Chem. Pharm. Bull. 15(6) 854~862 (1967)

UDC 547.963.07

106. Haruaki Yajima, Osamu Nishimura, Koichi Kawasaki, and Yoshio Okada : Studies on Peptides. XIV.*1,2 Synthesis of Lysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteinylhistidine, a Peptide related to Horse Heart Cytochrome C.

(*Faculty of Pharmaceutical Sciences, Kyoto University*3)

A partially protected hexapeptide, lysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteinylhistidine, related to the heme portion of horse heart cytochrome C was described. In addition, S-benzylcysteinylalanylglutaminyl-S-benzylcysteine, lysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine and some S-benzylcysteine peptides were prepared.

(Received October 6, 1966)

Since Margoliash, Tuppy and their colleagues[1] elucidated the entire amino acid sequence of horse heart cytochrome C, the structures of such type of important electron-transfering enzymes from various species have been determined.[2] As previously indicated by Theorell,[3] common structural feature of cytochrome C is that the two sulfhydryl groups of two cysteine residues are involved in the thioether linkages between the protein part and the heme.[4~6] Exhaustive hydrolysis of cytochrome C gave a product, so called porphyrin C, which was initially examined by Theorell[7,8] and Zeile and Meyer.[9] Recent investigations[10~12] have led to the conclusion that the structure of porphyrin C could be represented as 2,4,α,α'-bis(S-L-cysteine)mesoporphyrin IX, where the two sulfhydryl groups of two cysteine residues are attached to the α-carbon atoms of the vinyl side chains of protoporphyrin. The stereochemical nature, however, is still remained to be verified.

Several methods are now available to introduce the sulfhydryl group of cysteine into the side chains of protoporphyrin to form an adduct, so called porphyrin C type compound. Among those, the reaction between the dibromide of hematoporphyrin[15] and cysteine seems to be one of the choice to prepare this type of compound. This reaction was first studied by Theorell in 1939,[8] later revised by Zeile and Meyer[9] and Neiland and Tuppy.[10] It was recently adopted for the synthesis of hemopeptide by Gnichtel, *et al.*[13,14] On the other hand, the preparation of such type of compound by

*1 Part XIII. This Bulletin, **15**, 504 (1967).
*2 Peptides and peptide derivatives mentioned in this paper are of the L-configuration.
*3 Sakyo-ku, Kyoto (矢島治明, 西村 紀, 川崎紘一, 岡田芳男).

1) E. Margoliash, E. L. Smith, G. Kreil, H. Tuppy : Nature, **192**, 1121 (1961).
2) See review articles; E. Margoliash, A. Schejter : Advances in Protein Chem., **21**, 113 (1966), Academic Press, N. Y. and K. Narita : Seikagaku, **38**, 49 (1966).
3) H. Theorell : Biochem. Z., **298**, 242 (1938).
4) C. L. Tsou : Biochem. J., **49**, 367 (1951).
5) H. Tuppy, G. Bode : Monatsh., **85**, 1024, 1182 (1954).
6) H. Tuppy, S. Paleus : Acta Chem. Scand., **9**, 353 (1955).
7) H. Theorell : Enzymologia, **4**, 192 (1937).
8) *Idem* : Biochem. Z., **301**, 201 (1939).
9) K. Zeile, H. Meyer : Hope-Seyler's Z. Physiol. Chim., **262**, 178 (1939-1940).
10) J. B. Neilands, H. Tuppy : Biochim. Biophys. Acta, **38**, 351 (1960).
11) S. Sano, S. Granick : J. Biol. Chem., **236**, 1173 (1961).
12) S. Sano, N. Nanzyo, C. Rimington : Biochem. J., **93**, 270 (1964).
13) H. Gnichtel, W. Lantsch : Ber., **98**, 1647 (1965).
14) H. Gnichtel, H. Preptow : *Ibid.*, **98**, 2266 (1965).
15) R. Willstätter, M. Fischer : Hoppe-Seyler's Z. Physiol. Chim., **87**, 440 (1913).

the reaction of protoporphyrinogen, a reduced form of protoporphyrin, with cysteine in neutral or slightly acidic condition elaborated quite recently by Sano and Granick[11] and later confirmed by Popper and Tuppy[16] is an attractive procedure, Sano, *et al.*,[17] after a model experiment to yield the adduct of cysteinylglycylglycylcysteine,[18] reported the successful recombination between the heme and the separated protein part of cytochrome C to regenerate its activity by this method. These informations have brought the light to elucidate the chemical nature of the interesting bonds between the protein and the prosthetic group of cytochrome C and further to explore the relationship between the chemical structure and its biological functions in terms of phenomenon of hemochromogen[19~21] and peroxidase activity[22] as well as cytochrome C activity itself.

By the present time, several synthetic peptides around the heme portion of cytochrome C were reported, the partial synthesis of a peptic[23] or tryptic fragment,[24] preparation of cysteinylglylcylglycylcysteinylhistidine hemopeptide[25] and S-*p*-methoxybenzylcysteinylalanylglutaminyl-S-*p*-methoxybenzylcysteine.[26] Utility of trypsin-pronase treated hemopeptide for further extension of pepide chain was proposed by Inouye and Sakakibara.[27] However, in view of combination reactions between peptides and the heme, we have selected to prepare the hexapeptide, lysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteinylhistidine (I), since as shown in Fig 1, the presence of two basic amino acid residues, lysine and histidine in both sides of the two cysteinyl groups in cytochrome C from various species is now under our particular attention considering the possible interaction between the iron of the heme and these two basic groups. We have also prepared S-benzylcysteinylalanylglutaminyl-S-benzylcysteine (II), lysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine (III), and a number of S-benzylcysteine peptides. These peptides were treated with sodium in liquid ammonia to remove S-protecting groups[28~30] and the resulting peptides were converted to the corresponding mercuric salts. Combination of these peptides with protoporphyrinogen is in progress in Dr. S. Sano's laboratory.*[4] In this paper, the syntheses of the peptides mentioned above are recorded.

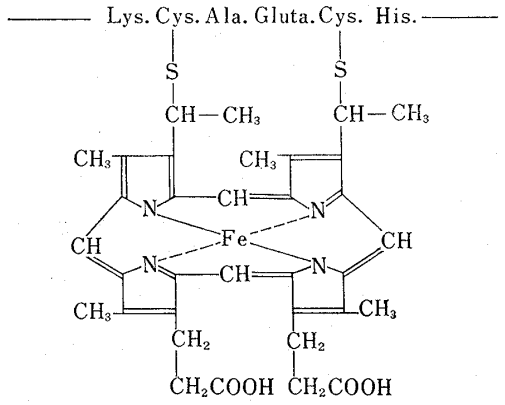

Fig. 1. Amino Acid Sequence around the Heme Portion of Horse Heart Cytochrome C.

