Studies of the Synthetic Model Enzyme. The Synthesis of Cyclo(His-Glu-Cys-D-Phe-Gly)$_2$ as the Esterase Model[1]

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(Received December 2, 1972)

To study the mechanism of enzyme action, cyclo(His–Glu–Cys–D–Phe–Gly)$_2$ was prepared as an esterase model from the corresponding linear decapeptide by the azide methods. The esterase-like activity of this cyclic decapeptide in reaction to *p*-nitrophenyl acetate was three times greater than that of the linear decapeptide, the curve of the catalytic coefficient *versus* the different pH is of a bell type, and the optimum pH was recorded at about 7.6. The kinetics of the hydrolysis obeyed Michaelis-Menten's typical equation.

In spite of all our knowledge of the structures of enzymes and the kinetics of enzyme-catalyzed reactions, we are still far from an adequate understanding of the mechanisms of enzyme action. To answer this problem, approaches have been made by many workers through synthetic research into model enzymes. In these cases, linear amino acid peptide[2] or polyamino acid,[3] with active sites of amino acid residues, have mostly been used as the model substances, these studies attained their first purpose to some extent.

In the present study, the authors proposed to synthesize the cyclic peptide as a model enzyme; it has a relatively low molecular weight and will have hydrolytic activity. For the above purpose, histidine, cysteine, and glutamic acid were chosen as the active-site amino acids of synthetic cyclic peptide; they are arranged along the cyclic decapeptide chain with an antiparallel structure, as is shown in Fig. 1.

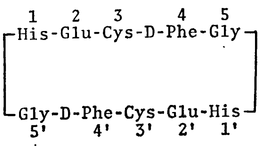

Fig. 1. Enzyme model (I)

Moreover, the cyclic decapeptide (I) could be successfully obtained by the removal of the protecting groups, followed by the cyclization of the linear decapeptide azide by using the high-dilution method. The hydrolytic activity of the synthetic model peptide has been investigated in detail by using the linear and cyclic decapeptide.

Synthesis of the Linear Decapeptide Derivative. The synthetic route of the linear decapeptide derivative (XV) is summarized in Figs. 2 and 3. Boc-dipeptide methyl ester (II) was prepared by the DCC method; then II was converted into tripeptide (IV) by the azide method. Boc-pentapeptide (X) was successively obtained from the tripeptide (V), Boc–Glu(OBzl)–OSu (VI), and Boc–His(*im*-Dnp)–OSu (IX) by the succinimide ester method. The preparation of Boc-decapeptide (XV) from X and the free pentapeptide (XII) failed, because of the difficulties of isolating the decapeptide from the reaction mixture. Also, the pure XV could be obtained by the coupling of IX and nonapeptide (XIV), which has been prepared from the Boc-tetrapeptide *p*-nitrophenyl ester (XI) and free pentapeptide (XII).

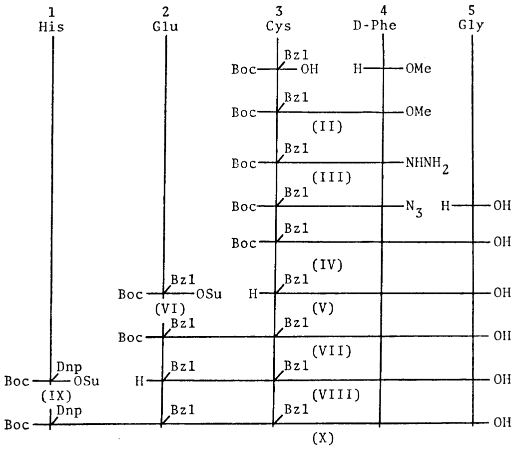

Fig. 2. Synthesis of Boc-pentapeptide (X)

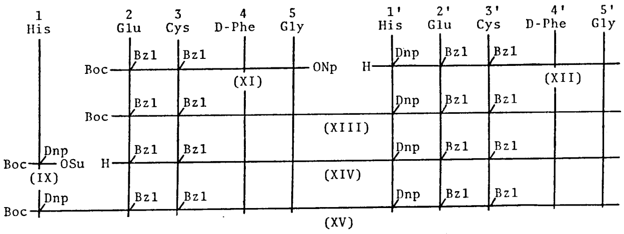

Fig. 3. Synthesis of Boc-decapeptide (XV)

The Cyclization of Linear Decapeptide Derivatives. Three different methods, using the *p*-nitrophenyl ester, *N*-hydroxysuccinimide ester, and azide, were used in the cyclization procedure (Fig. 4); the results are summarized in Table 1. The Boc-decapeptide *p*-nitrophenyl ester was obtained in a higher yield by the di-*p*-nitrophenyl sulfite method than by the DCC method, but the cyclization did not proceed satisfactorily. The Boc-decapeptide succinimide ester could be prepared in good yield by the DCC method, but

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[1] The abbreviations used for the amino acid residues, peptides and other abbreviations are based on the proposals by J. M. Stewart and J. D. Young, "Solid Phase Peptide Synthesis", W. H. Freeman and Co., San Francisco (1969).

