFA, BrCN→ chromatographic anal.

*a*) The amine component was used as the free base, without the addition of triethylamine.
*b*) The coupling was carried out in the presence of 1 eq. of triethylammonium chloride.
*c*) The mean value of triplicate measurements and the standard error of the mean; Control value (1.6%), racemization during acid hydrolysis, is deducted from the calculated value.
*d*) N,N′-dicyclohexylcarbodiimide: J. C. Sheehan and G. P. Hess, *J. Am. Chem. Soc.*, **77**, 1067 (1955).
*e*) N-Hydroxy-5-norbornene-2,3-dicarboximide: M. Fujino, S. Kobayashi, M. Obayashi, T. Fukuda, S. Shinagawa, and O. Nishimura, *Chem. Pharm. Bull. (Tokyo)*, **22**, 1857 (1974).
*f*) N-Hydroxysuccinimide: F. Weygand, D. Hoffmann, and E. Wünsch, *et al.*, *Z. Naturforsch.*, **b 21**, 426 (1966).
*g*) 1-Hydroxybenzotriazole: W. König and R. Geiger, *Chem. Ber.*, **103**, 788 (1970).
*h*) N-Ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline: B. Belleau and G. Malek, *J. Am. Chem. Soc.*, **90**, 1651 (1968).
*i*) 6-Chloro-1-*p*-chlorobenzene sulfonyloxybenzotriazole: M. Itoh, H. Nojima, J. Notani, D. Hagiwara, and K. Takai, *Tetrahedron Lett.*, **1974**, 3089.
*j*) Triphenylphosphine+2,2′-dipyridyldisulfide: T. Mukaiyama, R. Matsueda, and M. Suzuki, *Tetrahedron Lett.*, **1970**, 1901.
*k*) Diphenyl phosphorazidate: T. Shioiri, K. Ninomiya, and S. Yamada, *J. Am. Chem. Soc.*, **94**, 6203 (1972).
*l*) Diethylphosphorocyanidate: T. Shioiri, Y. Yokoyama, Y. Kasai, and S. Yamada, *Tetrahedron*, **32**, 2211 (1976).
*m*) T. Curtius, *Chem. Ber.*, **35**, 3226 (1902).
*n*) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).
*o*) M. Zaoral, *Collect. Czech. Chem. Commun.*, **30**, 1853 (1965).
*p*) G. W. Anderson J.E. Zimmerman, and F.M. Callahan, *J. Am. Chem. Soc.*, **88**, 1338 (1966).
*q*) Y. Isowa, T. Nagasawa, K. Kuroiwa, and K. Narita, Japan Patent 51-110093 (1976).

7) P. Sieber and B. Riniker, "Peptide, 1971," ed. by H. Nesvadba, North-Holland Publishing Co., Amsterdam, 1973, p. 49.
8) M. Bodanszky and L.E. Conklin, *J. Chem. Soc., Chem. Commun.*, **1967**, 773.

determination of the amount of alloisoleucine after total acid hydrolysis. We employed BOC-Leu-Ile-OH and H-Asn-Leu-OBu$^t$ respectively as a carboxy and an amine component for coupling. The results obtained under the standard coupling conditions are summarized in Table I, together with their references.

Next, we attempted to develop a similar test using threonine residue instead of isoleucine. Threonine residue in peptides gives the allo-isomer by epimerization at the $\alpha$-carbon. As shown in Table II, however, the threonine residue at the C-terminus of the carboxy component may resist formation of the oxazolone during the carboxy-activation process, the residue being relatively stable against racemization. Note that in the case of the threonine peptides only a small amount of D-allothreonine peptides was obtained, even by the DCC coupling method without any additives.

Table II. Extent of Racemization at Threonine Residue during Peptide Bond Formation between BOC-Asp(OBzl)-Thr-OH and Several Dipeptide Esters

| Amine component | Coupling method | |
|---|---|---|
| | HONB-DCC (%) | DCC (%) |
| H-Val-Thr-OEt | 2.0 | 3.4 |
| H-Ala-Gly-OBu$^t$ | 1.1 | 2.8 |
| H-Leu-Leu-OBu$^t$ | — | 0.6 |
| H-Asn-Leu-OBu$^t$ | 1.0 | 2.0 |

Extent of racemization is defined as 100 × D-allo-Thr/(D-allo-Thr+Thr): D-Allo-Thr is detected under the conditions, pH 5.42; 150 cm column; temp., 55°, by an amino acid analyzer. The values are given without deduction of the control value of acid hydrolysis.

