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Amino Acids and Peptides. I.[1] Synthesis of a Model Peptide related to Iron-Sulfur Protein

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A model peptide for iron-sulfur protein, Z–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–OH (III), and its cyclo derivative (IV) were synthesized by stepwise fragment condensation procedures. The deblocked (III) and (IV) formed chelate complexes with iron and sulfur.

Keywords——cysteine peptide; chelate formation; fragment condensation; iron-sulfur peptide; absorption spectrum

Iron-sulfur proteins, such as ferredoxin[3] and adrenodoxin,[4] contribute to electron transfer system and act by the formation of a chelate of cysteine residues in the protein with iron and sulfur. Examination of the amino acid sequences of iron-sulfur proteins shows that cysteine residues occupy a sequence of the form –Cys–X–Y–Cys–(X, Y; other amino acids), and at least one such sequence is found in the protein. This sequence is considered to be important for the chelate formation. For example, one suggested chelate form is shown in Fig. 1.[5] Investigation of chelate formation of iron-sulfur proteins is very interesting, but it is not easy to obtain sufficient protein from natural sources. Yajima *et al.*[6] reported the synthesis of a pentapeptide, H–Ser–Cys–Val–Ser–Cys–OH, related to clostridical ferredoxin and Sugiura *et al.*[7] reported chelate formation of that peptide with iron and sulfur. We sought to establish a synthetic procedure giving a good yield of model peptide containing the –Cys–X–Y–Cys- sequence in a facile manner, in order to investigate chelate formation. We adopted the benzyl group,[8] which is removable on exposure to sodium in liq. ammonia, as a thiol protecting group for cysteine residue during the synthesis, and synthesized the following dodecapeptide (III), as a model peptide for iron-sulfur proteins, *via* the hexapeptide (I) and nonapeptide (II).

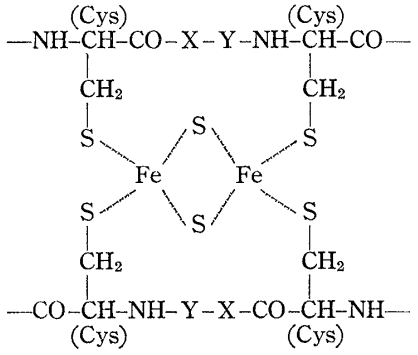

Fig. 1. A Possible Chelate Structure of Iron-Sulfur Protein

1) Amino acids, peptides and their derivatives mentioned in this paper are of the L-configuration. Abbreviations used are those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature: *Biochem.*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1976). Z=benzyloxycarbonyl, Bzl=benzyl, -ONp=*p*-nitrophenyl ester. Other abbreviations used in this paper are: DCC=dicyclohexylcarbodiimide, TFA=trifluoroacetic acid, Tris=tri(hydroxymethyl)aminomethane.

2) Location: *Arise Ikawadani-cho, Tarumi-ku, Kobe, 673, Japan.*

3) L.E. Montenson, R.C. Valentine, and J.E. Carnahan, *Biochem. Biophys. Res. Commun.*, **7**, 448 (1962).

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6) H. Yajima, N. Shirai, and Y. Kiso, *Chem. Pharm. Bull.* (Tokyo), **19**, 1900 (1971).

7) Y. Sugiura and H. Tanaka, *Biochem. Biophys. Res. Commun.*, **46**, 335 (1972).

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Z–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–OH (I)

Z–Ala–Cys(Bzl)–Gly–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–OH (II)

Z–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–OH (III)