[2] P. Cruickshank and J. D. Sheehan, *J. Amer. Chem. Soc.*, **86**, 2070 (1964); I. Photaki, V. Bardakos, A. W. Lake, and G. Low, *J. Chem. Soc., C*, **1968**, 1860.

[3] a) E. Katchalski, G. D. Fasman, E. Simons, E. R. Blout, F. R. N. Gurd, and W. L. Koltum, *Arch. Biochem. Biophys.*, **88**, 361 (1960). b) J. Noguchi and T. Saito, "Polyamino Acids, Polypeptides and Proteins", ed. by M. A. Stahmann. The Univ. Wisconsin Press, Madison (1962), p. 313.

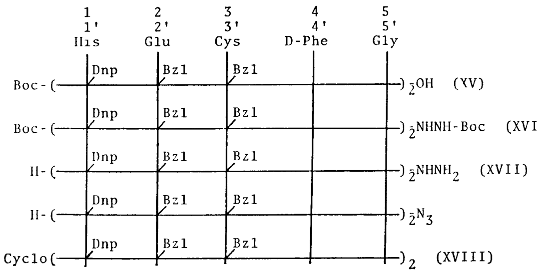

Fig. 4. Synthesis of Cyclic decapeptide (XVIII) by azide method

its active ester was not cyclized and the cyclic decapeptide derivative (XVIII) was not obtained. It was, therefore, concluded that the cyclization by the azide was the most favorable method in the present experiment, as is shown in Table 1.

Table 1. The results of the cyclization reaction

Method	Reaction temp. (°C)	Reaction time (hr)	Cyclo decapeptide (%)
ONp ester	60	6	52
OSu ester	rt	12	trace
Azide	0	24	87

XVIII was purified by the use of ion-exchange and gel filtration. The pure cyclic decapeptide (XVIII) was identified by thin-layer chromatography, by elemental analysis, and by molecular-weight measurements.

The Removal of the Protecting Groups from the Linear and Cyclic Decapeptide Derivatives. The benzyl ester and *s*-benzyl groups were removed from XV and XVIII by treating them with anhydrous hydrogen fluoride, and the dinitrophenyl group of the imidazole was removed by use of 2-mercaptoethanol[4] as is shown in Fig. 5.

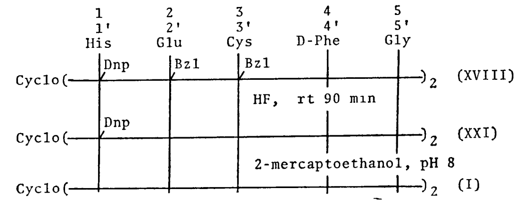

Fig. 5. Removal of the protecting groups from cyclic decapeptide (XVIII)

The free linear (XXIII) and cyclic decapeptide (I) thus obtained were purified with Sephadex G-15, and their purity was checked by means of a ninhydrin reaction, a Pauly reaction, and a nitroprusside reaction on thin-layer chromatography and paper electrophoresis. No disulfide groups were detected by the Ellman[5] and iodoacetamide methods.[6]

Hydrolytic Activity of the Synthetic Peptides, as Studied Using p-*Nitrophenyl Acetate as the Substrate.* The measurement of the catalytic action of the cyclic and linear decapeptide against *p*-nitrophenyl acetate was carried out by the method of Katchalski *et al.*;[3] the results are summarized in Table 2.

Table 2. Hydrolysis of *p*-nitrophenyl acetate in 1/15M phosphate buffer, pH 7.73, containing 3.23% dioxane and DMF (v/v) at 24°C. [*p*-nitrophenyl acetate] = 3.23×10^{-5}M.

	Concn of catalyst	k_1	c	k_2
Catalyst	mol l^{-1} $\times10^4$	min^{-1} $\times10^3$	mol l^{-1}	$l\ \text{mol}^{-1}$ min
none		1.75		
His·HCl	5.37	2.44	5.37	1.33
Cys·HCl	5.37	20.69	5.37	32.20
I·2AcOH	3.00	13.13	6.00	19.61
XXIII·3AcOH	3.00	5.26	6.00	6.05
Chymotrypsin				10^4

The value of the catalytic coefficient of the cyclic decapeptide was greater than those of the linear decapeptide and histidine hydrochloride, but smaller than that of cysteine hydrochloride. The results show that the active sites of the amino acid placed opposite to each in the cyclic molecule have a more effective interaction between the catalyst and the substrate than those of the linear peptide.

The initial rate of hydrolysis was determined at several concentrations of the substrate under a constant concentration of I; it was found that the reaction rate increased in parallel with the substrate concentration at a relatively lower concentration, but thereafter gradually reached the maximum rate, as is shown in Table 3.

