Dehydrooligopeptides. XIII. Selective Enzymatic Hydrolysis of *N*-Protected α-Dehydroglutamic Acid Diesters and Their Analogs Using Papain as Catalyst[1)]

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Since the α-ester of α,γ-dimethyl *N*-benzyloxycarbonyl-α-dehydroglutamate (ΔGlu) was found to be hydrolyzed using papain, a variety of *N*-protected α-dehydroglutamates and their analogs, both esters of which were the same or different from each other, were newly synthesized and subjected to a similar hydrolysis. Although the substrates, with bulkier ester group at the γ- as well as α-positions, were found to become difficult to hydrolyze, the kind and size of the *N*-protecting group did not effect the hydrolysis. Moreover, it was found that the length of side chain of the substrate greatly influenced hydrolysis. The present study suggests that papain may become a useful tool for the hydrolysis and coupling of ΔGlu with α-amino acid or peptide.

During about the past two decades, the many successes regarding the synthesis of a number of peptides by utilizing proteolytic enzymes, such as papain and α-chymotrypsin A etc., have contributed great epochal effects to the field of organic synthetic chemistry.[2-5)] Even the complex macromolecules of useful peptide hormones could be synthesized under considerably mild conditions by the widely developed enzymatic synthetic method.[6-8)]

On the contrary, it was hardly believed up to now that the unusual α-amino acid, α-dehydroamino acid (DHA) ester, could be similarly hydrolyzed or coupled with L-α-amino acid (AA) enzymatically. Recently, however, a very selective hydrolysis of the α-ester moiety of α,γ-dimethyl *N*-benzyloxycarbonyl-α-dehydroglutamate [Cbz-ΔGlu(OMe)-OMe; **1b**] and novel peptide formation with L-Leu-NHPh by using papain (EC 3.4.22.2) have for the first time been successful and reported briefly.[9,10)] Since it was thought to be necessary to reexamine and widely develop a similar hydrolysis, we undertook a further detailed study. That is, many kinds of α-dehydroglutamates and their analogs, in which two carboxyl groups and an amino group are protected with various *C*- and *N*-protecting groups, were newly synthesized and the effects of the three protecting groups and the influence of the length of the side chain to enzymatic hydrolysis were thoroughly studied. Regarding the results, the substrate specificity of papain was broadened considerably.

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 EPI-G2 spectrometer. The ^{1}H NMR spectra were measured with a JEOL LMN-PS-100 spectrometer in a $CDCl_3$ or DMSO-d_6 solution with tetramethylsilane used as the internal standard.

Enzyme. Papain (2.8 units mg^{-1}), purchased from Sigma Chemical Co., U. S. A., was used without further purification.

***N*-Protected α-Dehydroglutamates (1) and Their Analogs.** Cbz-ΔGlu(OMe)-OMe (**1b**), Cbz-ΔGlu(OEt)-OMe (**1c**), and Cbz-ΔGlu(OBzl)-OMe (**1e**) (Bzl=benzyl) were prepared by the γ-esterification of Cbz-ΔGlu-OMe (**1a**)[1)] with an appropriate alcohol, according to a reported method.[1)] Cbz-ΔGlu(OEt)-OEt (**1f**) and Cbz-ΔGlu(OBzl)-OBzl (**1g**) were also obtained similarly by the α,γ-diesterification of Cbz-ΔGlu-OH (**2a**)[11)] with EtOH or BzlOH. Cbz-ΔGlu(OBu^t)-OMe (**1d**) (Bu^t= *t*-butyl) was derived by the treatment of **1a** with isobutene in a sealed tubes.[12)] Similarly, Cbz-ΔGlu(OBu^t)-OBu^t(**1h**) was also obtained from **2a** and isobutene. In addition, *N*-methoxycarbonyl (Moc)-ΔGlu(OMe)-OMe (**1i**), *N*-ethoxycarbonyl (Eoc)-ΔGlu(OMe)-OMe (**1j**), *N*-isopropoxycarbonyl (Ipo)-ΔGlu(OMe)-OMe (**1k**), and *N*-*n*-butoxycarbonyl (Nbc)-ΔGlu(OMe)-OMe (**1l**) were prepared by the condensation of α,γ-dimethyl α-oxoglutarate with an appropriate alkyl carbamate.[1)] As an analog of the ΔGlu derivative, Cbz-ΔAsp(OMe)-OMe (**1m**) was obtained by the elimination of Cbz-β-(hydroxy)aspartic acid dimethyl ester.[13)] Furthermore, 2-(*N*-Cbz-amino)-2-hexenedioic acid dimethyl ester [Cbz-ΔAhx(OMe)-OMe; **1n**] and 2-(*N*-Cbz-amino)-2-heptenedioic acid dimethyl ester [Cbz-ΔAhp(OMe)-OMe; **1o**] were also derived by the condensation of 2-oxohexanedioic acid dimethyl ester and 2-oxoheptanedioic acid dimethyl ester with benzyl carbamate, respectively, by the usual method.[1)] The identification data of **1**, thus obtained, are presented in Tables 1 and 2.