To synthesize these peptides, stepwise fragment condensation of tripeptides was employed. Two different tripeptides, Z–Gly–Cys(Bzl)–Gly–$NHNH_2$ and Z–Ala–Cys(Bzl)–Gly–$NHNH_2$ were used for construction of the dodecapeptide (III). Gly and Ala in each fragment were used as "diagnostic amino acids" to check the purity of the synthetic peptides. The conception of a "diagnostic amino acid" was introduced by Hofmann *et al.*[9] to check the purity of a synthetic peptide synthesized by fragment condensation. The Ala: Gly ratio in a peptide synthesized by fragment condensation is used as an indication of the purity of that peptide. For example, the hexapeptide (I) contains 1 Ala and 3 Gly, the nonapeptide (II) containes 2 Ala and 4 Gly, and the dodecapeptide (III) contains 2 Ala and 6 Gly. If the synthetic peptides contain impurities derived from the carboxyl or amino component, this would be reflected in the Ala: Gly ratio of the acid hydrolysate. The carbobenzoxy derivative of an S-benzyl cysteine peptide usually shows a high *Rf* value on thin layer chromatography in various solvent systems, so checking the purity is sometimes difficult. In the present experiments, purity checking with the "diagnostic amino acids" was more effective than a thin layer chromatographic procedure. The carboxyl terminal tripeptide, Z–Ala–Cys(Bzl)–Gly–OH, was synthesized by the *p*-nitrophenyl ester method,[10] by condensation of Z–Ala–OH[11] with H–Cys(Bzl)–Gly–OH[12] derived from the corresponding Z-derivative by HBr treatment. This tripeptide was deblocked with HBr and the resulting peptide was coupled by the azide method with Z–Gly–Cys(Bzl)–Gly–$NHNH_2$, which was synthesized by the condensation of Z–Gly–OH[13] with H–Cys(Bzl)–Gly–OMe[14] using the mixed anhydride method,[15] followed by hydrazinolysis. The amino acid ratio in an acid hydrolysate of the resulting hexapeptide (I) was 1.00 Ala: 3.03 Gly. The hexapeptide (I), after deblocking with HBr, was coupled by the azide method with Z–Ala–Cys(Bzl)–Gly–$NHNH_2$, which was synthesized by the coupling of Z–Ala–OH with H–Cys(Bzl)–Gly–OMe using the mixed anhydride method, followed by hydrazinolysis. The amino acid ratio in an acid hydrolysate of the resulting nonapeptide (II) was 1.00 Ala: 2.06 Gly. The nonapeptide (II), after deblocking with HBr, was coupled with Z–Gly–Cys(Bzl)–Gly–$NHNH_2$ by the azide method to form the dodecapeptide (III). The amino acid ratio in an acid hydrolysate of the dodecapeptide (III) was 1.00 Ala: 3.06 Gly. The observed amino acid ratios in acid hydrolysates of (I), (II) and (III) suggested that these peptides were sufficiently pure, and that no significant side reaction, such as Curtius rearrangement, had occurred in the azide method.

Another synthetic route to the dodecapeptide (III), by fragment condensation of two hexapeptides, was tried. Z–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–$NHNH_2$ which was synthesized from Z–Gly–Cys(Bzl)–Gly–$NHNH_2$ and H–Ala–Cys(Bzl)–Gly–OMe by the azide method, followed by hydrazinolysis, was coupled with H–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–OH by the azide method to form the dodecapeptide (III). This route, however, gave a poorer coupling yield, and purification was not easier. Furthermore, there was no "diagnostic amino acid" for the dodecapeptide (III) synthesized by this route. The two hexapeptides, the acyl component and the amino component, had the same amino acid ratios as the dodecapeptide (III) and thus checking of the purity of the product (III) was rather difficult in this route.

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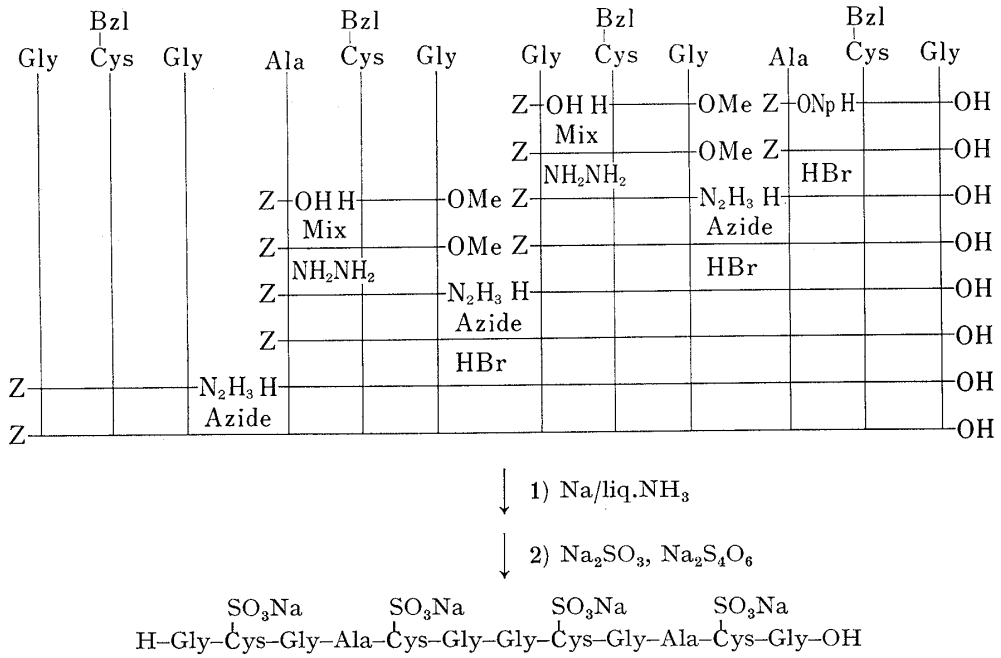

Fig. 2. Synthetic Scheme for the Dodecapeptide

Mix: mixed anhydride method. Azide: azide method.