Table 3. Effect of concentration on the rate (V) of *p*-nitrophenyl acetate in 1/15M phosphate buffer, pH 7.73, containing 3.23% dioxane and DMF (v/v) at 24°C. [decapeptide-1] = 47.6 μM

[NPA] $\times10^4$	V $\times10^6$ (M min^{-1})
0.78	0.15
0.92	0.19
1.94	0.42
3.95	0.75
7.81	1.73
15.68	3.36
23.39	4.10

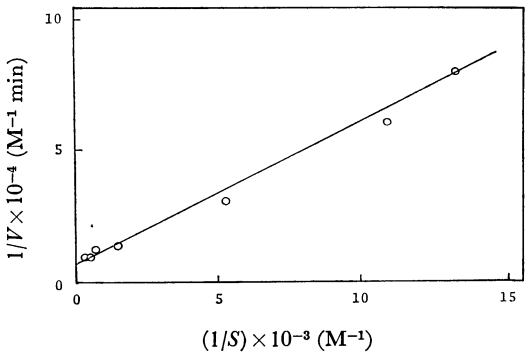

Fig. 6. Lineweaver-Burk plot for the hydrolysis of *p*-nitrophenyl acetate catalysed by Cyclic decapeptide (I) under condition indicated in Table 3

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[4] S. Shaltiel and M. Fridkin, *Biochemistry*, **9**, 5122 (1970).

[5] G. Ellman, *Arch. Biochem. Biophys.*, **82**, 70 (1959).

[6] S. Akabori, T. Kaneko, and K. Narita, "Chemistry of Proteines", Vol. 1, Kyoritsu Shuppan CO., Tokyo, (1969), p. 338.

Moreover, the Lineweaver-Burk plot of the data in Table 3 gives a straight line, as is shown in Fig. 6; the Michaelis-Menten constant, K_m, and the catalytic constant, K_{cat}, were 2.7 mM and 0.168 min^{-1} respectively. When the catalytic coefficient, K_2, was plotted against different pH values in the presence of a large excess of the substrate, the curve of K_2 was of a bell type and has an optimum pH at about 7.6, as is shown in Fig. 7.

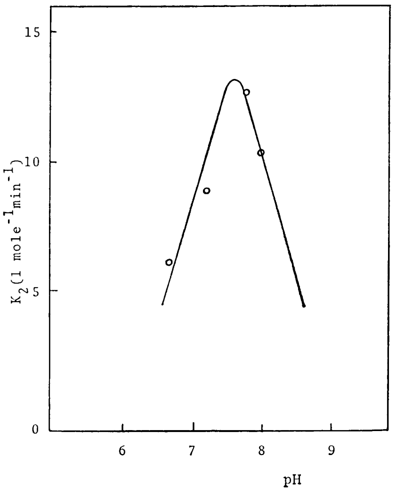

Fig. 7. PH dependance of catalytic coefficient for hydrolysis of *p*-nitrophenyl acetate by the cyclic decapeptide (I) in 1/15 M phosphate buffer containing 3.23% dioxane and DMF (v/v) at 24°C: [NPA]=1.57 mM, [Decapeptide-I]=2.37 mM

From the above results, it may be concluded that the cyclic decapeptide behaves as an acid-base type catalyst; it seems that the reaction proceeds through a soft binding intermediate (the so-called E-S complex), as in a usual enzyme system. The kinase-like activity will be investigated by using this same cyclic decapeptide in the near future.

Experimental

All the melting points are uncorrected. The infrared spectra were measured on a Hitachi Infrared Spectrophotometer, EPI-G3. The kinetics were followed with a Hitachi Photo-Electric Spectrophotometer, EPU-2. The purity of the compound was confirmed by thin-layer chromatography on Kieselgel G.

*Boc–Cys(SBzl)–*D*–Phe–OMe (II).* Boc–Cys(SBzl)–OH[7] (from the cyclohexylamine salt; 19.8 g, 50 mmol) was treated with DCC (11.4 g, 55 mmol) and H–D–Phe–OMe (from hydrochloride; 11.9 g, 55 mmol) in methylene chloride (250 ml) at 0 °C. After the reaction mixture had been allowed to stand overnight in the refrigerator, acetic acid (2 ml) was added and the mixture was stirred for 15 min. The dicyclohexyl urea thus produced was then filtered off, and the filtrate was washed with M sodium bicarbonate, M hydrochloric acid, and water successively, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. II was obtained from ethyl acetate–*n*-hexane in a 90.5% yield (21.4 g); mp 79 °C, $[\alpha]_D^{23}$ −33.3° (*c* 2.0, AcOH).

Found: C, 63.40; H, 6.90; N, 6.01; S, 6.89%. Calcd for $C_{25}H_{32}N_2O_5S$: C, 63.53; H, 6.83; N, 5.93; S, 6.78%.