Methyl 2-(*N*-Cbz-Amino)-5-hydroxy-2-pentenoate (1p). To a suspension of Cbz-ΔGlu(OMe)-OH (**2b**) (3 g, 10 mmol) and $NaBH_4$ (2.32 g, 61 mmol) in Bu^{t}OH (15 ml) was added MeOH, with stirring, at 60 °C until it became a transparent solution. After stirring for 3 h, the resulting solution was neutralized with 1 M-HCl (1 M=1 mol dm^{-3}) and concentrated under reduced pressure to give a crude residue, which was dissolved in ethyl acetate (30 ml). The resultant solution was washed with brine and dried over anhydrous Na_2SO_4. Concentration in vacuo gave crystals, which were recrystallized from $CHCl_3$ to give 2-(*N*-Cbz-amino)-5-hydroxy-2-pentenoic acid (**2p**) as colorless needles. Yield 80%, mp 114—115 °C. IR (KBr) 3250 (NH), 1655 (C=C) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ=8.57 (bs, 1H, NH), 6.48 (t, 1H, *J*=7.0 Hz, -CH=). Found: C, 60.34; H, 6.04; N, 5.11%. Calcd for $C_{14}H_{17}NO_5$ C, 60.20; H, 6.14; N, 5.02%. Subsequently, the obtained **2p** (2.1 g, 7.9 mmol) was treated with MeOH (20 ml)

Table 1. The Yields and Melting Points of ROOC-$(CH_2)_n$CH=C$\langle^{COOR'}_{NHCOOX}$ (**1**)

Compound					Yield	Mp	Formula	Found (Calcd)/%		
No.	n	X	R	R'	%	θ_m/°C		C	H	N
1b	1	Bzl	Me	Me	70	82—83[a]	$C_{15}H_{17}NO_6$	58.74 (58.63	5.50 5.78	4.53 4.56)
1c	1	Bzl	Et	Me	95	62—63[b]	$C_{16}H_{19}NO_6$	60.03 (59.80	5.78 5.96	4.37 4.36)
1d	1	Bzl	Bu^t	Me	81	Syrup	$C_{18}H_{23}NO_6$			4.20 (4.01)
1e	1	Bzl	Bzl	Me	69	Syrup	$C_{21}H_{21}NO_6$	66.00 (65.78	5.24 5.52	3.42 3.65)
1f	1	Bzl	Et	Et	87	55—56[b]	$C_{17}H_{21}NO_6$	60.69 (60.88	6.26 6.31	4.18 4.18)
1g	1	Bzl	Bu^t	Bu^t	66	62—63[c]	$C_{27}H_{25}NO_6$	70.33 (70.57	5.40 5.48	3.21 3.05)
1h	1	Bzl	Bzl	Bzl	60	71—72[c]	$C_{21}H_{29}NO_6$	64.40 (64.43	7.40 7.47	3.29 3.68)
1i	1	Me	Me	Me	52	82—83[d]	$C_9H_{13}NO_6$	46.61 (46.75	5.61 5.67	6.04 6.06)
1j	1	Et	Me	Me	80	42—43[b]	$C_{10}H_{15}NO_6$	48.86 (48.97	6.03 6.17	5.70 5.71)
1k	1	Pr^i	Me	Me	80	90—91[c]	$C_{11}H_{17}NO_6$	50.92 (50.96	6.51 6.61	5.56 5.40)
1l	1	Bu^n	Me	Me	86	Syrup	$C_{12}H_{19}NO_6$	52.88 (52.74	7.00 7.01	5.21 5.13)
1m[e]	0	Bzl	Me	Me	72	Syrup				
1n	2	Bzl	Me	Me	80	Syrup	$C_{16}H_{19}NO_6$	60.62 (60.88	6.19 6.31	4.03 4.18)
1o	3	Bzl	Me	Me	70	52—53[c]	$C_{17}H_{21}NO_6$	60.07 (59.80	6.09 5.96	4.58 4.36)

