Dehydrooligopeptides. XVIII. Enzymatic Hydrolysis and Coupling of Dehydrodipeptide Esters Containing α-Dehydroamino Acid Residue by Using Papain[1)]

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The enzymatic hydrolysis of *N*-protected dehydrodipeptide methyl esters (**2**) (Protect-ΔAA-AA-OMe) was first achieved, despite the requisite of the only neutral and large proteinic L-α-amino acid (AA) in the case using papain. Furthermore, the reverse enzymatic coupling of the *C*-component **2** with *N*-component α-amino acid anilides or dehydrodipeptide esters containing dehydrovaline (ΔVal) 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 dehydropeptide with *N*-component α-amino acid, peptide, or dehydropeptide.

In the recent papers[2—5)] we have reported on the very selective enzymatic hydrolysis of α-ester of *N*-benzyloxycarbonyl-α-dehydroglutamic acid dimethyl ester [Cbz-ΔGlu(OMe)-OMe; **1**] and the effective peptide synthesis of **1** with L-α-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[6)] and coupling[7)] of an unusual α- or β-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 dehydropeptide ester containing an unusual α-dehydroamino acid (DHA, ΔAA) residue at the P_2 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-α-amino acid (AA) residue, such as phenylalanine (Phe), leucine (Leu), or valine (Val), at P_2.

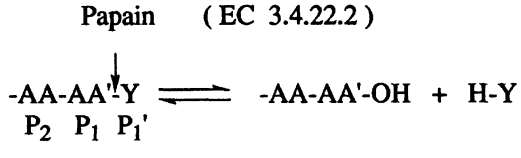


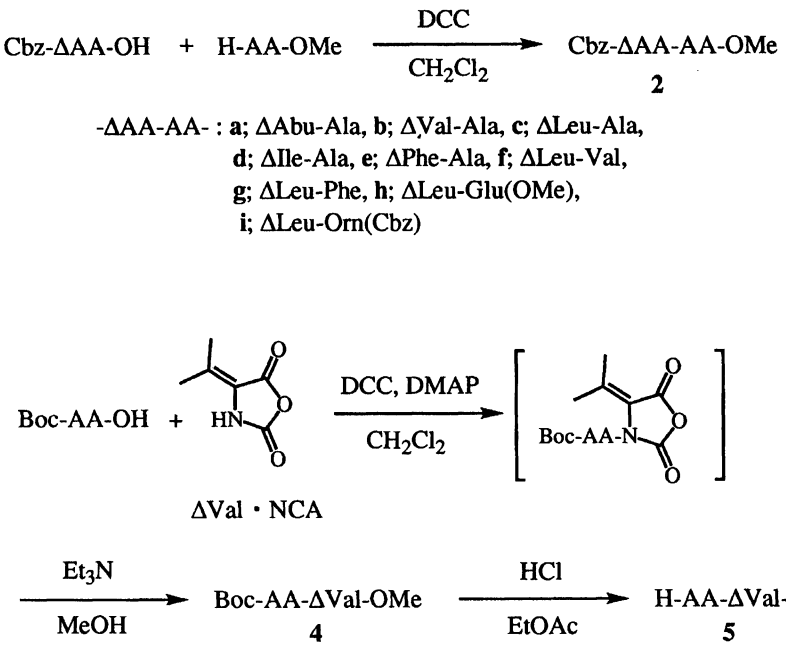
Fig. 1.

In connection with a comprehensive examination of the enzymatic dehydropeptide synthesis and the further development of a new catalytic action of cheaper and readily accessible papain in the organic synthesis, two kinds of Δ^1- and Δ^2-dehydrodipeptides[8)] as the substrate were chosen. That is, various Cbz-(*Z*)-Δ^1-dehydrodipeptide methyl esters (**2**: Cbz-ΔAA-AA-OMe) as a carboxyl (*C*-) component were prepared by the condensation of *Z*-form Cbz-ΔAA-OH[9)] with H-L-AA-OMe by the usual dicyclohexylcarbodiimide (DCC) method (Scheme 1). On the other hand, *N*-*t*-butoxycarbonyl (Boc)-AA-ΔVal-OMe (**4**) was derived by the coupling of *N*-carboxy-dehydrovaline anhydride (ΔVal·NCA)[10)] with Boc-L-AA-OH and the subsequent ring opening with MeOH in one pot, according to a previously reported method[11)] (Scheme 1). Then, the *C*-component **2** containing an unusual DHA residue at P_2 was subjected to enzymatic ester hydrolysis and coupling with the amine (*N*-) component α-amino acid anilide (**3**: H-AA-NHPh) or H-Δ^2-dehydrodipeptide methyl esters (**5**: H-AA-ΔVal-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 ^{1}H NMR spectra were



