

# Facile Synthesis of Cyclic Dipeptides and Detection of Racemization

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Several cyclic dipeptides were synthesized by refluxing methanolic solution of dipeptide methyl esters in high yield and purity. Cyclic dipeptides prepared by the Fischer method, the Nitecki method, and the present methanol-reflux method were compared with respect to yield and optical purity. The methanol-reflux method usually gave good results. High-performance liquid chromatography was effectively applied to detect racemization.

Various natural cyclic dipeptides (2,5-piperazinedione derivatives) are known by their diverse biological activities such as antibiotic,<sup>1)</sup> bitter taste,<sup>2)</sup> plant growth retardation,<sup>3,4)</sup> and inhibition of hormone secretion.<sup>5)</sup> With regard to synthetic cyclic dipeptides, Izumiya *et al.* observed highly asymmetric induction on hydrogenation of cyclic dehydrodipeptides.<sup>6)</sup> Shiba *et al.* studied structure-activity relationships of bitter cyclic dipeptides.<sup>7)</sup> Kopple *et al.* utilized cyclic dipeptides as a model for studying peptide conformation.<sup>8,9)</sup>

Cyclic dipeptides have been prepared by the Fischer method: Aminolysis of dipeptide ester in methanolic ammonia.<sup>10)</sup> This method is simple to apply and usually gives high yield. Nitecki *et al.*, however, pointed out appreciable racemization derived from this procedure and proposed another method.<sup>11)</sup> Deprotection of Boc-dipeptide methyl ester by formic acid and subsequent reflux of the deprotected ester formate with a mixture of *s*-butyl alcohol and toluene.<sup>12)</sup> This Nitecki method was reported to be less prone to racemization. Lee *et al.*<sup>13)</sup> reported that a side reaction occurred at the side chain of a serine residue in the course of the Nitecki method. Thus, both synthetic methods seemed to envisage undesired side reactions.

It is well-known that dipeptide esters spontaneously

cyclize to form cyclic dipeptides upon hydrogenolysis of a benzyloxycarbonyl protecting group. As a modification of this reaction, reflux of methanolic solution of dipeptide esters has been tried in the present study to facilitate cyclization, and experimental conditions have been examined to improve yield and optical purity of products.

For detection of diastereomers of cyclic dipeptides, Nitecki *et al.*<sup>11)</sup> and Westley *et al.*<sup>14)</sup> used NMR, TLC, and gas liquid partition chromatography. These methods are easy to operate but precision and sensitivity are insufficient to detect a small amount of racemates. Lee *et al.*<sup>13)</sup> and Kanmera *et al.*<sup>15)</sup> reported detection of racemization by the Manning-Shimohigashi method.<sup>6,16,17)</sup> This method using an amino acid analyzer can be applied with high precision, but the procedure is so complicated that an unexpected error might be possible. Recently, high-performance liquid chromatography has come into use in peptide chemistry.<sup>18,19)</sup> This technique is expected to give a simple and convenient method for racemization test.

We report here convenient synthesis of cyclic dipeptides by refluxing methanolic solution of dipeptide methyl esters. Rapid and highly sensitive detection of racemization by HPLC<sup>12)</sup> is also described.