*Boc–Cys(SBzl)–*D*–Phe–NHNH$_2$ (III).* 80% hydrazine hydrate (18.15 g, 300 mmol) was added to a solution of II (14.16 g, 30 mmol) in methanol (80 ml), and the solution was stirred for 30 min in an ice bath. After the reaction mixture had been kept at room temperature for one day, the addition of water to this reaction mixture gave white crystals. III was yielded; 13.9 g (98%); mp 110 °C, $[\alpha]_D^{23}$ −23.0° (*c* 2.0, AcOH).

Found: C, 60.92; H, 6.87; N, 11.59; S, 6.96%. Calcd for $C_{24}H_{32}N_4O_4S$: C, 60.99; H, 6.83; N, 11.80; S, 6.78%.

*Boc–Cys(SBzl)–*D*–Phe–Gly–OH (IV).* Into a solution of III (16.54 g, 35 mmol) in DMF (300 ml) and M hydrochloric acid (70 ml), was added, drop by drop, M sodium nitrite (35 ml, 35 mmol) under cooling at −15 °C. After stirring for 40 min, the reaction mixture was neutralized by the addition of triethylamine. A solution of glycine (7.88 g, 105 mmol), triethylamine (14.65 ml, 105 mmol), and water (35 ml) was added, drop by drop, into an azide solution at −10 °C. After the reaction mixture had then been stirred at −10 °C for 2 hr and allowed to stand overnight in a refrigerator, the mixture was acidified to pH 2 with 2 M hydrochloric acid, the precipitate appeared was collected and washed with water. The product was recrystallized from methanol–water. IV was yielded; 17.3 g (95.5%); mp 186.7—187 °C, $[\alpha]_D^{23}$ −22.0° (*c* 0.5, pyridine).

Found: C, 60.59; H, 6.55; N, 8.07; S, 6.50%. Calcd for $C_{20}H_{33}N_3O_6S$: C, 60.57; H, 6.45; N, 8.15; S, 6.22%.

*H–Cys(SBzl)–*D*–Phe–Gly–OH·HCl (V·HCl).* The dry hydrogen chloride was bubbled through a suspended solution of IV (7.73 g, 15 mmol) in ethyl acetate (90 ml) for 30 min at 0 °C; the solution was then allowed to stand in an ice bath for an additional 30 min. After the solvent had been removed under reduced pressure, anhydrous ether was added to the residual oil. The crystals (V·HCl) produced were obtained in the theoretical yield; mp 183—184 °C. $[\alpha]_D^{23}$ +16.0° (*c* 0.5, AcOH).

Found: C, 55.82; H, 5.87; N, 9.18; S, 7.05; Cl, 7.74%. Calcd for $C_{21}H_{26}N_3O_4SCl$: C, 55.81; H, 5.80; N, 9.30; S, 7.09; Cl, 7.84%.

Boc–Glu(OBzl)–OSu (VI). Boc–Glu(OBzl)–OH[7] (from dicyclohexylamine salt, 25.94 g, 50 mmol) was treated with HOSu (6.33 g, 55 mmol) and DCC (11.4 g, 55 mmol) in methylene chloride (100 ml) at −5 °C while being stirred for 5 hr. After the reaction mixture had then been allowed to stand overnight in a refrigerator and acetic acid (1 ml) had been added, the dicyclohexyl urea thus produced was filtered off and the filtrate was washed with 0.5 M sodium bicarbonate, 0.5 M hydrochloric acid, and water successively, dried over anhydrous sodium sulfate, and concentrated under reduced pressure. The residual oil was crystallized from 2-propanol; 17.97 g (83.6%); mp 101 °C. $[\alpha]_D^{23}$ −23.4° (*c* 2.0, dioxane). ν_{max}^{KBr} 1815, 1785, 1740 cm^{-1} (ester).

Found: C, 58.35; H, 6.18; N, 6.58%. Calcd for $C_{21}H_{26}N_2O_7$: C, 58.06; H, 6.08; N, 6.48%.

*Boc–Glu(OBzl)–Cys(SBzl)–*D*–Phe–Gly–OH (VII).* The solid VI (13.1 g, 30 mmol) was added to a solution of V(from hydrochloride, 14.94 g, 33 mmol) and triethylamine (9.24 ml, 66 mmol) in 65% dioxane (100 ml) at room temperature. After stirring for 48 hr, the reaction mixture was acidified to pH 2 with M hydrochloric acid. The precipitate thus formed was extracted with ethyl acetate; the ethyl acetate solution was dried over anhydrous sodium sulfate and was concentrated under reduced pressure. The residual product was crystallized from methanol–water. VII was

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[7] E. Schnabel, *Ann. Chem.*, **702**, 188 (1968); E. Schnabel, H. Herzog, P. Hoffmann, E. Klauke, and I. Ugi, *ibid.*, **716**, 175 (1968).

thus yielded; 19.3 g (88%); mp 154—154.5 °C, $[\alpha]_D^{23}$ −1.83° (*c* 0.5, pyridine).

