

# Dehydrooligopeptides. XVIII. Enzymatic Hydrolysis and Coupling of Dehydrodipeptide Esters Containing $\alpha$ -Dehydroamino Acid Residue by Using Papain<sup>1)</sup>

Chung-gi Shin,\* Takeshi Kakusho, Kazushige Arai, and Masashi Seki

Laboratory of Organic Chemistry, Faculty of Technology, Kanagawa University,  
Rokkakubashi, Kanagawa-ku, Yokohama 221

(Received June 23, 1995)

The enzymatic hydrolysis of *N*-protected dehydrodipeptide methyl esters (**2**) (Protect- $\Delta$ AA-AA-OMe) was first achieved, despite the requisite of the only neutral and large proteinic L- $\alpha$ -amino acid (AA) in the case using papain. Furthermore, the reverse enzymatic coupling of the *C*-component **2** with *N*-component  $\alpha$ -amino acid anilides or dehydrodipeptide esters containing dehydrovaline ( $\Delta$ Val) residue was also successful. Consequently, the present study suggests that the proteolytic enzyme papain is able to become a very useful tool for peptide synthesis by a coupling of the *C*-component dehydrodipeptide with *N*-component  $\alpha$ -amino acid, peptide, or dehydrodipeptide.

In the recent papers<sup>2–5)</sup> we have reported on the very selective enzymatic hydrolysis of  $\alpha$ -ester of *N*-benzyloxycarbonyl- $\alpha$ -dehydroglutamic acid dimethyl ester [Cbz- $\Delta$ Glu(OMe)-OMe; **1**] and the effective peptide synthesis of **1** with L- $\alpha$ -amino acid anilide (H-AA-NHPh; **3**) by the catalytic action of thiol protease papain (EC 3.4.22.2).

So far, a great number of enzymatic reactions, such as, the optical resolution<sup>6)</sup> and coupling<sup>7)</sup> of an unusual  $\alpha$ - or  $\beta$ -amino acid derivatives by using proteases, have been reported. However, there has been no report on enzymatic hydrolysis or a peptide bond formation reaction of the dehydrodipeptide ester containing an unusual  $\alpha$ -dehydroamino acid (DHA,  $\Delta$ AA) residue at the P<sub>2</sub> position, as illustrated in Fig. 1.

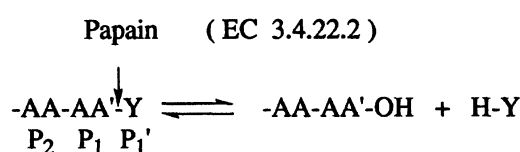
Generally, in the cases of normal enzymatic ester hydrolysis and peptide bond formation of appropriate peptide esters, it is well-known that papain generally requires only a neutral and large proteinic L- $\alpha$ -amino acid (AA) residue, such as phenylalanine (Phe), leucine (Leu), or valine (Val), at P<sub>2</sub>.

In connection with a comprehensive examination of the enzymatic dehydrodipeptide synthesis and the further development of a new catalytic action of cheaper and readily accessible papain in the organic synthesis, two kinds of  $\Delta^1$ - and  $\Delta^2$ -dehydrodipeptides<sup>8)</sup> as the substrate were chosen. That is, various Cbz-(*Z*)- $\Delta^1$ -dehydrodipeptide methyl esters (**2**: Cbz- $\Delta$ AA-AA-OMe) as a carboxyl (*C*-) component were prepared by the condensation of *Z*-form Cbz- $\Delta$ AA-OH<sup>9)</sup> with H-L-AA-OMe by the usual dicyclohexylcarbodiimide (DCC) method (Scheme 1). On the other hand, *N*-*t*-butoxycarbonyl (Boc)-AA- $\Delta$ Val-OMe (**4**) was derived by the coupling of *N*-carboxy-dehydrovaline anhydride ( $\Delta$ Val-NCA)<sup>10)</sup> with Boc-L-AA-OH and the subsequent ring opening with MeOH in one pot, according to a previously reported method<sup>11)</sup> (Scheme 1). Then, the *C*-component **2** containing an unusual DHA residue at P<sub>2</sub> was subjected to enzymatic ester hydrolysis and coupling with the amine (*N*-) component  $\alpha$ -amino acid anilide (**3**: H-AA-NHPh) or H- $\Delta^2$ -dehydrodipeptide methyl esters (**5**: H-AA- $\Delta$ Val-OMe), which was derived by deprotection of the Boc group of **4** with HCl.

In the present paper we wish to report on the first achievement of the enzymatic ester hydrolysis of **2** and the peptide bond formation of **2** with **3** or **5**.

## Experimental

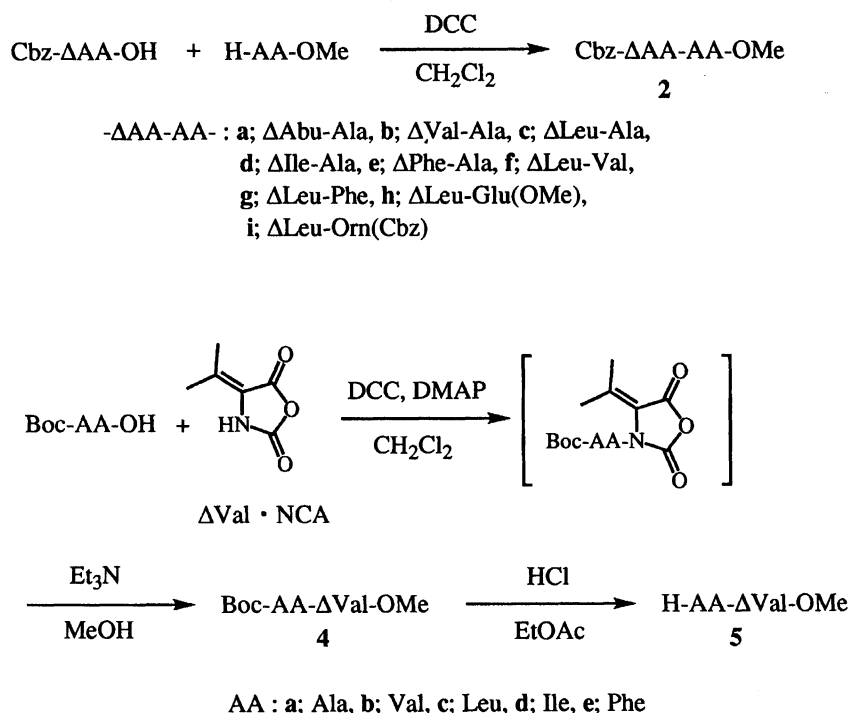
**General.** The melting points were determined with a Yamato Mp-21 micro melting-point apparatus, and were uncorrected. The IR spectra were recorded with a Hitachi 270-30 spectrometer in KBr. The <sup>1</sup>H NMR spectra were