TABLE 1. YIELDS AND PHYSICAL CONSTANTS OF CYCLIC DIPEPTIDES

| Compound                     | Yield<br>% | Mp<br>$\theta_m/^\circ\text{C}$ , decomp | $[\alpha]_D^{20}$ <sup>a)</sup> / $^\circ$ | Formula                                                       | Found(%)<br>(Calcd(%)) |              |                |
|------------------------------|------------|------------------------------------------|--------------------------------------------|---------------------------------------------------------------|------------------------|--------------|----------------|
|                              |            |                                          |                                            |                                                               | C                      | H            | N              |
| <i>cyclo</i> (-L-Val-L-Val-) | 64         | 272—273                                  | —62.0<br>( <i>c</i> 0.5, AcOH)             | C <sub>10</sub> H <sub>18</sub> O <sub>2</sub> N <sub>2</sub> | 60.37<br>(60.58)       | 9.31<br>9.15 | 14.13<br>14.13 |
| <i>cyclo</i> (-L-Val-D-Val-) | 45         | >300                                     |                                            | C <sub>10</sub> H <sub>18</sub> O <sub>2</sub> N <sub>2</sub> | 60.43<br>(60.58)       | 9.24<br>9.15 | 14.14<br>14.13 |
| <i>cyclo</i> (-L-Ala-L-Ala-) | 80         | 276—280                                  | —22.8<br>( <i>c</i> 0.5, AcOH)             | C <sub>6</sub> H <sub>10</sub> O <sub>2</sub> N <sub>2</sub>  | 50.78<br>(50.70)       | 7.17<br>7.09 | 19.49<br>19.71 |
| <i>cyclo</i> (-L-Ala-D-Ala-) | 88         | 256—259                                  |                                            | C <sub>6</sub> H <sub>10</sub> O <sub>2</sub> N <sub>2</sub>  | 50.84<br>(50.70)       | 7.14<br>7.09 | 19.51<br>19.71 |
| <i>cyclo</i> (-L-Ser-L-Ser-) | 77         | 231—233                                  | —54.0<br>( <i>c</i> 0.1, DMF)              | C <sub>6</sub> H <sub>10</sub> O <sub>4</sub> N <sub>2</sub>  | 41.32<br>(41.38)       | 5.93<br>5.79 | 15.97<br>16.08 |
| <i>cyclo</i> (-L-Ser-D-Ser-) | 74         | 261—264                                  |                                            | C <sub>6</sub> H <sub>10</sub> O <sub>4</sub> N <sub>2</sub>  | 41.33<br>(41.38)       | 5.88<br>5.79 | 15.99<br>16.08 |
| <i>cyclo</i> (-L-Pro-L-Pro-) | 64         | 141—142                                  | —148<br>( <i>c</i> 1, AcOH)                | C <sub>10</sub> H <sub>14</sub> O <sub>2</sub> N <sub>2</sub> | 61.54<br>(61.84)       | 7.35<br>7.27 | 14.20<br>14.42 |
| <i>cyclo</i> (-L-Pro-D-Pro-) | 83         | 176—177                                  |                                            | C <sub>10</sub> H <sub>14</sub> O <sub>2</sub> N <sub>2</sub> | 61.53<br>(61.84)       | 7.34<br>7.27 | 14.24<br>14.42 |

a)  $[\alpha]_D$  of all *cyclo*(-L-AA-D-AA-) should be 0°, therefore measurement was not carried out.

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TABLE 2. SEPARATION CONDITIONS BY HPLC<sup>a)</sup>

| Compound                             | Filler;<br>LiChrosorb | Eluent<br>(ratio)                                                                   | Column<br>(cm) | Pressure<br>kg cm <sup>-2</sup> | Retention<br>time/min |      | Absorption<br>intensity<br>ratio<br>(LL/DL) |
|--------------------------------------|-----------------------|-------------------------------------------------------------------------------------|----------------|---------------------------------|-----------------------|------|---------------------------------------------|
|                                      |                       |                                                                                     |                |                                 | LL                    | DL   |                                             |
| <i>cyclo</i> (-L-Leu-(L and D)-Leu-) | PR-18                 | H <sub>2</sub> O-CH <sub>3</sub> CN<br>(8 : 2)                                      | 0.26 × 15      | 300                             | 7.5                   | 5.4  | 1.06                                        |
| <i>cyclo</i> (-L-Val-(L and D)-Val-) | RP-18                 | H <sub>2</sub> O-CH <sub>3</sub> CN<br>(9 : 1)                                      | 0.26 × 15      | 300                             | 4.0                   | 2.3  | 0.98                                        |
| <i>cyclo</i> (-L-Ala-(L and D)-Ala-) | RP-8                  | H <sub>2</sub> O                                                                    | 0.4 × 25       | 150                             | 10.3                  | 7.8  | 1.05                                        |
| <i>cyclo</i> (-L-Ser-(L and D)-Ser-) | RP-18                 | KH <sub>2</sub> PO <sub>4</sub> -<br>Na <sub>2</sub> HPO <sub>4</sub> <sup>b)</sup> | 0.26 × 50      | 450                             | 31.7                  | 27.4 | 1.03                                        |

a) Other conditions are as follows: Hitachi 635A liquid chromatograph; flow rate, 1 ml/min; wavelength, 210 nm; temperature, 20 °C. b) 3 M (1 M = 1 mol dm<sup>-3</sup>) at pH 6.6.