16) T. L. Popper, H. Tuppy : Acta Chem. Scand., **17**, supple. 1, 47 (1963).
17) S. Sano, K. Tanaka : J. Biol. Chem., **239**, 3109 (1964).
18) S. Sano, K. Ikeda, S. Sakakibara : Biochem. Biophys. Res. Comm., **15**, 284 (1964).
19) H. Theorell, A. Akesson : J. Am. Chem. Soc., **63**, 1804 (1941).
20) K. G. Paul : Acta Chem. Scand., **5**, 389 (1951).
21) A. Ehrenberg, H. Theorell : *Ibid.*, **9**, 1193 (1955).
22) See review article; E. Margoliash : Heamatin Enzyme, Ed. by J. E. Falk, R. Lemberg, R. K. Morton, p. 259 (1961), Pergamon press, N. Y.
23) Y. Shimonishi, Y. Nobuhara, S. Sakakibara, S. Akabori : Proceeding of the Meeting of Japan Chem. Soc., **17**, 281 (1964).
24) D. Theodoropoulos, I. Souchleris : Acta Chim. Acad. Sci. Hung., **44**, 183 (1965).
25) S. Inouye, S. Sakakibara, S. Akabori : Bull. Chem. Soc. Japan, **37**, 713 (1964).
26) S. Akabori, K. Okawa, F. Sakiyama, N. Takabayashi, T. Yamakawa, H. Shimonishi : Proceeding of the Meeting of Japan Chem. Soc., **13**, 261 (1960).
27) S. Inouye, S. Sakakibara : *Ibid.*, **18**, 302 (1965).
28) V. du Vigneaud, L. F. Audrieth, H. S. Loring : J. Am. Chem. Soc., **52**, 4500 (1930).
29) R. H. Sifferd, V. du Vigneaud : J. Biol. Chem., **108**, 753 (1935).
30) J. L. Wood, V. du Vigneaud : *Ibid.*, **130**, 109 (1939).

For the synthesis of I, S-protected tetrapeptide, S-benzylcysteinylalanylglutaminyl-S-benzylcysteine (II) was first prepared as a key intermediate for further extension of peptide chains. The mixed anhydride[31] or dicyclohexylcarbodiimide (DCC)[32] procedure was mainly used for elongation of peptide chain and the *p*-nitrophenyl ester method[33,34] was also adopted. Benzyloxycarbonyl groups of synthetic intermediates were removed by hydrogen bromide in anhydrous glacial acetic acid.[35] Under these conditions, the amide group of glutamine residue remained intact, as previously demonstrated,[36] throughout the syntheses.

S-Benzylcysteine methyl ester was condensed with N^{α}-benzyloxycarbonylglutamine by DCC to give N^{α}-benzyloxycarbonylglutaminyl-S-benzylcysteine methyl ester, which was converted to glutaminyl-S-benzylcysteine methyl ester hydromide. This dipeptide ester condensed with N^{α}-benzyloxycarbonylalanine to give N^{α}-benzyloxycarbonylalanylglutaminyl-S-benzylcysteine methyl ester, which was saponified by sodium hydroxide to give N^{α}-benzyloxycarbonylalanylglutaminyl-S-benzylcysteine in relatively low yield. It is known that exposure of S-benzylcysteinyl peptides to the action of sodium hydroxide causes some undesired side reactions.[37~41] Therefore this protected tripeptide was prepared in better yield than the above procedure by direct coupling of N^{α}-benzyloxy carbonylalanine with glutaminyl-S-benzylcyteine. The latter dipeptide was derived from its N-protected dipeptide, which was prepared by coupling N^{α}-benzyloxycarbonyl glutamine and a triethylammonium salt of S-benzylcysteine *via* the mixed anhydride method. This procedure avoided the danger of the action of sodium hydroxide.

The debenzyloxycarbonylated tripeptide, alanylglutaminyl-S-benzylcysteine was condensed with N^{α}-benzyloxycarbonyl-S-benzylcysteine to form N^{α}-benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine. Treatment of this protected tetrapeptide either with hydrogen bromide or with diazomethane followed by hydrazine gave S-benzylcysteinylalanylglutaminyl-S-benzylcysteine (II) or N^{ε}-benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine hydrazide. The former tetrapeptide (II) gave, after reaction with N^{α},N^{ε}-dibenzyloxycarbonyllysine,[42] a protected pentapeptide, N^{α},N^{ε}-dibenzyloxycarbonyllysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine, which was converted to lysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine (III) by hydrogen bromide in acetic acid followed by neutralization with triethylamine.

The above tetrapeptide hydrazide was converted to its azide, which was allowed to react with the triethylammonium salt of histidine to yield the protected pentapeptide, N^{α}-benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteinylhistidine (IV). This was also prepared in better yield than the above procedure by the stepwise elongation method starting from histidine. Protected synthetic intermediates are less soluble in a dilute acetic acid solution as well as in usual organic solvents. Therefore, crude reaction mixtures were washed with 10% acetic acid to remove unchanged amino

*4 S. Sano, M. Kuriha, O. Nishimura, H. Yajima: Symposium on Cytochromes. VIIth International Congress of Biochemistry, Tokyo (1967) in press.

31) J.R. Vaughan, R. Osato: J. Am. Chem. Soc., **74**, 676 (1952).
32) J.C. Sheehan, G.P. Hess: *Ibid.*, **77**, 1067 (1955).
33) M. Bodanszky: Nature, **175**, 685 (1955).
34) M. Bodanszky, V. du Vigneaud: *Ibid.*, **183**, 1324 (1959).
35) G.W. Anderson, J. Boldinger, A.D. Welcher: J. Am. Chem. Soc., **74**, 5309 (1952).
36) M. Bodanszky, V. du Vigneaud: *Ibid.*, **81**, 5688 (1959).
37) J.A. MacLaren: Australian J. Chem., **11**, 360 (1958).
38) H.T. Clark, J.M. Inouye: J. Biol. Chem., **94**, 541 (1931).
39) B.H. Nicolet: J. Am. Chem. Soc., **53**, 3066 (1931).
40) V. du Vigneaud, M.F. Bartlett, A. Jöhl: *Ibid.*, **79**, 5572 (1957).
41) J.A. MacLaren, W.E. Savige, J.M. Swan: Australin J. Chem., **11**, 345 (1958).
42) M. Bergmann, L. Zervas, W.F. Ross: J. Biol. Chem., **111**, 245 (1935).

components in every step and then recrystallized from relatively large excess of methanol to remove unchanged N^{α}-benzyloxycarbonylamino acids. These procedures gave the well characterized intermediates.