AA: Phe, Leu, Val

Y: Amino acid, Ester, Amide

Fig. 1.



Scheme 1.

measured with a JEOL EX 90 spectrometer in a  $\text{CDCl}_3$  solution with tetramethylsilane used as the internal standard. The specific rotations were measured in a 0.5 dm tube using a JASCO DIP-4 polarimeter in MeOH (Japan Spectroscopic Co., Ltd.). High performance liquid-chromatography (HPLC) analyses and separations with a Hitachi 638-50 liquid chromatograph apparatus were performed on an SLE-04H (40×250 mm) column using LiChroSorb RP-18.

**Enzyme.** Papain (2.8 unit  $\text{mg}^{-1}$ , crude powder, P3375), purchased from Sigma Chemical Co., U.S.A., was used without further purification.

**Cbz- $\Delta$ AA-AA-OMe (**2**) as the *C*-Component.** A solution of Cbz- $\Delta$ AA-OH (4.0 mmol) and H-AA-OMe (4.4 mmol) in  $\text{CH}_2\text{Cl}_2$  (5 ml) in the presence of DCC (4.4 mmol) was stirred at  $-10^\circ\text{C}$  overnight. Dicyclohexylurea deposited as insoluble material was removed and the filtrate was concentrated in vacuo to give a syrupy residue. The residue was dissolved in EtOAc (30 ml), and the resulting solution was washed successively with a saturated aqueous  $\text{NaHCO}_3$  solution (50 ml), 1 M HCl (50 ml) (1 M = 1 mol dm $^{-3}$ ), and then brine (50 ml), and finally dried over anhydrous  $\text{MgSO}_4$ . Concentration in vacuo gave crude crystals, which were recrystallized from EtOAc-hexane to give **2** as colorless needles. The yields, melting points, and physical constants (IR,  $^1\text{H}$ NMR, and specific rotation) of **2** are summarized in Table 1.

**H-AA-NHPh (3) as the *N*-Component.**  $\alpha$ -Amino acid anilides were prepared by the usual method.<sup>5)</sup>

**Boc-AA- $\Delta$ Val-OMe (4) as the Precursor of the *N*-Component.** To a solution of  $\Delta$ Val-NCA (5.0 mmol) and an appropriate Boc-AA-OH (5.5 mmol) in  $\text{CH}_2\text{Cl}_2$  (30 ml) was added, with stirring, DCC (5.5 mmol) under cooling for 20 min. After adding 4-(dimethylamino)pyridine (DMAP; 0.5 mmol) and stirring for 3 h, MeOH (4 ml) and  $\text{Et}_3\text{N}$  (6.0 mmol) were further added, with stirring, to the resulting

solution. The reaction mixture was stirred continuously at room temperature overnight. Concentration in vacuo gave a residue, which was dissolved in EtOAc (30 ml). After any insoluble material was filtered off, the filtrate was washed successively with 1 M HCl (30 ml), saturated aqueous NaHCO<sub>3</sub> solution (30 ml), and then brine (30 ml), and finally dried over anhydrous MgSO<sub>4</sub>. Once again, the concentration in vacuo gave crude crystals, which were recrystallized from EtOAc–hexane to give **4** as colorless needles. The yields, melting points, and physical constants (IR, <sup>1</sup>H NMR, and specific rotation) of **4** are summarized in Table 2.

**Enzymatic Ester Hydrolysis of 2.** **Typical Procedure:** A suspension (2.5 ml) of an appropriate **2** [200 mM (1 M=1 mol dm<sup>-3</sup>)] and papain (30 g dm<sup>-3</sup>) in the presence of 2-mercaptoethanol (0.1 ml) in McIlvaine buffer was incubated, with shaking, at pH 8.0 and at 35 °C for 24 h. The reaction mixture was diluted with water (20 ml) and then acidified with 1 M HCl. The resulting solution was extracted three times with EtOAc (10 ml×3) and the combined extracts were washed with brine (30 ml) and dried over anhydrous MgSO<sub>4</sub>. Concentration in vacuo gave a crude syrup or crystals, which were purified by the HPLC method using a mixture of MeOH and water (7:3 v/v) to give colorless crystals. Recrystallization from EtOAc–hexane gave Cbz-(Z)-Δ<sup>1</sup>-dehydrodipeptides (**6**: Cbz-ΔAA-AA-OH) as colorless needles. The yields and physical constants of **6** are summarized in Table 3.