a) Colorless needles from $CHCl_3$. b) Colorless needles from hexane–ethyl acetate. c) Colorless prisms from hexane–ethyl acetate. d) Colorless needles from diisopropyl ether. e) Lit, 13.

Table 2. The IR and ^{1}H NMR Spectral Data of **1**

Compound No.	IR, ν/cm^{-1} in KBr		^{1}H NMR, δ in $CDCl_3$	
	-NH-	-C=C-	-CH= (J/Hz)	-NH-
1b	3250	1665	6.76t (7.0)	6.60bs
1c	3275	1655	6.71t (7.0)	6.63bs
1d	3340	1670	6.78t (7.0)	6.69bs
1e	3370	1665	6.76t (7.0)	6.60bs
1f	3280	1665	6.78t (7.0)	6.77bs
1g	3290	1655	6.64t (7.0)	6.54bs
1h	3345	1645	6.87t (7.0)	6.64bs
1i	3340	1670	6.68t (7.0)	6.80bs
1j	3340	1655	6.64t (7.0)	6.98bs
1k	3270	1670	6.76t (7.5)	6.38bs
1l	3330	1670	6.60t (7.0)	6.90bs
1m	3305	1640	5.44s	9.63s
1n	3330	1660	6.51t (7.0)	6.70bs
1o	3335	1660	6.46t (7.0)	7.02bs

in the presence of $SOCl_2$ (0.64 ml), with stirring, at −10 °C for 30 min. The reaction solution was further stirred at room temperature overnight and concentrated in vacuo. The obtained residue was dissolved in ethyl acetate (30 ml) and the resulting solution was washed with successively saturated $NaHCO_3$ aqueous solution and brine and then dried over anhydrous Na_2SO_4. Concentration in vacuo gave a yellow syrup, which was purified on a silica-gel column using a mixture of $CHCl_3$ and acetone (10 : 1 v/v) as the eluent. The eluate was concentrated in vacuo to give **1p** as a yellow syrup. Yield 82%. IR 3330 (NH), 1660 (C=C) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ=7.00 (bs, 1H, NH), 6.59 (t, 1H, J=7.0 Hz, -CH=). Found: C, 59.03; H, 5.65; N, 5.33%. Calcd for $C_{13}N_{15}NO_5$: C, 58.86; H, 5.70; N, 5.28%.

General Enzymatic Hydrolysis of 1. A suspension (5 ml) of an appropriate **1** [100 mM 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 structures and yields of the hydrolyzates were identified and determined by HPLC on a Hitachi 630-50 model (column 4ϕ×250 mm). The eluent (MeOH : H_2O=60 : 40 v/v) was run with a flow rate of 1 ml min^{-1}. The peaks were determined at 230 nm by comparing the retention times with those of authentic compounds prepared chemically.[1,11] The peak intensities were used to calculate the relative concentrations. Furthermore, to isolate pure **2**, the reaction solution was treated with a saturated $NaHCO_3$ aqueous solution (50 ml) and washed twice with ethyl acetate (60 ml). The aqueous layer was acidified to pH 2.0 with 6 M-HCl and extracted three times with ethyl acetate (100 ml). The combined extracts were washed twice with brine (60 ml) and dried over anhydrous Na_2SO_4. Concentration in vacuo gave crystals or syrup, which were recrystallized from an appropriate organic solvent or purified on a silica-gel column using a mixture of $CHCl_3$ and acetone (10 : 1 v/v) as the eluent to give γ-alkyl esters of *N*-protected α-dehydroglutamic acids (**2a**—**2d** and **2i**—**l**) and their analogs (**2n** and **2o**). The identification data of **2** are summarized in Tables 3 and 4.

