Chemical Synthesis of Antigenic Determinants in Hen Egg-White Lysozyme. I. Heneicosapeptide Corresponding to Positions 34—54 in the Primary Structure

Takayuki MITAKI and Yasutsugu SHIMONISHI

Institute for Protein Research, Osaka University, Yamada-kami, Suita, Osaka 565

(Received October 18, 1976)

For elucidating one of the antigenic determinants of hen egg-white lysozyme close to its catalytic site, heneicosapeptide was synthesized by a conventional method. This peptide, phenylalanylglutamylserylasparaginylphenylalanylasparaginylthreonylglutaminylalanylthreonylasparaginylarginylasparaginylthreonylaspartylglycylserylthreonylaspartyltyrosylglycine, corresponds to positions 34—54 of the amino acid sequence of hen egg-white lysozyme.

Two methods have been used in studies on the antigenic structures of hen egg-white lysozyme (HEL). One is screening of proteolytic digests of HEL for immunologically active peptides;[1-7] characterization of these peptides then provides various informations about the structures of the antigenic determinants. This method has the disadvantages that fractions of digests may be contaminated with small quantities of immunologically active materials and that it cannot be used to study the minimal structures of antigenic determinants for activity and the functions of the individual amino acid residue. The second method is chemical synthesis of antigenic structures of HEL.[8-10] This method does not have the disadvantages of the other method, because it can give pure substance with natural structure and also various artificial analogs, whereas it cannot be used alone for determining the antigenic determinants.

Sakato *et al.*[4] found that certain peptides isolated from thermolytic digests of HEL, *i.e.*, P_{Ib} [sequence[11,12] Val^{29} to Gly^{54} and Val^{109} to Trp^{123} linked by a single disulfide (Cys^{30} and Cys^{115}) bond], P_{Ba-1} (sequence Phe^{34} to Gly^{54}) and P_{Ba-2} (sequence Phe^{38} to Gln^{57}), are immunologically active, and described that the antigenic determinant of HEL included in these peptides is in the sequence Phe^{38} to $Gly.^{54}$ It seemed interesting to characterize this determinant, because these immunoreactive peptides are close to a catalytic site of HEL and because antibody populations directed to them also neutralize the enzymatic activity of HEL.[4] The second method described above seemed most suitable for examining this antigenic determinant of HEL, because various peptides related to this determinant could not be isolated from enzymatic digests of HEL. Studies on this have been carried out in collaboration[13] with Prof. T. Amano and his group of the Research Institute for Microbial Diseases, Osaka University. This paper reports the synthesis of a heneicosapeptide, phenylalanylglutamylserylasparaginylphenylalanylasparaginylthreonylglutaminylalanylthreonylasparaginylarginylasparaginylthreonylaspartylglycylserylthreonylaspartyltyrosylglycine corresponding to P_{Ba-1}, which includes two residues of the catalytic sites of HEL (Glu^{35} and Asp^{52}).[14]

This peptide was synthesized from appropriately protected smaller fragments by the azide coupling reaction. In planning the synthesis, easy ways of purifying peptide intermediates and the ultimate removal of protecting groups with liquid hydrogen fluoride[15] were considered. For the synthesis, heneicosapeptide (sequence Phe^{34} to Gly^{54}) was divided into four subfragments (P_1—P_4), as shown in Figs. 1—4. For synthesis of subfragment P_1, N^{α}-benzyloxycarbonyl-γ-*t*-butylglutamic acid *N*-hydroxysuccinimide ester[16] was condensed with serine methyl ester. Then N^{α}-benzyloxycarbonyl-γ-*t*-butylglutamylserine methyl ester (I) was hydrogenated catalytically and the resulting peptide ester was not isolated but coupled directly with benzyloxycarbonylphenylalanine[17] by dicyclohexylcarbodiimide to give benzyloxycarbonylphenylalanyl-γ-*t*-butylglutamylserine methyl ester (II). The protected tripeptide methyl ester was converted to the corresponding hydrazide (III). Benzyloxycarbonylphenylalanyl-γ-*t*-butylglutamylserine azide obtained from compound III

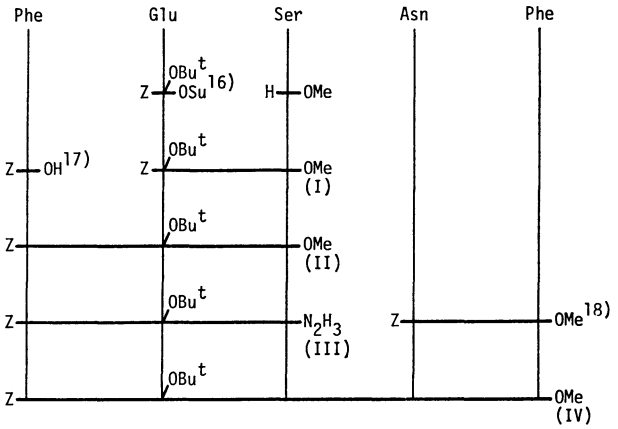

Fig. 1. Scheme for synthesis of subfragment P_1 (sequence 34—38)[28]

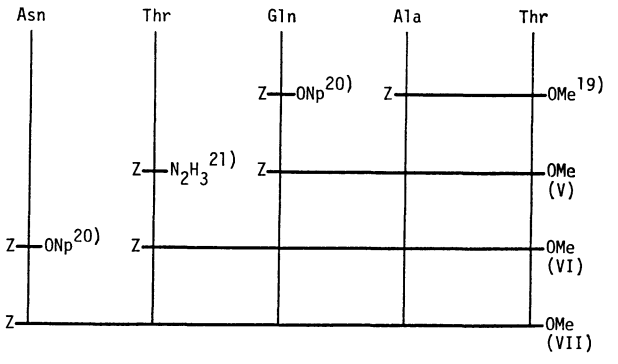

Fig. 2. Scheme for synthesis of subfragment P_2 (sequence 39—43).

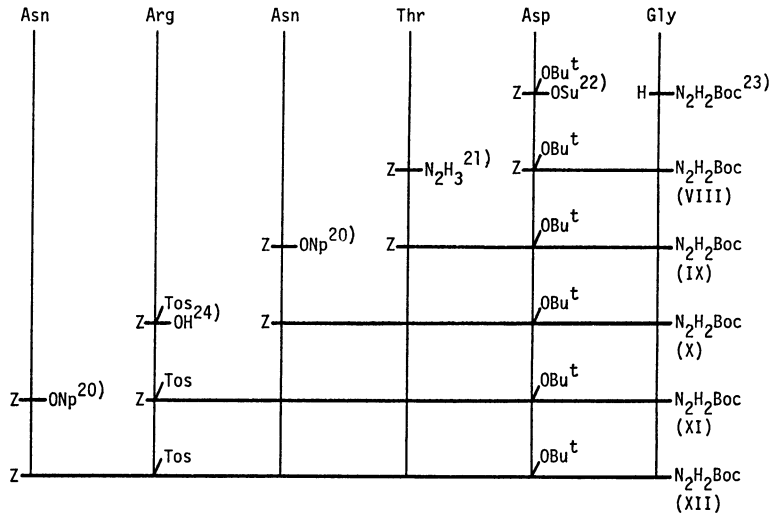

Fig. 3. Scheme for synthesis of subfragment P_3 (sequence 44—49).