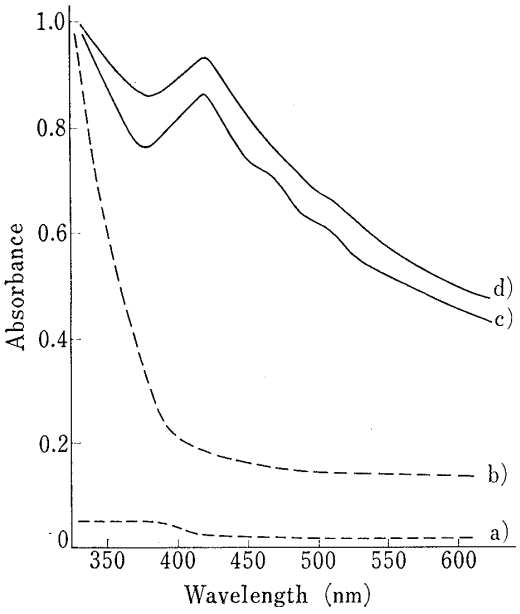

Fig. 3. Absorption Spectra of the Deblocked Dodecapeptides (III, IV) with Iron and Sulfur

a) A mixture of $HSCH_2CH_2OH$ (7×10^{-4} mol/ml), $FeCl_3$ (2.7×10^{-4} mol/ml) and Na_2S (3.8×10^{-4} mol/ml).
b) Deblocked III (5×10^{-4} mol/ml).
c) Deblocked III (4.5×10^{-6} mol/ml) with $FeCl_3$ and Na_2S.
d) Deblocked IV (1.1×10^{-6} mol/ml) with $FeCl_3$ and Na_2S.

Benzyloxycarbonyl and benzyl groups of (III) were removed with sodium in liq. ammonia and the deblocked peptide was converted to the sulfonate with sodium sulfite in the presence of sodium tetrathionate according to a procedure reported by Bailey *et al.*[16] One reason for conversion of (III) to the sulfonate was that sulfonate was removable with mercaptoethanol, which was used as a reagent for chelate formation. Therefore we expected that sulfonate could be used for chelate formation without first deblocking the sulfo group.

Chelate formation of (III) with iron and sulfur was tried according to a procedure reported by Taniguchi *et al.*[17] (III) was deblocked with sodium in liq. ammonia and the product was dissolved in a mixture of Tris buffer (pH 7.5) and mercaptoethanol. Sodium sulfide and ferric chloride were added to the solution and the mixture was kept at 0° for 2 hr. The resulting precipitate was removed by centrifugation and the absorption spectrum of the brown-colored supernatant was measured. The spectra are shown in Fig. 3. Neither the deblocked peptide dissolved in Tris buffer nor a mixture of the reagents (mercaptoethanol, sodium

16) J.L. Baily and R.D. Cole, *J. Biol. Chem.*, **234**, 1733 (1959).
17) T. Taniguchi and T. Kitamura, *Biochem.*, **14**, 5573 (1975).

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sulfide and ferric chloride) in Tris buffer showed an absorption peak around 400 nm at the concentrations used. The brown-colored solution of the deblocked peptide with sodium sulfide and ferric chloride did show an absorption peak at 420 nm, suggesting chelate formation of the peptide with iron and sulfur. Iron-sulfur proteins have absorption peaks between 300—500 nm arising from chelate formation of the protein with iron and sulfur. For example, spinach ferredoxin has absorption peaks at 325, 420 and 465 nm,[18] while *Bacillus* ferredoxin has an absorption peak at 400 nm.[18] Okada and Kawasaki[19] synthesized two heptapeptides, Leu–Ala–Cys–Ser–Thr–Cys–His and Leu–Gly–Cys–Gln–Ile–Cys–Leu, corresponding to positions 50—56 and 90—96 of bovine adrenodoxin, and reported an absorption peak of the peptides with iron and sulfur at 415 nm. Thus, the absorption peak of the deblocked (III) with iron and sulfur at 420 nm indicates chelate formation of the peptide with iron and sulfur. The absorption peak at 420 nm gradually disappeared at room temperature and the peak disappeared completely after 40 hr.