## Results and Discussion

### Separation Conditions of Diastereomers by HPLC.

Table 1 shows cyclic dipeptides prepared in this study as model peptides. *cyclo*(-L-Leu-L-Leu-), *cyclo*(-L-Leu-D-Leu-), *cyclo*(-L-Phe-L-Phe-), and *cyclo*(-L-Phe-D-Phe-) were also prepared according to the literature.<sup>11)</sup> Separation conditions by HPLC were examined. Satisfactory separation was obtained by reversed-phase chromatography with LiChrosorb RP-8 and RP-18 as filler, experimental conditions being shown in Table 2. Diastereomers of *cyclo*(-L-Ser-(L and D)-Ser-) were separated by phosphate buffer better than by water. Generally, LiChrosorb RP-18 shows longer retention time than LiChrosorb RP-8 does. On the other hand, unsatisfactory results were obtained for adsorption chromatography with Hitachi gel #3040 and reversed-phase chromatography with gels of large particle size. *cyclo*(-L-Pro-(L and D)-Pro-) could not be satisfactorily separated.

### Experimental Conditions for the MeOH-reflux Method.

Reaction conditions for the MeOH-reflux method were studied by the use of *cyclo*(-L-Leu-(L and D)-Leu-) as a model. Degree of racemization is given by racemization (%) = [LD%/(LL% + LD%)] × 100. A rough estimate of reaction was given in terms of the reaction time elapsed until a TLC spot of the starting ester had disappeared.

**Effect of Methyl and Ethyl Ester Groups.** Cyclization of H-L-Leu-L-Leu-OMe and H-L-Leu-L-Leu-OEt in MeOH was compared in order to study the effect of methyl and ethyl groups on reaction rate, racemization, and yield of *cyclo*(-L-Leu-L-Leu-). The reaction time, racemization, and yield were 80 h, 1.2%, and 76% for methyl, and 160 h, 1.9%, and 65% for ethyl

ester, respectively. Therefore, methyl ester was used hereafter.

**Effect of Reflux Solvent.** Table 3 shows the effect of reflux solvent. The reaction rate and racemization increased with the raise of the boiling point of a solvent. Severe racemization was detected in the case of *s*-BuOH and a side reaction was suggested by TLC. Racemization was negligible for MeOH and EtOH, and MeOH gave higher yield. Based on these results, reflux of methanolic solution of dipeptide methyl ester is recommended for facile synthesis of cyclic dipeptides.

**Effect of Salt on Racemization.** Boc-L-Leu-L-Leu-OMe was deprotected with HCOOH, subsequently neutralized with NEt<sub>3</sub>, and the methanolic solution of the mixture was refluxed. The same procedure was repeated without salt, and the degree of racemization was compared. When 1.2, 1.0, and 0 equivalent of HCOOH·NEt<sub>3</sub> were present, 2.3, 1.8, and 1.2% racemizations were observed respectively. Clearly, excess of acid and base should be avoided.

**Effect of Side Chain of Amino Acid Residues on Cyclization Rate.** The effect of the size of a side chain in H-dipeptide-OMe on reaction rate was studied. When H-Gly-L-Ala-OMe, H-L-Ala-L-Ala-OMe, and H-L-Leu-L-Ala-OMe were used as starting compounds, the reaction times to give a cyclic dipeptide were 10, 20, and 24 h, respectively. For H-Gly-L-Leu-OMe and H-L-Leu-L-Leu-OMe, 24 and 80 h, respectively. As expected, cyclization rate decreased with the increase of the size of a side chain.