**Enzymatic Coupling of 2c with 3 or 5. Typical Procedure:** A suspension (4 ml) of Cbz- $\Delta$ Leu-Ala-OMe (**2c**) (50 mM), an appropriate H-AA-NHPh (**3**) or H-AA- $\Delta$ Val-OMe (**5**) (150 mM) and papain (7.5 g dm<sup>-3</sup>) in the presence of 2-mercaptoethanol (0.1 ml) in McIlvaine buffer was incubated, with shaking, at pH 6.0 and at 35 °C for 24 h. The intermediate (**5**) was derived from Boc-AA- $\Delta$ Val-OMe (**4**: 150 mM) by treating with EtOAc (1 ml) saturated

Table 1. Yields, Physical Constants, and Spectral Data of **2**

| Compd No.              | Yield % | Mp <sup>b)</sup><br>$\theta_m$ /°C | Formula                                                       | Found (%) (Calcd) |                |                | IR, $\nu$ /cm <sup>-1</sup> |       | <sup>1</sup> H NMR, $\delta$ |                                 |                     | $[\alpha]_D^{25}$ /<br>(c 1.0, in MeOH) |             |
|------------------------|---------|------------------------------------|---------------------------------------------------------------|-------------------|----------------|----------------|-----------------------------|-------|------------------------------|---------------------------------|---------------------|-----------------------------------------|-------------|
|                        |         |                                    |                                                               | C                 | H              | N              | NH—                         | CONH— | C=C—                         | CONH—                           | CH=                 |                                         | $\alpha$ -H |
| <b>2a</b>              | 55      | 67—68                              | C <sub>16</sub> H <sub>20</sub> N <sub>2</sub> O <sub>5</sub> | 60.21<br>(59.99)  | 6.51<br>(6.29) | 8.51<br>(8.75) | 3315                        | 1700  | 1665                         | 6.79d (7.0)<br>7.30—7.50m (+Ph) | 6.47q<br>(7.0)      | 4.59dq<br>(7.0,8.0)                     | —10.70      |
| <b>2b</b>              | 70      | Syrup                              | C <sub>17</sub> H <sub>22</sub> N <sub>2</sub> O <sub>5</sub> | 61.23<br>(61.06)  | 6.81<br>(6.63) | 8.08<br>(8.38) | 3250                        | 1705  | 1670                         | 6.50br s<br>6.76d (7.0)         | —                   | 4.57dq<br>(6.0,9.0)                     | —8.72       |
| <b>2c</b>              | 86      | 74—75                              | C <sub>18</sub> H <sub>24</sub> N <sub>2</sub> O <sub>5</sub> | 62.10<br>(62.05)  | 6.92<br>(6.94) | 8.18<br>(8.04) | 3250                        | 1690  | 1670                         | 6.13br s<br>6.68d (8.0)         | 6.08d<br>(10.0)     | 4.52dq<br>(6.0,9.0)                     | —1.50       |
| <b>2d<sup>a)</sup></b> | 66      | 82—83                              | C <sub>18</sub> H <sub>24</sub> N <sub>2</sub> O <sub>5</sub> | 61.96<br>(62.05)  | 7.07<br>(6.94) | 8.77<br>(8.04) | 3235                        | 1695  | 1670                         | 6.04br s<br>6.70d (8.0)         | —                   | 4.60dq<br>(6.0,9.0)                     | —2.32       |
| <b>2e</b>              | 70      | 63—64                              | C <sub>21</sub> H <sub>22</sub> N <sub>2</sub> O <sub>5</sub> | 65.66<br>(65.95)  | 5.75<br>(5.80) | 7.23<br>(7.33) | 3310                        | 1710  | 1660                         | 6.94d (8.0)<br>7.09—7.35m (+Ph) | 7.04—7.14m<br>(+Ph) | 4.58dq<br>(7.0,8.0)                     | +15.44      |
| <b>2f</b>              | 65      | 75—76                              | C <sub>20</sub> H <sub>28</sub> N <sub>2</sub> O <sub>5</sub> | 63.57<br>(63.81)  | 7.40<br>(7.50) | 7.36<br>(7.44) | 3335                        | 1705  | 1670                         | 6.10br s<br>6.62d (7.0)         | 6.20d<br>(10.0)     | 4.48dd<br>(6.0,9.0)                     | —1.25       |
| <b>2g</b>              | 70      | 89—90                              | C <sub>24</sub> H <sub>29</sub> N <sub>2</sub> O <sub>5</sub> | 67.60<br>(67.75)  | 6.61<br>(6.87) | 6.72<br>(6.58) | 3315                        | 1700  | 1665                         | 6.12br s<br>6.70d (7.0)         | 6.27d<br>(10.0)     | 4.52dd<br>(7.0,9.0)                     | —3.37       |
| <b>2h</b>              | 80      | 68—70                              | C <sub>21</sub> H <sub>28</sub> N <sub>2</sub> O <sub>5</sub> | 60.10<br>(59.99)  | 6.75<br>(6.71) | 6.69<br>(6.66) | 3240                        | 1690  | 1670                         | 6.85d (8.0)<br>7.37—7.55m (+Ph) | 6.26d<br>(10.0)     | 4.67dd<br>(7.0,9.0)                     | —19.59      |
| <b>2i</b>              | 61      | Syrup                              | C <sub>28</sub> H <sub>35</sub> N <sub>2</sub> O <sub>5</sub> | 63.70<br>(63.98)  | 6.82<br>(6.71) | 8.07<br>(8.00) | 3240                        | 1690  | 1670                         | 6.72d (7.0)<br>7.33—7.60m (+Ph) | 6.26d<br>(10.5)     | 4.62dd<br>(7.0,9.0)                     | —6.98       |

a) Mixture of (E)- and (Z)- isomers in 7:3 ratio. b) Colorless needles from EtOAc-hexane.

with dry HCl gas. The deposited colorless crystals were collected by filtration. The filtrate was acidified with 1 M HCl and further diluted with water (30 ml) and then extracted three times with EtOAc (10 ml×3). The combined extracts were washed with brine (30 ml), and then dried over anhydrous MgSO<sub>4</sub>. Concentration in vacuo gave additional crude crystals. The combined crystals were recrystallized from EtOAc-hexane to give Cbz-(Z)- $\Delta^1$ -dehydrotripeptide anilides (**7**: Cbz- $\Delta$ Leu-Ala-AA-NHPh) and Cbz-(Z)- $\Delta^{1,4}$ -dehydrotetrapeptide methyl esters<sup>8)</sup> (**8**: Cbz- $\Delta$ Leu-Ala-AA- $\Delta$ Val-OMe) as colorless needles. The yields and physical constants are summarized in Tables 4 and 5.

