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Synthesis of a Tetradecapeptide corresponding to Sequence 50—63 of Adrenodoxin from Bovine Adrenal Cortex and Formation of Its Iron-Sulfur Complex[1)]

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A tetradecapeptide, Z–Leu–Ala–Cys(Bzl)–Ser–Thr–Cys(Bzl)–His–Leu–Ile–Phe–Glu–Gln–His–Ile–OH (A) corresponding to sequence 50—63 of adrenodoxin from bovine adrenal cortex was synthesized. The deblocked peptide obtained from A formed an iron-sulfur complex which showed a broad peak at 418 nm.

Keywords——synthesis of tetradecapeptide; partial structure of adrenodoxin; Na in liquid ammonia; iron-sulfur complex; absorption spectra

Adrenodoxin, one of the non-heme iron-sulfur proteins, is a component of the electron transport system in the adrenal cortex. The structure of the iron-sulfur center in the enzyme was elucidated by Tanaka *et al.*,[3)] as shown in Fig. 1.

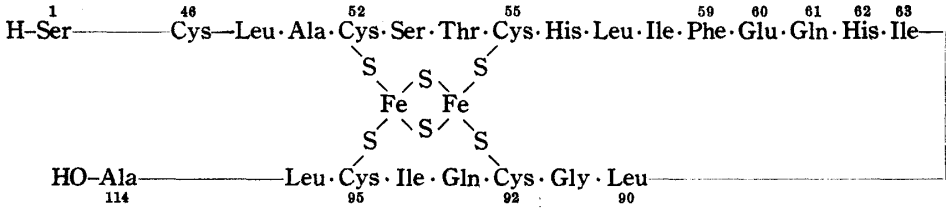

Fig. 1. Possible Chelate Structure of Bovine Adrenodoxin

In our laboratory, the synthesis of cysteine-containing peptides related to adrenodoxin is under way with the aim of synthesizing the active center of that enzyme. In our previous

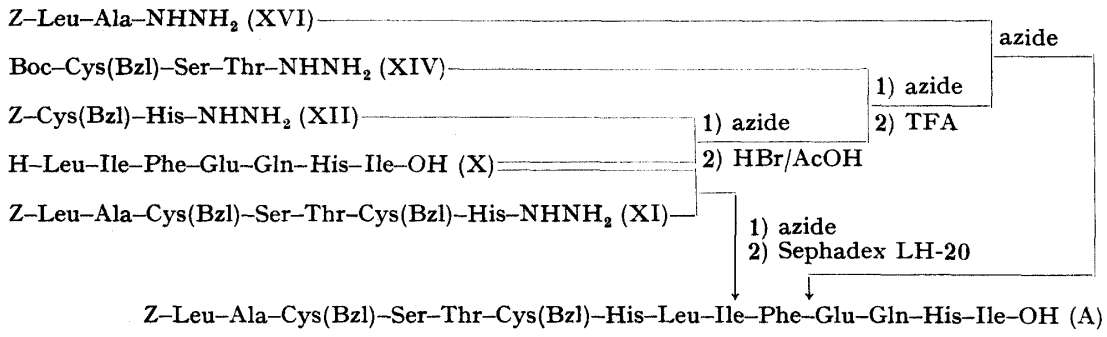

Fig. 2. Synthetic Scheme for the Protected Tetradecapeptide (A)

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: *Biochemistry*, **5**, 2485 (1966); *ibid.*, **6**, 362 (1967); *ibid.*, **11**, 1726 (1972). Z=benzyloxycarbonyl, *t*-Boc=*tert*-butyloxycarbonyl, OBu^t=*tert*-butyl ester, Bzl=benzyl, ONp=*p*-nitrophenyl ester, TPP=triphenylphosphite.

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

3) M. Tanaka, M. Haniu, and K.T. Yasunobu, *J. Biol. Chem.*, **248**, 1141 (1973).