The protected pentapeptide thus obtained by two alternate routes was again treated with hydrogen bromide to give S-benzylcysteinylalanylglutaminyl-S-benzylcysteinylhistdine, which was subsequently condensed with N^{α},N^{ε}-dibenzyloxycarbonyllysine to give the protected hexapeptide, N^{α},N^{ε}-dibenzyloxycarbonyllysyl-S-benzylcysteinylalanyl glutaminyl-S-benzylcysteinylhistidine. Treatment of this protected hexapeptide with hydrogen bromide followed by neutralization gave S-protected hexapeptide (I). The peptide thus obtained produced a single spot on paper chromatography in two different solvent systems. Acid hydrolysis gave the constituent amino acids in the ratios predicted by theory where glutamine was detected as glutamic acid and S-benzylcysteine was partially destroyed. Leucine aminopeptidase (LAP)[43] digestion of I thus obtained

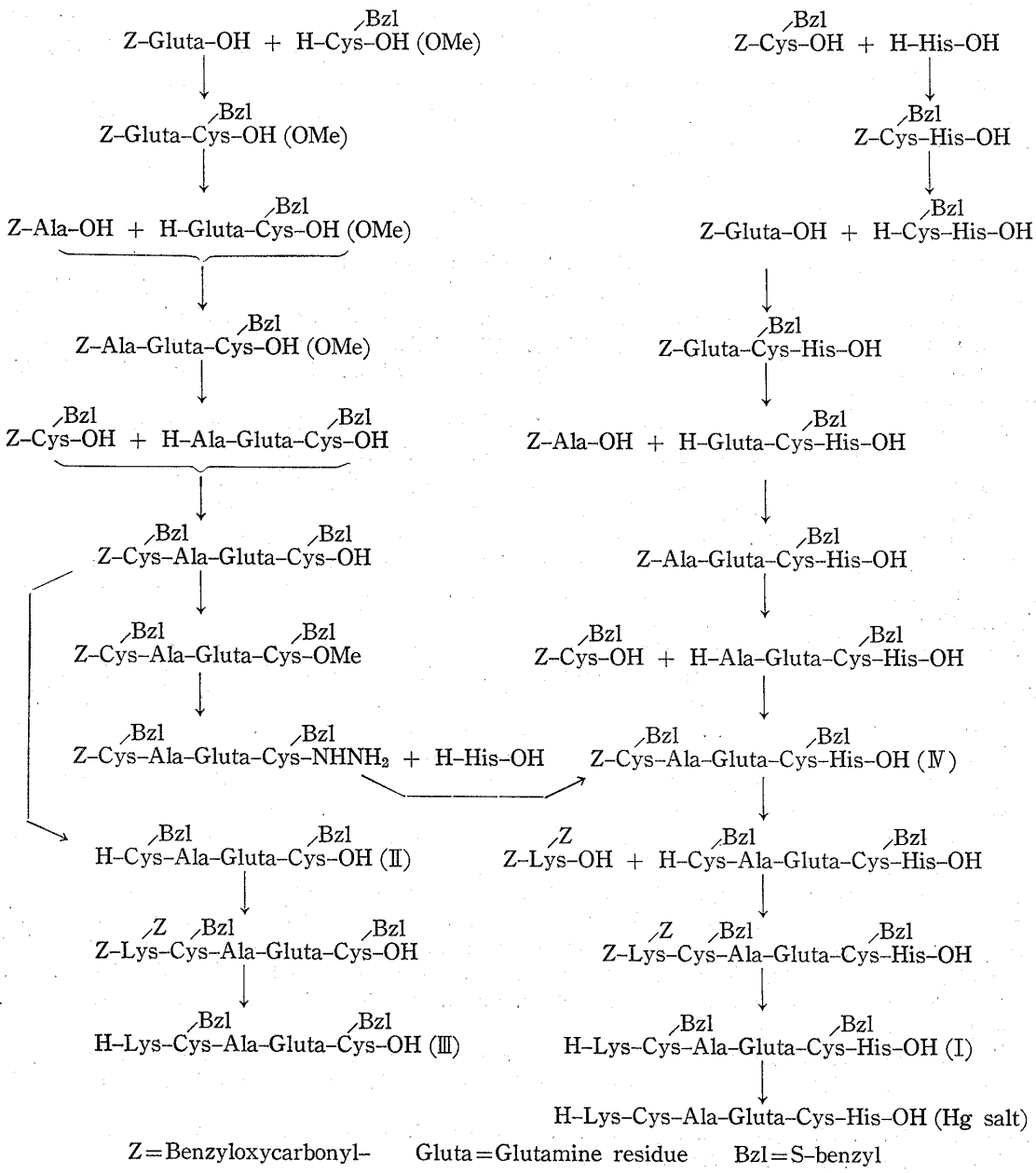

Chart 1. Synthetic Scheme of the Hexapeptide

43) Partially purified LAP was prepared according to the procedure of D. H. Spackman, E. L. Smith, D. M. Brown: J. Biol. Chem., **212**, 255 (1955).

gave the constituent amino acids in nearly equal molar ratios where glutamine which forms pyrolidone carboxylic acid could not be detected by ninhydrin technique[44] and S-benzylcysteine was detected as a peak right ahead of the lysine peak in the chromatogram. These results seem enough to demonstrate that our synthetic S-protected peptide is homogeneous.

Synthetic peptides, I, II, III and IV were then treated with sodium in liquid ammonia to remove the S-protecting groups and the resulting peptides were converted to the corresponding mercuric salts. For testing of homogeneity, each peptide salt was treated with hydrogen sulfide and the resulting S-free peptide, after treatment with performic acid,[45] was hydrolyzed by acid. Amino acid ratios in the hydrolysate were quite identical with those predicted by theory where two moles of cysteic acids were detected per mole of peptide.

Two basic amino acid residues, histidine and lysine, as mentioned above, located near the heme of cytochrome C might have an important role to direct the reaction between peptides and porphyrin nucleus. Therefore, in addition to the synthesis of I, a few model S-benzylcysteinylpeptides, with or without of these basic residues were synthesized. Syntheses of hemopeptides with those functional groups mentioned in this paper will be summarized in the future.

Experimental

Analytical procedures and general experimental methods employed in this investigation are essentially the same as described in the IV[46] of this series. Rf^1 values refer to the system of Partridge.[47] Rf^2 values refer to the system of 2-butanol-ammonia[48] and are expressed as a multiple of the distance traveled by a phenylalanine marker under identical conditions. Glacial AcOH was distilled over triacetyl boric acid.[49] The following abbreviations are used; Lys=lysine, Cys=cysteine, Ala=alanine, Gluta=glutamine, His=histidine, and $CySO_3H$=cysteic acid.

N^{α},N^{ε}-Dibenzyloxycarbonyllysyl-S-benzylcysteine Methyl Ester——N^{α},N^{ε}-Dibenzyloxycarbonyllysine[41] (0.81 g.) and S-benzylcysteine methyl ester[30] (prepared from 0.61 g. of the hydrobromide and 0.28 ml. of triethylamine) were dissolved in acetontrile (10 ml.). Under ice-cooling, DCC (0.45 g.) was added and the solution was stirred at room temperature for 24 hr. After filtration, the solvent was evaporated and the residue was dissolved in AcOEt. The organic phase was successively washed with 5% NH_4OH, 1 *N* HCl and H_2O, dried over Na_2SO_4, and evaporated to give a solid mass which was recrystallized from MeOH; yield 0.90 g. (65%), m.p. 144~147°, $[\alpha]_D^{18}$ −37.2° (c=1.0, MeOH). *Anal.* Calcd. for $C_{33}H_{39}O_7N_3S$: C, 63.8; H, 6.3; N, 6.7. Found: C, 63.5; H, 6.2; N, 6.7.