The dodecapeptide sulfonate was also tested for chelate formation, but no absorbance due to a chelate was observed. The sulfo groups of cysteine residues were not removable with mercaptoethanol under the conditions used.

In an attempt to obtain a more stable chelate compound, (III) was cyclized and the cyclic peptide (IV) was tested for chelate formation. (III) was converted to the *p*-nitrophenyl ester with DCC and *p*-nitrophenol, and the product was treated with HBr to remove Z group. The dodecapeptide *p*-nitrophenyl ester hydrobromide was cyclized in pyridine and the crude product was treated with Dowex 50 (H^+) to remove linear peptide, followed by purification by Sephadex LH-20 column chromatography.

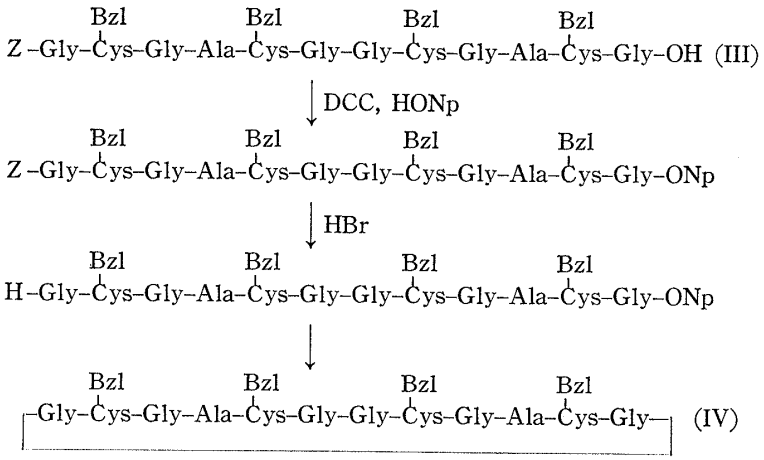

Fig. 4. Synthetic Scheme for the Cyclo Peptide

HONp: *p*-nitrophenol.

The protected cyclic peptide (IV) was quite insoluble except in DMF or DMSO, so estimation of the molecular weight of (IV) was attempted by mass spectrometry, though in the case of polypeptides there are often difficulties due to poor volatility. The mass spectrum of the cyclic peptide (IV) (molecular weight 1258) was measured with a Hitachi M-60 mass spectrometer but no peak above the *m/e* 1000 range was observed. To increase its volatility, nitrogens in amide bonds of (IV) were methylated with methyl iodide and sodium hydride according to a procedure reported by Cheung *et al.*[20] The methylated material showed *m/e*: 1424, 1351, 1324, and so on (120°, 40 e.v. ionizing voltage). The computer-controlled recorder

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19) Y. Okada, C. Kawasaki, M. Okinaka, and K. Kawasaki, *Chem. Pharm. Bull.* (Tokyo), **27**, 1360 (1979).
20) S.T. Cheung and N. Leo Benoitor, *Can. J. Chem.*, **55**, 916 (1977).

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was not corrected above the *m/e* 600 range because the standard sample (tetrameric phosphonitrilate) for the *m/e* 600—1500 range did not show good agreement with the reported spectrum.[21] M^+ 1426, which corresponds to the molecular weight of the methylated cyclic dodecapeptide, was not observed but the peak at *m/e* 1424 suggested that the cyclic peptide (IV) was the dodecapeptide, not a dimer or trimer of the dodecapeptide. Details of this and of a further investigation of the mass spectrum of the cyclic peptide will be published elsewhere.

(IV) was treated with sodium in liq. ammonia to remove benzyl groups and the resulting peptide was tested for chelate formation with iron and sulfur. An absorption similar to that of the chelate form of the linear dodecapeptide was observed and an absorption peak was also observed at 420 nm at a peptide concentration 4.48×10^{-6} mol/ml. In comparison with the linear dodecapeptide, the cyclic peptide formed a chelate at lower concentrations, but the absorption peak also disappeared at room temperature after 40 hr. Cyclization of the linear dodecapeptide (III) did not result in the formation of a more stable chelate.

We are carrying out further studies to isolate the chelate compound and to synthesize a more stable chelate compound.

Experimental

Melting points are uncorrected. Optical rotations were measured with an automatic polarimeter, model DIP-180 (Japan Spectroscopic Co., Ltd.). The amino acid compositions of acid hydrolysates and AP-M digests were determined with a JEOL JLC-6AH amino acid analyzer. Absorption spectra were recorded with a Hitachi 323 spectrophotometer. Solvents were evaporated off *in vacuo* at a temperature of 40° in a rotary evaporator. Solvent systems for ascending thin-layer chromatography on silica gel (E. Merck) are indicated as follows: Rf_1 *n*-BuOH, AcOH, H_2O (4: 1: 5, upper phase); Rf_2 *n*-BuOH, pyridine, AcOH, H_2O (4: 1: 1: 2); Rf_3 $CHCl_3$, MeOH, H_2O (8: 3: 1, lower phase) ;Rf_4 AcOEt, benzene (1: 1).