Although these racemization tests are convenient, unexpected racemization can occur during total acid hydrolysis of the resulting peptides or degradation of the activated intermediates under the usual work-up. To avoid these demerits, a more suitable method for determining epimerization was developed. The new test consists of coupling BOC-Ala-Met-Leu-OH and the *tert*-butyl ester of amino acid (leucine, isoleucine or aspartic acid) and detection of the dipeptide diastereomers derived by cleavage with cyanogen bromide[9] at the

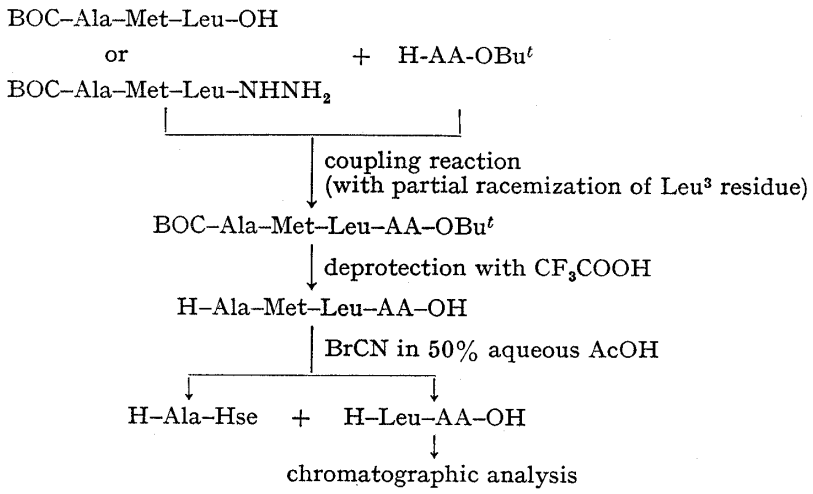


Fig. 1. The Scheme for the Racemization Test

H-AA-OBu$^t$=H-Leu-OBu$^t$, H-Ile-OBu$^t$ or H-Asp(OBu$^t$)$_2$,
AA=Leu, Ile or Asp, Hse=homoserine or homoserine-lactone.

9) E. Gross and B. Witkop, *J. Am. Chem. Soc.*, **83**, 1510 (1961).

carbonyl part of the methionine residue of the tetrapeptides which result from the deprotection with TFA of the protected tetrapeptides, as illustrated in Fig. 1.

Although the crude products, BOC-Ala-Met-Leu-AA-OBu$^t$, may contain some concomitants such as unreacted components and/or an activated intermediate, these concomitants do not hinder the determination of epimerization at the leucine[3] residue in the tetrapeptides, because the dipeptides (H-Leu-Leu-OH, H-Leu-Ile-OH and H-Leu-Asp-OH) are the final products to be subjected to chromatographic analyses and are derived only from the corresponding tetrapeptides.

As shown in Table III, the diastereomeric mixture containing leucine, isoleucine or aspartic acid as the C-terminal residue was separated completely by the analyzer under the conditions given in the Table. The measurable limitation of these methods, studied with an artificial mixture of both isomers, was approximately 1%.

Table III. Conditions of Amino Acid Analysis and Elution Volumes of L- and D-Isomers

| Compound | Column size (cm) | Elution buffer[a] (pH) | Elution volume[b] | |
|---|---|---|---|---|
| | | | L-L | D-L (isomer) |
| H-Leu-Leu-OH | 50 | 5.28 | 57 | 74 |
| H-Leu-Ile-OH | 50 | 5.28 | 52 | 68 |
| H-Leu-Asp-OH | 20 | 3.25 | 89 | 71 |

*a*) Column temperature, 55°. *b*) 60 ml/hour.

These procedures were applied to examine the influence of various known coupling reagents or coupling methods on epimerization, and the results are summarized in Table I together with the corresponding references. The results indicated that the amine component substantially influences the extent of racemization when the same carboxy component is used. In our experiments, aspartic acid di-*tert*-butyl ester exerted considerably more influence on the racemization even in the absence of triethylammonium chloride. This may be due to the great bulkiness of di-*tert*-butyl aspartate. To add excess base was also found unsuitable as indicated by several other investigators[4,6]; even the coupling was achieved by the standard azide method. Using these test systems, we surprisingly found that hte DCC-additive (Eintopf) method, especially the HONB–DCC method, was more suitable than the standard azide coupling in avoiding racemization.