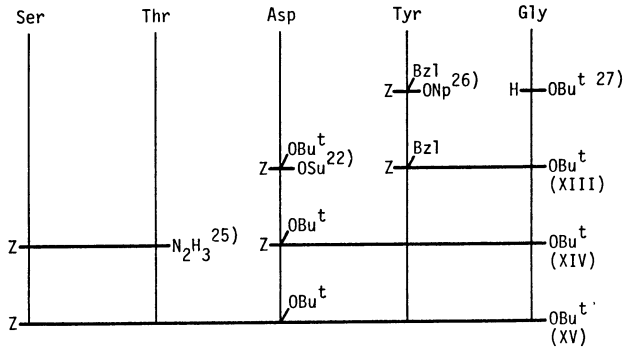

Fig. 4. Scheme for synthesis of subfragment P_4 (sequence 50—54).

was not isolated but allowed to react directly with asparaginylphenylalanine methyl ester, which was obtained by removal of the benzyloxycarbonyl group from the protected peptide[18] by catalytic hydrogenation. In this way benzyloxycarbonylphenylalanyl-γ-*t*-butylglutamylserylasparaginylphenylalanine methyl ester (IV) was obtained without difficulty.

Subfragment P_2 was synthesized using benzyloxycarbonylalanylthreonine methyl ester[19] as starting material by a stepwise elongation procedure. The benzyloxycarbonyl group was removed by catalytic hydrogenation and the resulting peptide ester was not isolated but coupled directly with benzyloxycarbonylglutamine *p*-nitrophenyl ester[20] to yield benzyloxycarbonylglutaminylalanylthreonine methyl ester (V). The protecting group was cleaved by catalytic hydrogenation and the tripeptide methyl ester was condensed with benzyloxycarbonylthreonine azide prepared from its corresponding hydrazide[21] to give benzyloxycarbonylthreonylglutaminylalanylthreonine methyl ester (VI). The benzyloxycarbonyl group was removed by catalytic hydrogenation and the tetrapeptide methyl ester thus obtained was coupled with benzyloxycarbonylasparagine *p*-nitrophenyl ester[20] to give benzyloxycarbonylasparaginylthreonylglutaminylalanylthreonine methyl ester (VII).

Subfragment P_3 was constructed using benzyloxycarbonyl-β-*t*-butylaspartylglycine *t*-butoxycarbonylhydrazide (VIII), which was prepared by coupling benzyloxycarbonyl-β-*t*-butylaspartic acid *N*-hydroxysuccinimide ester[22] with glycine *t*-butoxycarbonylhydrazide.[23] The benzyloxycarbonyl group of compound VIII was removed by catalytic hydrogenation and the resulting peptide *t*-butoxycarbonylhydrazide was coupled with benzyloxycarbonylthreonine azide to yield benzyloxycarbonylthreonyl-β-*t*-butylaspartylglycine *t*-butoxycarbonylhydrazide (IX). The protecting group for amino terminus was removed by catalytic hydrogenation and the chain elongated by further three single steps using benzyloxycarbonylasparagine *p*-nitrophenyl ester,[20] N^{α}-benzyloxycarbonyl-N^{ω}-tosylarginine[24] and dicyclohexylcarbodiimide in the presence of *N*-hydroxysuccinimide, and benzyloxycarbonylasparagine *p*-nitrophenyl ester[20] for acylation. Thus, benzyloxycarbonylasparaginyl-N^{ω}-tosylarginylasparaginylthreonyl-β-*t*-butylaspartylglycine *t*-butoxycarbonylhydrazide (XII) was obtained.

Subfragment P_4 (XV) was synthesized by coupling benzyloxycarbonylserylthreonine azide with β-*t*-butylaspartyltyrosylglycine *t*-butyl ester, which was obtained by removal of the benzyloxycarbonyl group from the protected tripeptide ester (XIV) by catalytic hydrogenation, without isolation. The tripeptide derivative (XIV) was prepared by condensation of benzyloxycarbonyl-β-*t*-butylaspartic acid *N*-hydroxysuccinimide ester[22] with tyrosylglycine *t*-butyl ester, which was prepared by removal of the protecting groups from benzyloxycarbonyl-*O*-benzyltyrosylglycine *t*-butyl ester (XIII) by catalytic hydrogenation. The dipeptide derivative (XIII) was obtained by condensation of benzyloxycarbonyl-*O*-benzyltyrosine *p*-nitrophenyl ester[26] with glycine *t*-butyl ester.[27]

The couplings of subfragment P_1 with P_2 and of subfragment P_3 with P_4 were carried out separately, as shown in Fig. 5. The hydrazide (XVI) of subfragment P_1 was converted to the corresponding azide and coupled with pentapeptide methyl ester, which was prepared by removal of the benzyloxycarbonyl group of subfragment P_2 by catalytic hydrogenation. The decapeptide

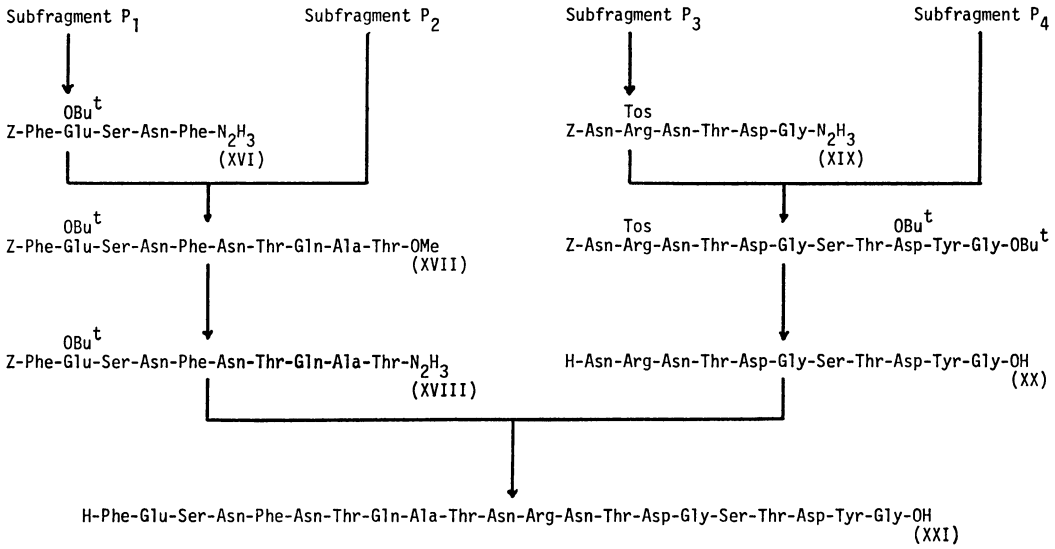

Fig. 5. Scheme for synthesis of the heneicosapeptide (sequence 34—54).