Z-Ala-Cys(Bzl)-Gly-OH——A solution of Z–Cys(Bzl)–Gly–OH[12] (4 g) in a mixture of TFA (4 ml) and anisole (0.4 ml) was treated with 25% HBr/AcOH (10 ml), and the mixture was stirred at room temperature for 45 min. Dry ether was added and the resulting precipitate was collected by filtration, washed with ether and dried. This dipeptide hydrobromide was dissolved in DMF (50 ml) and the solution was adjusted to pH 8 with Et_3N. Z–Ala–ONp[22] (10.22 g) was added and the mixture was stirred overnight at room temperature. The solvent was evaporated off and the residue was dissolved in 5% Na_2CO_3. The solution was washed 3 times with ether and acidified with conc. HCl. The resulting precipitate was collected by filtration, washed with water and dried. Recrystallized from MeOH; yield 4.2 g (90%), mp 180°, $[\alpha]_D^{26}$ −23.6° (c=1.0, MeOH), Rf_1 0.70. *Anal.* Calcd. for $C_{23}H_{27}N_3O_6S$: C, 58.1; H, 5.7; N, 8.9. Found: C, 58.3; H, 5.7; N, 8.9.

Z-Gly-Cys(Bzl)-Gly-OMe——Z–Cys(Bzl)–Gly–OMe[15] (6 g) was treated with 25% HBr/AcOH (12 ml) containing anisole (0.5 ml) at room temperature for 45 min. Petroleum ether was added and the resulting syrupy precipitate was washed 5 times with petroleum ether by decantation. After drying over KOH pellets, the material was dissolved in THF (50 ml) and the solution was adjusted to pH 8 with Et_3N.

A mixed anhydride, prepared from Z–Gly–OH[13] (5.4 g) with Et_3N (3.97 ml) and ethylchloroformate (2.71 ml) in THF (50 ml) at −10°, was added to the solution and the mixture was stirred for 3 hr. The solvent was evaporated off and the residue was dissolved in AcOEt. The AcOEt layer was washed with 0.1 N HCl, water, 5% $NaHCO_3$ and water successively, then dried over Na_2SO_4. The AcOEt was evaporated off and the residue was recrystallized from AcOEt/ether; yield 5.4 g (79%), mp 67°, $[\alpha]_D^{26}$ −29.4° (c=1.0, MeOH), Rf_3 0.75. *Anal.* Calcd. for $C_{23}H_{27}N_3O_6S$: C, 58.3; H, 5.8; N, 8.9. Found: C, 58.5; H, 5.7; N, 8.9.

Z-Gly-Cys(Bzl)-Gly-NHNH$_2$——A solution of Z–Gly–Cys(Bzl)–Gly–OMe (2 g) in MeOH (8 ml) was treated with hydrazine hydrate (0.42 ml), and the mixture was stirred at room temperature overnight. The resulting precipitate was collected by filtration and recrystallized from EtOH; yield 1.7 g (85%), mp 137—139°, $[\alpha]_D^{26}$ −24.8° (c=1.0, EtOH), Rf_3 0.71. *Anal.* Calcd. for $C_{22}H_{27}N_5O_5S$: C, 55.8; H, 5.8; N, 14.8. Found: C, 55.6; H, 5.6; N, 14.5.

Z-Ala-Cys(Bzl)-Gly-OMe——Z–Cys(Bzl)–Gly–OMe (5 g) was deblocked with HBr and coupled with Z–Ala–OH[11] (4 g) by the mixed anhydride method in the manner described for Z–Gly–Cys(Bzl)–Gly–OMe; yield 3.8 g (65%), mp 146°, $[\alpha]_D^{25}$ −33.8° (c=1.0, DMF), Rf_3 0.80. *Anal.* Calcd. for $C_{24}H_{29}N_3O_6S$: C, 59.1; H, 6.0; N, 8.6. Found: C, 59.3; H, 6.3; N, 8.5.