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paper,[4] we reported the synthesis of two heptapeptides corresponding to sequences 50—56 and 90—96 of adrenodoxin (see Fig. 1) and the formation of their iron-sulfur complexes, both of which showed absorption maxima at 415 nm due to the iron-sulfur chromophore.

The present report describes the synthesis of a tetradecapeptide, Z–Leu–Ala–Cys(Bzl)–Ser–Thr–Cys(Bzl)–His–Leu–Ile–Phe–Glu–Gln–His–Ile–OH (A), corresponding to sequence 50—63 of adrenodoxin and the formation of an iron-sulfur complex with deblocked A. The synthetic scheme for A is illustrated in Fig. 2. The benzyl group, removable on exposure to sodium in liquid ammonia,[5] was selected as a protecting group for the sulfhydryl group of cysteine and the Z or *t*-Boc group was used as the α-amino protecting group for amino acids employed. A was synthesized by two different methods. One was azide coupling[6] of the C-terminal heptapeptide, H–Leu–Ile–Phe–Glu–Gln–His–Ile–OH (X), and Z–Leu–Ala–Cys(Bzl)–Ser–Thr–Cys(Bzl)–His–$NHNH_2$ (XI), which was derived from Z–Leu–Ala–Cys(Bzl)–Ser–Thr–Cys(Bzl)–His–OH[4] by hydrazine hydrate treatment through its methyl ester. The other was azide coupling of X with relatively small peptides, Z–Cys(Bzl)–His–$NHNH_2$ (XII),