Scheme 1.

measured with a JEOL EX 90 spectrometer in a $CDCl_3$ 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 mg^{-1}, crude powder, P3375), purchased from Sigma Chemical Co., U.S.A., was used without further purification.

Cbz-ΔAA-AA-OMe (2) as the *C*-Component. A solution of Cbz-ΔAA-OH (4.0 mmol) and H-AA-OMe (4.4 mmol) in CH_2Cl_2 (5 ml) in the presence of DCC (4.4 mmol) was stirred at −10 °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 $NaHCO_3$ solution (50 ml), 1 M HCl (50 ml) (1 M=1 mol dm^{-3}), and then brine (50 ml), and finally dried over anhydrous $MgSO_4$. 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, ^1H NMR, and specific rotation) of **2** are summarized in Table 1.

H-AA-NHPh (3) as the *N*-Component. α-Amino acid anilides were prepared by the usual method.[5)]

Boc-AA-ΔVal-OMe (4) as the Precursor of the *N*-Component. To a solution of ΔVal·NCA (5.0 mmol) and an appropriate Boc-AA-OH (5.5 mmol) in CH_2Cl_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 Et_3N (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 $NaHCO_3$ solution (30 ml), and then brine (30 ml), and finally dried over anhydrous $MgSO_4$. 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, ^1H 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^{-3})] and papain (30 g dm^{-3}) 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 $MgSO_4$. 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^1-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-ΔLeu-Ala-OMe (**2c**) (50 mM), an appropriate H-AA-NHPh (**3**) or H-AA-ΔVal-OMe (**5**) (150 mM) and papain (7.5 g dm^{-3}) 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-ΔVal-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[b] θ_m/°C	Formula	Found (%) (Calcd) C	H	N	IR, ν/cm^{-1} –NH–	–CONH–	–C=C–	^{1}H NMR, δ –CONH–	–CH= (J/Hz)	α-H	$[\alpha]_D^{25}$/° (c 1.0, in MeOH)
2a	55	67—68	$C_{16}H_{20}N_2O_5$	60.21	6.51	8.51	3315	1700	1665	6.79d (7.0)	6.47q	4.59dq	−10.70
				(59.99	6.29	8.75)		1540		7.30—7.50m (+Ph)	(7.0)	(7.0,8.0)	
2b	70	Syrup	$C_{17}H_{22}N_2O_5$	61.23	6.81	8.08	3250	1705	1670	6.50br s	—	4.57dq	−8.72
				(61.06	6.63	8.38)		1510		6.76d (7.0)		(6.0,9.0)	
2c	86	74—75	$C_{18}H_{24}N_2O_5$	62.10	6.92	8.18	3250	1690	1670	6.13br s	6.08d	4.52dq	−1.50
				(62.05	6.94	8.04)		1525		6.68d (8.0)	(10.0)	(6.0,9.0)	
2d[a]	66	82—83	$C_{18}H_{24}N_2O_5$	61.96	7.07	7.87	3235	1695	1670	6.04br s	—	4.60dq	−2.32
				(62.05	6.94	8.04)		1510		6.70d (8.0)		(6.0,9.0)	
2e	70	63—64	$C_{21}H_{22}N_2O_5$	65.66	5.75	7.23	3310	1710	1660	6.94d (8.0)	7.04—7.14m	4.58dq	+15.44
				(65.95	5.80	7.33)		1530		7.09—7.35m (+Ph)	(+Ph)	(7.0,8.0)	
2f	65	75—76	$C_{20}H_{28}N_2O_5$	63.57	7.40	7.36	3335	1705	1670	6.10br s	6.20d	4.48dd	−1.25
				(63.81	7.50	7.44)		1540		6.62d (7.0)	(10.0)	(6.0,9.0)	
2g	70	89—90	$C_{24}H_{29}N_2O_5$	67.60	6.61	6.72	3315	1700	1665	6.12br s	6.27d	4.52dd	−3.37
				(67.75	6.87	6.58)		1540		6.70d (7.0)	(10.0)	(7.0,9.0)	
2h	80	68—70	$C_{21}H_{28}N_2O_5$	60.10	6.75	6.69	3240	1690	1670	6.85d (8.0)	6.26d	4.67dd	−19.59
				(59.99	6.71	6.66)		1520		7.37—7.55m (+Ph)	(10.0)	(7.0,9.0)	
2i	61	Syrup	$C_{28}H_{35}N_2O_5$	63.70	6.82	8.07	3240	1690	1670	6.72d (7.0)	6.26d	4.62dd	−6.98
				(63.98	6.71	8.00)		1560		7.33—7.60m (+Ph)	(10.5)	(7.0,9.0)	