Found: C, 62.05; H, 6.28; N, 7.39; S, 4.57%. Calcd for $C_{38}H_{46}N_4O_9S$: C, 62.11; H, 6.31; N, 7.62; S, 4.36%.

H-Glu(OBzl)-Cys(SBzl)-D-Phe-Gly-OH·HCl (VIII·HCl). The dry hydrogen chloride was bubbled through a suspended solution of VII (11.01 g, 15 mmol) in dioxane (140 ml) at 0 °C for 30 min and was then allowed to stand in an ice bath for an additional 30 min. After the solvent had been removed under reduced pressure, anhydrous ether was added to the residue. White crystals were obtained in the theoretical yield; mp 178—178.5 °C. $[\alpha]_D^{23}$ +1.0° (*c* 0.5, AcOH).

Found: C, 59.25; H, 5.73; N, 8.17; S, 4.63; Cl, 5.20%. Calcd for $C_{33}H_{39}N_4O_7SCl$: C, 59.05; H, 5.86; N, 8.35; S, 4.78; Cl, 5.28%.

*Boc-His(*im*-Dnp)-OSu (IX).* Boc-His(*im*-Dnp)-OH[8] (1.58 g, 3.7 mmol) was treated with HOSu (472 mg, 4.1 mmol) and DCC (846 mg, 4.1 mmol) in dimethoxyethane (20 ml) and methylene chloride (10 ml) at 0 °C while being stirred for 10 hr. From the reaction mixture, IX was obtained by the method used in the case of VI. The product was recrystallized from ethyl acetate–ether–*n*-hexane. IX was obtained in an 83% (1.59 g) yield; mp 146—146.5 °C. $[\alpha]_D^{23}$ −3.37° (*c* 0.9, DMF). ν_{max}^{KBr} 1819, 1780, 1745 cm^{-1} (ester).

Found: C, 47.27; H, 4.25; N, 15.32%. Calcd for $C_{21}H_{22}N_6O_{10}\cdot H_2O$: C, 47.02; H, 4.51; N, 15.67%.

*Boc-His(*im*-Dnp)-Glu(OBzl)-Cys(SBzl)-D-Phe-Gly-OH (X).* Into a solution of VIII·HCl (6.4 g, 9 mmol) in DMF (20 ml) and water (2 ml), we stirred a solution of triethylamine (2.5 ml, 18 mmol) in DMF (5 ml) over a 20 min period at 0 °C; solid IX (7.33 g, 13.5 mmol) was then added to the above solution. After the reaction mixture had been stirred for 48 hr at room temperature, the reaction mixture was acidified to pH 2 with M hydrochloric acid, and water was added. The yellow precipitate was recrystallized from methanol–methylene chloride–ether. The yellow crystals were thus obtained in an 87.6% (8.18 g) yield; mp 153—154 °C. $[\alpha]_D^{23}$ −12.5° (*c* 0.3, DMF).

Found: C, 55.93; H, 5.18; N, 11.53; S, 3.10%. Calcd for $C_{50}H_{59}N_9O_{16}S$: C, 55.91; H, 5.54; N, 11.74; S, 2.98%.

Boc-Glu(OBzl)-Cys(SBzl)-D-Phe-Gly-ONp (XI). A solution of VII (7.35 g, 10 mmol) and *p*-nitrophenol (2.78 g, 20 mmol) in methylene chloride (10 ml) and acetonitrile (10 ml) was treated with DCC (2.27 g, 11 mmol) at 0 °C for 4 hr with stirring. After the dicyclohexyl urea thus produced had been filtered off, the reaction mixture was washed with M sodium bicarbonate, 0.1 M hydrochloric acid, and water successively, and then dried over anhydrous sodium sulfate. The solvent was evaporated under reduced pressure. The residual crystals were recrystallized from methylene chloride–ether–*n*-hexane (6.74 g, 78.7%); mp 127—128 °C, $[\alpha]_D^{24}$ −5.8° (*c* 0.3, DMF), R_f[9] 0.92 (aqueous AcOEt). ν_{max}^{KBr} 1780 cm^{-1} (ester).

Found: C, 61.53; H, 5.83; N, 7.88; S, 4.01%. Calcd for $C_{44}H_{49}N_5O_{11}S$: C, 61.74; H, 5.77; N, 8.18; S, 3.75%.

*H-His(*im*-Dnp)-Glu(OBzl)-Cys(SBzl)-D-Phe-Gly-OH·HCl (XII·HCl).* The dry hydrogen chloride gas was bubbled through a solution of X (1.15 g, 1.1 mmol) in ethylacetate (30 ml) containing anisole (1.5 ml) for 10 min at 0 °C; the reaction mixture was then allowed to stand at room temperature for 30 min. After the solvent had been evaporated under reduced pressure, anhydrous ether was added; the crystals thus formed were collected in the theoretical yield. The products were used immediately for the next reaction.