Table 3. The Yields and Melting Points of ROOC-$(CH_2)_n$CH=C$\langle^{COOH}_{NHCOOX}$ (2)

Compound				Yield	Mp	Formula	Found (Calcd)/%		
No.	*n*	R	X	%	θ_m/°C		C	H	N
2a	1	H	Bzl	38	146—147[c]	$C_{13}H_{13}NO_6$	56.19	4.97	4.92
							(55.91	4.70	5.02)
2b	1	Me	Bzl	94	129—130[d]	$C_{14}H_{15}NO_6$	57.58	5.12	4.80
							(57.33	5.16	4.78)
2c	1	Et	Bzl	86[a] 51[b]	132—133[d]	$C_{15}H_{17}NO_6$	58.43	5.45	4.49
							(58.63	5.58	4.56)
2d	1	Bu^t	Bzl	23	Syrup	$C_{17}H_{21}NO_6$	60.78	6.05	4.12
							(60.88	6.31	4.18)
2i	1	Me	Me	Quant	127—128[e]	$C_8H_{11}NO_6$	43.91	5.03	6.35
							(44.24	5.11	6.45)
2j	1	Me	Et	Quant	130—131[d]	$C_9H_{13}NO_6$	46.77	5.58	5.86
							(46.75	5.67	6.06)
2k	1	Me	Pr^i	Quant	154—155[d]	$C_{10}H_{15}NO_6$	48.67	5.93	5.41
							(48.97	6.17	5.71)
2l	1	Me	Bu^n	Quant	119—120[d]	$C_{11}H_{17}NO_6$	51.05	6.63	5.33
							(50.96	6.61	5.40)
2n	2	Me	Bzl	47	116—117[f]	$C_{14}H_{15}NO_6$	57.48	5.31	4.57
							(57.33	5.16	4.78)
2o	3	Me	Bzl	11	78—79[f]	$C_{15}H_{17}NO_6$	58.78	5.69	4.31
							(58.63	5.58	4.56)

a) From **1c**. b) From **1f**. c) Colorless needles from $CHCl_3$–ethyl acetate. d) Colorless needles from ethyl acetate. e) Colorless needles from hexane–ethyl acetate. f) Colorless prisms from hexane–ethyl acetate.

Table 4. The IR and ^{1}H NMR Spectral Data of **2**

Compound No.	IR, ν/cm^{-1} in KBr		^{1}H NMR, δ in DMSO-d_6		
	-NH-	-C=C-	-COOH	-CH= (*J*/Hz)	-CH$_2$CH=
2a	3250	1655	8.56bs	6.50t (7.0)	3.19d
2b	3285	1660	8.41bs[a]	6.88t (7.0)	3.29d
2c	3290	1665	9.21bs[a]	6.86t (7.0)	3.26d
2d	3305	1655	7.58bs	6.76t (7.0)	3.22d
2i	3300	1660	8.59bs	6.44t (7.0)	3.28d
2j	3305	1660	8.54bs	6.42t (7.0)	3.28d
2k	3300	1660	8.41bs	6.38t (7.0)	3.24d
2l	3280	1665	8.46bs	6.39t (7.0)	3.24d
2n	3285	1660	8.65bs	6.37t (7.0)	2.58—2.20m
2o	3290	1650	8.59bs	6.37t (7.0)	1.70—1.48m

a) Measured in $CDCl_3$.

Enzymatic Hydrolysis of 1p. Quite similarly, the hydrolysis of **1p** with papain was worked up to give **2p**, which was completely in accord with the compound derived by the reduction of **2b** with $NaBH_4$. Yield 80%.