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 $MgSO_4$. Concentration in vacuo gave additional crude crystals. The combined crystals were recrystallized from EtOAc–hexane to give Cbz-(*Z*)-Δ^1-dehydrotripeptide anilides (**7**: Cbz-ΔLeu-Ala-AA-NHPh) and Cbz-(*Z*)-$\Delta^{1,4}$-dehydrotetrapeptide methyl esters[8] (**8**: Cbz-ΔLeu-Ala-AA-ΔVal-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**[12] 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**[4] as the substrate.

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

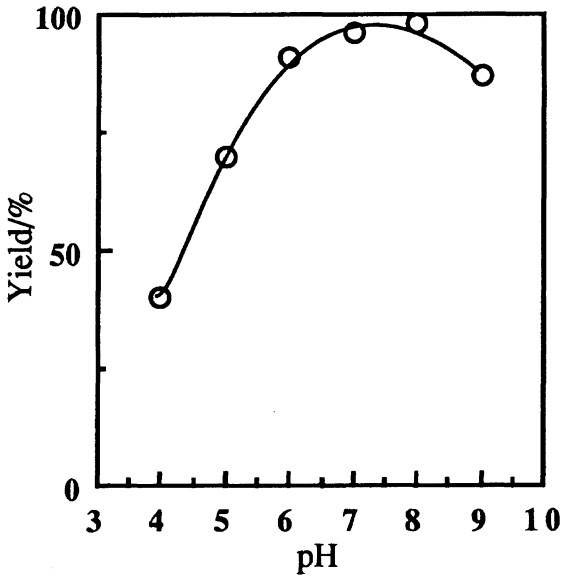


Fig. 2. Effect of pH on the yields of **6c**. The reaction mixture (2.5 ml), containing 200 mM **2c** and 30 g dm^{-3} papain (2.8 units·mg^{-1}) in McIlvaine buffer, was shaken at various pH's at 35 °C for 24 h.

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

Compd No.	Yield %	Mp[a] θ_m/°C	Formula	Found (%) (Calcd) C	H	N	IR, ν/cm^{-1} –NH–	–CONH–	–C=C–	^{1}H NMR, δ –CONH– (J/Hz)	α-H	$[\alpha]_D^{25}$/° (c 1.0, in MeOH)
4a	64	114—115	$C_{14}H_{24}N_2O_5$	56.39	8.06	9.42	3360	1660	1680	4.96br s	4.24dd	−25.6
				(55.99	8.05	9.33)		1520		7.43br s	(7.0,7.0)	
4b	50	161—162	$C_{16}H_{28}N_2O_5$	58.33	8.78	8.54	3330	1660	1690	5.06d (8.6)	3.98dd	−28.3
				(58.52	8.59	8.53)		1520		7.32br s	(6.0,6.0)	
4c	74	83—84	$C_{17}H_{30}N_2O_5$	59.51	8.99	8.06	3260	1660	1710	4.94d (7.7)	4.18m	−23.9
				(59.63	8.83	8.18)		1520		7.50br s		
4d	58	141—142	$C_{17}H_{30}N_2O_5$	59.63	8.93	8.08	3330	1660	1690	5.10d (8.8)	3.98dd	−32.9
				(59.63	8.83	8.18)		1530		7.45br s	(6.5,6.5)	
4e	67	138—139	$C_{20}H_{28}N_2O_5$	63.39	7.47	7.47	3330	1660	1690	5.00d (7.5)	4.43dt	−24.1
				(63.81	7.50	7.44)		1530		7.14—7.39m (+Ph)	(7.0,8.0)	