derivative (XVII) obtained was converted to its corresponding hydrazide (XVIII). On the other hand, subfragment P_3 was treated with trifluoracetic acid and the corresponding hydrazide (XIX) obtained was coupled with serylthreonyl-β-*t*-butylaspartyltyrosylglycine *t*-butyl ester prepared from subfragment P_4 by Rudinger's azide method[29] to give a protected undecapeptide. Without further purification the peptide was treated with liquid hydrogen fluoride in an HF-apparatus[15] to remove its protecting groups. The free undecapeptide (XX), asparaginylarginylasparaginylthreonylaspartylglycylserylthreonylaspartyltyrosylglycine, was purified on a column of diethylaminoethyl-cellulose. The azide, prepared from the hydrazide (XVIII) by Rudinger's method,[29] was further coupled *in situ* with free undecapeptide (XX) to give protected heneicosapeptide. The crude heneicosapeptide was not purified due to its low solubility but was directly treated with hydrogen fluoride[15] to remove its protecting groups. The resulting free peptide was fractionated on a column of Sephadex G-25, as shown in Fig. 6, and the main fraction collected was purified further on a column of carboxymethyl-cellulose, as shown in Fig. 7.

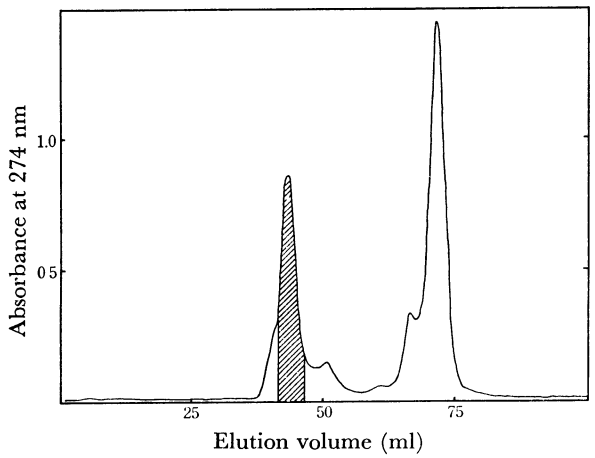

Fig. 6. Gel-filtration of crude heneicosapeptide (XXI) on Sephadex G-25 in 50% acetic acid.

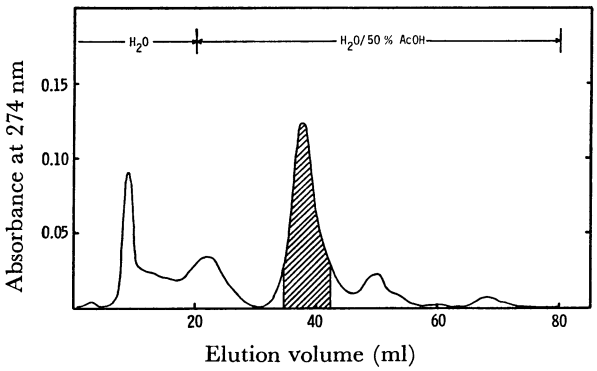

Fig. 7. Chromatogram of the main fraction shown in Fig. 6 on carboxymethyl-cellulose (H^+ cycle) using a linear gradient from water to 50% acetic acid.

The heneicosapeptide thus synthesized showed the same order of inhibitory activity in a precipitin reaction between ^{125}I-HEL and the purified *anti*-P_{Ib} antibody, as that reported elsewhere.[13] This confirms that one of the antigenic determinants of HEL is in the region from Phe^{34} to Gly^{54} of HEL. Chemical synthesis of the minimal structure necessary for inhibitory activity is now in progress.

Experimental

All chemicals were of reagent grade and they were used without further purification. The amino acids except glycine were of the L-configuration. Sephadex G-25 fine was purchased from Pharmacia Co. (Uppsala). Thin layer chromatography was performed on silica gel G (Merck) using the following solvent systems (volume ratios); chloroform: methanol: acetic acid (95: 5: 3), ethyl acetate: benzene (1: 1), 1-butanol: acetic acid: water (4: 1: 1), 1-butanol: acetic acid: pyridine: water (15: 3: 10: 12), phenol: water (3: 1) and chloroform: methanol: acetic acid: water (10: 10: 1: 10, lower phase). Paper electrophoresis was performed at pH 4.8 in 0.2 M pyridinium acetate buffer. Peptide derivatives were hydrolyzed in 6 M hydrochloric acid with phenol in sealed tubes for 24 h at 105 °C, and amino acids in the hydrolysates were examined in a Hitachi KLA-5

analyzer by the method of Moore *et al.*[30] Optical rotations were determined with a Perkin-Elmer Model 241 polarimeter. The purities of the peptide derivatives synthesized were confirmed by thin layer chromatography and paper electrophoresis and by the ratio of their constituent amino acids measured in acid hydrolysates by amino acid analysis. Melting points were measured by the capillary method and are given as uncorrected values.

N$^{\alpha}$-Benzyloxycarbonyl-γ-t-butylglutamylserine Methyl Ester (I). A solution of serine methyl ester hydrochloride (6.80 g, 44.0 mmol) in *N,N*-dimethylformamide (50 ml) was cooled to 0—5 °C. The chilled solution was mixed with triethylamine (6.2 ml) and then a solution of N$^{\alpha}$-benzyloxycarbonyl-γ-t-butylglutamic acid *N*-hydroxysuccinimide ester[16] (17.4 g, 40.1 mmol) in *N,N*-dimethylformamide (40 ml) and stirred for 2 h at room temperature. Then the mixture was concentrated *in vacuo*, and diluted with ethyl acetate. The diluted solution was washed successively with 0.1 M hydrochloric acid, 5% aqueous sodium hydrogencarbonate, and water, and dried over anhydrous sodium sulfate. The dried solution was concentrated *in vacuo* to a syrup, and the material was crystallized in a mixture of ethyl acetate and hexane; wt 15.7 g (89.2%). The crude product was recrystallized from ethyl acetate and hexane; wt 12.7 g (72.2%); mp 70—72 °C; $[\alpha]_D^{19}$ −5.3° (*c* 0.9, ethanol).