**Effect of Diastereomers in a Dipeptide Ester.** Cyclization of H-L-Leu-L-Leu-OMe and H-L-Leu-D-Leu-OMe was compared to examine diastereomeric difference. Reaction time, racemization, and yield of cyclization were 80 h, 1.2%, and 76% for H-L-Leu-L-Leu-OMe, and 70 h, 1.1%, and 67% for H-L-Leu-D-Leu-OMe. The effect of diastereomeric difference is only slight if any.

**Comparison of Synthetic Methods.** Cyclic dipeptides shown in Table 4 were prepared by the three methods, and racemization of these peptides except for *cyclo*(-L-Phe-L-Phe-) was detected by HPLC. Racemization of *cyclo*(-L-Phe-L-Phe-) was detected by the Manning-Shimohigashi method.<sup>16)</sup> The Fischer method occasionally gave low yield possibly because of the formation of dipeptide amide. The Fischer method caused appreciable racemization for *cyclo*(-L-Ser-L-Ser-), al-

TABLE 3. EFFECT OF REFLUX SOLVENT ON CYCLIZATION OF H-L-LEU-L-LEU-OME

| Solvent        | Bp<br>θ <sub>b</sub> /°C | Reaction rate | Racemization<br>(%) | Yield to produce<br>cyclic dipeptide |
|----------------|--------------------------|---------------|---------------------|--------------------------------------|
|                |                          | h             |                     | %                                    |
| MeOH           | 64                       | 80            | 1.2                 | 76                                   |
| EtOH           | 79                       | 70            | 0.8                 | 66                                   |
| <i>s</i> -BuOH | 100                      | 48            | >26                 | 15                                   |
| Toluene        | 110                      | 50            | 1.9                 | 75                                   |

TABLE 4. COMPARISON OF THE THREE METHODS

| Compound obtained            | Fischer method |              | Nitecki method |              | MeOH-reflux method |              |
|------------------------------|----------------|--------------|----------------|--------------|--------------------|--------------|
|                              | Yield          | Racemization | Yield          | Racemization | Yield              | Racemization |
|                              | %              | (%)          | %              | (%)          | %                  | (%)          |
| <i>cyclo</i> (-L-Leu-L-Leu-) | 70             | 1.5          | 71             | 5.5          | 76                 | 1.2          |
| <i>cyclo</i> (-L-Val-L-Val-) | 20             | 0.9          | 58             | 0.4          | 64                 | 0.6          |
| <i>cyclo</i> (-L-Ala-L-Ala-) | 72             | 0.4          | 85             | 0.3          | 81                 | 0.3          |
| <i>cyclo</i> (-L-Ser-L-Ser-) | 53             | 2.7          | 40             | 1.5          | 63                 | 1.4          |
| <i>cyclo</i> (-L-Phe-L-Phe-) | 86             | 0.6          | 89             | 0.6          | 93                 | 0.1          |

though detected racemization was much lower than reported by Nitecki *et al.* (5–40%).<sup>11)</sup> It should be noted that H-dipeptide-OMe was treated with methanolic ammonia in the shortest reaction time in this study, whereas reported reaction time was 1 d to 5 d.<sup>11)</sup>

The Nitecki method caused comparatively less racemization. In the case of *cyclo*(-L-Leu-L-Leu-), however, 5.5% racemization was observed. We can not explain this result at present. A side reaction was observed also in the case of *cyclo*(-L-Ser-L-Ser-) producing a mixture of three components. Each component separated by silica gel chromatography was found to be HCO-L-Ser-L-Ser-OMe (**1**), a formylated derivative (**2**) of **1**, and desired *cyclo*(-L-Ser-L-Ser-). This side reaction was observed only in the case of dipeptides containing serine.

Nitecki *et al.* presumed that racemization took place only on cyclization step.<sup>11)</sup> However, Fig. 1 shows that racemization proceeds mainly after cyclization. Cyclic dipeptide is found to be susceptible to racemization in the presence of excess base. Possible reason of racemization is keto-enol tautomerism resulted from deprotonation from  $\alpha$ -carbon.