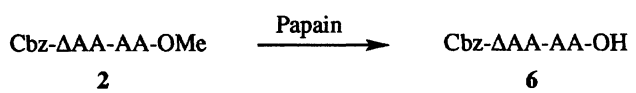
a) Colorless needles from EtOAc–hexane.

to 30 g dm^{-3} and the substrate concentration ranging widely from 20 to 400 mM. In all cases, the hydrolyzate Cbz-ΔLeu-Ala-OH (**6c**) was obtained in good yield, and particularly, the case using the enzyme concentration of 30 g dm^{-3} 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^{-3}), 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**: ΔAA-AA **a**; ΔAbu-Ala (ΔAbu=2-amino-2-butenoic acid), **b**; ΔVal-Ala, **d**; ΔIle-Ala, **e**; ΔPhe-Ala, **f**; ΔLeu-Val, **g**; ΔLeu-Phe, **h**; ΔLeu-Glu(OMe), **i**; ΔLeu-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-ΔAA-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 ΔAA residue at P_2 took place readily, and that the papain is a sufficiently potent catalyst for the hydrolysis of various Δ^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, ^{1}H NMR, 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,[4,5] the enzymatic ester hydrolysis of **2** using papain successfully proceeded.



Scheme 2.

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**,[5] 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-ΔVal-OMe (**5a**—**e**: AA **a**; Ala, **b**; Val, **c**; Leu, **d**; Ile, **e**; Phe), was tried in order to obtain the corresponding Cbz-ΔLeu-Ala-AA-NHPh (**7a**—**e**) and Cbz-ΔLeu-Ala-AA-ΔVal-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-ΔLeu-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^{-3}) 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 Δ^1-dehydrotripeptide formation.

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

Compd No.	Yield %	Mp[a] θ_m/°C	Formula	Found (%) (Calcd) C	H	N	IR, ν/cm^{-1} –NH–	–COOH–	–C=C–	^{1}H NMR, δ –CONH–	–COOH (J/Hz)	α-H	$[\alpha]_D^{25}$/° (c 1.0, in MeOH)
6a	Quant.	123—124	$C_{15}H_{18}N_2O_5$	58.71	5.93	9.00	3310	1725	1675	7.10d (6.0)	8.23s	4.50dq	+5.50
				(58.81	5.92	9.15)				7.26—7.34m (+Ph)		(6.0,9.0)	
6b	Quant.	135—136	$C_{16}H_{20}N_2O_5$	60.04	5.98	8.68	3305	1710	1670	6.35br s	8.32s	4.38dq	+19.86
				(59.99	6.29	8.74)				6.78d (7.0)		(6.0,8.0)	
6c	Quant.	Syrup	$C_{29}H_{45}N_3O_5$[c]	67.25	8.85	8.08	3305	1730	1670	6.95d (7.3)	10.42br s	4.63dd	+3.59
				(67.54	8.80	8.15)				7.16br s		(7.0,8.0)	
6d[b]	Quant.	Syrup	$C_{29}H_{45}N_3O_5$[c]	67.68	8.59	7.88	3305	1720	1675	6.55br s	8.21br s	4.60dd	−3.04
				(67.54	8.80	8.15)				6.92br s		(7.0,8.0)	
6f	Quant.	137—138	$C_{19}H_{26}N_2O_5$	62.57	7.35	7.45	3270	1700	1670	6.90br s	9.28s	4.54dd	−18.28
				(62.96	7.23	7.73)				7.44—7.60m (+Ph)		(6.0,8.0)	
6g	Quant.	Syrup	$C_{23}H_{27}N_2O_5$	66.95	6.81	6.77	3300	1705	1670	6.10br s	8.97s	4.52dd	−3.02
				(67.14	6.61	6.81)				6.72d (7.0)		(7.0,9.0)	
6h	Quant.	Syrup	$C_{19}H_{25}N_2O_6$	63.31	7.19	7.46	3310	1710	1675	7.22d (7.5)	9.34s	4.62m	−5.28
				(63.14	6.97	7.75)				7.35—7.55m (+Ph)			
6i	Quant.	Syrup	$C_{27}H_{33}N_3O_7$	63.18	6.73	7.98	3335	1720	1665	7.09d (7.0)	9.17s	4.58m	−0.73
				(63.39	6.50	8.21)				7.40—7.55m (+Ph)			