Z-Ala-Cys(Bzl)-Gly-NHNH$_2$——Z–Ala–Cys(Bzl)–Gly–OMe (2 g) was converted to the corresponding hydrazide in the usual manner; yield 1.5 g (75%), mp 192°, $[\alpha]_D^{25}$ −37.7° (c=1.0, DMF), Rf_3 0.48. *Anal.* Calcd. for $C_{23}H_{29}N_5O_5S$: C, 56.7; H, 6.0; N, 14.4. Found: C, 56.5; H, 6.0; N, 14.2.

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

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Z-Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly-OH (I)——A solution of Z–Ala–Cys(Bzl)–Gly–OH (4.9 g) in TFA (6 ml) containing anisole (0.3 ml) was treated with 25% HBr/AcOH (11.8 ml), and the mixture was stirred at room temperature for 45 min. Ether was added and the resulting precipitate was collected by centrifugation and dried over KOH pellets in a desiccator. This material was dissolved in DMF (10 ml) and the solution was adjusted to pH 8 with Et_3N.

Next, 7 N HCl/dioxane (5.2 ml) and *tert*-butyl nitrite (1.57 ml) were added successively to a solution of Z–Gly–Cys(Bzl)–Gly–$NHNH_2$ (5.8 g) in DMF (30 ml) at −10°, and the mixture was stirred for 15 min. The mixture was adjusted to pH 8 with Et_3N and was combined with the solution of H–Ala–Cys(Bzl)–Gly–OH described above. The reaction mixture was stirred for 48 hr in a cold room and the solvent was evaporated off. The residue was washed with 1% AcOH (3 times), water (3 times) and MeOH (4 times) in a mortar and precipitated from DMF/AcOEt; yield 6.9 g (73%), mp 215—219°, $[\alpha]_D^{26}$ −38.2° (c=1.1, DMF), Rf_1 0.71. *Anal.* Calcd. for $C_{37}H_{44}N_6O_9S_2$: C, 56.2; H, 5.7; N, 10.8. Found: C, 56.1; H, 5.7; N, 10.6. Amino acid ratio in an acid hydrolysate (6 N HCl, 24 hr): $Ala_{1.00}Gly_{3.03}$ (average recovery 89%).[23]

Z-Ala-Cys(Bzl)-Gly-Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly-OH (II)——(I) (1 g) was deblocked with HBr/AcOH in TFA in the presence of anisole. The deblocked (I) was coupled with Z–Ala–Cys(Bzl)–Gly–$NHNH_2$ (0.92 g) by the azide method and the product was purified in the manner described for (I); yield 1.2 g (66%), mp 229—230°, $[\alpha]_D^{26}$ −41.8° (c=1.0, DMF), Rf_1 0.84, Rf_2 0.86. *Anal.* Calcd. for $C_{52}H_{63}N_9O_{12}S_3$: C, 56.7; H, 5.8; N, 11.4. Found: C, 56.4; H, 5.6; N, 11.3. Amino acid ratio in an acid hydrolysate (6 N HCl, 24 hr): $Ala_{1.00}Gly_{2.06}$ (average recovery 86%).

Z-Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly-Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly-OH (III)——(II) (1 g) was deblocked with HBr/AcOH in TFA in the presence of anisole. The deblocked (II) was coupled with Z–Gly–Cys(Bzl)–Gly–$NHNH_2$ (0.64 g) by the azide method and the product was purified in the manner described for (I); yield 0.85 g (63%), mp 238—240°, $[\alpha]_D^{26}$ −33.2° (c=1.0, DMF), Rf_1 0.92, Rf_2 0.95. *Anal.* Calcd. for $C_{66}H_{80}N_{12}O_{15}S_4$: C, 56.2; H, 5.7; N, 11.9. Found: C, 56.3; H, 5.8; N, 12.1. Amino acid ratio in an acid hydrolysate (6 N HCl, 24 hr): $Ala_{1.00}Gly_{3.03}$ (average recovery 83%).

Z-Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly-OMe——Z–Ala–Cys(Bzl)–Gly–OMe (2 g) was deblocked with HBr/AcOH in the presence of anisole. The deblocked material was coupled with Z–Gly–Cys(Bzl)–Gly–$NHNH_2$ (1.9 g) by the azide method and the product was purified according to the procedure described for (I). After washing with 1% AcOH, water and AcOEt in a mortar, the material was treated with Dowex 50(H^+) in a mixture of DMF and MeOH (1:1). The solvent was evaporated off and the residue was precipitated from DMF/AcOEt; yield 2 g (63%), mp 165°, $[\alpha]_D^{26}$ −37.3° (c=1.0, DMF), Rf_1 0.83, Rf_2 0.89. *Anal.* Calcd. for $C_{38}H_{46}N_6O_9S_2$: C, 57.4; H, 5.8; N, 10.6. Found: C, 57.4; H, 5.8; N, 10.5. Amino acid ratio in an acid hydrolysate (6 N HCl, 24 hr): $Ala_{1.00}Gly_{3.00}$ (average recovery 88%).