Found: C, 57.64; H, 6.91; N, 6.40%. Calcd for $C_{21}H_{30}O_8N_2$: C, 57.52; H, 6.90; N, 6.39%.

Benzyloxycarbonylphenylalanyl-γ-t-butylglutamylserine Methyl Ester (II). Compound I (12.7 g, 29.0 mmol) was dissolved in methanol (500 ml), and hydrogenated over a 5% palladium–charcoal catalyst for 1.5 h at atmospheric pressure. The catalyst was filtered off and washed with a small volume of methanol. The filtrate and washings were combined and concentrated to a syrupy residue *in vacuo*. The residue was dissolved with benzyloxycarbonylphenylalanine[17] (8.70 g, 29.1 mmol) in *N,N*-dimethylformamide (70 ml), and the solution was cooled to −10 °C. Then a solution of dicyclohexylcarbodiimide (6.00 g, 29.1 mmol) in *N,N*-dimethylformamide (20 ml) was added and the mixture was stirred for one hour at the same temperature and then overnight at room temperature. The precipitate formed was filtered off and washed with *N,N*-dimethylformamide. The filtrate and washings were combined, concentrated to a small volume *in vacuo*, and diluted with ethyl acetate. The diluted solution was washed successively with 0.1 M hydrochloric acid, 5% aqueous sodium hydrogencarbonate, and water, and dried over anhydrous sodium sulfate. The dried solution was concentrated to a syrup under reduced pressure, and crude material was crystallized from ethyl acetate and hexane; wt 12.6 g (74.1%). It was recrystallized from ethyl acetate and hexane; wt 11.7 g (68.8%); mp 87.5—89 °C; $[\alpha]_D^{19}$ −14.9° (*c* 1.1, ethanol).

Found: C, 61.67; H, 6.87; N, 7.30%. Calcd for $C_{33}H_{39}O_9N_3$: C, 61.52; H, 6.71; N, 7.18%.

Benzyloxycarbonylphenylalanyl-γ-t-butylglutamylserine Hydrazide (III). A solution of compound II (11.7 g, 20.0 mmol) in methanol (200 ml) was cooled to 0—5 °C, and then 90% hydrazine hydrate (7 g) was added. The mixture was kept for 24 h at room temperature, and the precipitate formed was filtered off and washed with methanol; wt 10.9 g (91.6%); mp 171—173 °C.

Found: C, 58.85; H, 6.70; N, 11.65%. Calcd for $C_{29}H_{39}O_8N_5 \cdot 0.5H_2O$: C, 58.57; H, 6.78; N, 11.78%.

Benzyloxycarbonylphenylalanyl-γ-t-butylglutamylserylasparaginylphenylalanine Methyl Ester (IV). Benzyloxycarbonylasparaginylphenylalanine methyl ester[18] (6.41 g, 15.0 mmol) was dissolved in methanol (1 liter) and hydrogenated over a 5% palladium–charcoal catalyst for 3.5 h at atmospheric pressure. The catalyst was filtered off and washed with methanol. The filtrate and washings were combined and concentrated to a solid residue *in vacuo*. The residue was dissolved in *N,N*-dimethylformamide (50 ml), and cooled to −10 °C. Compound III (8.78 g, 14.8 mmol) was suspended in *N,N*-dimethylformamide (40 ml), cooled to −20 °C and mixed with 3.90 M HCl in dioxane (11.5 ml). The clear solution was mixed with isopentyl nitrite (2.10 ml) at the same temperature. After 10 min a solution of *N*-methylmorpholine (4.55 g) in *N,N*-dimethylformamide (10 ml) and then a cooled solution of the amino component were added. The mixture was stirred for 40 h in a refrigerator (0 °C). Then a solution of *N*-methylmorpholine (0.98 g) in *N,N*-dimethylformamide (2.1 ml) was added and the mixture was stirred for 48 h at the same temperature. Then, the solution was poured into large volume of water. The precipitate formed was collected by filtration; wt 10.2 g (79.7%). The crude product was reprecipitated from *N,N*-dimethylformamide and ethyl acetate; wt 8.70 g (68.0%); mp 214—215 °C; $[\alpha]_D^{27}$ −3.8° (*c* 1.1, *N,N*-dimethylformamide). Amino acid ratio: Asp, 1.00 (1); Ser, 0.93 (1); Glu, 1.05 (1); Phe, 2.00 (2).

Found: C, 60.51; H, 6.45; N, 9.92%. Calcd for $C_{43}H_{54}O_{12}N_6 \cdot 0.5H_2O$: C, 60.33; H, 6.47; N, 9.82%.

Benzyloxycarbonylglutaminylalanylthreonine Methyl Ester (V). Benzyloxycarbonylalanylthreonine methyl ester[19] (3.38 g, 10.0 mmol) was dissolved in ethanol (120 ml) and hydrogenated over a 5% palladium–charcoal catalyst. The catalyst was filtered off and the filtrate was concentrated to a syrup *in vacuo*. The syrup was dissolved with benzyloxycarbonylglutamine *p*-nitrophenyl ester[20] (3.34 g, 8.33 mmol) in *N,N*-dimethylformamide (25 ml). The solution was stirred for an hour at room temperature, and then concentrated to a syrup *in vacuo*. The syrup was triturated in ethyl acetate. The solid thus formed was collected by filtration, and recrystallized from methanol; wt 2.64 g (68.0%); mp 190.5—191 °C; $[\alpha]_D^{10}$ −7.6° (*c* 0.94, *N,N*-dimethylformamide).

Found: C, 53.80; H, 6.47; N, 12.09%. Calcd for $C_{21}H_{30}O_8N_4$: C, 54.07; H, 6.48; N, 12.01%.

Benzyloxycarbonylthreonylglutaminylalanylthreonine Methyl Ester (VI). Compound V (11.7 g, 25.1 mmol) was suspended in methanol (900 ml) and hydrogenated over a 5% palladium–charcoal catalyst at atmospheric pressure for 1.5 h. The catalyst was filtered off and washed with methanol. The filtrate and washings were combined and evaporated to dryness under reduced pressure. The residue was dissolved in a mixture of *N,N*-dimethylformamide (100 ml) and dimethyl sulfoxide (40 ml), and then cooled in ice–water. Benzyloxycarbonylthreonine hydrazide[21] (6.70 g, 25.1 mmol) was suspended in *N,N*-dimethylformamide (50 ml) and cooled to −25 °C. The chilled suspension became clear on adding 3.55 M HCl in dioxane (21.1 ml). The solution was mixed with isopentyl nitrite (3.50 ml) and stirred for 15 min at the same temperature. Then it was carefully mixed with a solution of triethylamine (10.5 ml) in *N,N*-dimethylformamide (10 ml). The mixture was combined with the above cooled solution of the hydrogenated product of compound V, and stirred for 48 h at 0 °C. The mixture was concentrated to a solid residue *in vacuo*, and collected with water; wt 10.4 g (72.7%). The crude material was reprecipitated from *N,N*-dimethylformamide and water; wt 9.30 g (65.0%); mp 241—243 °C; $[\alpha]_D^{20}$ −7.6° (*c* 1.1, *N,N*-dimethylformamide).