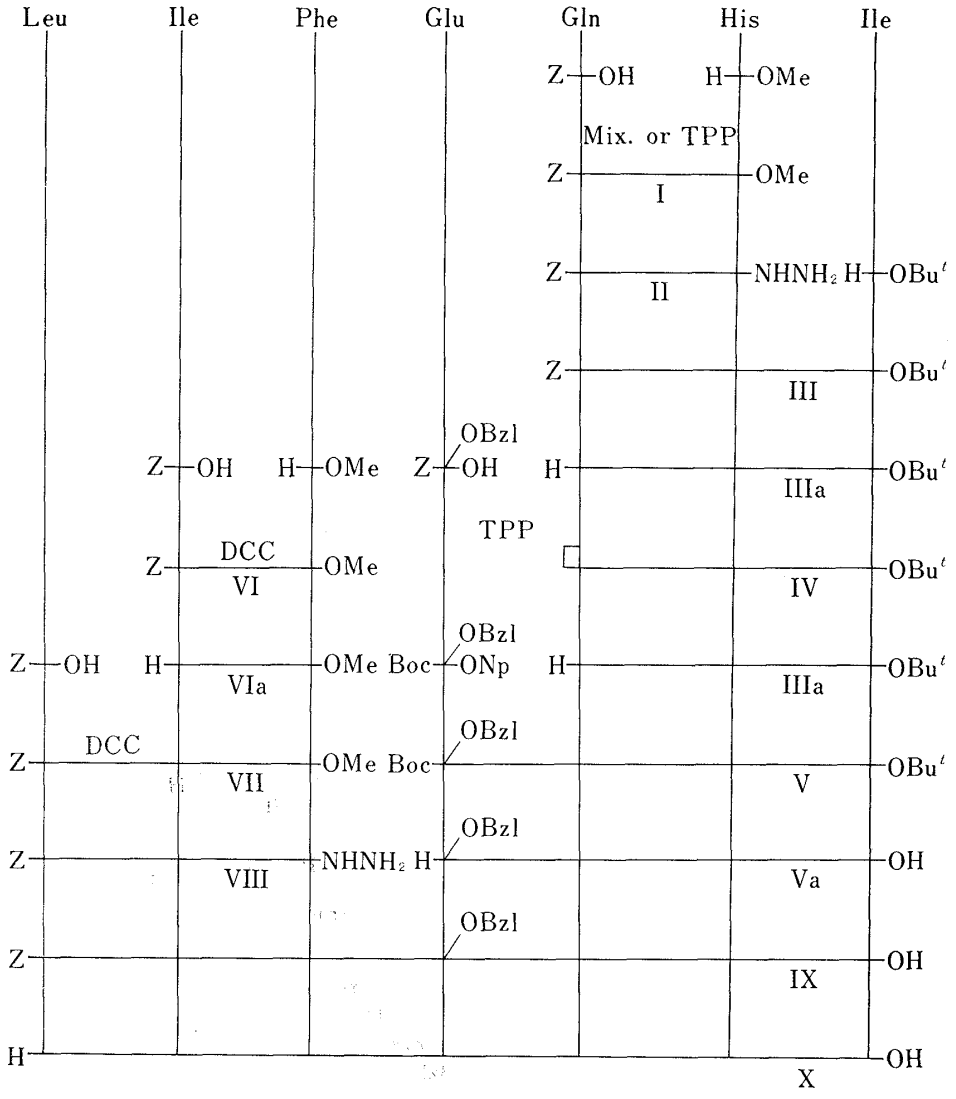

Fig. 3. Synthetic Route to the Heptapeptide (X)

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t-Boc–Cys(Bzl)–Ser–Thr–$NHNH_2$ (XIV)[4] and Z–Leu–Ala–$NHNH_2$ (XVI),[4] as shown in Fig. 2. These relatively small acylating agents, used in excess, could be readily removed by washing with organic solvent.

The synthetic route to X is illustrated in Fig. 3. Z–Gln–OH[7] and H–His–OMe[8] were coupled by a mixed anhydride procedure[9] or by the triphenylphosphite method[10] to give Z–Gln–His–OMe (I). I was converted to the corresponding hydrazide (II) by treatment with hydrazine hydrate in methanol. II was coupled with H–Ile–OBu^t [11] by the azide method to afford Z–Gln–His–Ile–OBu^t (III). Removal of the Z group of III by catalytic hydrogenation produced an oily tripeptide ester (IIIa). We attempted to couple *t*-Boc–Glu-(OBzl)–OH and IIIa by two different methods. First, a solution of *t*-Boc–Glu–(OBzl)–OH,[12] IIIa, triphenylphosphite and imidazole in DMF was stirred overnight at 40°. The main product was Pyr–His–Ile–OBu^t (IV) together with a small amount of the desired tetrapeptide, *t*-Boc–Glu(OBzl)–Gln–His–Ile–OBu^t (V). Next, *t*-Boc–Glu(OBzl)–ONp[13] was reacted with IIIa to afford V in good yield, with a trace of IV. Treatment of V with trifluoroacetic acid (TFA) cleaved the amino and carboxyl protecting groups at the same time to give H–Glu-(OBzl)–Gln–His–Ile–OH (Va). Z–Ile–OH[7] and H–Phe–OMe[14] were coupled by the DCC coupling method to give Z–Ile–Phe–OMe (VI), which was converted to the dipeptide amine (VIa) by catalytic hydrogenation. Z–Leu–OH[15] and VIa were coupled by the DCC procedure to afford Z–Leu–Ile–Phe–OMe (VII). VII was converted to the corresponding hydrazide (VIII) in the usual manner. Azide reaction between Va and VIII gave Z–Leu–Ile–Phe–Glu-(OBzl)–Gln–His–Ile–OH (IX). IX was hydrogenated over a palladium catalyst in 20% AcOH to afford H–Leu–Ile–Phe–Glu–Gln–His–Ile–OH (X).