N^{α},N^{ε}-Dibenzyloxycarbonyllysyl-S-benzylcysteine——A mixed anhydride was prepared from N^{α}, N^{ε}-dibenzyloxycarbonyllysine (10.85 g.), tri-*n*-butylamine (6.18 ml.) and ethyl chloroformate (2.4 ml.) in tetrahydrofuran (35 ml.). This solution was added to an ice-cold solution of S-benzylcysteine (7.95 g.) and triethylamine (5.20 ml.) in H_2O (75 ml.). The mixture was stirred at 0° for 1.5 hr. The solvent was evaporated and the residue was acidified with 1 *N* HCl. The resulting product was extracted with AcOEt which was washed successively with 1 *N* HCl, a solution of NaCl and H_2O. The organic solution was dried over Na_2SO_4 and evaporated. The residue was crystallized from MeOH; yield 14.14 g. (87%), m.p. 158~160°, $[\alpha]_D^{18}$ −12.1° (c=0.3, dimethylformamide=DMF). *Anal.* Calcd. for $C_{32}H_{37}O_7N_3S$: C, 63.2; H, 6.1; N, 6.9. Found; C, 63.2; H, 6.0; N, 7.1.

N^{α},N^{ε}-Dibenzyloxycarbonyllysyl-S-benzylcysteine Hydrazide Monohydrate——N^{α},N^{ε}-Dibenzyloxycarbonyllysyl-S-benzylcysteine methyl ester (1.60 g.) was dissolved in MeOH (150 ml.) and 80% hydrazine (2.5 ml.) was added. The solution was kept at room temperature for 40 hr. and the ensuing precipitate was collected and dried over H_2SO_4 *in vacuo*. The product was recrystallized from MeOH; yield 1.00 g. (64%) m.p. 174~176°, $[\alpha]_D^{27}$ −16.3° (c=0.8, DMF). *Anal.* Calcd. for $C_{32}H_{39}O_6N_5S \cdot H_2O$: C, 61.1; H, 6.3; N, 11.1. Found: C, 61.7; H, 6.5; N, 11.1.

Lysyl-S-benzylcysteine Dihydrobromide——N^{α},N^{ε}-Dibenzyloxycarbonyllysyl-S-benzylcysteine (0.35 g.) was treated with 2.6 *N* HBr in glacial AcOH (6.5 ml.) at 13° for 1.5 hr. The precipitate formed by addition

44) K. Hofmann, T. A. Thompson, H. Yajima, E. T. Schwartz, H. Inouye: J. Am. Chem. Soc., **82**, 3715 (1960).
45) S. Moore: J. Biol. Chem., **238**, 235 (1963).
46) H. Yajima, K. Kubo: This Bulletin, **13**, 759 (1965).
47) S. M. Partridge: Biochem. J., **42**, 238 (1948).
48) J. F. Roland, A. M. Gross: Anal. Chem., **26**, 502 (1954).
49) A. Pictet, A. Geleznoff: Ber., **36**, 2219 (1903).

of anhydrous ether was collected by filtration, washed with fresh ether and dried over KOH pellets and P_2O_5 *in vacuo*; yield quantitative, $[\alpha]_D^{18}$ −23.0° (c=1.0, MeOH), Rf^1 0.47, single ninhydrin positive spot. Amino acid ratios in a LAP digestion $Lys_{1.00}$ S-benzyl-$Cys_{1.00}$ (average recovery 93%). *Anal.* Calcd. for $C_{16}H_{25}O_3N_3S \cdot 2HBr$: C, 38.3; H, 5.4; N, 8.4. Found: C, 37.8; H, 5.7; N, 8.7.

N^α-Benzyloxycarbonyl-S-benzylcysteinyl-N^ε-benzyloxycarbonyllysine Methyl Ester——A mixed anhydride, prepared from N^α-benzyloxycarbonyl-S-benzylcysteine (3.80 g.) in iceo-cooled tetrahydrofuran (40 ml.) with triethylamine (1.5 ml.) and ethyl chloroformate (1.05 ml.) was added to a cold solution of N^ε-benzyloxycarbonyllysine methyl ester (prepared from 3.16 g. of the hydrochloride and 1.5 ml. of triethylamine) in tetrahydrofuran (20 ml.). The mixture was stirred in an ice-bath for 2 hr. The solvent was evaporated and the residue was dissolved in ether, which was washed successively with 5% NH_4OH, 1*N* HCl and H_2O, dried over Na_2SO_4 and evaporated. The resulting crystalline mass was collected by filtration and washed with ether; yield 5.30 g. (77%), m.p. 106~108°, $[\alpha]_D^{18}$ −22.4° (c=1.0, MeOH). *Anal.* Calcd. for $C_{33}H_{39}O_7N_3S$: C, 63.8; H, 6.3; N, 6.8. Found: C, 64.0; H, 6.6; N, 6.8.

N^α-Benzyloxycarbonyl-S-benzylcysteinyl-N^ε-benzyloxycarbonyllysine Amide——N^α-Benzyloxycarbonyl-S-benzylcysteinyl-N^ε-benzyloxycarbonyllysine methyl ester (2.00 g.) was dissolved in MeOH (20 ml.) and under cooling with dry-ice acetone, liquid NH_3 (approximately 10 ml.) was added and the solution was kept in a sealed tube at 20° for 72 hr. The solvent was evaporated and the resulting mass was collected and washed with hot MeOH; yield 1.30 g. (67%), m.p. 178~181°, $[\alpha]_D^{18}$ −16.5° (c=0.8, DMF). *Anal.* Calcd. for $C_{32}H_{38}O_6N_4S$: C, 63.3; H, 6.3; N, 9.2. Found: C, 63.2; H, 6.6; N, 9.4.

S-Benzylcysteinyllysine Amide Dihydrobromide——To a solution of N^α-benzyloxycarbonyl-S-benzylcysteinyl-N^ε-benzyloxycarbonyllysine amide (0.30 g.) in glacial AcOH (2 ml.) was added 4*N* HBr in glacial AcOH (1.4 ml.). A very hygroscopic powder was obtained by addition of anhydrous ether; yield 0.16 g. (80%), $[\alpha]_D^{15}$ +11.0° (c=1.2, MeOH). Rf^1 0.31, single ninhydrin positive spot.

N^α-Benzyloxycarbonyl-S-benzylcysteinylalanylglutamine——A mixed anhydride was prepared in the usual manner from N^α-benzyloxycarbonyl-S-benzylcysteine (1.31 g.) in anhydrous tetrahydrofuran (5 ml.) with tri-*n*-butylamine (0.96 ml.) and ethyl chloroformate (0.38 ml.). This solution was added to an ice-cold solution of alanylglutamine[50] (0.68 g.) and triethylamine (0.42 ml.) in H_2O (7 ml.). Stirring was continued for 1.5 hr., the solvent was evaporated and the residue was acidified with 1*N* HCl. The resulting precipitate was collected by filtration and washed with H_2O and then AcOEt. The product was recrystallized from 95% MeOH; yield 1.34 g. (82%), m.p. 154~156°, $[\alpha]_D^{25}$ −13.4° (c=1.1, DMF). *Anal.* Calcd. for $C_{24}H_{32}O_7N_4S$: C, 57.3; H, 5.9; N, 10.3. Found: C, 57.4; H, 6.0; N, 10.5.