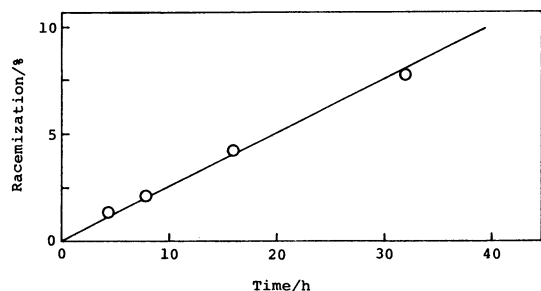


Fig. 1. Time-course of racemization of *cyclo*(-L-Leu-L-Leu-) in methanolic ammonia.

Although the MeOH-reflux method is recommendable compared to the Fischer method and the Nitecki method, it should be noted that Suzuki *et al.*<sup>20)</sup> have recently proposed a similar method, AcOH-catalyzed aminolysis of dipeptide esters, *s*-BuOH being used as a solvent. Additionally, Takahashi *et al.* described synthesis of cyclic dipeptides using *N*-carboxy amino acid and amino acid esters.<sup>21)</sup>

### Experimental

The melting points are uncorrected. TLC was carried out on silica gel G (Merck) with the following solvent systems:  $R_f^1$ ,  $\text{CHCl}_3$ -MeOH (5 : 1);  $R_f^2$ ,  $\text{CHCl}_3$ -MeOH-AcOH (50 : 10 :

2). The ratio in parentheses after a solvent system is indicated by volume. HPLC was carried out with a Hitachi 635A liquid chromatograph equipped with multi-wavelength UV monitor 635-0900. Solvents for HPLC were filtered and degassed before use. A Hitachi amino acid analyzer KLA-5 was used for the determination of amino acids and diastereomeric dipeptides under the following conditions: column,  $0.6 \times 55$  cm; jacket temperature, 55 °C; flow rate, 30 ml/h.  $^1\text{H}$  NMR spectra were obtained on a JEOL FX-90Q spectrometer with tetramethylsilane as an internal standard.

**Z-Dipeptide Esters.** *Z*-L-Val-L-Val-OMe: Preparation of this compound is described as an example of the preparation of *Z*-dipeptide esters. To a chilled solution of *Z*-L-Val-OH (1.51 g, 6 mmol), H-L-Val-OMe·HCl (1.01 g, 6 mmol) and  $\text{NEt}_3$  (0.84 ml, 6 mmol) in  $\text{CH}_2\text{Cl}_2$  (18 ml), was added DCC (1.30 g, 6.3 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 4 h and allowed to stand at room temperature overnight. After removal of *N,N'*-dicyclohexylurea, the filtrate was evaporated *in vacuo* and the residue was dissolved in EtOAc. The solution was washed successively with 2% HCl, 4%  $\text{NaHCO}_3$ , and water, dried ( $\text{Na}_2\text{SO}_4$ ), and evaporated. The residue was recrystallized from EtOAc-ether-petroleum ether; yield, 1.68 g (77%); mp 145–147 °C;  $[\alpha]_D^{20}$  –28.0° (*c* 1, MeOH);  $R_f^1$  0.88.

Found: C, 62.78; H, 8.00; N, 7.60%. Calcd for  $\text{C}_{19}\text{H}_{28}\text{O}_5\text{N}_2$ : C, 62.62; H, 7.74; N, 7.69%.

**Several Z-Dipeptide Esters:** Other compounds were prepared in a similar manner. New compounds prepared were as follows: *Z*-L-Leu-D-Leu-OMe, *Z*-L-Leu-D-Leu-OEt, *Z*-L-Ala-D-Ala-OEt, *Z*-L-Ser-D-Ser-OEt, *Z*-L-Pro-D-Pro-OMe, and *Z*-L-Phe-D-Phe-OMe.