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 No.	Yield %	Mp[a] θ_m/°C	Formula	Found (%) (Calcd) C	H	N	IR, ν/cm^{-1} –NH–	–CONH–	–C=C–	^{1}H NMR, δ –CONH– (J/Hz)	–CH=	$[\alpha]_D^{25}$/° [b]
7a	58	113—114	$C_{26}H_{32}N_4O_5$	64.85	6.95	11.85	3310	1668	1650	7.46d (6.0), 7.61br s	6.14d	−9.16 (c=0.84)
				(64.98	6.71	11.66)		1540		7.69br s, 9.06br s	(10.0)	
7b	33	196—197	$C_{28}H_{36}N_4O_5$	66.44	7.02	10.85	3316	1668	1650	7.36d (10.0), 7.74br s	5.94d	−21.80 (c=1.06)
				(66.12	7.13	11.02)		1539		7.82br s, 8.48br s	(10.0)	
7c	86	107—108	$C_{29}H_{38}N_4O_5$	66.46	7.60	10.48	3310	1665	1650	7.28d (13.0), 7.75br s	6.03d	−30.18 (c=1.00)
				(66.50	7.51	10.70)		1540		7.83br s, 8.72br s	(10.0)	
7d	68	191—192	$C_{29}H_{38}N_4O_5$	66.37	7.22	10.73	3292	1668	1655	7.45d (9.0), 7.71br s	5.98d	−21.39 (c=1.00)
				(66.52	7.51	10.70)		1539		7.79br s, 8.53br s	(10.0)	
7e	60	152—154	$C_{32}H_{36}N_4O_5$	68.67	6.80	10.03	3305	1668	1650	7.15d (15.0), 7.77br s	5.88d	−37.63 (c=0.86)
				(69.05	6.52	10.06)		1533		7.85br s, 8.61br s	(10.0)	

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

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

Compd No.	Yield %	Mp[a] θ_m/°C	Formula	Found (%) (Calcd) C	H	N	IR, ν/cm^{-1} –NH–	–CONH–	–C=C–	^{1}H NMR, δ –CONH– (*J*/Hz)	–CH=	$[\alpha]_D^{25}$/° [b]
8a	72	161—163	$C_{26}H_{36}N_4O_7$	60.14	7.01	10.54	3305	1665	1660	6.83d (5.3), 7.07br s	5.91d	−17.25 (c=0.86)
				(60.45	7.02	10.85)		1540		7.50d (6.5), 7.93br s	(10.0)	
8b	37	138—140	$C_{28}H_{40}N_4O_7$	61.98	7.35	10.09	3310	1670	1650	6.57br s, 7.23d (7.0)	6.11d	−33.84 (c=1.00)
				(61.74	7.40	10.29)		1545		7.35—7.55m (+Ph), 7.73br s	(10.0)	
8c	96	177—179	$C_{29}H_{42}N_4O_7$	62.55	7.79	10.08	3320	1660	1645	7.00d (4.8), 7.22br s	5.83d	−32.82 (c=1.00)
				(62.34	7.58	10.03)		1540		7.41d (6.5), 7.97br s	(10.0)	
8d	68	169—171	$C_{29}H_{42}N_4O_7$	62.50	7.76	10.08	3310	1665	1650	6.56d (5.1), 7.20d (6.5)	6.09d	−31.52 (c=1.00)
				(62.34	7.58	10.03)		1540		7.35—7.55m (+Ph), 7.71br s	(10.0)	
8e	78	197—199	$C_{32}H_{40}N_4O_7$	64.61	6.95	9.38	3305	1670	1650	6.20d (5.7), 6.37br s	5.85d	−48.64 (c=1.00)
				(64.85	6.80	9.45)		1540		7.20d (7.0), 7.87br s	(10.0)	