N^α-Benzyloxycarbonyl-S-benzylcysteinylalanylglutamine Methyl Ester——N^α-Benzyloxycarbonyl-S-benzylcysteinylalanylglutamine (0.50 g.) was dissolved in 98% MeOH and ethereal solution of diazomethane was added until the yellow color persisted and the mixture was kept for 1.5 hr. The excess of diazomethane was destroyed by the addition of a few drops of AcOH and the solution was evaporated to dryness *in vacuo* to give a crystalline mass which was recrystallized from MeOH; yield 0.23 g. (46%), m.p. 194~196°, $[\alpha]_D^{25}$ −23.8° (c=0.6, DMF). *Anal.* Calcd. for $C_{27}H_{34}O_7N_4S$: C, 58.1; H, 6.1; N, 9.9. Found: C, 58.3; H, 5.9; N, 10.0.

N^α-Benzyloxycarbonyl-S-benzylcysteinylalanylglutamine Hydrazide——N^α-Benzyloxycarbonyl-S-benzylcysteinylalanylglutamine methyl ester (0.38 g.) was dissolved in a mixture of DMF (10 ml.) and MeOH (10 ml.) and 80% hydrazine hydrate (0.70 ml.) was added. The solution was warmed at 60° for 1.5 hr. and kept at room temperature overnight. The solvent was removed *in vacuo* and the residue was precipitated from DMF with ether; yield 0.33 g. (85%), m.p. 204~210°, $[\alpha]_D^{25}$ −17.3° (c=0.2, DMF). *Anal.* Calcd. for $C_{26}H_{34}O_6N_6S$: C, 55.9; H, 6.1; N, 15.0. Found: C, 55.7; H, 6.4; N, 15.2.

N^α-Benzyloxycarbonylglutaminyl-S-benzylcysteine Methyl Ester——N^α-Benzyloxycarbonylglutamine (5.40 g.) and S-benzylcysteine methyl ester (prepared from 5.24 g. of the hydrobromide with 2.8 ml. of triethylamine) were dissolved in DMF (100 ml.). To this solution cooled to 0°, DCC (4.13 g.) was added and the mixture was stirred at room temperature overnight. Dicyclohexylurea was removed by filtration and the filtrate was concentrated. Ether was added to the residue and the resulting crystalline product was collected by filtration, washed with three portions of 5% NH_4OH, 1*N* HCl and H_2O and dried *in vacuo*. The material was precipitated twice from DMF with ether and finally recrystallized from AcOH; yield 7.73 g. (79%), m.p. 202~203°, $[\alpha]_D^{25}$ −25.9° (c=0.6, DMF). *Anal.* Calcd. for $C_{24}H_{33}O_6N_3S$: C, 59.1; H, 6.0; N, 8.6. Found: C, 58.9; H, 6.3; N, 8.8. (lit.[51] m.p. 201°, $[\alpha]_D^{25}$ −28.0° in DMF, lit.[52] m.p. 204°, $[\alpha]_D^{19}$ −30.3° in AcOH).

N^α-Benzyloxycarbonylglutaminyl-S-benzylcysteine——A mixed anhydride, prepared from N^α-benzyloxycarbonylglutamine (2.70 g.) in tetrahydrofuran (40 ml.) with triethylamine (1.40 ml.) and ethyl chloroformate (0.96 ml.), was added to a solution of S-benzylcysteine (2.21 g) and triethylamine (1.40 g.) in H_2O (50 ml.). The mixture was stirred in an ice-bath for 3 hr., when the solvent was evaporated and 1*N* HCl was added to the residue. The resulting solid was collected, washed with 1*N* HCl, and H_2O and recrystal-

50) E. Sondheimer, R. W. Holley: J. Am. Chem. Soc., **74**, 2816 (1954).
51) D. T. Gish, P. G. Katsoyannis, P. G. Hess, R. J. Stedman: J. Am. Chim. Soc., **78**, 5954 (1956).
52) R. A. Boissonnas, S. Guttmann, P. A. Jaquenoud, J. P. Waller: Helv. Chim. Acta, **39**, 1421 (1956).

lized from MeOH; yield 3.34 g. (71%), m.p. 201~202°, $[\alpha]_D^{24}$ −16.5°(c=1.0, DMF). *Anal.* Calcd. for $C_{23}H_{27}O_6N_3S$: C, 58.3; H, 5.7; N, 8.9. Found: C, 58.1; H, 5.9; N, 9.0.

N^{α}-Benzyloxycarbonylalanylglutaminyl-S-benzylcysteine Methyl Ester——N^{α}-Benzyloxycarbonylglutaminyl-S-benzylcysteine methyl ester (14.63 g.) in AcOH (30 ml.) was treated with 4.7*N* HBr in AcOH (19 ml.) at room temperature for 1 hr. Glutaminyl-S-benzylcysteine methyl ester hydromide was precipitated by addition of anhydrous ether and dried over P_2O_5 and KOH pellets *in vacuo*; yield 15.50 g. (100%), Rf^1 0.67. This hydrobromide was dissolved in MeOH and triethylamine (6.9 ml.) was added. The solvent was evaporated at 35°. DMF (100 ml.) was added and insoluble triethylamine hydrobromide was removed by filtration. The filtrate was mixed with a solution of N^{α}-benzyloxycarbonylalanine (11.66 g.) in DMF (50 ml.) and DCC (10.32 g) was added under ice-cooling. The mixture was stirred at room temperature overnight. Dicyclohexylurea was removed by filtration and the solvent was evaporated *in vacuo*. Ether was added to the residue and the resulting solid was collected, washed with 5% NH_4OH, 1*N* HCl and H_2O and recrystallized from MeOH; yield 13.90 g. (61%), m.p. 198~200°, $[\alpha]_D^{25}$ −29.4°(c=0.7, DMF). *Anal.* Calcd. for $C_{27}H_{34}O_7N_4S$: C, 58.1; H, 6.1; N, 10.0. Found: C, 57.8; H, 6.4; N, 10.0.