**Boc-Dipeptide Esters.** *Boc*-L-Ala-L-Ala-OMe: Coupling of *Boc*-L-Ala-OH (0.95 g, 5 mmol) and H-L-Ala-OMe·HCl (0.70 g, 5 mmol) by DCC (1.03 g, 5 mmol) was carried out as described above. The product was recrystallized from EtOAc-ether-petroleum ether; yield, 1.03 g (75%); mp 108–109 °C;  $[\alpha]_D^{20}$  –57.8° (*c* 1, MeOH);  $R_f^2$  0.79.

Found: C, 52.55; H, 8.18; N, 10.25%. Calcd for  $\text{C}_{12}\text{H}_{22}\text{O}_5\text{N}_2$ : C, 52.54; H, 8.08; N, 10.21%.

**Several Boc-Dipeptide Esters:** Known *Boc*-dipeptide esters such as *Boc*-L-Leu-L-Leu-OMe were prepared similarly. New compound prepared was *Boc*-L-Val-D-Val-OMe.

**Authentic Cyclic Dipeptides.** *cyclo*(-L-Val-L-Val-): *Z*-L-Val-L-Val-OMe (182 mg, 0.5 mmol) was dissolved in MeOH (5 ml) and hydrogenolyzed in the presence of Pd black. After the catalyst was filtered off, the filtrate was evaporated *in vacuo* and the residue was dissolved in MeOH (10 ml). The solution was refluxed for 110 h and evaporated *in vacuo*. The residue was recrystallized from MeOH-ether; yield, 63 mg (64%); physical constants being shown in Table 1.

**Several Cyclic Dipeptides:** Other compounds were prepared by the same procedure. Yields and physical constants are shown in Table 1.

**Separation of Cyclic Dipeptide Diastereomers by HPLC.**

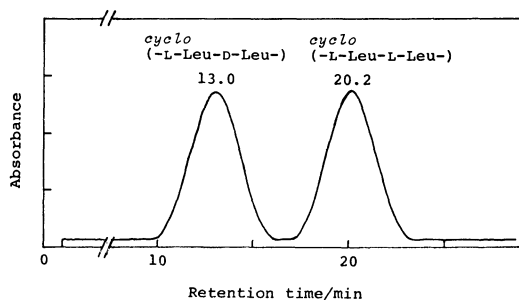


Fig. 2. Separation of cyclic dipeptide diastereomers by adsorption HPLC. Column, Hitachi gel #3040, 0.26  $\times$  50 cm; eluent, hexane-EtOH (9:1); flow rate, 1 ml/min; wavelength, 210 nm.

a) *Reversed-phase HPLC*: Hitachi Gel #3050 (particle size, 20–25  $\mu$ m), #3053 (10  $\mu$ m), LiChrosorb RP-8 (5  $\mu$ m), and LiChrosorb RP-18 (5  $\mu$ m) were used as filler, and aqueous mixtures such as water-MeOH, water-MeOH-*i*-PrOH, water-CH<sub>3</sub>CN, and water-CH<sub>3</sub>CN-*i*-PrOH were tested as solvent systems. Optimal conditions are shown in Table 2. In the case of *cyclo*(-L-Ser-L,D-Ser-), phosphate buffer was used, which was tested over the wide range of concentrations at pH 6.6. The filler was filled by slurry method.

b) *Adsorption HPLC*: Solvent systems were tested by TLC using various two or three component systems with MeOH, EtOH, *i*-PrOH, hexane, and CH<sub>3</sub>CN. The solvent system, hexane-EtOH, gave good results for the separation of *cyclo*(-L-Leu-L-Leu-) as shown in Fig. 2. However, the separation of other cyclic dipeptides were unsuccessful.

c) *Determination of Racemization*: Separation conditions for four cyclic dipeptides were shown in Table 2. Ten  $\mu$ l of a sample solution, concentration of which was about 1 mg/ml, was applied to HPLC and analyzed. All values of racemization were corrected with the ratio of absorption intensity.