Even in our study, some coupling reagents assessed gave irregular results in regard to the extent of racemization. Therefore, when such methods are used for practical peptide synthesis, the degree of racemization of the resulting peptides prepared by fragment assembly should be checked using methods such as that of Manning and Moore[10] and/or the L- and D-amino acid oxidase.[11]

## Experimental

All melting points were taken in open capillaries and uncorrected. Rotations were determined with a Perkin-Elmer Model 141 polarimeter. Amino acid analyses were performed with a Hitachi KLA-3B amino acid analyzer. Evaporations were carried out in a rotary evaporator under reduced pressure at a temperature of 35—40°. The purity of the products was tested by thin-layer chromatography (TLC) using Merck precoated silica gel plate $60F_{254}$. Solvent systems used were: $CHCl_3$–MeOH (19: 1, $Rf^1$) and $CHCl_3$–MeOH–AcOH (9: 1: 0.5, $Rf^2$).

**BOC-Leu-Ile-OH**——To a solution of H–Ile–OH (15.7 g, 120 mmol) and TEA (15 ml) in a mixture of dioxane (30 ml), THF (50 ml) and $H_2O$ (150 ml) was added BOC–Leu–ONB (39.4 g, 100 mmol) and the

10) J.M. Manning and S. Moore, *J. Biol. Chem.*, **243**, 5591 (1968).
11) S. Ishii and B. Witkop, *J. Am. Chem. Soc.*, **85**, 1832 (1963).

mixture was stirred for 3 hr at 5° and for additional 16 hr at room temperature. After concentration to about half of its original volume, the mixture was neutralized with 1 N HCl (110 ml) and then the product was extracted with AcOEt (600 ml). After washings and evaporation, the residue was crystallized from $H_2O$: 29.9 g (87%), mp 120—122°, $[\alpha]_D^{21}$ −25.9° ($c$=0.51 in EtOH), $Rf^2$ 0.61. *Anal.* Calcd. for $C_{17}H_{32}N_2O_5$: C, 59.28; H, 9.37; N, 8.13. Found: C, 59.81; H, 9.63; N, 8.12.

**BOC-Leu-Ile-NHNH$_2$**——To a solution of BOC–Leu–Ile–OEt[12] (9.7 g, 26 mmol) in MeOH (60 ml) was added hydrazine hydrate (12.6 ml) and the mixture was allowed to stand at room temperature for 3 days. The crystals formed were collected by filtration and then washed well with MeOH: 8.65 g (92%), mp 165—166°, $[\alpha]_D^{21}$ −65.8° ($c$=0.48 in EtOH), $Rf^2$ 0.63. *Anal.* Calcd. for $C_{17}H_{34}N_4O_4$: C, 56.96; H, 9.56; N, 15.63. Found: C, 56.99; H, 9.87; N, 15.62.

**BOC-Met-Leu-OH·DCHA**——BOC–Met–Leu–OH was obtained from BOC–Met–OH and H–Leu–OH by the HONB activated ester method by essentially the same procedure described above, and the product was converted to the corresponding DCHA salt by the usual manner: 55.4%, mp 148—149.5°, $[\alpha]_D^{23}$ −24.3° ($c$=0.975 in MeOH), $Rf^2$ 0.57. *Anal.* Calcd. for $C_{28}H_{53}N_3O_5S$: C, 61.84; H, 9.82; N, 7.73; S, 5.90. Found: C, 61.69; H, 9.99; N, 7.71; S, 5.81.

**BOC-Met-Leu-OMe**——To a solution of H–Leu–OMe·HCl (18.2 g, 100 mmol), TEA (14.0 ml) and BOC–Met–OH (24.9 g, 100 mmol) in a mixture of acetonitrile (150 ml) and $CH_2Cl_2$ (150 ml) was added DCC (22.7 g, 110 mmol) at 0°. The mixture was stirred at 0° for 4 hr and at room temperature for additional 12 hr. After filtration and evaporation, the residue was dissolved in AcOEt (500 ml) and the solution was washed with 4% $NaHCO_3$, 0.2 N HCl and $H_2O$, then dried over anhydr. $Na_2SO_4$. The solution was evaporated to dryness and the residue was crystallized from AcOEt-pet. ether: 31.6 g (84%), mp 110—112°, $[\alpha]_D^{22}$ −37.3° ($c$=1.13 in MeOH), $Rf^1$ 0.73. *Anal.* Calcd. for $C_{17}H_{32}N_2O_5S$: C, 54.23; H, 8.57; N, 7.44; S, 8.52. Found: C, 54.49; H, 8.45; N, 7.47; S. 8.55.

**BOC-Ala-Met-Leu-OH**——This compound was prepared from BOC–Ala–ONB and H–Met–Leu–OH by essentially the same procedure described above and the material was crystallized from ether: 68%, mp 171—172°, $[\alpha]_D^{24}$ −49.1° ($c$=0.95 in MeOH), $Rf^2$ 0.55. *Anal.* Calcd. for $C_{19}H_{35}N_3O_6S$: C, 52.63; H, 8.14; N, 9.69; S, 7.40. Found: C, 52.57; H, 8.15; N, 9.69; S, 7.40.