N^{α}-Benzyloxycarbonylalanylglutaminyl-S-benzylcysteine Monohydrate——a) N^{α}-Benzyloxycarbonylalanylglutaminyl-S-benzylcysteine methyl ester (5.59 g.) in a mixture of MeOH (30 ml.) and DMF (20 ml.) was treated with 1*N* NaOH (12 ml.) at room temperature for 1 hr. The product was isolated in the usual manner and recrystallized from MeOH; yield 3.35 g. (65%), m.p. 198~199°, $[\alpha]_D^{25}$ −15.0°(c=1.0, DMF). *Anal.* Calcd. for $C_{26}H_{32}O_7N_4S\cdot H_2O$: C, 55.5; H, 6.1; N, 10.0; S, 5.7. Found: C, 55.4; H, 6.4; N, 10.3; S, 5.5.

b) N^{α}-Benzyloxycarbonylglutaminyl-S-benzylcysteine (23.7 g.) was suspended in AcOH (30 ml.) and 4.1*N* HBr in AcOH (40 ml.) was added. After 1 hr. dry ether was added and the resulting precipitate was collected, washed with ether and dried over KOH pellets *in vacuo*. To a solution of this solid in H_2O (250 ml.), triethylamine (33 ml.) was added followed by a solution of a mixed anhydride of N^{α}-benzyloxycarbonylalanine (prepared from 23.3 g. of the acid component with 14 ml. of triethylamine and 9.6 ml. of ethyl chloroformate) in tetrahydrofuran (100 ml.). After stirring for 3 hr., the solvent was evaporated and the residue was acidified with 1*N* HCl. The resulting solid was collected, washed with H_2O and recrystallized from MeOH; yield 15.8 g. (58%), m.p. 194~198°, $[\alpha]_D^{25}$ −17.9°(c=0.7, DMF). *Anal.* Calcd. for $C_{26}H_{32}O_7N_4S$: C, 57.3; H, 5.9; N, 10.3. Found: C, 57.5; H, 6.1; N, 10.0.

Alanylglutaminyl-S-benzylcysteine Dihydrate——N^{α}-Benzyloxycarbonylalanylglutaminyl-S-benzylcysteine (0.55 g.) was dissolved in 1*N* HBr in AcOH (4 ml.). After 1 hr. at room temperature, dry ether was added and the resulting precipitate was collected by filtration, dried over KOH pellets *in vacuo* and dissolved in EtOH (10 ml.). The solution was neutralized with triethylamine. The precipitate formed on standing in a refrigerator was collected and recrystallized from H_2O; yield 0.14 g. (34%), m.p. 241~243°(decomp.), $[\alpha]_D^{29}$ −46.7°(c=0.2, 1*N* HCl), Rf^1 0.66, Rf^2 0.94. Amino acid ratios in an acid hydrolysate: $Ala_{1.00}$ $Glu_{1.03}$ S-benzyl-$Cys_{0.88}$ (average recovery 90%), amino acid ratios in a LAP digestion: $Ala_{1.00}$ S-benzyl-$Cys_{0.88}$ (average recovery 99%, Gluta was not determined). *Anal.* Calcd. for $C_{18}H_{26}O_5N_4S\cdot 2H_2O$: C, 50.5; H, 6.6; N, 13.1. Found: C, 50.2; H, 6.7; N, 13.2.

N^{α}-Benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine——N^{α}-Benzyloxycarbonylalanylglutaminyl-S-benzylcysteine (10.92 g.) in AcOH (30 ml.) was treated with 4.1*N* HBr in AcOH (20 ml.). After 1 hr., anhydrous ether was added and the precipitate was collected, washed with ether, and dried over KOH pellets *in vacuo*. To an ice-cold solution of this solid (Rf^1 0.66) and triethylamine (8.4 ml.) in H_2O (100 m.) was added a solution of a mixed anhydride of N^{α}-benzyloxycarbonyl-S-benzylcysteine (prepared from 13.81 g. of the acid component with 5.6 ml. of triethylamine and 3.8 ml. of ethyl chloroformate) in tetrahydrofuran (100 ml.). After stirring for 3 hr., the solvent was evaporated and the residue was acidified with 1*N* HCl. The resulting solid was collected, washed with 1*N* HCl and H_2O and recystallized from MeOH; 7.66 g. (52%), m.p. 208~210°, $[\alpha]_D^{26}$ −27.0°(c=0.7, DMF). Amino acid ratios in an acid hydrolysate $Ala_{1.00}$ $Glu_{1.02}$ (S-benzylcysteine was not determined, average recovery 99%). *Anal.* Calcd. for $C_{36}H_{43}O_8N_5S_2$: C, 58.6; H, 5.9; N, 9.5. Found: C, 58.5; H, 5.8; N, 9.6.

S-Benzylcysteinylalanylglutaminyl-S-benzylcysteine (II)——N^{α}-Benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine (0.37 g.) was dissolved in 1.4*N* HBr in AcOH (3 ml.). After 1 hr., dry ether was added and the precipitate was washed with ether, dried over KOH pellets *in vacuo* and dissolved in EtOH. The solution was neutralized with triethylamine. The precipitate formed on standing in a refrigerator was collected and recrystallized from H_2O; yield 0.16 g. (53%), m.p. 188~190°, $[\alpha]_D^{28}$ −33.3°(c=0.1, DMF), Rf^1 0.75, Rf^2 1.09. Amino acid ratios in an acid hydrolysate $Ala_{1.00}$ $Glu_{1.05}$ (S-benzylcysteine was not determined, average recevery 94%). *Anal.* Calcd. for $C_{29}H_{37}O_6N_6S_2$: C, 52.6; H, 6.8; N, 11.0. Found: C, 52.4; H, 6.8; N, 11.1.

N^{α}-Benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine Methyl Ester——N^{α}-Benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine (0.25 g.) in a mixture of DMF (2 ml.) and MeOH (10 ml.) was treated with ethereal diazomethane in the usual manner. The solvent was removed by evaporation and the residue was precipitated from DMF with ether; yield 0.22 g. (95%), m.p. 221~222°, $[\alpha]_D^{27}$ −30.8°(c=1.0, DMF). *Anal.* Calcd. for $C_{37}H_{45}O_8N_6S_2$: C, 59.1; H, 6.0; N, 9.3. Found: C, 58.8; H, 6.3; N, 9.4.

N^{α}-Benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine Hydrazide——N^{α}-Benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine methyl ester (3.76 g) was dissolved in a mixture of MeOH (100 ml.) and DMF (50 ml.). After addition of 80% hydrazine hydrate (2.4 ml.), the solution was kept at room temperature for 48 hr. The resulting precipitate was collected and reprecipitated from DMF with ether; yield 3.24 g. (86%), m.p. 236~239°, $[\alpha]_D^{28}$ −25.6° (c=0.4, DMF). *Anal.* Calcd. for $C_{36}H_{43}O_7N_5S_2$: C, 57.5; H, 6.0; N, 13.1. Found: C, 57.4; H, 6.3; N, 12.9.

N^{α},N^{ε}-Dibenzyloxycarbonyllysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine——N^{α}-Benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine (3.69 g.) in AcOH (20 ml.) was treated with 4.1*N* HBr in AcOH (7.5 ml.) for 1 hr. The precipitate formed by addition of ether was collected and dried over KOH pellets *in vacuo*. To a solution of this solid and triethylamine (2.1 ml.) in H_2O (100 ml.) was added a mixed anhydride of N^{α},N^{ε}-dibenzyloxycarbonyllysine (prepared from 4.14 g. of the acid component in 50 ml. of tetrahydrofuran with 1.4 ml. of triethylamine and 0.96 ml. of ethyl chloroformate). The mixture was stirred in an ice-bath for 3 hr., then the solvent was evaporated *in vacuo*. The residue was acidified with 1*N* HCl and the resulting solid was recrystallized from MeOH; yield 3.20 g. (64%), m.p. 195~197°, $[\alpha]_D^{25}$ −24.3°(c=0.7, DMF). Amino acid ratios in an acid hydrolysate S-benzyl-$Cys_{1.74}$ $Lys_{1.00}$ $Ala_{1.00}$ $Glu_{1.00}$ (average recovery 96%). *Anal.* Calcd. for $C_{50}H_{61}O_{11}N_7S_2$: C, 60.1; H, 6.2; N, 9.8. Found: C, 60.0; H, 6.4; N, 9.7.