*Boc-Glu(OBzl)-Cys(SBzl)-D-Phe-Gly-His(*im*-Dnp)-Glu(OBzl)-Cys(SBzl)-D-Phe-Gly-OH (XIII).* The solid XI (486 mg, 0.568 mmol) was added to a solution of XII·HCl (609 mg, 0.625 mmol) and triethylamine (0.26 ml, 1.875 mmol) in DMF (5 ml). After the reaction mixture had been stirred for 3 days at room temperature, it was acidified to pH 2 by the use of M hydrochloric acid in an ice bath and the precipitate thus formed was collected by filtration. The recrystallization from DMF–water gave a pure XIII; 881 mg, (92.7% yield); mp 187 °C (decomp.), $[\alpha]_D^{22}$ −4.2° (*c* 0.4, DMF). R_f[9] 0.80 (PW), 0.80 (BAW).

Found: C, 59.58; H, 5.44; N, 10.86; S, 4.03%. Calcd for $C_{87}H_{93}N_{13}O_{21}S_2$: C, 59.59; H, 5.60; N, 10.86; S, 3.83%.

*H-Glu(OBzl)-Cys(SBzl)-D-Phe-Gly-His(*im*-Dnp)-Glu(OBzl)-Cys(SBzl)-D-Phe-Gly-OH (XIV).* The dry hydrogen chloride gas was bubbled through a solution of XIII (1 g, 0.6 mmol) in dioxane (20 ml) and methylene chloride (20 ml) containing anisole (1.5 ml) for 30 min in an ice bath. After the reaction mixture had then been allowed to stand for 1.5 hr, the solution was concentrated under reduced pressure. The addition of anhydrous ether gave yellow crystals of XIV·HCl in the theoretical yield. Free XIV was obtained by the isoelectric point precipitation method; mp 175—177 °C. $[\alpha]_D^{23}$ −21.0° (*c* 1.1, DMF).

Found: C, 58.00; H, 5.43; N, 11.68; S, 3.63%. Calcd for $C_{78}H_{89}N_{13}O_{21}S_2$: C, 58.23; H, 5.58; N, 11.32; S, 3.99%.

*Boc-(His(*im*-Dnp)-Glu(OBzl)-Cys(SBzl)-D-Phe-Gly)$_2$-OH (XV).* Into a solution of XIV·HCl (2.168 g, 1.347 mmol) in DMF (10 ml) and water (1 ml), we stirred triethylamine (0.376 ml, 2.7 mmol) over a 20 min period in an ice bath. Solid IX (1.4 g, 2.7 mmol) was then added to the above solution. After the reaction mixture had been stirred at room temperature for an additional 3 days, the reaction mixture was acidified to pH 2 by the addition of M hydrochloric acid and the precipitate thus fromed was collected by filtration. The recrystallization form DMF–water gave pure yellow crystals in a 90% yield (2.37 g); mp 200 °C (decomp.). $[\alpha]_D^{24}$ −10.7° (*c* 0.3, DMF). R_f[9] 0.84 (PW), 0.86 (BAW).

Found: C, 56.63; H, 4.98; N, 12.13; S, 3.53%. Calcd for $C_{95}H_{105}N_{18}O_{28}S_2$: C, 56.71; H, 5.31; N, 12.53; S, 3.19%.

*Boc-(His(*im*-Dnp)-Glu(OBzl)-Cys(SBzl)-D-Phe-Gly)$_2$-NH-NH-Boc (XVI).* Into a solution of XV (1.96 g, 1 mmol), Boc–NHNH$_2$ (146 mg, 1.1 mmol), HOSu (230 mg, 2 mmol) in DMF (10 ml), we stirred, drop by drop, a solution of DCC (247 mg, 1.2 mmol) in DMF (3 ml) at 0 °C; the reaction mixture was then allowed to stand for 72 hr in a refrigerator. After the addition of acetic acid (0.5 ml), the dicyclohexyl urea thus produced was filtered off and was washed with cold DMF (3 ml). The filtrate was concentrated under reduced pressure, and the residual oily product was scratched by anhydrous ether. The powder-like product was recrystallized from DMF–water in a 97% yield (2.0 g); mp 192—192.3 °C (decomp.). $[\alpha]_D^{23}$ −6.53° (*c* 0.45, DMF). R_f[9] 0.73 (aqueous AcOEt).

Found: C, 58.05; H, 5.80; N, 13.32; S, 3.37%. Calcd for $C_{100}H_{110}N_{20}O_{26}S_2$: C, 57.96; H, 5.35; N, 13.52; S, 3.09%.