X was coupled with Z–Leu–Ala–Cys(Bzl)–Ser–Thr–Cys(Bzl)–His–$NHNH_2$ (XI) by the azide procedure. Using this method, it was difficult to obtain the desired tetradecapeptide (A) in good yield because A and the starting materials were quite insoluble in DMF, and only a small amount of A could be isolated in a pure form by repeated gel-filtration on Sephadex LH-20 using DMF due to the overlapping of A and the starting materials in the eluate. Construction of A by condensation of X with relatively small peptides, XII, XIV and XVI gave fairly good results. Z–Cys(Bzl)–OH and H–His–OMe were coupled by the DCC or mixed anhydride method to give Z–Cys(Bzl)–His–OMe, which was converted to the corresponding hydrazide (XII). XII was coupled with X by the azide procedure to afford Z–Cys(Bzl)–His–Leu–Ile–Phe–Glu–Gln–His–Ile–OH (XIII) in good yield. Treatment of XIII with hydrogen bromide in acetic acid cleaved the N-protecting group and furnished the corresponding hydrobromide salt (XIIIa). *t*-Boc–Cys(Bzl)–Ser–Thr–$NHNH_2$ (XIV) and XIIIa were joined by the azide procedure to give *t*-Boc–Cys(Bzl)–Ser–Thr–Cys(Bzl)–His–Leu–Ile–Phe–Glu–Gln–His–Ile–OH (XV). TFA treatment of XV eliminated the N-protecting group and generated the corresponding trifluoroacetate salt (XVa). Finally, Z–Leu–Ala–$NHNH_2$ (XVI) and XVa were coupled to give A. This material was identical with the tetradecapeptide obtained by the coupling of X and XI (melting point, *Rf* values on thin layer chromatography and optical rotation).

To check the optical purity of the constituent amino acids of A, it was converted to the corresponding S-sulfonated derivative by reduction with sodium in liquid ammonia and oxidative sulfitolysis, then purified by gel-filtration on Sephadex G-25 using 5% AcOH.

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Digestion of the S-sulfonated tetradecapeptide with APM[16] followed by amino acid analysis gave molar ratios in good agreement with the theoretically expected values. It thus appeared that the synthetic chain was completely digested by the enzyme, indicating that the configuration of the constituent amino acids was retained during the synthetic process.

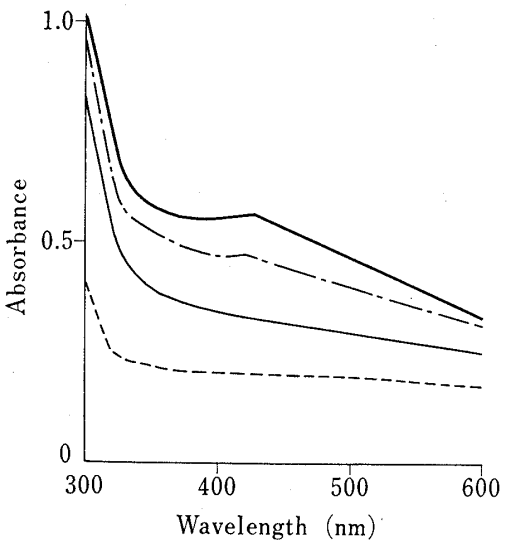

Fig. 4. Absorption Spectra of the Iron-Sulfur Complex of the Deblocked Peptide Obtained from A in 10 mM Tris buffer (pH 7.5)

———, deblocked A (50 mg, 0.026 mM), mercaptoethanol (41.3 mg, 0.53 mM), Na_2S (0.20 mM), and $FeCl_3$ (0.13 mM), 0°, 2 hr. ——, 0°, 3 hr. ——, 0°, 15 hr. ------, mercaptoethanol (41.3 mg, 0.53 mM), Na_2S (0.20 mM), $FeCl_3$ (0.13 mM).

An attempt to form an iron-sulfur complex with deblocked A was made as described previously.[4] Namely, A was treated with sodium in liquid ammonia. After removal of the ammonia, the residue was mixed at 0° with Na_2S and $FeCl_3$ in 10 mM Tris buffer (pH 7.5) containing mercaptoethanol. After 2 hr, the mixture was centrifuged. Absorption spectra of the brown-colored supernatant are shown in Fig. 4. In our previous paper,[4] we reported that the iron-sulfur complexes formed from cysteine-containing heptapeptides showed absorption maxima at 415 nm due to the iron-sulfur chromophore and that this peak was still observed after 24 hr at 0°. The brown-colored supernatant obtained in the present study showed a broad peak at around 418 nm which might be due to the iron-sulfur chromophore.[17] After 15 hr at 0°, this peak disappeared. We deduce that the conformation of the tetradecapeptide is less suitable for the formation of iron-sulfur complex than those of the heptapeptides reported previously,[4] and that the C-terminal portion of the tetradecapeptide destabilized the complex in some way relative to the complexes of the heptapeptides.