## Results and Discussion

**Enzymatic Ester Hydrolysis of 2.** To obtain the optimal conditions for the ester hydrolysis, the hydrolysis of **2c**<sup>12)</sup> was reexamined in regard to: 1) the effect of pH; 2) the effect of the reaction time; and 3) those of the concentrations of both the enzyme and substrate.

Firstly, the pH was changed from 4 to 9 to examine the ester hydrolytic ability of **2c**. The yield of the hydrolyzate **6c** gradually increased as the pH was increased from 4, and surprisingly, reached an almost 100% at around pH 8, although the optimal pH of esterase action of papain in McIlvaine buffer has been believed to be around 7. Figure 2 indicates that a rather alkaline pH was preferred for the hydrolysis of **2**.

Secondly, the time course of the hydrolysis of **2c** at the optimal pH 8.0 was examined. As a result, the hydrolyzate was obtained almost quantitatively within 10 h. It was found that the hydrolysis was considerably rapid compared with the case of **1**<sup>4)</sup> as the substrate.

Finally, the reaction was performed by various combinations of the enzyme concentration ranging from 5

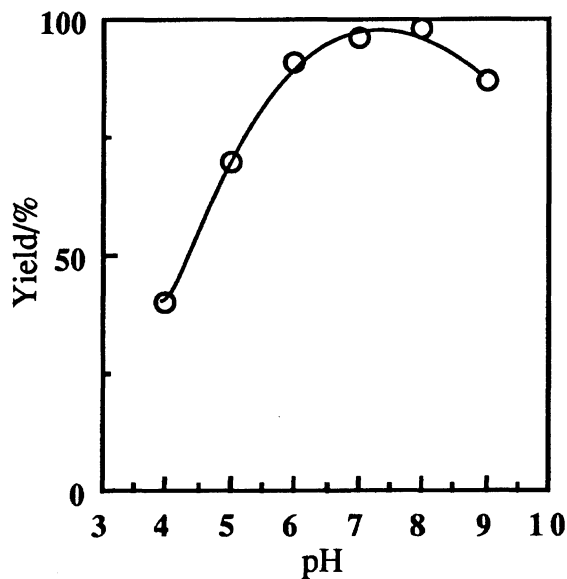


Fig. 2. Effect of pH on the yields of **6c**. The reaction mixture (2.5 ml), containing 200 mM **2c** and 30 g dm<sup>-3</sup> papain (2.8 units·mg<sup>-1</sup>) in McIlvaine buffer, was shaken at various pH's at 35 °C for 24 h.

Table 2. Yields, Physical Constants, and Spectral Data of 4

| Compd No. | Yield % | Mp <sup>a)</sup> $\theta_m/^\circ\text{C}$ | Formula                                                       | Found (%) (Calcd) |                |                | IR, $\nu/\text{cm}^{-1}$ |              |       | <sup>1</sup> HNMR, $\delta$     |                     | $[\alpha]_D^{25}/^\circ$<br>(c 1.0, in MeOH) |
|-----------|---------|--------------------------------------------|---------------------------------------------------------------|-------------------|----------------|----------------|--------------------------|--------------|-------|---------------------------------|---------------------|----------------------------------------------|
|           |         |                                            |                                                               | C                 | H              | N              | -NH-                     | -CONH-       | -C=C- | -CONH-<br>(J/Hz)                | $\alpha$ -H         |                                              |
| 4a        | 64      | 114—115                                    | C <sub>14</sub> H <sub>24</sub> N <sub>2</sub> O <sub>5</sub> | 56.39<br>(55.99)  | 8.06<br>(8.05) | 9.42<br>(9.33) | 3360                     | 1660<br>1520 | 1680  | 4.96br s<br>7.43br s            | 4.24dd<br>(7.0,7.0) | -25.6                                        |
| 4b        | 50      | 161—162                                    | C <sub>16</sub> H <sub>28</sub> N <sub>2</sub> O <sub>5</sub> | 58.33<br>(58.52)  | 8.78<br>(8.59) | 8.54<br>(8.53) | 3330                     | 1660<br>1520 | 1690  | 5.06d (8.6)<br>7.32br s         | 3.98dd<br>(6.0,6.0) | -28.3                                        |
| 4c        | 74      | 83—84                                      | C <sub>17</sub> H <sub>30</sub> N <sub>2</sub> O <sub>5</sub> | 59.51<br>(59.63)  | 8.99<br>(8.83) | 8.06<br>(8.18) | 3260                     | 1660<br>1520 | 1710  | 4.94d (7.7)<br>7.50br s         | 4.18m               | -23.9                                        |
| 4d        | 58      | 141—142                                    | C <sub>17</sub> H <sub>30</sub> N <sub>2</sub> O <sub>5</sub> | 59.63<br>(59.63)  | 8.93<br>(8.83) | 8.08<br>(8.18) | 3330                     | 1660<br>1530 | 1690  | 5.10d (8.8)<br>7.45br s         | 3.98dd<br>(6.5,6.5) | -32.9                                        |
| 4e        | 67      | 138—139                                    | C <sub>20</sub> H <sub>28</sub> N <sub>2</sub> O <sub>5</sub> | 63.39<br>(63.81)  | 7.47<br>(7.50) | 7.47<br>(7.44) | 3330                     | 1660<br>1530 | 1690  | 5.00d (7.5)<br>7.14—7.39m (+Ph) | 4.43dt<br>(7.0,8.0) | -24.1                                        |

a) Colorless needles from EtOAc-hexane.

to 30 g dm<sup>-3</sup> and the substrate concentration ranging widely from 20 to 400 mM. In all cases, the hydrolyzate Cbz- $\Delta$ Leu-Ala-OH (**6c**) was obtained in good yield, and particularly, the case using the enzyme concentration of 30 g dm<sup>-3</sup> and the substrate concentration of 200 mM was found to be the most effective and the yield was almost quantitative. As can be seen from the above results, the enzymatic hydrolysis of **2c** requires a considerably short reaction time and comparatively higher concentrations of both the enzyme and substrate under a slightly alkaline pH.