*Cyclo(His(*im*-Dnp)-Glu(OBzl)-Cys(SBzl)-D-Phe-Gly)$_2$ (XVIII).* *a)* Via *the Azide Method*: The dry hydrogen chloride gas was bubbled through a suspended solution of XVI (1.036 g, 0.5 mmol) in anhydrous dioxane (20 ml) con-

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[8] F. Chillemi and R. B. Merrifield, *Biochemistry*, **8**, 4344 (1969).

[9] The thin layer chromatography solvent system. aqueous AcOEt: water saturated with ethyl acetate, PW: pyridine–water (4:1 v/v), BAW: *n*-butanol–acetic acid–water (4:1:1 v/v), BAPW: *n*-butanol-acetic acid–pyridine–water (4: 1: 2: 1 v/v).

taining anisole (1 ml) for 30 min at 0 °C. After the reaction mixture had been allowed to stand for 45 min at room temperature, the solvent was removed under reduced pressure; the addition of anhydrous ether to the residual oil gave a pure yellow product of decapeptide hydrazide di-hydrochloride (XVII·2HCl) in the theoretical yield.

Found: C, 52.27; H, 5.20; N, 13.86; S, 3.39%. Calcd for $C_{90}H_{110}N_{20}O_{29}S_2Cl_2$: C, 52.19; H, 5.35; N, 13.52; S, 3.10%.

The hydrazide was used immediately in the following cyclization procedure. Into a solution of XVII·2HCl (850 mg, 0.415 mmol) in DMF (10 ml) and acetic acid (10 ml), we stirred M sodium nitrite (0.45 ml, 0.45 mmol) and M hydrochloric acid (0.415 ml, 0.415 mmol) cooling at −10—−15 °C over a 60 min period. The reaction mixture was added, drop by drop, into cold pyridine (320 ml) at 0 °C over a 5 min. After the reaction mixture was stirred for an additional 24 hr at 0 °C. After the solvent had been evaporated under reduced pressure and water had been added to the residual oil, the product was collected by filtration and was dissolved in a mixed solvent of methanol–methylene chloride–DMF–water (17: 32: 5: 6 v/v). The solution was then treated with IRC-50 (1.5×25 cm) and IR-45 (1.5×25 cm) columns. The effluent (200 ml) was concentrated under reduced pressure, and the products were purified with Sephadex LH-20 by a mixed solvent of DMF–methylene chloride (1: 1 v/v). XVIII was obtained as crystals from DMF–water in an 87.6% yield (610 mg); mp 165—166.5 °C. $[\alpha]_D^{23}$ −21.4° (*c* 0.35, DMF). R_f9) 0.94 (PW), 0.88 (BAW).

Found: C, 55.34; H, 5.12; N, 12.46; S, 3.61%. Calcd for $C_{90}H_{90}N_{18}O_{22}S_2 \cdot 6H_2O$: C, 55.49; H, 5.28; N, 12.94; S, 3.29%. Mol wt Found:10) 1880. Calcd for $C_{90}H_{90}N_{18}O_{22}S_2$: 1840.

b) Via the ONp Method: Di-*p*-nitrophenyl sulfite (4 g, 12.9 mmol) was added to a solution of XV (587 mg, 0.33 mmol) in pyridine (4 ml). After the mixture had been allowed to stand for 4 days at room temperature, the solvent was evaporated under reduced pressure. The residual product (XIX) was collected by using anhydrous ether (432mg); ν_{max}^{KBr} 1775 cm^{-1} (ester). XIX was treated with formic acid (5 ml) containing anisole (1 ml) at 20 °C. After the reaction mixture had been allowed to stand for 4 hr, the solution was concentrated under reduced pressure, and the *p*-nitrophenyl ester formate was immediately dissolved in DMF (10 ml) containing a small amount of acetic acid. The DMF solution was stirred, drop by drop, into prydine (148 ml) at 60 °C over a 4 hr period; the stirring was then continued for an additional 2 hr. Cyclic decapeptide (XVIII) was obtained in a 52% yield (334 mg) by the purification procedure described in a).

c) Via the OSu Method: The solution of XV (1.96 g, 1 mmol) and HOSu (253 mg, 2.2 mmol) in DMF (10 ml) was treated with DCC (453 mg, 2.2 mmol) at 0 °C for 4 hr. After the dicyclohexyl urea had been filtered off, the reaction mixture was concentrated under reduced pressure and the product (XX) was collected by using anhydrous ether; ν_{max}^{KBr} 1820, 1785, and 1742 cm^{-1} (C=O and ester). The active ester was dissolved in TFA (10 ml) containing anisole (1 ml). After the mixture had been allowed to stand at 0 °C for 2 hr, the solvent was removed under reduced pressure. The addition of anhydrous ether to the residual product gave decapeptide succinimide ester trifluoroacetate. The active ester was used immediately in the following cyclization procedure. A solution of the active ester dissolved in DMF (10 ml) containing 6 drops of acetic acid was added drop by drop into anhydrous pyridine (755 ml) at room temperature over a period 12 hr with stirring. The reaction mixture was also treated by the same purification procedure as has been described in a), but XVIII was not isolated; it was only detected by thin-layer chromatography.