Experimental

General experimental methods employed here were essentially the same as those described in the previous paper[4] of this series. Thin layer chromatography was performed on silica gel (Kieselgel G, Merck). Rf^1 and Rf^2 values refer to the systems of *n*-butanol, AcOH and H_2O (4: 1: 5), and *n*-butanol, pyridine, AcOH and H_2O (4: 1: 1: 2), respectively.

Z-Gln-His-OMe (I)——(a) Mixed Anhydride: A mixed anhydride prepared from Z–Gln–OH (5.6 g) with triethylamine (2.8 ml) and ethyl chloroformate (1.9 ml) at −10° in tetrahydrofuran (THF) (40 ml) and dioxane (40 ml) was added to a cold solution of H–His–OMe (prepared from 4.8 g of H–His–OMe·2HCl and 5.6 ml of triethylamine) in N,N-dimethylformamide (DMF) (20 ml). After stirring in an ice-bath for 3 hr, the solvent was removed and the residue was dissolved in 5% Na_2CO_3. The solution was stored in a refrigerator overnight and the precipitate formed was collected by filtration and recrystallized from MeOH; yield 8.0 g (92%), mp 176—178°, $[\alpha]_D^{23}$ −10.7° (c=1.0, MeOH), Rf^1 0.57, Rf^2 0.72. *Anal.* Calcd. for $C_{20}H_{25}N_5O_6$: C, 55.7; H, 5.84; N, 16.2. Found: C, 55.5; H, 5.69; N, 16.1.

(b) Triphenylphosphite: Z–Gln–OH (5.6 g), H–His–OMe (prepared from 4.8 g of H–His–OMe·2HCl and 5.6 ml of triethylamine), triphenylphosphite (9.9 g) and imidazole (2.2 g) were dissolved in DMF (30 ml). The reaction mixture was stirred at 40° for 18 hr. After removal of the solvent, the residue was dissolved in 5% Na_2CO_3 (30 ml) and AcOEt (30 ml). After storage in a refrigerator overnight, the precipitate was collected by filtration and recrystallized from MeOH; yield 6.8 g (79%), mp 178—180°, $[\alpha]_D^{23}$ −11.0° (c=1.0, MeOH), Rf^1 0.57, Rf^2 0.72. *Anal.* Calcd. for $C_{20}H_{25}N_5O_6$: C, 55.7; H, 5.84; N, 16.2. Found: C, 55.4; H, 5.87; N, 16.0.

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Z-Gln-His-NHNH$_2$ (II)——Hydrazine hydrate (80%, 8.2 ml) was added to a solution of I (4.5 g) in MeOH (80 ml). After 10 hr at room temperature, the solvent was removed and the residue was crystallized from EtOH. The crystalline material was collected by filtration, washed with EtOH and ether and recrystallized from MeOH; yield 4.0 g (88.9%), mp 189—190°, $[\alpha]_D^{23}$ −6.9° (c=1.0, DMSO), Rf^1 0.29, Rf^2 0.64. *Anal.* Calcd. for $C_{19}H_{25}N_7O_5$: C, 52.9; H, 5.84; N, 22.7. Found: C, 52.6; H, 5.78; N, 22.5.