Results and Discussion

In order to examine and to further develop the new catalytic action of cheaper and readily accessible papain, various DHA esters (**1**) as the substrate were submitted to the enzymatic reaction.

First of all, in particular, to obtain optimal conditions the hydrolysis of **1b** was thoroughly reexamined, since a preliminary run using papain had already been achieved to give **2b**[9] almost quantitatively. In addition to a detailed examination of the effect of the pH,[9] the effects of the reaction time and concentration of both the enzyme and substrate were studied, as shown in Figs. 1—4.

The pH of the reaction solution was changed from 3 to 9, although the optimal pH value of esterase action of papain in a McIlvaine buffer is well-known to be around 7. As a result, the yield of **2b** increased abruptly over the pH range from 6 to 8, and reached more than 90% at pH 8. Consequently, the α-ester hydrolysis of **1b** was found to be extremely dependent upon the pH. That is, Fig. 1 indicates that a rather alkaline pH may be preferred for the such hydrolysis of **1**. Subsequently, the time course of a similar hydrolysis of **1b** at 8.0 was also examined. As Fig. 2 shows, the hydrolysis is comparatively slow and the yield of the hydrolyzate reached ultimately more than 90% over 24 h.

Concerning the amount of papain, the enzyme con-

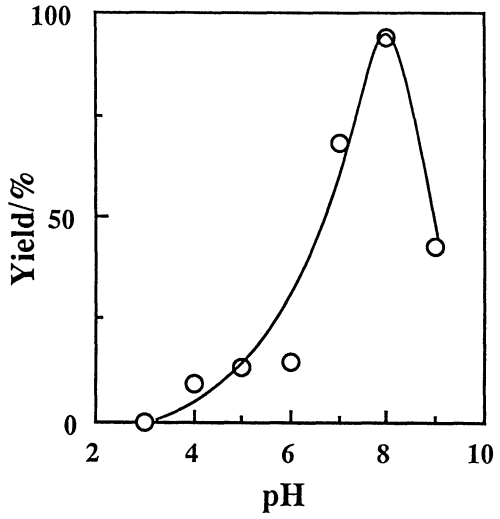


Fig. 1. Effect of pH. The reaction mixture (5 ml), containing 200 mM **1b** and 30 g dm^{-3} papain (2.8 units mg^{-1}) in McIlvaine buffer, was shaken at various pH's at 35 °C for 24 h.

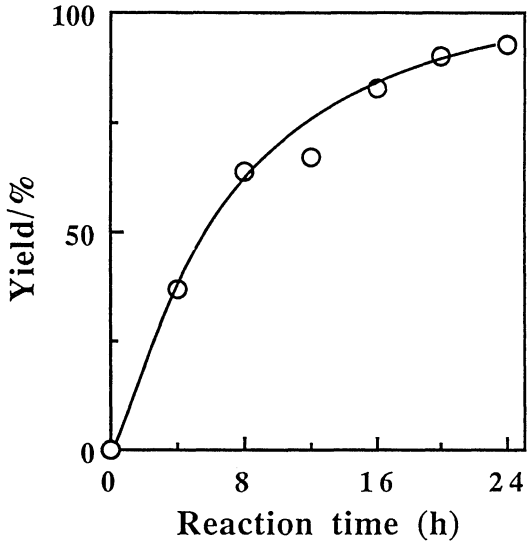


Fig. 2. Effect of reaction time. The reaction mixture (5 ml), containing 200 mM **1b** and 30 g dm^{-3} papain (2.8 units mg^{-1}) in McIlvaine buffer, was shaken at pH 8 at 35 °C for various hours.