The optimal conditions of the hydrolysis of **2c** [papain (30 g dm<sup>-3</sup>), substrate (200 mM) in McIlvaine buffer at pH 8.0 and at 35 °C for 10 h] was applied to a similar hydrolysis of all of the substrates [**2a**—**i**:  $\Delta$ AA-AA **a**;  $\Delta$ Abu-Ala ( $\Delta$ Abu=2-amino-2-butenic acid), **b**;  $\Delta$ Val-Ala, **d**;  $\Delta$ Ile-Ala, **e**;  $\Delta$ Phe-Ala, **f**;  $\Delta$ Leu-Val, **g**;  $\Delta$ Leu-Phe, **h**;  $\Delta$ Leu-Glu(OMe), **i**;  $\Delta$ Leu-Orn(Cbz)], as shown in Scheme 2. Consequently, the hydrolyses of eight kinds of **2a**—**i** were fully carried out to give the desired Cbz- $\Delta$ AA-AA-OH (**6a**—**i**) in almost quantitative yields.

From the results, contrary to the expectation, very interestingly, it was found that the enzymatic ester hydrolysis of **2** having an unusual  $\Delta$ AA residue at P<sub>2</sub> took place readily, and that the papain is a sufficiently potent catalyst for the hydrolysis of various  $\Delta^1$ -dehydrodipeptide esters. Therefore, the peptide bond formation of **2**, which is reverse to the ester hydrolysis, is also expected.

The yields, melting points, and physical constants (IR, <sup>1</sup>HNMR, and specific rotation) of **6** are summarized in Table 3. In conclusion, similarly as in the case of the hydrolysis of **1**, which has been reported in recent papers,<sup>4,5)</sup> the enzymatic ester hydrolysis of **2** using pa-

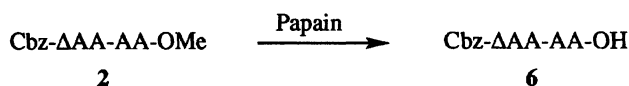
pain successfully proceeded.

**Peptide Formation: Enzymatic Coupling of 2c with 3 or 5.** From these results obtained so far, the peptide bond formation of **2**, which is a reverse reaction to the peptide hydrolysis, is also expected. According to the coupling reaction of **1** with **3**,<sup>5)</sup> the following enzymatic coupling reactions were investigated. The coupling of **2c** with H-AA-NHPh (**3a**—**e**: AA **a**; Ala, **b**; Val, **c**; Leu, **d**; Ile, **e**; Phe) or H-AA- $\Delta$ Val-OMe (**5a**—**e**: AA **a**; Ala, **b**; Val, **c**; Leu, **d**; Ile, **e**; Phe), was tried in order to obtain the corresponding Cbz- $\Delta$ Leu-Ala-AA-NHPh (**7a**—**e**) and Cbz- $\Delta$ Leu-Ala-AA- $\Delta$ Val-OMe (**8a**—**e**).

Firstly, as in the case of the hydrolytic reaction, the effects of the pH were examined in order to obtain optimal yield of the peptides **7c** and **8c**. As a result, in the case using an equimolar **2c** and *N*-component **3c**, the yield of the condensation product, Cbz- $\Delta$ Leu-Ala-Leu-NHPh (**7c**), was found to gradually increase in the range of the pH from 5 to 8, and to come up to the highest at a pH of around 6 (Fig. 2). Unfortunately, however, the yield was comparatively low and did not exceed 41%.

At this point, we turned our attention to change the relative amount of the *N*-components to the *C*-components, because in this reaction system the hydrolysis must simultaneously proceeds and the ratio of the substrates at the reaction site of enzyme has importance. Consequently, the experiment upon increasing the amount of the *N*-component **3c** from 1 to 5 equivalents to a *C*-component **2c** in the presence of papain (7.5 g dm<sup>-3</sup>) at pH 6.0 and at 35 °C was carried out. As we expected, the yield reached 86% at the component ratio (*N/C*) of 3.0, as shown in Figs. 3 and 4.

From the above results, it can be concluded that the formation of **7c** from **2c** and **3c** also depends upon the pH, and especially upon the *N*-component substrate concentration. Namely, contrary to the case of the ester hydrolysis of **2c**, it was found that a rather acidic pH is favorable to  $\Delta^1$ -dehydrotripeptide formation.



Scheme 2.