*Cyclo(His-Glu-Cys-*D*-Phe-Gly)$_2$·2AcOH (I·2AcOH). i) Removal of the Benzyl Ester and the* S*-Benzyl Group*: XVIII (1.27 g, 0.7 mmol) was treated with anhydrous HF (10 ml) containing anisole (1.5 ml) at room temperature for 90 min. After the reaction mixture had been concentrated under reduced pressure, the residual oil was dissolved in aqueous acetic acid and the residual anisole was extracted with ether from the aqueous acetic acid layer. Also, the acetic acid layer was treated with an IR-45 (1.5×30 cm, acetate form) column. The effluent (100 ml) was concentrated under reduced pressure; the addition of peroxide-free anhydrous ether to the residual oil gave a white powder (XXI).

ii) Removal of the Dinitrophenyl Group: XXI was dissolved in DMF (10 ml), and the solution was adjusted to pH 8 with 10% sodium carbonate. After 2-mercaptoethanol (60 ml)4) had been added to the above solution, the reaction mixture was allowed to stand at room temperature for 3 hr. The reaction mixture was then neutralized by acetic acid and was concentrated under reduced pressure. The residual product was immediately treated by the use of a Sephadex G-15 (5×50 cm) column by using 50% aqueous acetic acid. When the effluent was concentrated under reduced pressure and the residue was treated with peroxide-free ether, the free cyclic decapeptide (I) was obtained in a 64.9% yield (606 mg). $[\alpha]_D^{23}$ +3.5° (*c* 0.3, DMF). The purity of I was checked by thin-layer chromatography on Kieselgel G and by paper electrophoresis. The thin-layer chromatography of I showed a single spot; R_f9) 0.56 (BAW): Pauly reaction, nitroprusside reaction positive; ninhydrin negative. On the paper electrophoresis, I moved as a single band toward the cathode; R_{His} 0.24.11) No disulfide bond at all was detected in I by either the Ellman5) or iodoacetamide method.6)

Found: C, 45.64; H, 5.94; N, 13.71; S, 4.73%. Calcd for $C_{50}H_{62}N_{14}O_{14}S_2 \cdot 2AcOH \cdot 8H_2O$: C, 45.95; H, 6.14; N, 13.89; S, 4.54%.

*H-(His-Glu-Cys-*D*-Phe-Gly)$_2$-OH·3AcOH (XXIII·3AcOH).* XV (964 mg, 0.5 mmol) was treated with anhydrous HF (15 ml) containing anisole (1.5 ml) at room temperature for 1 hr. After the reaction mixture had been treated in the same way as in the case of the cyclic decapeptide (I), the debenzylated product (XXII) was obtained in a 78% yield (609 mg). XXII (151 mg, 0.1 mmol) was treated with 2-mercaptoethanol (1.56 g, 20 mmol) in DMF (5 ml) at pH 8. XXIII was purified by the same method used for the cyclic decapeptide (I). XXIII·3AcOH was thus obtained in a 44.7% yield (65 mg). $[\alpha]_D^{23}$ +1.7° (*c* 0.3, DMF). R_f9) 0.08 (BAW). R_{His} 0.58.11) The amount of the free thiol group was measured by the Ellman method.5)

Found: C, 46.03; H, 5.74; N, 13.56; S, 4.70%. Calcd for $C_{50}H_{64}N_{14}O_{15}S_2 \cdot 3AcOH \cdot 6H_2O$: C, 46.27; H, 6.10; N, 13.49; S, 4.43%.

Kinetic Method of Catalytic Activity. A solution of a 1/15 M phosphate buffer containing dioxane and DMF was used for all the kinetic measurements, and all the experiments were carried out at 24±1 °C. DMF was used to increase the solubility of the peptide (I or XXIII) in the buffer solution.

The linear (XXIII) and the cyclic decapeptide (I) were

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10) Molecular weight was measured by a Knauer Thermoelectric Cooling Unit for cryoscopic measurements using formic acid as a solvent.

11) Solvent system: pH 1.8 (HCOOH–AcOH–MeOH–water, 1: 3: 6: 10 v/v), 1000 v/20 cm, 1 hr, Toyo Roshi No. 50.

weighed and dissolved in DMF (1 ml) and the buffer (29 ml). The buffer solution was pretreated with nitrogen gas to remove the oxygen in the solution. A solution (0.1 ml) of *p*-nitrophenyl acetate in peroxide-free dioxane was added to the peptide solution (3 ml). The reaction mixture was rapidly stirred and the liberated *p*-nitrophenol was detected by the use of a Hitachi Photo-Electric Spectrophotometer (at 400 nm[3]).