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

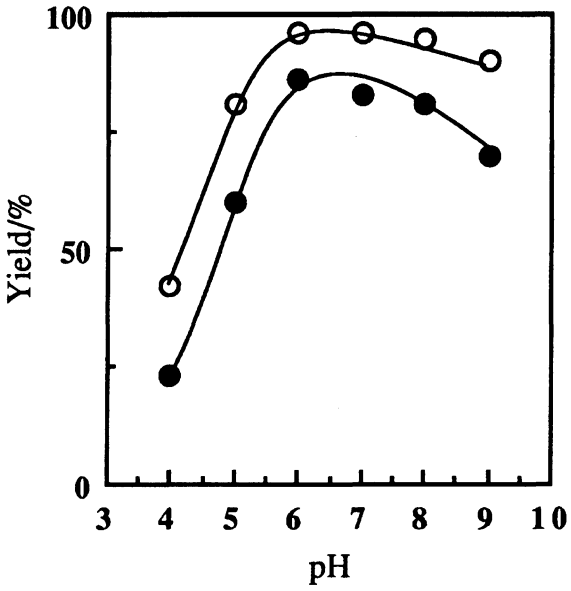


Fig. 3. Effects of pH on the yields of **7c** (●) and **8c** (○). The reaction mixture (4 ml), containing 50 mM **2c**, and 150 mM **3c** or **5c**, and 7.5 g dm^{-3} papain (2.8 units·mg^{-1}) in McIlvaine buffer, was shaken at various pH's at 35 °C for 24 h.

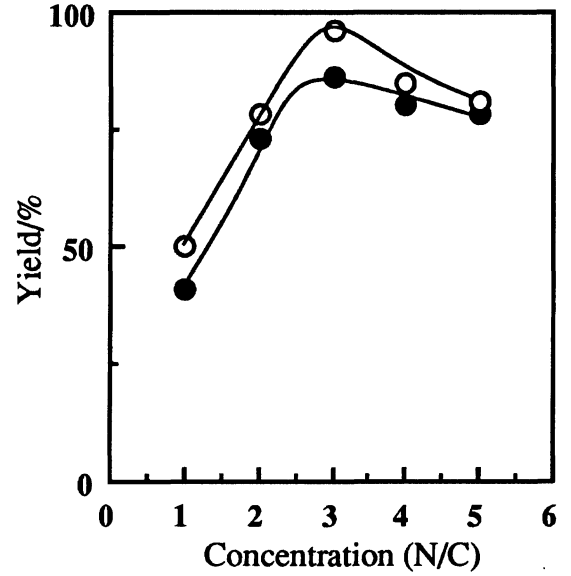
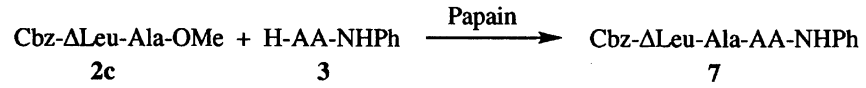


Fig. 4. Effects of *N*-component concentration on the yields of **7c** (●) and **8c** (○). The reaction mixture (4 ml), containing 50 mM **2c**, various concentrations of **3c** or **5c**, and 7.5 g dm^{-3} papain (2.8 units·mg^{-1}) in McIlvaine buffer, was shaken at pH 6 at 35 °C for 24 h.

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.

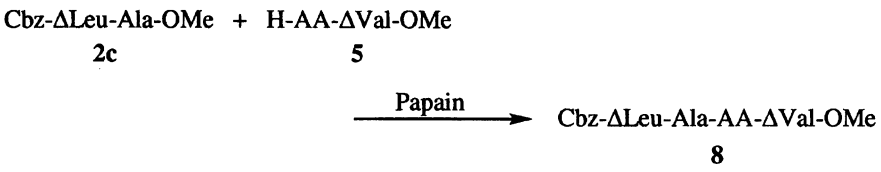
In the case of peptide formation, the effect of the con-



2c **3** **7**

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

Scheme 3.



2c **5** **8**

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 $g\,dm^{-3}$, the enzyme concentration at 7.5 $g\,dm^{-3}$ provided the highest yield. A small amount of enzyme was required for the coupling reaction compared with that (30 $g\,cm^{-3}$) 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 Δ^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, ^{1}H NMR, 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.

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