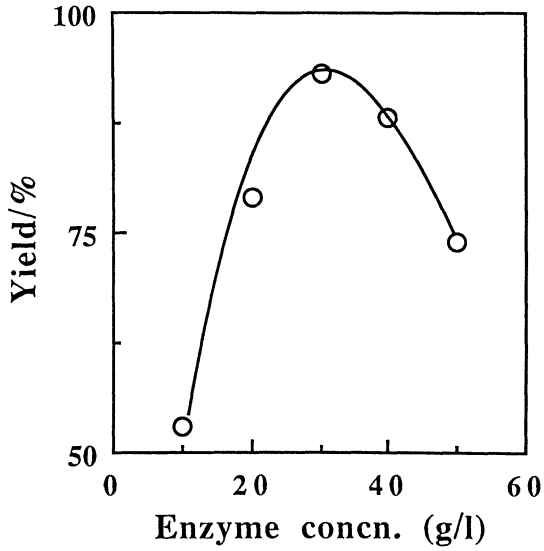


Fig. 3. Effect of enzyme concentration. The reaction mixture (5 ml), containing 200 mM **1b** and various concentrations of papain in McIlvaine buffer, was shaken at pH 8 at 35 °C for 24 h.

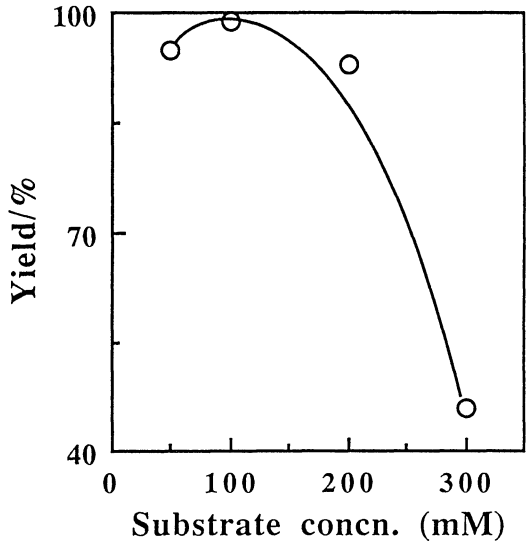


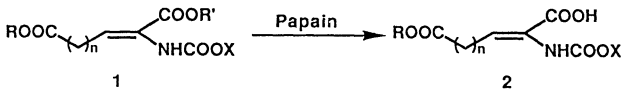
Fig. 4. Effect of substrate concentration. The reaction mixture (5 ml), containing various concentrations of **1b** and 30 g dm^{-3} papain (2.8 units mg^{-1}) in McIlvaine buffer, was shaken at pH 8 at 35 °C for 24 h.

centration at around 30 g dm^{-3} provided the highest yield. Fig. 3 indicates that a considerably large amount of enzyme is required for the hydrolysis. In addition, Fig. 4 indicates that a substrate concentration ranging from 50 to 150 mM affords a very high yield of more than 95%. Consequently, the substrate concentration of 100 mM was found to be the most effective.

As can be seen from Figs. 1—4, the results show that the enzymatic hydrolysis of **1b** requires a considerably prolonged reaction time and higher concentration of enzyme, but closely depends upon a comparatively lower substrate concentration under a slightly alkaline pH.

The optimal conditions of the hydrolysis of **1b** [papain (30 g dm^{-3}), substrate (100 mM) in McIlvaine buffer at pH 8.0 and at 35 °C for 24 h], was applied to a similar hydrolysis of all the substrates (**1**), according to Scheme 1.

From the results and the fact that other DHA esters have also been hydrolyzed,[14] our present study indicates that papain is a potent catalyst for the hydrolysis of various DHA esters. The structural characteristics of



1 2

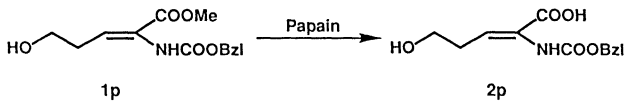
No.	n	X	R	R'
1a	1	Bzl	H	Me
1b	1	Bzl	Me	Me
1c	1	Bzl	Et	Me
1d	1	Bzl	Bu^t	Me
1e	1	Bzl	Bzl	Me
1f	1	Bzl	Et	Et
1g	1	Bzl	Bu^t	Bu^t
1h	1	Bzl	Bzl	Bzl
1i	1	Me	Me	Me
1j	1	Et	Me	Me
1k	1	Pr^i	Me	Me
1l	1	Bu^n	Me	Me
1m	0	Bzl	Me	Me
1n	2	Bzl	Me	Me
1o	3	Bzl	Me	Me