Z-Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly-$NHNH_2$——A solution of Z–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–OMe (848 mg) in DMF (3 ml) was treated with hydrazine hydrate (0.1 ml), and the mixture was stirred at room temperature for 40 hr. Ether was added and the resulting precipitate was collected by filtration, washed with ether and dried. This was washed with water (3 times) and MeOH (3 times) in a mortar, and dried; yield 736 mg (92%), mp 189°, $[\alpha]_D^{22}$ −30.1° (c=1.0, DMSO), Rf_1 0.70, Rf_2 0.78. *Anal.* Calcd. for $C_{37}H_{46}N_8O_8S_2$: C, 55.9; H, 5.8; N, 14.1. Found: C, 55.6; H, 5.6; N, 14.3. Amino acid ratio in an acid hydrolysate (6 N HCl, 24 hr): $Ala_{1.00}Gly_{3.02}$ (average recovery 85%).

Z-Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly-Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly-OH (III)——(I) (780 mg) was deblocked with HBr/AcOH in the manner descrived for the synthesis of (II). The deblocked (I) was dissolved in DMF (4 ml) and the solution was adjusted to pH 8 with Et_3N.

Next, 7 N HCl/dioxane (0.39 ml) and *tert*-butyl nitrite (0.11 ml) were added successively to a solution of Z–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–$NHNH_2$ (730 mg) in a mixture of DMSO (1 ml) and DMF (2 ml) at −15°, and the mixture was stirred for 15 min. This azide solution was adjusted to pH 8 with Et_3N and was combined with the solution of deblocked (I) described above. The reaction mixture was stirred for 48 hr in a cold room and the solvent was evaporated off. The residue was washed with 5% AcOH (3 times), water (3 times) and MeOH (4 times) in a mortar. The material was then treated with Dowex 50(H^+) in DMF (10 ml) and the DMF was evaporated off. The residue was precipitated from DMF/AcOEt and dissolved in DMF (2 ml). The solution was applied to a Sephadex LH-20 column (1×165 cm) and the column was developed with DMF. Fractions of 2 ml were collected and were tested with H_2PtCl_6-KI reagent. Fractions 31—33 were pooled and concentrated. The residue was washed with MeOH and dried; yield 380 mg (29%), mp 237—241°, $[\alpha]_D^{26}$ −32.7° (c=1.0, DMSO), Rf_1 0.92, Rf_2 0.95. *Anal.* Calcd. for $C_{66}H_{80}N_{12}O_{15}S_4$: C, 56.2; H, 5.7; N, 11.7. Found: C, 56.4; H, 5.9; N, 11.7. Amino acid ratio in an acid hydrolysate (6 N HCl, 24 hr): $Ala_{1.00}Gly_{2.99}$ (average recovery 87%).

Z-Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly-Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly-ONp——*p*-Nitrophenol (74 mg) and DCC (95 mg) were added to a solution of (III) (500 mg) in DMF (10 ml) at −10° and the mixture was stirred for 48 hr in a cold room. The resulting precipitate was filtered off and the filtrate was concentrated. The residue was washed with MeOH and precipitated from DMF/AcOEt; yield 348 mg (65%), mp 250°, $[\alpha]_D^{26}$

23) Cys(Bzl) was not detected.

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$-34.0°$ ($c=1.0$, DMSO), Rf_1 0.91, Rf_2 0.93. *Anal.* Calcd. for $C_{72}H_{83}N_{13}O_{17}S_4$: C, 56.5; H, 5.5; N, 11.9. Found: C, 56.7; H, 5.6; N, 11.6.