Table 3. Yields, Physical Constants, and Spectral Data of **6**

| Compd<br>No.           | Yield<br>% | Mp <sup>a)</sup><br>θ <sub>m</sub> /°C | Formula                                                                     | Found (%) (Calcd) |                |                | IR, ν/cm <sup>−1</sup> |        |       | <sup>1</sup> H NMR, δ           |                        |                     | [α] <sub>D</sub> <sup>25</sup> /°<br>(c 1.0, in MeOH) |
|------------------------|------------|----------------------------------------|-----------------------------------------------------------------------------|-------------------|----------------|----------------|------------------------|--------|-------|---------------------------------|------------------------|---------------------|-------------------------------------------------------|
|                        |            |                                        |                                                                             | C                 | H              | N              | −NH−                   | −COOH− | −C=C− | −CONH−                          | −COOH<br>(J/Hz)        | α-H                 |                                                       |
|                        |            |                                        |                                                                             |                   |                |                |                        |        |       |                                 |                        |                     |                                                       |
| <b>6a</b>              | Quant.     | 123—124                                | C <sub>15</sub> H <sub>18</sub> N <sub>2</sub> O <sub>5</sub>               | 58.71<br>(58.81)  | 5.93<br>(5.92) | 9.00<br>(9.15) | 3310                   | 1725   | 1675  | 7.10d (6.0)<br>7.26—7.34m (+Ph) | 8.23s<br>(6.0,9.0)     | 4.50dq<br>(6.0,9.0) | +5.50                                                 |
| <b>6b</b>              | Quant.     | 135—136                                | C <sub>16</sub> H <sub>20</sub> N <sub>2</sub> O <sub>5</sub>               | 60.04<br>(59.99)  | 5.98<br>(6.29) | 8.68<br>(8.74) | 3305                   | 1710   | 1670  | 6.35br s<br>6.78d (7.0)         | 8.32s<br>(6.0,8.0)     | 4.38dq<br>(6.0,8.0) | +19.86                                                |
| <b>6c</b>              | Quant.     | Syrup                                  | C <sub>29</sub> H <sub>45</sub> N <sub>3</sub> O <sub>5</sub> <sup>c)</sup> | 67.25<br>(67.54)  | 8.85<br>(8.80) | 8.08<br>(8.15) | 3305                   | 1730   | 1670  | 6.95d (7.3)<br>7.16br s         | 10.42br s<br>(7.0,8.0) | 4.63dd<br>(7.0,8.0) | +3.59                                                 |
| <b>6d<sup>b)</sup></b> | Quant.     | Syrup                                  | C <sub>29</sub> H <sub>45</sub> N <sub>3</sub> O <sub>5</sub> <sup>c)</sup> | 67.68<br>(67.54)  | 8.59<br>(8.80) | 7.88<br>(8.15) | 3305                   | 1720   | 1675  | 6.55br s<br>6.92br s            | 8.21br s<br>(7.0,8.0)  | 4.60dd<br>(7.0,8.0) | −3.04                                                 |
| <b>6f</b>              | Quant.     | 137—138                                | C <sub>19</sub> H <sub>26</sub> N <sub>2</sub> O <sub>5</sub>               | 62.57<br>(62.96)  | 7.35<br>(7.23) | 7.45<br>(7.73) | 3270                   | 1700   | 1670  | 6.90br s<br>7.44—7.60m (+Ph)    | 9.28s<br>(6.0,8.0)     | 4.54dd<br>(6.0,8.0) | −18.28                                                |
| <b>6g</b>              | Quant.     | Syrup                                  | C <sub>23</sub> H <sub>27</sub> N <sub>2</sub> O <sub>5</sub>               | 66.95<br>(67.14)  | 6.81<br>(6.61) | 6.77<br>(6.81) | 3300                   | 1705   | 1670  | 6.10br s<br>6.72d (7.0)         | 8.97s<br>(7.0,9.0)     | 4.52dd<br>(7.0,9.0) | −3.02                                                 |
| <b>6h</b>              | Quant.     | Syrup                                  | C <sub>19</sub> H <sub>25</sub> N <sub>2</sub> O <sub>6</sub>               | 63.31<br>(63.14)  | 7.19<br>(6.97) | 7.46<br>(7.75) | 3310                   | 1710   | 1675  | 7.22d (7.5)<br>7.35—7.55m (+Ph) | 9.34s<br>(7.0,9.0)     | 4.62m<br>(7.0,9.0)  | −5.28                                                 |
| <b>6i</b>              | Quant.     | Syrup                                  | C <sub>27</sub> H <sub>33</sub> N <sub>3</sub> O <sub>7</sub>               | 63.18<br>(63.39)  | 6.73<br>(6.50) | 7.98<br>(8.21) | 3335                   | 1720   | 1665  | 7.09d (7.0)<br>7.40—7.55m (+Ph) | 9.17s<br>(7.0,9.0)     | 4.58m<br>(7.0,9.0)  | −0.73                                                 |

a) Colorless needles from EtOAc–hexane. b) Mixture of (*E*)- and (*Z*)-isomers in 7 : 3 ratio. c) Obtained as dicyclohexylamine salt.Table 4. Yields, Physical Constants, and Spectral Data of **7**