Scheme 1.

the substrates (**1**) to the substrate specificity of papain is discussed below. Particularly, as can be seen from Table 3, in the cases of **1b**, **1c**, and **1f**, when the α,γ-diester groups were comparatively small in size, the α-ester alone was readily hydrolyzed to give the corresponding γ-methyl ester (**2b**) in 94% yield and γ-ethyl ester [Cbz-ΔGlu(OEt)-OH; (**2c**)] in 85% from **1c** and 51% from **1f** yields, respectively. However, a similar hydrolysis of the γ-free ΔGlu-OMe (**1a**) could not easily give **2a**[1] in only 38% yield.

On the other hand, since the sizes of the γ-ester (**1d** and **1e**) and α,γ-diesters (**1g** and **1h**) gradually become bulky, hydrolysis was found to become more difficult. In the case of **1d**, the hydrolysis proceeded ultimately to give Cbz-ΔGlu(OBu^t)-OH (**2d**) in 23% yield, while in the case of a bulkier γ-benzyl ester (**1e**) hydrolysis didn't take place. Accordingly, as is naturally expected, the bulky α,γ-di-*t*-butyl (**1g**) and α,γ-dibenzyl esters (**1h**) were by no means hydrolyzed. As a result, it can be seen the size and bulkiness of the γ-ester (X), as well as those of the α-ester (Y), are very important factors for the hydrolysis of such substrates [Cbz-ΔGlu(OX)-OY] with papain, except for **1a**.

In addition, besides the above-two *C*-protecting groups, the *N*-protecting groups of **1** are also thought to be another important factor. It is necessary to investigate whether or not the *N*-protecting group influences this type of hydrolysis. Being kept the both sides as methyl esters, the substrates (**1**) protected with five kinds of *N*-protecting groups [that is, from the smallest Moc (**1i**), Eoc (**1j**), Ipc (**1k**), Nbc (**1l**) to the largest Cbz group (**1b**)] were synthesized, and then applied to a similar hydrolysis. As a result, all of the esterase actions took place beautifully to give the corresponding *N*-protected ΔGlu(OMe)-OH (**2i**—**l**) in ca 91% yields. These results clearly indicate that such enzymatic hydrolysis is not affected by the size of the *N*-protecting groups.



1p 2p

Scheme 2.

However, interestingly, it was found that the length of the side chain of **1** was greatly related to the hydrolysis, as shown in Table 3. Namely, dehydroaspartate (**1m**) was by no means hydrolyzed, whereas dehydroglutamate (**1b**) was hydrolyzed almost quantitatively; then, the longer the side chain from **1b** to **1o** via **1n** became, the yield decreased abruptly.

Furthermore, since a similar hydrolysis of **1b** having a hydroxyl group at the terminus of the side chain was also achieved to give 2-(*N*-Cbz-amino)-5-hydroxy-2-pentenoic acid (**2p**) in 80% yield (Scheme 2), it seems that the existence of the γ-ester group in ΔGlu derivatives is not necessarily essential; that of the electronegative group near the γ-position, however, may be rather important. On the other hand, it is of particular interest that the results of the hydrolysis are not affected by the kinds and sizes of the *N*-protecting groups at all, but by the length of the side chain.

On the contrary, however, in the case of the treatment of **1b** with α-chymotrypsin A, interestingly, it has been already revealed that the γ-methyl ester is selectively hydrolyzed.[15]

In conclusion, besides being surprised that the enzymatic hydrolysis of the DHA ester occurred, it is very available synthetically that only the α-ester of **1** could be enzymatically hydrolyzed, whereas the selective hydrolysis of **1** was extremely difficult by the chemical method.[9] Furthermore, the results indicate that the reverse reaction, i.e., peptide-bond formation, can also be catalyzed with papain. In fact, the enzymatic synthesis of various dehydroglutamyldipeptides were successful. After all, the present study suggested that papain has become a sufficiently useful tool for organic synthesis.

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