Cyclo[Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly-Gly-Cys(Bzl)-Gly-Ala-Cys(Bzl)-Gly] (IV)——A solution of Z–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–Gly–Cys(Bzl)–Gly–Ala–Cys(Bzl)–Gly–ONp (100 mg) in TFA (1 ml) containing 1% anisole was treated with 25% HBr/AcOH (0.15 ml), and the mixture was stirred at room temperature for 45 min. Dry ether was added and the resulting precipitate was collected by centrifugation, washed with ether and dried over KOH pellets. This material was dissolved in DMF (10 ml) containing 1% AcOH, and the solution was added to pyridine (60 ml) at 55° over a period of 6 hr. The mixture was then stirred at 55° for 2 hr and at 30° for 36 hr. The solvent was evaporated off and the residue was washed with AcOEt and water. This material was treated with Dowex 50(H^+) in DMF and the DMF was evaporated off. The residue was dissolved in DMF (1 ml) again and the solution was applied to a Sephadex LH-20 column (1×170 cm). The column was developed with DMF and the 2 ml fractions were tested with H_2PtCl_6-KI reagent. Fractions 30—33 were pooled and concentrated. The residue was precipitated from DMF/ether; yield 30 mg (36%), mp 245°, $[\alpha]_D^{26}$ $-53.5°$ ($c=1.0$, DMSO), Rf_1 0.92, Rf_2 0.95; the ninhydrin test was negative and the H_2PtCl_6-KI test was positive. *Anal.* Calcd. for $C_{58}H_{72}N_{12}O_{12}S_4$: C, 55.4; H, 5.8; N, 13.8. Found: C, 55.2; H, 5.7; N, 13.8.

Methylation of the Cyclic Peptide (IV)——Sodium hydride (50%, 18 mg) in Bayol 85 and methyl iodide (54 mg) were added to a solution of the cyclic peptide (IV) (20 mg) in DMF (1 ml), and the mixture was stirred at 40° for 24 hr. The mixture was then applied to a Sephadex LH-20 column (1×170 cm) equilibrated with DMF. The column was eluted with DMF at a flow rate of 2 ml/15 min. Fractions of 2 ml were collected and each fraction was tested with H_2PtCl_6-KI reagent. Fractions 30—32 were pooled and concentrated. The residue was precipitated from DMF/ether; 13 mg (57%), mp 152°, Rf_1 0.95, 0.00 (trace), Rf_2 0.95, 0.00 (trace), $[\alpha]_D^{22}$ $-125.0°$ ($c=0.4$, DMF). *Anal.* Calcd. for $C_{70}H_{93}N_{12}O_{12}S_4$: C, 59.0; H, 6.8; N,11.8. Found: C, 58.6; H, 6.5; N, 11.5.

H-Gly-Cys(SO_3Na)-Gly-Ala-Cys(SO_3Na)-Gly-Gly-Cys(SO_3Na)-Gly-Ala-Cys(SO_3Na)-Gly-OH——A solution of the protected dodecapeptide (III) (100 mg) in liq. ammonia (100 ml) was treated with sodium at the boiling point of ammonia until the solution remained blue for 5 sec. The blue color was discharged with ammonium chloride and the ammonia was evaporated off. The residue was dried in a desiccator and dissolved in 0.2 M Tris buffer (pH 9.4, 2 ml). Sodium sulfite (314 mg) and sodium tetrathionate (100 mg) were added and the mixture was stirred at 37° for 24 hr. The mixture was then applied to a Sephadex G-25 column (1×170 cm) and the column was developed with 10% AcOH. Fractions of 2 g were collected and each fraction was tested with ninhydrin. Fractions 40—43 were pooled and lyophilized. The resulting fluffy powder was dissolved in water (1.5 ml) and the solution was applied to a Sephadex G-25 column (1×170 cm) equilibrated with water. Fractions of 2 ml were collected and tested with ninhydrin. Fractions 40—43 were pooled and lyophilized; yield 65 mg (74%), Fluffy hygroscopic powder, $[\alpha]_D^{26}$ $-32.7°$ ($c=1.0$, H_2O), Rf_1 0.05, Rf_2 0.20. Amino acid ratios in an aminopeptidase-M digest[24] (40 hr): $Ala_{1.00}Gly_{2.94}Cys(SO_3Na)_{1.70}$[25] (average recovery 71%).

Chelate Formation——The protected dodecapeptide (III) (100 mg) was deblocked with sodium in liq. ammonia in the manner described above. After the removal of ammonia, the residue was dissolved in a mixture of 10 mM Tris buffer (pH 7.5, 4 ml) and mercaptoethanol (0.22 ml) and the mixture was kept at room temperature for 30 min. Sodium sulfide nonahydrate (367 mg) and ferric chloride hexahydrate (289 mg) were added and the mixture was kept at 0° for 2 hr. The precipitate was removed by centrifugation and the supernatant was used for measurement of the absorption spectrum. The result is shown in Fig. 3. Chelate formation with the cyclic dodecapeptide (IV) was done in the same manner. The concentrations of the peptides were determined as follows. A definite volume of the brown-colored supernatant was taken and concentrated. The residue was hydrolyzed with HCl and the hydrolysate was analyzed. From the results, the peptide concentration was calculated.

24) K. Hofmann, F.M. Finn M. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).
25) Cysteic acid was used as a standard for calculation.

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