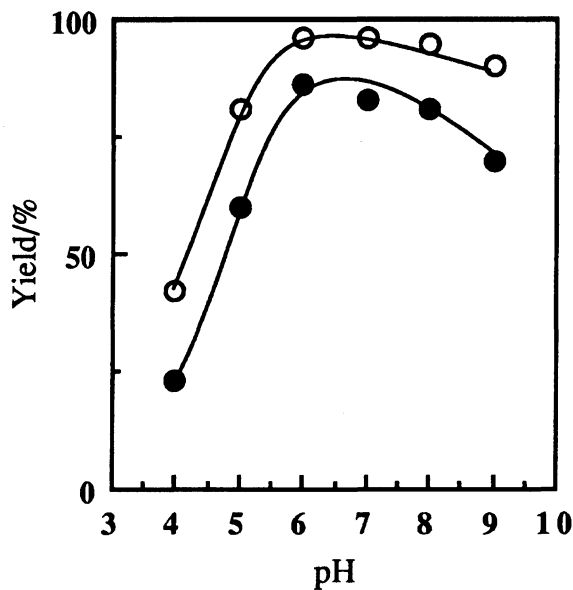
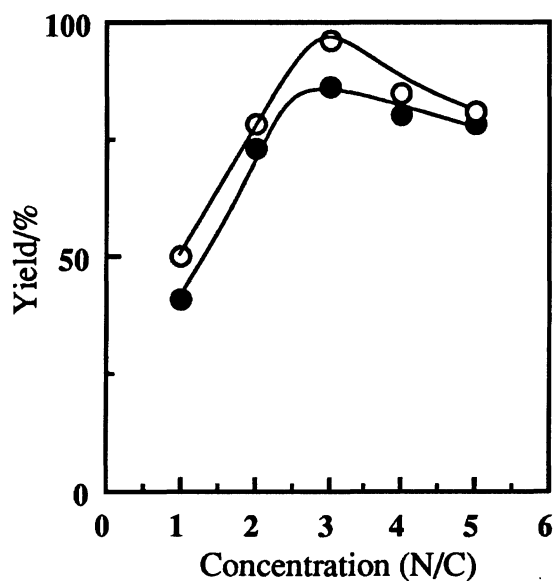
| Compd<br>No. | Yield<br>% | Mp <sup>a)</sup><br>$\theta_m/^{\circ}\text{C}$ | Formula                                                       | Found (%) (Calcd) |              |                 | IR, $\nu/\text{cm}^{-1}$ |              |       | <sup>1</sup> H NMR, $\delta$                 |                 | $[\alpha]_D^{25}/^{\circ}\text{ b)}$ |
|--------------|------------|-------------------------------------------------|---------------------------------------------------------------|-------------------|--------------|-----------------|--------------------------|--------------|-------|----------------------------------------------|-----------------|--------------------------------------|
|              |            |                                                 |                                                               | C                 | H            | N               | -NH-                     | -CONH-       | -C=C- | -CONH-                                       | -CH=            |                                      |
| <b>7a</b>    | 58         | 113—114                                         | C <sub>26</sub> H <sub>32</sub> N <sub>4</sub> O <sub>5</sub> | 64.85<br>(64.98)  | 6.95<br>6.71 | 11.85<br>11.66) | 3310                     | 1668<br>1540 | 1650  | 7.46d (6.0), 7.61br s<br>7.69br s, 9.06br s  | 6.14d<br>(10.0) | −9.16 (c=0.84)                       |
| <b>7b</b>    | 33         | 196—197                                         | C <sub>28</sub> H <sub>36</sub> N <sub>4</sub> O <sub>5</sub> | 66.44<br>(66.12)  | 7.02<br>7.13 | 10.85<br>11.02) | 3316                     | 1668<br>1539 | 1650  | 7.36d (10.0), 7.74br s<br>7.82br s, 8.48br s | 5.94d<br>(10.0) | −21.80 (c=1.06)                      |
| <b>7c</b>    | 86         | 107—108                                         | C <sub>29</sub> H <sub>38</sub> N <sub>4</sub> O <sub>5</sub> | 66.46<br>(66.50)  | 7.60<br>7.51 | 10.48<br>10.70) | 3310                     | 1665<br>1540 | 1650  | 7.28d (13.0), 7.75br s<br>7.83br s, 8.72br s | 6.03d<br>(10.0) | −30.18 (c=1.00)                      |
| <b>7d</b>    | 68         | 191—192                                         | C <sub>29</sub> H <sub>38</sub> N <sub>4</sub> O <sub>5</sub> | 66.37<br>(66.52)  | 7.22<br>7.51 | 10.73<br>10.70) | 3292                     | 1668<br>1539 | 1655  | 7.45d (9.0), 7.71br s<br>7.79br s, 8.53br s  | 5.98d<br>(10.0) | −21.39 (c=1.00)                      |
| <b>7e</b>    | 60         | 152—154                                         | C <sub>32</sub> H <sub>36</sub> N <sub>4</sub> O <sub>5</sub> | 68.67<br>(69.05)  | 6.80<br>6.52 | 10.03<br>10.06) | 3305                     | 1668<br>1533 | 1650  | 7.15d (15.0), 7.77br s<br>7.85br s, 8.61br s | 5.88d<br>(10.0) | −37.63 (c=0.86)                      |

a) Colorless needles from EtOAc–hexane. b) Measured in MeOH.

Table 5. Yields, Physical Constants, and Spectral Data of **8**

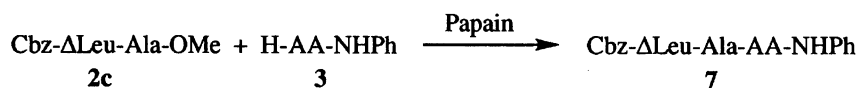
| Compd No. | Yield % | Mp <sup>a)</sup><br>$\theta_m/^\circ\text{C}$ | Formula                                                       | Found (%) (Calcd) |              |                | IR, $\nu/\text{cm}^{-1}$ |        |       | <sup>1</sup> H NMR, $\delta$                           |                 | $[\alpha]_D^{25}/^\circ$ b) |
|-----------|---------|-----------------------------------------------|---------------------------------------------------------------|-------------------|--------------|----------------|--------------------------|--------|-------|--------------------------------------------------------|-----------------|-----------------------------|
|           |         |                                               |                                                               | C                 | H            | N              | -NH-                     | -CONH- | -C=C- | -CONH-<br>(J/Hz)                                       | -CH=            |                             |
| <b>8a</b> | 72      | 161—163                                       | C <sub>26</sub> H <sub>36</sub> N <sub>4</sub> O <sub>7</sub> | 60.14<br>(60.45)  | 7.01<br>7.02 | 10.54<br>10.85 | 3305                     | 1665   | 1660  | 6.83d (5.3), 7.07br s<br>7.50d (6.5), 7.93br s         | 5.91d<br>(10.0) | -17.25 (c=0.86)             |
| <b>8b</b> | 37      | 138—140                                       | C <sub>28</sub> H <sub>40</sub> N <sub>4</sub> O <sub>7</sub> | 61.98<br>(61.74)  | 7.35<br>7.40 | 10.09<br>10.29 | 3310                     | 1670   | 1650  | 6.57br s, 7.23d (7.0)<br>7.35—7.55m (+Ph), 7.73br s    | 6.11d<br>(10.0) | -33.84 (c=1.00)             |
| <b>8c</b> | 96      | 177—179                                       | C <sub>29</sub> H <sub>42</sub> N <sub>4</sub> O <sub>7</sub> | 62.55<br>(62.34)  | 7.79<br>7.58 | 10.08<br>10.03 | 3320                     | 1660   | 1645  | 7.00d (4.8), 7.22br s<br>7.41d (6.5), 7.97br s         | 5.83d<br>(10.0) | -32.82 (c=1.00)             |
| <b>8d</b> | 68      | 169—171                                       | C <sub>29</sub> H <sub>42</sub> N <sub>4</sub> O <sub>7</sub> | 62.50<br>(62.34)  | 7.76<br>7.58 | 10.08<br>10.03 | 3310                     | 1665   | 1650  | 6.56d (5.1), 7.20d (6.5)<br>7.35—7.55m (+Ph), 7.71br s | 6.09d<br>(10.0) | -31.52 (c=1.00)             |
| <b>8e</b> | 78      | 197—199                                       | C <sub>32</sub> H <sub>40</sub> N <sub>4</sub> O <sub>7</sub> | 64.61<br>(64.85)  | 6.95<br>6.80 | 9.38<br>9.45   | 3305                     | 1670   | 1650  | 6.20d (5.7), 6.37br s<br>7.20d (7.0), 7.87br s         | 5.85d<br>(10.0) | -48.64 (c=1.00)             |

a) Colorless needles from EtOAc-hexane. b) Measured in MeOH.