N^{α},N^{ε}-Dibenzyloxycarbonyllysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine Methyl Ester——N^{α},N^{ε}-Dibenzyloxycarbonyllysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine (0.25 g.) in MeOH (20 ml.) was treated with diazomethane in the usual manner. The resulting product was recrystallized from MeOH; yield 0.18 g. (70%), m.p. 203~204°, $[\alpha]_D^{26}$ −17.0°(c=0.5, DMF). *Anal.* Calcd. for $C_{51}H_{63}O_{11}N_7S_2$: C, 60.4; H, 6.3; N, 9.7. Found: C, 60.6; H, 6.5; N, 9.9.

Lysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine Tetrahydrate (III)——N^{α},N^{ε}-Dibenzyloxycarbonyllysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine (0.50 g.) in AcOH (2 ml.) was treated with 2.7*N* HBr in AcOH (2 ml.). The precipitate formed by addition of anhydrous ether was collected and dissolved in EtOH. The solution was neutralized with triethylamine. The crystalline product formed on standing in a refrigerator was recrystallized from H_2O and acetone; yield 0.33 g. (90%), m.p. 201~206°, $[\alpha]_D^{29}$ −34.0° (c=0.4, H_2O), Rf^1 0.60, Rf^2 0.97. Amino acid ratios in an acid hydrolysate; $Lys_{1.00}$ S-benzyl-$Cys_{1.85}$ $Ala_{1.00}$ $Glu_{1.07}$ (average recovery 96%). Amino acid ratios in a LAP digestion; $Lys_{1.00}$ S-benzyl-$Cys_{2.00}$ $Ala_{1.08}$ (Gluta was not determined, average recovery 86%). *Anal.* Calcd. for $C_{34}H_{49}O_7N_7S_2\cdot 4H_2O$: C, 50.8; H, 7.2; N, 12.2. Found: C, 50.7; H, 7.0; N, 11.8.

N^{α}-Benzyloxycarbonyl-S-benzylcysteinylhistidine Hemihydrate——A mixed anhydride, prepared from N^{α}-benzyloxycarbonyl-S-benzylcysteine (9.84 g.) in tetrahydrofuran (100 ml.) with triethylamine (3.2 ml.) and ethyl chloroformate (2.9 ml.) was added to an ice-cold solution of histidine hydrochloride (8.38 g.) and triethylamine (11.2 ml.) in H_2O (100 ml.) After the mixture was stirred for 2 hr., the solvent was evaporated and the residue was acidified with AcOH to form a solid which was collected by filtration, washed with AcOEt and H_2O and recrystallized from MeOH; yield 7.80 g. (50%), m.p. 156~158°, $[\alpha]_D^{25}$ −26.6° (c=1.2, DMF). *Anal.* Calcd. for $C_{24}H_{26}O_5N_4S\cdot\frac{1}{2}H_2O$; C, 58.6; H, 5.5; N, 11.4. Found: C, 58.4; H, 6.0; N, 11.5. The hydrochloride, m.p. 156~159°. *Anal.* Calcd. for $C_{24}H_{26}O_5N_4S\cdot HCl$: C, 55.5; H, 5.6; N, 10.5. Found: C, 55.5; H, 5.6; N, 10.9.

N^{α}-Benzyloxycarbonylglutaminyl-S-benzylcysteinylhistidine Monohydrate——N^{α}-Benzyloxycarbonyl-S-benzylcysteinylhistidine (33.8 g.) in AcOH (50 ml.) was treated with 4.1*N* HBr in AcOH (85 ml.) for 1 hr. The precipitate formed by addition of anhydrous ether was collected, and dried over KOH pellets *in vacuo*. To a solution of this solid and triethylamine (29.4 ml.) in DMF (200 ml.) was added a mixed anhydride of N^{α}-benzyloxycarbonylglutamine (prepared from 29.7 g. of the acid component in 150 ml. of tetrahydrofuran and 50 ml. of DMF with 15.4 ml. of triethylamine and 10.5 ml. of ethyl chloroformate). The mixture was stirred for 3 hr., then the solvent was evaporated *in vacuo*. The residue was acidified with AcOH to form a solid, which after washing with AcOEt and H_2O, was recrystallized from MeOH; yield 31.1 g. (73%), m.p. 191~194°, $[\alpha]_D^{26}$ −22.5°(c=0.9, DMF), Rf^1 0.75, single Pauly positive spot. *Anal.* Calcd. for $C_{29}H_{34}O_7N_6S\cdot H_2O$: C, 55.4; H, 5.8; N, 13.4. Found: C, 55.7; H, 6.0; N, 13.3.

N^{α}-Benzyloxycarbonylalanylglutaminyl-S-benzylcysteinylhistidine Monohydrate——N^{α}-Benzyloxycarbonylglutaminyl-S-benzylcysteinylhislidine (3.05 g.) in AcOH (5 ml.) was treated with 4.1*N* HBr in AcOH (10 ml.) for 1 hr. The product (Rf^1 0.40) was isolated in the usual manner. To a solution of this solid in 80% DMF (50 ml.) was added triethylamine (2.8 ml.) followed by N^{α}-benzyloxycarbonylalanine *p*-nitrophenyl ester[53] (1.75 g.). After 24 hr., at room temperature, the solvent was evaporated *in vacuo* and the residue was acidified with AcOH. The resulting solid was washed with AcOEt and H_2O and recrystallized from MeOH; yield 1.93 g. (55%), m.p. 195~200°, $[\alpha]_D^{27}$ −26.7° (c=0.9, DMF). Rf^1 0.73, single Pauly positive spot. *Anal.* Calcd. for $C_{32}H_{39}O_8N_7S_2\cdot H_2O$: C, 54.9; H, 5.9; N, 14.0. Found: C, 54.6; H, 6.2; N, 13.9.

N^{α}-Benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteinylhistidine Monohydrate (IV)——a) A mixed anhydride, prepared from N^{α}-benzyloxycarbonyl-S-benzylcysteine (1.04 g.) in tetrahydrofuran

53) M. Goodman, K. C. Stueben: J. Am. Chem. Soc., **81**, 3980 (1959).