*Effects of Factors on Formation of Cyclic Dipeptide by the MeOH-reflux Method.* a) *Effect of Ester Groups*: Z-L-Leu-L-Leu-OMe or Z-L-Leu-L-Leu-OEt (0.5 mmol each) dissolved in MeOH (5 ml) was treated with H<sub>2</sub>/Pd and the solution was refluxed as described in the section of Authentic Cyclic Dipeptides.

b) *Effect of Salt on Racemization*: After deprotection of Boc-L-Leu-L-Leu-OMe with HCOOH, the solution was evaporated and dried *in vacuo* overnight. After neutralization with NEt<sub>3</sub>, the residue was dissolved in MeOH and the solution was refluxed for 72 h. After the solution was evaporated, the residue was dissolved in MeOH and applied to HPLC in order to detect racemization.

c) *Effect of Reflux Solvent*: H-L-Leu-L-Leu-OMe (0.5 mmol), which was prepared by hydrogenolysis of Z-L-Leu-L-Leu-OMe, was dissolved in 5 ml each of MeOH, EtOH, *s*-BuOH, or toluene, and each solution was refluxed. Effect of each solvent on racemization and yield were compared, the results being shown in Table 3.

*Determination of Racemization during the Formation of Cyclic Dipeptides.* a) *Fischer Method*: Z-L-Leu-L-Leu-OMe (0.5 mmol) was dissolved in MeOH (5 ml) and hydrogenolyzed.

The filtrate was evaporated, the residue was dissolved in methanolic ammonia (5 ml) and allowed to stand for 12 h, and the solution was evaporated. The residue was dissolved in MeOH (20 ml), a portion of which was applied to HPLC. The rest of the solution was evaporated and the residue was recrystallized from MeOH-ether; yield, 79 mg (70%). Other cyclic dipeptides were prepared by the same procedure, and the yield and racemization are shown in Table 4.

b) *Nitecki Method*: Boc-L-Leu-L-Leu-OMe (0.5 mmol) was dissolved in HCOOH (2.5 ml) and the solution was allowed to stand for 24 h at room temperature. After evaporation of the solution, the residue was dried *in vacuo* overnight and dissolved in a mixture of *s*-BuOH (5 ml) and toluene (3 ml). After the solution was refluxed for 6 h and evaporated, the residue was dissolved in MeOH (20 ml). A portion of the methanolic solution was evaporated and the residue was recrystallized from MeOH-ether; yield, 71%. Other cyclic dipeptides were prepared by the same procedure, and yields and racemization are shown in Table 4.

c) *MeOH-reflux Method*: Cyclic dipeptides were prepared by the same procedure as described in the section of Authentic Cyclic Dipeptides. Yields and racemization are shown in Table 4.

*Side Reaction in the Nitecki Method.* Boc-L-Ser-L-Ser-OMe (168 mg, 0.5 mmol) was dissolved in HCOOH (3 ml) and the solution was allowed to stand for 6 h. After evaporation of the solution, the residue was dried, and dissolved in a mixture of *s*-BuOH (5 ml) and toluene (3 ml). The solution was refluxed for 5 h, evaporated, and the residue (65 mg) was obtained as a mixture of three components, which were separated to component HCO-Ser-Ser-OMe (**1**), a *O*-formylated derivative (**2**) of **1**, and *cyclo*(-L-Ser-L-Ser-) by silica gel chromatography under the following conditions: column size, 1.1  $\times$  50 cm; eluent, CHCl<sub>3</sub>-MeOH (9:1); fraction volume, 0.7 ml, flow rate, 7 ml/h. Separated compounds were recrystallized from MeOH-ether and afforded **1** and **2**. Yield of **1**, 17 mg (13%); mp 153 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$ =8.02 (s, CHO). Yield of **2**, 7 mg, (5%); mp 138–142 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>)  $\delta$ =8.02 (s, CHO), 8.20 (s, CHO). Yield of *cyclo*(-L-Ser-L-Ser-), 38 mg (40%); mp 231–233 °C (dec). The structures of **1** and **2** were assessed by the results of <sup>1</sup>H NMR as HCO-Ser-Ser-OMe and its *O*-formylated derivative. However, the formylated hydroxyl group was not identified.