Fig. 3. Effects of pH on the yields of **7c** (●) and **8c** (○). The reaction mixture (4 ml), containing 50 mM **2c**, and 150 mM **3c** or **5c**, and 7.5 g dm<sup>-3</sup> papain (2.8 units·mg<sup>-1</sup>) in McIlvaine buffer, was shaken at various pH's at 35 °C for 24 h.Fig. 4. Effects of *N*-component concentration on the yields of **7c** (●) and **8c** (○). The reaction mixture (4 ml), containing 50 mM **2c**, various concentrations of **3c** or **5c**, and 7.5 g dm<sup>-3</sup> papain (2.8 units·mg<sup>-1</sup>) in McIlvaine buffer, was shaken at pH 6 at 35 °C for 24 h.

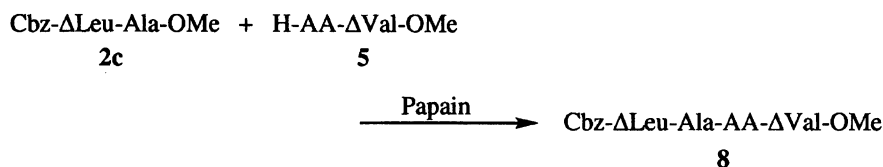
The time course of the peptide synthesis under a similar *C*- and *N*-component ratio (*N/C*=3) at pH 6.0 and at 35 °C indicated that the peptide formation proceeded more slowly than the corresponding hydrolysis (within 10 h for the completion); the highest yield was obtained after 24 h.

In the case of peptide formation, the effect of the con-



AA : a; Ala, b; Val, c; Leu, d; Ile, e; Phe

Scheme 3.



AA : a; Ala, b; Val, c; Leu, d; Ile, e; Phe

Scheme 4.

centration of the enzyme was further studied. From the results of an examination regarding the concentration of papain from 5.0 to 15.0 g dm<sup>-3</sup>, the enzyme concentration at 7.5 g dm<sup>-3</sup> provided the highest yield. A small amount of enzyme was required for the coupling reaction compared with that (30 g cm<sup>-3</sup>) in the hydrolysis. It was also found that the C-component substrate concentration was important. After examining the concentration ranging from 50 mM to 100 mM, 50 mM of the C-component concentration was found to be the most effective, the yield of 7c eventually reached the highest value of 86%.

The above results show that the enzymatic coupling of 2c with 3c requires: 1) a slightly acidic pH; 2) a considerably prolonged reaction time; and 3) a lower concentration of enzyme, and depends upon both the ratio and concentration of the C- and N-components. The obtained conditions were applicable to all similar peptide formation reactions of 2c with 3a–e to  $\Delta^1$ -dehydrotripeptides (7a–e), as shown in Scheme 3, with 5c to give 8c (96%), and with 5a–e to  $\Delta^{1,4}$ -dehydrotetrapeptides (8a–e), as shown in Scheme 4.

The yields, melting points, and physical constants (IR, <sup>1</sup>HNMR, and specific rotation) of 7 and 8 are summarized in Tables 4 and 5. It should be emphasized that all of the condensation products, 7 and 8, were deposited as crystals from an aqueous buffer solution during the coupling reactions, which makes the purification of the product from the starting material much easier.

In conclusion, the present study showed that, from a synthetic standpoint of view, papain was widely useful and served as a tool for the ester hydrolyses of dehydropeptide esters and their coupling reaction with the

other amino acid, peptide, and dehydropeptide derivatives under mild conditions.

## References

- 1) Part XVII: Y. Nakamura, M. Hirai, K. Tamotsu, Y. Yonezawa, and C. Shin, *Bull. Chem. Soc. Jpn.*, **68**, 1369 (1995).
- 2) C. Shin, N. Takahashi, and Y. Yonezawa, *Chem. Lett.*, **1988**, 2001.
- 3) C. Shin and N. Takahashi, *Chem. Lett.*, **1989**, 747.
- 4) C. Shin, N. Takahashi, and M. Seki, *Bull. Chem. Soc. Jpn.*, **64**, 3575 (1991).
- 5) C. Shin, M. Seki, T. Kakusho, and N. Takahashi, *Bull. Chem. Soc. Jpn.*, **66**, 2048 (1993).
- 6) For examples: J. B. West, W. J. Hennen, J. L. Lalonde, J. A. Bibbs, Z. Zhong, E. F. Meyer, Jr., and C-H. Wong, *J. Am. Chem. Soc.*, **112**, 5313 (1990); J-M. Fang and C-H. Wong, *Synlett*, **1994**, 393.
- 7) For examples: M. Ota, N. Nio, and Y. Ariyoshi, *Biosci. Biotech. Biochem.*, **57**, 808 (1993); M. Schuster, B. Munoz, and C-H. Wong, *Tetrahedron Lett.*, **34**, 1247 (1993).
- 8) In this paper, the symbols  $\Delta^1$ ,  $\Delta^2$ , and  $\Delta^{1,4}$  indicate the position number of double bond of DHA residue from the N-terminus in sequence.
- 9) C. Shin, T. Yamada, and Y. Yonezawa, *Tetrahedron Lett.*, **24**, 2175 (1983).
- 10) C. Shin, Y. Yonezawa, and M. Ikeda, *Bull. Chem. Soc. Jpn.*, **59**, 3578 (1986).
- 11) C. Shin, Y. Yonezawa, K. Unoki, and J. Yoshimura, *Tetrahedron Lett.*, **1979**, 1049; Y. Yonezawa, C. Shin, Y. Ono, and J. Yoshimura, *Bull. Chem. Soc. Jpn.*, **59**, 3573 (1980).
- 12) C. Shin, M. Seki, and H. Tano, "The National Meeting of the Chemical Society of Japan," Yokohama, April 1991, Abstr., No. 3D104.