Found: C, 52.82; H, 6.62; N, 12.34%. Calcd for $C_{25}H_{37}O_{10}N_5$: C, 52.90; H, 6.57; N, 12.34%.

Benzyloxycarbonylasparaginylthreonylglutaminylalanylthreonine Methyl Ester (VII). Compound VI (8.00 g, 14.1 mmol) was suspended in methanol (750 ml) and hydrogenated over a

5% palladium–charcoal catalyst at atmospheric pressure for 3 h. The catalyst was filtered off and the filtrate was evaporated to dryness *in vacuo*. The residue was dissolved in a mixture of *N,N*-dimethylformamide (40 ml) and dimethyl sulfoxide (20 ml), and mixed with benzyloxycarbonylasparagine *p*-nitrophenyl ester[20] (5.40 g, 14.0 mmol). The solution was stirred overnight at room temperature, and then poured into a large volume of ethyl acetate. The precipitate formed was collected by filtration; wt 9.20 g (94.0%). The crude material was dissolved in a boiling mixture of *N,N*-dimethylformamide (200 ml) and water (100 ml), and insoluble material was filtered off. The filtrate was mixed with ethyl acetate (250 ml), and allowed to stand overnight in a refrigerator. The precipitate formed was collected by filtration; wt 6.70 g (68.4%); mp 245—246 °C (decomp); $[\alpha]_D^{27}$ −21.0° (*c* 1.0, *N,N*-dimethylformamide). Amino acid ratio: Asp, 1.00 (1); Thr, 2.00 (2); Glu, 1.07 (1); Ala, 1.00 (1).

Found: C, 50.38; H, 6.39; N, 13.96%. Calcd for $C_{29}H_{43}O_{12}N_7 \cdot 0.5H_2O$: C, 50.43; H, 6.42; N, 14.20%.

N^{α}-Benzyloxycarbonyl-β-t-butylaspartylglycine t-Butoxycarbonylhydrazide (VIII). A solution of glycine *t*-butoxycarbonylhydrazide[23] (14.5 g, 76.7 mmol) in *N,N*-dimethylformamide (150 ml) was mixed with benzyloxycarbonyl-β-*t*-butylaspartic acid *N*-hydroxysuccinimide ester[22] (32.2 g, 76.7 mmol). The solution was stirred for 50 min at room temperature and concentrated to a syrup *in vacuo*, and this was dissolved in ethyl acetate. The solution was washed successively with 0.1 M hydrochloric acid, 5% aqueous sodium hydrogencarbonate, and water, and then dried over anhydrous sodium sulfate. The dried solution was concentrated to an oily residue *in vacuo*, and this was treated with hexane to give an amorphous powder; wt 35.8 g (94.2%); mp 60—65 °C.

Benzyloxycarbonylthreonyl-β-t-butylaspartylglycine t-Butoxycarbonylhydrazide (IX). Compound VIII (35.3 g, 71.3 mmol) was dissolved in methanol (1 liter) and hydrogenated over a 5% palladium–charcoal catalyst at atmospheric pressure. The catalyst was filtered off and the filtrate was concentrated to a syrup *in vacuo*. This was dissolved in *N,N*-dimethylformamide (170 ml). On the other hand, benzyloxycarbonylthreonine hydrazide[21] (19.1 g, 71.5 mmol) was suspended in *N,N*-dimethylformamide (200 ml) and cooled to −30 °C. To the cooled suspension were added 3.90 M HCl in dioxane (55.0 ml) and then isopentyl nitrite (10.1 ml). After 15 min a solution of *N*-methylmorpholine (21.7 g) in *N,N*-dimethylformamide (20 ml) and then the solution obtained above were added. The mixture was stirred for 48 h at 0 °C, and then diluted with ethyl acetate. The diluted mixture was washed successively with 0.1 M hydrochloric acid, 5% aqueous sodium hydrogencarbonate, and water, and dried over anhydrous sodium sulfate. The dried solution was concentrated to a syrup *in vacuo*. The syrup was triturated in ethyl acetate and ether; wt 25.7 g (60.5%); mp 107—109 °C; $[\alpha]_D^{10}$ −19.3° (*c* 1.0, ethyl acetate).

Found: C, 54.53; H, 7.03; N, 11.69%. Calcd for $C_{27}H_{41}O_{10}N_5$: C, 54.44; H, 6.94; N, 11.76%.

Benzyloxycarbonylasparaginylthreonyl-β-t-butylaspartylglycine t-Butoxycarbonylhydrazide (X). Compound IX (10.7 g, 18.0 mmol) was dissolved in methanol (700 ml) and hydrogenated over a 5% palladium–charcoal catalyst at atmospheric pressure. The catalyst was filtered off and washed with methanol. The filtrate and washings were combined and concentrated to a syrup under reduced pressure, and the syrup was dissolved with benzyloxycarbonylasparagine *p*-nitrophenyl ester[20] (8.40 g, 21.7 mmol) in *N,N*-dimethylformamide (40 ml). The solution was stirred overnight at room temperature, and then concentrated to a residue under reduced pressure. The residue was triturated in ethyl acetate and ether; wt 11.3 g (85.0%). The powder obtained was reprecipitated from ethanol and water; wt 7.70 g (57.9%); mp 121—125 °C; $[\alpha]_D^{27}$ −22.1° (*c* 1.0, *N,N*-dimethylformamide).

Found: C, 50.33; H, 6.74; N, 13.02%. Calcd for $C_{31}H_{47}O_{12}N_7 \cdot 1.5H_2O$: C, 50.53; H, 6.84; N, 13.31%.

N^{α}-Benzyloxycarbonyl-N^{ω}-tosylarginylasparaginylthreonyl-β-t-butylaspartylglycine t-Butoxycarbonylhydrazide (XI). N^{α}-Benzyloxycarbonyl-N^{ω}-tosylarginine[24] (3.70 g, 7.99 mmol) and *N*-hydroxysuccinimide (0.92 g, 8.0 mmol) were dissolved in tetrahydrofuran (6 ml). The solution was cooled to −10 °C and then mixed with dicyclohexylcarbodiimide (1.70 g, 8.25 mmol). The solution was stirred for one hour at −10 °C and then for one hour at room temperature. The precipitate formed was filtered off and the filtrate was concentrated to a solid residue *in vacuo*. The residue was dissolved in *N,N*-dimethylformamide (10 ml) with the material which was obtained by hydrogenation of compound X (3.60 g, 4.89 mmol) over a 5% palladium–charcoal catalyst at atmospheric pressure. The solution was stirred for 4 h at room temperature and then concentrated to a small volume *in vacuo*. The residue was diluted with ethyl acetate and washed with 0.1 M hydrochloric acid, and water, and then dried over anhydrous sodium sulfate. The dried solution was concentrated to a gelatinous solid *in vacuo*, and this was collected by filtration; wt 4.50 g (89.3%). The crude material was reprecipitated from ethanol and water; wt 3.30 g (65.5%); mp 148—151 °C; $[\alpha]_D^{10}$ −19.2° (*c* 1.1, *N,N*-dimethylformamide). Amino acid ratio: Asp, 1.94 (2); Thr, 0.92 (1); Gly, 1.00 (1); Arg, 1.02 (1).