In the case of H-L-Ser-L-Ser-OEt also, ethyl esters **1'** (mp 153–155 °C) and **2'** (mp 116–118 °C) corresponding to **1** and **2** were obtained by the same procedure. *N*-formyl and *N,O*-diformyl nature of **1'** and **2'** were ascertained by elemental analyses and <sup>1</sup>H NMR.

*Degree of Racemization in the Fischer Method.* *cyclo*(-L-Leu-L-Leu-) (10 mg) was dissolved in methanolic ammonia (2 ml), and the solution was allowed to stand at room temperature. After 4, 8, 16, and 32 h, an aliquot of the solution was taken and evaporated. The residue was dissolved in MeOH (5 ml), and the solution was applied to HPLC, the results being shown in Fig. 1.

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## References

- 1) T. Kamiya, S. Maeno, M. Hashimoto, and Y. Mine, *J. Antibiot.*, **25**, 576 (1972).
- 2) K. Nunami and T. Shiba, *Proc. 11th Symposium on Peptide Chem.*, p. 97 (1973).
- 3) Y. S. Chen, *Bull. Agric. Chem. Soc. Jpn.*, **24**, 379 (1960).
- 4) Y. Kodaira, *Agric. Biol. Chem.*, **25**, 261 (1961).
- 5) K. Bauer, K. K. Graf, A. F. Bauman, S. Beier, A. T. Vidal, and H. Kleinkauf, *Nature*, **274**, 174 (1978).
- 6) N. Izumiya, S. Lee, T. Kanmera, and H. Aoyagi, *J. Am. Chem. Soc.*, **99**, 8346 (1977).
- 7) T. Shiba, *Farumashia*, **12**, 313 (1976).
- 8) K. D. Kopple and D. H. Marr, *J. Am. Chem. Soc.*, **89**, 6193 (1967).

- 9) K. D. Kopple and M. Ohnishi, *J. Am. Chem. Soc.*, **91**, 962 (1969).
  - 10) E. Fischer, *Ber.*, **39**, 2893 (1906).
  - 11) D. E. Nitecki, B. Halpern, and J. W. Westley, *J. Org. Chem.*, **33**, 864 (1968).
  - 12) Abbreviations according to IUPAC-IUB Commission, *J. Biol. Chem.*, **247**, 977 (1972), are used throughout. Additional abbreviations: DCC, dicyclohexylcarbodiimide; DMF, *N,N*-dimethylformamide; HPLC, high-performance liquid chromatography.
  - 13) S. Lee, T. Kanmera, H. Aoyagi, and N. Izumiya, *Int. J. Pept. Protein Res.*, **13**, 207 (1979).
  - 14) J. W. Westley, V. A. Close, D. E. Nitecki, and B. Halpern, *Anal. Chem.*, **40**, 1888 (1968).
  - 15) T. Kanmera, S. Lee, H. Aoyagi, and N. Izumiya, *Int. J. Pept. Protein Res.*, **16**, 280 (1980).
  - 16) Y. Shimohigashi, S. Lee, and N. Izumiya, *Bull. Chem. Soc. Jpn.*, **49**, 3280 (1976).
  - 17) J. M. Manning and S. Moore, *J. Biol. Chem.*, **243**, 5591 (1968).
  - 18) E. P. Kroeff and D. J. Pietrzyk, *Anal. Chem.*, **50**, 1353 (1978).
  - 19) S. Terabe, R. Konaka, and K. Inoue, *J. Chromatogr.*, **172**, 163 (1979).
  - 20) K. Suzuki, Y. Sasaki, N. Endo, and Y. Mihara, "Peptide Chemistry," ed by K. Okawa, Protein Research Foundation, Osaka (1980), p. 11.
  - 21) T. Takahashi, M. Kaneko, and M. Oya, *Nippon Nogei Kagaku Kaishi*, **56**, 277 (1982).
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