(20 ml.) with triethylamine (0.4 ml.) and ethyl chloroformate (0.3 ml.) was added to a solution of alanylglutaminyl-S-benzylcysteinylhistidine hydrobromide (prepared from 1.36 g. of the corresponding N-protected tetrapedtide by the treatment with 3 ml. of 4.1*N* HBr-AcOH) and triethylamine (0.84 ml.) in DMF (20 ml.). Stirring was continued for 3 hr., when the solvent was evaporated and the residue was acidified with AcOH. The resulting precipitate, after washing with AcOEt and H_2O, was recrystallized from MeOH; yield 0.90 g. (67%), m.p. 198~201°, $[\alpha]_D^{28}$ −30.2°(c=1.0, DMF), Rf^1 0.75, single Pauly positive spot. *Anal.* Calcd. for $C_{42}H_{50}O_9N_8S_2 \cdot H_2O$; C, 56.5; H, 5.9; N, 12.6. Found: C, 56.3; H, 6.0; N, 12.3.

b) To an ice-cold solution of N^{α}-benzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteine hydrazide (0.38 g.) in AcOH (15 ml.), 1*N* HCl (2.5 ml.) was added followed by a solution of $NaNO_2$ (0.09 g.) in H_2O (3 ml.). After 15 min., a saturated solution of NaCl (50 ml.) was added. The precipitate collected by filtration was added to a solution of histidine (0.21 g.) and triethylamine (0.14 ml.) in H_2O (15 ml.) After 48 hr., the solvent was evaporated and the residue was dissolved in 5% NH_4OH. After filtration, the filtrate was acidified with AcOH. The resulting product was collected and recrystallized from AcOH; yield 0.10 g. (24%), m.p. 188~191°.

N^{α},N^{ε}-Dibenzyloxycarbonyllysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteinylhistidine Monohydrate——A mixed anhydride prepared from N^{α},N^{ε}-dibenzyloxycarbonyllysine (0.33 g.) in tetrahydrofuran (10 ml.) with triethylamine (0.11 ml.) and ethyl chloroformate (0.08 ml.) was added to a solution of S-benzylcysteinylalanylglutaminyl-S-benzylcysteinylhisthidine hydrobromide (prepared from 0.35 g. of the corresponding N-protected pentapeptide by the treatment with 1 ml. of 4.1*N* HBr-AcOH) and triethylamine (0.17 ml.) in DMF (15 ml.). The mixture was stirred for 3 hr. The solvent was evaporated *in vacuo* and the residue was acidified with AcOH. The resulting solid was washed with AcOEt and H_2O and precipitated from DMF with H_2O; yield 0.45 g. (98%), m.p. 209~213°, $[\alpha]_D^{25.5}$ −31.1°(c=0.6, DMF). Amino acid ratios in an acid hydrolysate $Lys_{0.93}$ S-benzyl-$Cys_{1.63}$ $Ala_{0.97}$ $Glu_{1.09}$ $His_{1.00}$ (average recovery 95%). *Anal.* Calcd. for $C_{56}H_{68}O_{12}N_{10}S_2 \cdot H_2O$: C, 57.3; H, 6.2; N, 11.9. Found: C, 57.3; H, 6.4; N, 12.1.

Lysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteinylhistidine Hexahydrate (I)——N^{α},N^{ε}-Dibenzyloxycarbonyllysyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteinylhistidine (0.47 g.) in AcOH (2 ml.) was treated with 4.1*N* HBr in AcOH (2 ml.) for 1 hr. The product formed by addition of dry ether was collected, dried over KOH pellets *in vacuo* and then dissolved in EtOH. The solution was neutralized with triethylamine and the precipitate formed on standing in a refrigerator was recrystallized from H_2O and acetone; yield 0.43 g. (78%), m.p. 160~165°, $[\alpha]_D^{27}$ −37.9°(c=1.0, 1*N* HCl), Rf^1 0.78, Rf^2 1.26, single ninhydrin and Pauly positive spot. Amino acid ratios in an acid hydrolysate $Lys_{0.92}$ S-benzyl-$Cys_{1.60}$ $Ala_{1.05}$ $Glu_{1.10}$ $His_{1.00}$ (average recovery 95%). Amino acid ratios in a LAP digest $Lys_{1.17}$ S-benzyl-$Cys_{1.96}$ $Ala_{1.12}$ $His_{1.00}$ (average recovery 86%, Gluta was not determined). *Anal.* Calcd. for $C_{40}H_{56}O_8N_{10}S_2 \cdot 6H_2O$: C, 49.2; H, 7.0; N, 14.3. Found: C, 49.5; H, 6.9; N, 14.1.

The Mercuric Salts of the Synthetic Peptides——The S-benzyl groups of synthetic peptides were removed by sodium in liquid ammonia in essentially the same procedure as described by du Vigneaud, *et al.*[28] As an example, N^{α},N^{ε}-dibenzyloxycarbonyl-S-benzylcysteinylalanylglutaminyl-S-benzylcysteinylhistidine (0.57 g.) was dissolved in liquid ammonia (approximately 100 ml.) and sodium was added in small pieces with stirring until a permanent blue color remained. A few pieces of NH_4Cl was added and the ammonia was allowed to evaporate to dryness. A slow stream of nitrogen was passed on the residue to remove the last trace of ammonia. The residue was dissolved in H_2O and the pH of the solution was adjusted to 6.0 with AcOH. A solution of mercuric acetate (25%, 10 ml.) was added and the resulting precipitate was collected by centrifugation and washed with H_2O; yield 0.85 g. This excess yield is probably due to complex formation with mercuric ion with the peptide besides S-mercuric salt, since it is known that mercuric ion formes polymorphologic complex with peptides depending upon its pH of the solution.[54] This salt (50 mg.) was suspended in 50% AcOH and H_2S was passed to the suspension and the solution, after centrifugation, was evaporated to dryness. The nitroprusside test[55] was used for detection of the free sulfhydryl groups. The residue was treated with performic acid as described by Moore[45] then hydrolyzed with 6*N* HCl. The Hg content and the result of the acid hydrolysis of the treated peptides were as follows: (recovery was corrected from Hg content): peptide from I (Hg 48%), $Lys_{0.80}$ $CySO_3H_{1.87}$ $Ala_{1.00}$ $Glu_{1.00}$ $His_{0.80}$ (average recovery 91%); peptide from II (Hg 58%), $CySO_3H_{2.37}$ $Ala_{1.00}$ $Glu_{0.94}$ (93%); pertide from III (Hg 40%), $Lys_{0.83}$ $CySO_3H_{1.81}$ $Ala_{1.00}$ $Glu_{0.97}$ (92%); peptide from IV (Hg 45%), $CySO_3H_{1.96}$ $Ala_{1.00}$ $Glu_{1.00}$ $His_{0.63}$ (96%).

This work reported here was performed in collaboration with Dr. Seiyo Sano, Department of Public Health, Faculty of Medicine, Kyoto University, with support from the National Institute of Health (Grant No. GM 11793-01Al). The authors express their sincere appreciation to Prof. Shojiro Uyeo of Faculty of Pharmaceutical Sciences, Kyoto University for his encouragement during the course of this invertigation. They are also indebted to Miss Y. Kanayama, Department of Public Health, Faculty of Medicine, Kyoto University for amino acid alalysis.

54) R. Cecil, J. R. McPhee: Advances in Protein Chemistry, **14**, 255 (1959).
55) G. Toennies, J. J. Kolb: Anal. Chem., **23**, 823 (1951).