Found: C, 51.38; H, 6.59; N, 15.00; S, 3.09%. Calcd for $C_{44}H_{65}O_{15}N_{11}S \cdot 0.5H_2O$: C, 51.35; H, 6.46; N, 14.97; S, 3.12%.

Benzyloxycarbonylasparaginyl-N^{ω}-tosylarginylasparaginylthreonyl-β-t-butylaspartylglycine t-Butoxycarbonylhydrazide (XII). Compound XI (3.25 g, 3.16 mmol) was dissolved in methanol (300 ml) and hydrogenated over a 5% palladium–charcoal catalyst at atmospheric pressure. The catalyst was filtered off and the filtrate was concentrated to a syrup under reduced pressure. The syrup was dissolved with benzyloxycarbonylasparagine *p*-nitrophenyl ester[20] (1.41 g, 3.64 mmol) in *N,N*-dimethylformamide (7 ml). The solution was stirred for 24 h at room temperature, and then concentrated to a residue *in vacuo*. The residue was collected with ethanol; wt 1.80 g (50.0%). The crude product was reprecipitated from *N,N*-dimethylformamide and ethanol; wt 1.60 g (44.4%); mp 190—191 °C; $[\alpha]_D^{23}$ −24.4° (*c* 1.0, *N,N*-dimethylformamide). Amino acid ratio: Asp, 3.09 (3); Thr, 1.02 (1); Gly, 1.00 (1); Arg, 0.96 (1).

Found: C, 50.35; H, 6.48; N, 16.03; S, 2.80%. Calcd for $C_{58}H_{71}O_{17}N_{13}S \cdot 0.5H_2O$: C, 50.45; H, 6.34; N, 15.93; S, 2.80%.

Benzyloxycarbonyl-O-benzyltyrosylglycine t-Butyl Ester (XIII). Glycine *t*-butyl ester, prepared by the hydrogenation of benzyloxycarbonylglycine *t*-butyl ester[27] (11.8 g, 44.5 mmol) over a 5% palladium–charcoal catalyst at atmospheric pressure, was dissolved with benzyloxycarbonyl-*O*-benzyltyrosine *p*-nitrophenyl ester[26] (19.0 g, 36.0 mmol) in *N,N*-dimethylformamide (60 ml). The solution was stirred overnight at room temperature, and then concentrated to a syrup *in vacuo*. The syrupy residue was dissolved in ethyl acetate, and washed successively with 0.1 M hydrochloric acid, 5% aqueous sodium hydrogencarbonate, and water, and then dried over anhydrous sodium sulfate. The dried solution was concentrated to a crystalline residue *in vacuo*; wt 15.8 g (84.5%); mp 120—121 °C; $[\alpha]_D^{13}$ −13.4° (*c* 1.0, ethanol).

Found: C, 69.33; H, 6.60; N, 5.35%. Calcd for $C_{30}H_{34}$-

O_6N_2: C, 69.48; H, 6.61; N, 5.40%.

N^{α}-Benzyloxycarbonyl-β-t-butylaspartyltyrosylglycine t-Butyl Ester (XIV). Compound XIII (14.3 g, 27.6 mmol) was dissolved in methanol (500 ml) and hydrogenated over a 5% palladium-charcoal catalyst at atmospheric pressure. The catalyst was filtered off and washed with methanol. The filtrate and washings were combined and concentrated to a syrup *in vacuo*. The syrup was dissolved with benzyloxycarbonyl-β-t-butylaspartic acid *N*-hydroxysuccinimide ester[22] (12.8 g, 30.5 mmol) in *N,N*-dimethylformamide (50 ml). The solution was stirred for 24 h at room temperature, and then diluted with ethyl acetate. The diluted solution was washed successively with 0.1 M hydrochloric acid, 5% aqueous sodium hydrogencarbonate, and water, and then dried over anhydrous sodium sulfate. The dried solution was concentrated to a syrup *in vacuo*. The syrup was washed with a mixture of ethyl acetate and hexane, and triturated in hexane. The crude material obtained was reprecipitated from ether and hexane; wt 11.2 g (67.5%); mp 97—99 °C; $[\alpha]_D^{14}$ −35.4° (*c* 1.0, ethanol).

Found: C, 62.19; H, 7.02; N, 6.86%. Calcd for $C_{31}H_{41}O_9N_3$: C, 62.09; H, 6.89; N, 7.01%.

Benzyloxycarbonylserylthreonyl-β-t-butylaspartyltyrosylglycine t-Butyl Ester (XV). Benzyloxycarbonylserylthreonine hydrazide[25] (4.61 g, 13.0 mmol) was suspended in *N,N*-dimethylformamide (40 ml) and cooled to −20 °C. Then 3.90 M HCl in dioxane (10 ml) was added. The resulting clear solution was mixed with isopentyl nitrite (1.82 ml) and stirred for 20 min at the same temperature. Then a solution of *N*-methylmorpholine (1.37 g) in *N,N*-dimethylformamide (5 ml) was added. The solution was mixed with a solution in *N,N*-dimethylformamide (30 ml) of the material obtained by hydrogenation of compound XIV (8.40 g, 14.0 mmol) over a 5% palladium-charcoal catalyst. The solution was stirred for 48 h at 0 °C, and then poured into cold water. The precipitate formed was collected by filtration; wt 6.70 g (64.4%). The crude product was reprecipitated from *N,N*-dimethylformamide and water; wt 6.00 g (57.5%); mp 174—176 °C; $[\alpha]_D^{24}$ −13.5° (*c* 1.0, *N,N*-dimethylformamide). Amino acid ratio: Asp, 1.11 (1); Thr, 1.05 (1); Ser, 1.02 (1); Gly, 1.00 (1); Tyr, 0.97 (1).

Found: C, 57.33; H, 6.85; N, 8.93%. Calcd for $C_{38}H_{53}O_{13}N_5 \cdot 0.5H_2O$: C, 57.27; H, 6.83; N, 8.79%.