**BOC-Ala-Met-Leu-OMe**——The BOC-group of BOC–Met–Leu–OMe (15.06 g, 40 mmol) was removed by treatment with TFA and the resulting TFA salt was dissolved in AcOEt (100 ml). To this solution were added BOC–Ala–ONB (14.0 g, 40 mmol) and TEA (6 ml) and the mixture was stirred for 16 hr at room temperature. The reaction mixture was washed with 4% $NaHCO_3$ and 0.2 N HCl and dried over anhydr. $Na_2SO_4$, then evaporated. The residue was crystallized from AcOEt–pet. ether: 15.6 g (87%), mp 100—101°, $[\alpha]_D^{26}$ −54.5° ($c$=0.55 in MeOH), $Rf^1$ 0.47. *Anal.* Calcd. for $C_{20}H_{37}N_3O_6S$: C, 53.67; H, 8.33; N, 9.39; S, 7.16. Found: C, 53.54; H, 8.27; N, 9.46; S, 7.11.

**BOC-Ala-Met-Leu-NHNH$_2$**——To a solution of BOC–Ala–Met–Leu–OMe (4.5 g, 10 mmol) in MeOH (20 ml) was added hydrazine hydrate (2.5 ml) and the solution was allowed to stand at room temperature for 20 hr. The reaction mixture was evaporated and the residue was crystallized from aqueous MeOH: 4.2 g (93%), mp 214—215°, $[\alpha]_D^{26}$ −58.4° ($c$=0.5 in MeOH), $Rf^2$ 0.46. *Anal.* Calcd. for $C_{19}H_{37}N_5O_5S$: C, 50.98; H, 8.33; N, 15.65; S, 7.16. Found: C, 50.95; H, 8.43; N, 15.71; S, 7.07.

**Procedure of a Modified Bodanszky and Conklin Test**——BOC–Leu–Ile–OH or BOC–Leu–Ile–NHNH$_2$ (2 mmol) was condensed with H–Asn–Leu–OBu$^t$ (2 mmol), which was prepared from Z–Asn–Leu–OBu$^t$[13] by hydrogenolysis, by the various coupling methods to be essentially the same procedure described in the literatures cited in Table I. The reaction mixture was diluted with AcOEt (100 ml) and the mixture was washed with 4% $NaHCO_3$ and 0.2 N HCl, then dried over anhydr. $Na_2SO_4$. After evaporation the residue was dried well *in vacuo*. A part of the residue (*ca.* 500 mg) was applied to a column (2.5×3.5 cm) of silica gel, which was eluted with a mixture of $CHCl_3$ and MeOH (97: 3). The fractions (14—56 ml) were collected and evaporated to give a powder. A part of the powder (*ca.* 5 mg) was hydrolyzed with 5.7 N HCl at 110° for 24 hr. The hydrolysate was analyzed with a Hitachi amino acid analyzer. According to the Bodanszky and Conklin method[8] the extent of epimerization was expressed.

The test using the threonine-peptide was carried out by essentially the same procedure described above.

**Procedure of the New Racemization Test**——BOC–Ala–Met–Leu–OH or BOC–Ala–Met–Leu–NHNH$_2$ (0.62 mmol) was coupled to H–AA–OBu$^t$ (Leu, Ile or Asp *tert*-butyl ester) (0.62 mmol) under the condition mentioned in Table I. The reaction mixture was diluted with AcOEt (50 ml) and then washed with 4% $NaHCO_3$ and 0.2 N HCl, dried over anhydr. $Na_2SO_4$. After evaporation the residue was dried well *in vacuo*, and a part of the residue (20 mg) was treated with TFA (2 ml) for 30 min at room temperature to remove the protecting groups. After removal of TFA by evaporation, the residue was dissolved in 50% AcOH (2 ml), and to this solution was added BrCN (50—100 mg). The mixture was kept stand for 60—120 min at room temperature and then the mixture was lyophilized. The resulting product was analyzed with an amino acid analyzer under the condition shown in Table III.

12) E. Schroder, *Ann. Chem.*, **711**, 227 (1968).
13) R.O. Studer and W. Lergier, *Helv. Chim. Acta*, **48**, 460 (1965).

**The Limitation of Measurement**——The measurable limit of D-Leu–Ile and Leu-Ile was studied with a synthetic mixture of both the isomers. When the mixture containing 1.07% D-isomer was analyzed, the measurable peak of the D-isomer was observed and the content of the D-isomer in the mixture, which was calculated according to the formula: (D-isomer)/(L-isomer+D-isomer)×100, was 1.13%.

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