Benzyloxycarbonylphenylalanyl-γ-t-butylglutamylserylasparaginyl-phenylalanine Hydrazide (XVI). A solution of compound IV (4.23 g, 4.90 mmol) in *N,N*-dimethylformamide (50 ml) was cooled to 0—5 °C in an ice-bath, and then 80% hydrazine hydrate (2 g) was added. The solution was stirred for 24 h at room temperature, and then the precipitate formed was collected by filtration and washed with ethanol. The product was boiled in hot ethanol, and then cooled and filtered; wt 4.15 g (100%); mp 238—240 °C (decomp).

Found: C, 59.61; H, 6.49; N, 13.29%. Calcd for $C_{42}H_{54}O_{11}N_8$: C, 59.56; H, 6.43; N, 13.23%.

Benzyloxycarbonylphenylalanyl-γ-t-butylglutamylserylasparaginyl-phenylalanylasparaginylthreonylglutaminylalanylthreonine Methyl Ester (XVII). Compound XVI (1.27 g, 1.50 mmol) was suspended in *N,N*-dimethylformamide (4 ml) and cooled to −20 °C. Then 3.90 M HCl in dioxane (1.15 ml) and isopentyl nitrite (0.212 ml) were added. The mixture was stirred for 80 min at the same temperature and insoluble material was filtered off. The filtrate was mixed with *N*-methylmorpholine (0.46 g) and then with a solution in a mixture of *N,N*-dimethylformamide (5 ml) and dimethyl sulfoxide (1 ml) of the material obtained by hydrogenation of compound VII (1.36 g, 1.97 mmol) over a 5% palladium-charcoal catalyst at atmospheric pressure. The mixture was stirred for 48 h at 0 °C, and then poured into cold water. The precipitate formed was filtered off and washed with 0.1 M hydrochloric acid and water; wt 1.60 g (77.3%). The crude material was reprecipitated repeatedly from *N,N*-dimethylformamide and water; wt 1.04 g (50.2%); mp 233 °C (sintered) and 248 °C (decomp); $[\alpha]_D^{18}$ −10.7° (*c* 0.42, dimethyl sulfoxide). Amino acid ratio: Asp, 1.98 (2); Thr, 1.93 (2); Ser, 0.89 (1); Glu, 2.02 (2); Ala, 0.97 (1); Phe, 2.00 (2).

Found: C, 54.65; H, 6.44; N, 13.08%. Calcd for $C_{63}H_{87}O_{21}N_{13} \cdot H_2O$: C, 54.81; H, 6.50; N, 13.19%.

Benzyloxycarbonylphenylalanyl-γ-t-butylglutamylserylasparaginyl-phenylalanylasparaginylthreonylglutaminylalanylthreonine Hydrazide (XVIII). Compound XVII (0.92 g, 0.67 mmol) was dissolved in a mixture of dimethyl sulfoxide (60 ml) and *N,N*-dimethylformamide (10 ml) and mixed with 80% hydrazine hydrate (5 g). The solution was stirred for 48 h at room temperature, concentrated to a small volume *in vacuo*, and mixed with ethanol. The precipitate formed was boiled in ethanol and collected by filtration; wt 0.87 g (94%); mp 240 °C (sintered) and 246—247 °C (decomp).

Found: C, 53.22; H, 6.55; N, 14.74%. Calcd for $C_{62}H_{87}O_{20}N_{15} \cdot 2H_2O$: C, 53.25; H, 6.56; N, 15.02%.

Benzyloxycarbonylasparaginyl-N^{ω}-tosylarginylasparaginylthreonyl-aspartylglycine Hydrazide Trifluoracetate (XIX). Compound XII (0.60 g, 0.53 mmol) was dissolved in cold trifluoracetic acid (8 ml). The solution was stirred for 40 min at room temperature, and then concentrated to a syrup *in vacuo*. The syrup was triturated in ether; wt 0.57 g (95.0%).

Asparaginylarginylasparaginylthreonylaspartylglycylserylthreonyl-aspartyltyrosylglycine (XX). Compound XIX (0.57 g, 0.52 mmol) was suspended in *N,N*-dimethylformamide (2 ml), cooled to −20 °C and mixed with 3.90 M HCl in dioxane (0.40 ml). The clear solution was mixed with isopentyl nitrite (0.074 ml) and stirred for 20 min at the same temperature. Then, the solution was mixed with *N*-methylmorpholine (0.32 g) and with a solution in *N,N*-dimethylformamide (1 ml) of the material (0.41 g, 0.63 mmol) prepared by hydrogenation of compound XV over a 5% palladium-charcoal catalyst. The solution was stirred for 48 h at 0 °C and poured into cold 0.1 M hydrochloric acid. The precipitate formed was collected by filtration; wt 0.64 g (77%). The crude product was reprecipitated from *N,N*-dimethylformamide and ethanol; wt 0.53 g (64%). Amino acid ratio: Asp, 4.35 (4); Thr, 2.05 (2); Ser, 0.99 (1); Gly, 2.00 (2); Tyr, 0.92 (1); Arg+Orn, 0.76 (1). The protected peptide (161.1 mg) was steeped in anisole (0.46 ml) and dissolved in cold trifluoracetic acid (10 ml). The solution was stirred for 60 min at room temperature, and then concentrated to a syrup *in vacuo*. The syrup was triturated in ether; 140.3 mg. The powder and anisole (0.5 ml) were put into the Daiflon cylinder of an HF-reaction apparatus.[15] Anhydrous hydrogen fluoride (5 ml) was distilled into the cylinder cooled to −78 °C in a Dry Ice and methanol bath. The contents of the cylinder were stirred for 60 min at −15 °C, and then the hydrogen fluoride was evaporated off *in vacuo*. The residue was dissolved in 1 M acetic acid and washed with ether. Then, the solution was applied to a column of Amberlite IR-45 (acetate cycle) and eluted with 1 M acetic acid. The eluate was lyophilized; wt 111.8 mg. The lyophilized material (35.0 mg) was dissolved in water (1 ml) and charged on a column of diethylaminoethyl-cellulose (OH^- cycle, 0.7×10 cm). The column was eluted with a linear gradient from water (150 ml) to 0.2 M ammonium acetate (150 ml). The fractions (from 33 ml to 58 ml) of the eluate with absorption at 274 nm were collected and lyophilized; wt 29.3 mg. Thin layer chromatography: R_f 0.18 in 1-butanol: acetic acid: pyridine: water (15: 3: 10: 12, by volume) and 0.08 in phenol: water (3: 1, by volume);

paper electrophoresis: $R_{f,ala}$ 1.7 (0.2 M acetic acid, 14 V/cm); $[\alpha]_D^{18}$ −33.5° (*c* 0.55, water). Amino acid ratio: Asp, 4.06 (4); Thr, 1.94 (2); Ser, 0.93 (1); Gly, 2.00 (2); Tyr, 0.89 (1); Arg, 1.03 (1).

Found: C, 43.35; H, 6.18; N, 17.63%. Calcd for $C_{46}H_{70}O_{22}N_{16}\cdot 4H_2O$: C, 43.39; H, 6.14; N, 17.43%.

Phenylalanylglutamylserylasparaginylphenylalanylasparaginylthreonylglutaminylalanylthreonylasparaginylarginylasparaginylthreonylaspartylglycylserylthreonylaspartyltyrosylglycine (XXI). Compound XVIII (0.82 g, 0.59 mmol) was suspended in a mixture of dimethyl sulfoxide (3.3 ml) and *N,N*-dimethylformamide (1 ml), cooled to −10 °C and mixed with 4.74 M HCl in dioxane (0.45 ml) and isopentyl nitrite (0.09 ml). The mixture was stirred for 60 min at −20 °C and then mixed with a solution of compound XX (0.53 g, 0.42 mmol) in dimethyl sulfoxide (2.1 ml) and *N,N*-dimethylformamide (0.5 ml), and then with a solution of *N*-methylmorpholine (0.26 g) in dimethyl sulfoxide (0.5 ml). The mixture was stirred for 3 days at 0 °C, and then poured into cold 0.1 M hydrochloric acid. The precipitate formed was collected by filtration and dried over P_2O_5; wt 0.84 g. The crude product (403.1 mg) and anisole (0.62 ml) were treated with hydrogen fluoride (5 ml) at −10 °C for 60 min in an HF-reaction apparatus.[15] Hydrogen fluoride was distilled off, and the residue was dissolved in aqueous formic acid and lyophilized; wt 411.8 mg. The freeze-dried powder was dissolved in 50% acetic acid, charged on a column of Sephadex G-25 (2×130 cm) and eluted using the same solvent. The fractions shaded in Fig. 6, with an absorption at 274 nm, were collected and lyophilized; wt 93.5 mg. The lyophilized powder (46.4 mg) was dissolved in water and charged on a column of carboxymethyl-cellulose (H^+ cycle, 15×170 mm). The column was eluted with a linear gradient from water (150 ml) to 50% acetic acid (150 ml). The portions shaded in Fig. 7 with an absorption at 274 nm were collected and lyophilized; wt 22.4 mg; R_f (TLC) 0.18 in 1-butanol: acetic acid: pyridine: water (15: 3: 10: 12, by volume); $R_{f,ala}$ (paper electrophoresis) 1.1 (0.2 M acetic acid, 14 V/cm). Amino acid ratio: Asp, 5.84 (6); Thr, 3.85 (4); Ser, 1.81 (2); Glu 2.08 (2); Ala, 1.00 (1); Gly, 1.92 (2); Tyr, 0.87 (1); Phe, 1.92 (2); Arg, 1.00 (1).

References

1) S. Shinka, M. Imanishi, N. Miyagawa, T. Amano, M. Inoue, and A. Tsugita, *Biken J.*, **10**, 89 (1967).

2) H. Fujio, M. Imanishi, K. Nishioka, and T. Amano, *Biken J.*, **11**, 207 (1968).

3) R. Arnon and M. Sela, *Proc. Natl. Acad. Sci. U. S. A.*, **62**, 163 (1969).

4) N. Sakato, H. Fujio, and T. Amano, *Biken. J.*, **15**, 135 (1972).

5) M. Z. Atassi, A. F. S. A. Habeeb, and K. Ando, *Biochim. Biophys. Acta*, **303**, 203 (1973).

6) H. Fujio, R. E. Martin, Y. -M. Ha, N. Sakato, and T. Amano, *Biken J.*, **17**, 73 (1974).

7) Y. -M. Ha, H. Fijio, N. Sakato, and T. Amano, *Biken J.*, **18**, 47 (1975).

8) R. Arnon, E. Maron, M. Sela, and C. B. Anfinsen, *Proc. Natl. Acad. Sci. U. S. A.*, **68**, 1450 (1971).

9) M. Z. Atassi, J. Koketsu, and A. F. S. A. Habeeb, *Biochim. Biophys. Acta*, **420**, 358 (1976).

10) M. Z. Atassi, C. -L. Lee, and R. -C. Pai, *Biochim. Biophys. Acta.*, **427**, 745 (1976).

11) I. Jauregui-Adell, J. Jolles, and P. Jolles, *Biochim. Biophys. Acta*, **107**, 97 (1965).

12) R. E. Canfield and A. K. Liu, *J. Biol. Chem.*, **240**, 1997 (1965).

13) H. Fujio, Y. Takagaki, Y. -M. Ha, T. Amano, T. Mitaki, and Y. Shimonishi, Proc. 6th Ann. Meeting of the Japanese Soc. Immunol. Abstracts: p. 388 (1976).

14) D. C. Phillips, *Scientific American*, **215**, 78 (1966).

15) S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, and H. Sugihara, *Bull. Chem. Soc. Jpn.*, **40**, 2164 (1967).

16) J. Beacham, G. Cupuis, F. M. Finn, H. T. Storey, C. Yanaihara, N. Yanaihara, and K. Hofmann, *J. Am. Chem. Soc.*, **93**, 5526 (1971).

17) W. Grassmann and E. Wünsch, *Chem. Ber.*, **91**, 462 (1958).

18) W. L. Haas, E. V. Krumkalns, and K. Gerzon, *J. Am. Chem. Soc.*, **88**, 1988 (1966).

19) Th. Wieland and R. Sarges, *Justus Liebigs Ann. Chem.*, **658**, 181 (1962).

20) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 2504 (1959).

21) E. Schröder and H. Gibian, *Justus Liebigs Ann. Chem.*, **655**, 211 (1962).

22) K. Hofmann, W. Haas, M. J. Smithers, and G. D. Zanetti, *J. Am. Chem. Soc.*, **87**, 631 (1965).

23) A. M. Felix and R. B. Merrifield, *J. Am. Chem. Soc.*, **92**, 1385 (1970).

24) J. Ramachandran and C. H. Li, *J. Org. Chem.*, **27**, 4006 (1962).

25) F. Marchiori, R. Rocchi, and E. Scoffone, *Gazz. Chim. Ital.*, **93**, 834 (1963).

26) M. Itoh, *Chem. Pharm. Bull.*, **18**, 784 (1970).

27) G. W. Anderson and F. M. Callahan, *J. Am. Chem. Soc.*, **82**, 3359 (1960).

28) The abbreviations used in this report are those recommended by IUPAC-IUB: *J. Biol. Chem.*, **247**, 977 (1972).

29) J. Honzl and J. Rudinger, *Collect. Czech. Chem. Commun.*, **26**, 2333 (1961).

30) S. Moore, D. H. Spackman, and W. Stein, *Anal. Chem.*, **30**, 1185 (1958).