Z-Gln-His-Ile-OBu^t (III)——The entire operation was carried out in a cold room at 4°. First, 7 N HCl in dioxane (3 ml), followed by isoamylnitrite (1.34 ml), was added to a solution of II (4.0 g) in DMF (20 ml) cooled to −15°. After 5 min, triethylamine (2.8 ml) was added. This solution was added to a cold solution of H–Ile–OBu^t (prepared from 3.0 g of Z–Ile–OBu^t by hydrogenolysis over a palladium catalyst) in DMF (10 ml). The reaction mixture was stirred for 48 hr and evaporated to dryness. The residue was dissolved in AcOEt (30 ml) and 5% Na_2CO_3 (30 ml). After 18 hr at 0°, the gelatinous precipitate was collected by filtration and recrystallized from MeOH and AcOEt; yield 4.4 g (80%), mp 189—191°, $[\alpha]_D^{23}$ −33.4° (c=1.0, MeOH), Rf^1 0.61. *Anal.* Calcd. for $C_{29}H_{42}N_6O_7 \cdot H_2O$: C, 57.6; H, 7.33; N, 13.9. Found: C, 57.3; H, 7.12; N, 13.8.

Reaction of *t*-Boc-Glu(OBzl)-OH and H-Gln-His-Ile-OBu^t——(a) Triphenylphosphite: H–Gln–His–Ile–OBu^t (IIIa) (prepared from 1.0 g of III by hydrogenolysis over a palladium catalyst, Rf^1 0.29, ninhydrin and Pauly stain), *t*-Boc–Glu(OBzl)–OH (0.6 g), imidazole (0.18 g) and triphenylphosphite (0.82 g) were dissolved in DMF (6 ml). The reaction mixture was stirred at 40° for 18 hr. After removal of the solvent, the oily residue was washed with ether 3 times and applied to a silica gel column (2.5×17 cm), which was eluted with $CHCl_3$ (500 ml), 5% MeOH in $CHCl_3$ (1000 ml) and then 10% MeOH in $CHCl_3$ (500 ml). A small amount of tetrapeptide, *t*-Boc–Glu(OBzl)–Gln–His–Ile–OBu^t (V) was eluted with 5% MeOH in $CHCl_3$, mp 135—136°, Rf^1 0.68. With 10% MeOH in $CHCl_3$, Pyr–His–Ile–OBu^t (IV) was obtained; yield 0.5 g (65%), mp 203—204°, $[\alpha]_D^{23}$ −20.4° (c=1.0, MeOH), Rf^1 0.40. *Anal.* Calcd. for $C_{21}H_{33}N_5O_5$: C, 57.9; H, 7.64; N, 16.1. Found: C, 58.0; H, 7.77; N, 15.8, Amino acid ratios in an acid hydrolysate: Glu 1.1; His 1.0; Ile 1.0 (average recovery 85%).

(b) *p*-Nitrophenyl Ester Method: IIIa (prepared from 1.5 g of III by hydrogenolysis over a palladium catalyst) and *t*-Boc–Glu(OBzl)–ONp (1.24 g) were mixed in DMF (15 ml) and the solution was stirred at room temperature overnight. The solvent was removed by evaporation and the residue was extracted with AcOEt. The extract was washed with 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. Ether was added to the residue to give Z–Glu(OBzl)–Gln–His–Ile–OBu^t (V) in crystalline form, which was collected by filtration and recrystallized from AcOEt and ether; yield 1.6 g (80%), mp 143—145°, $[\alpha]_D^{23}$ −33.0° (c=1.0, MeOH), Rf^1 0.68. *Anal.* Calcd. for $C_{38}H_{57}N_7O_{10}$: C, 59.1; H, 7.44; N, 12.7. Found: C, 59.0; H, 7.67; N, 12.6, Amino acid ratios in an acid hydrolysate: Glu 1.9; His 0.8; Ile 1.0 (average recovery 85%).

Z-Ile-Phe-OMe (VI)——N,N′-Dicyclohexylcarbodiimide (DCC) (7.4 g) was added to a cold solution of H–Phe–OMe (prepared from 6.45 g of H–Phe–OMe·HCl and 4.2 ml of triethylamine) and Z–Ile–OH (7.95 g) in CH_3CN (50 ml). The reaction mixture was stirred at room temperature overnight. After removal of urea derivative and the solvent, the residue was extracted with AcOEt. The extract was washed successively with 1 N HCl, 5% Na_2CO_3 and water, dried over Na_2SO_4 and concentrated. Ether was added to the residue to afford a white crystalline material, which was collected by filtration and recrystallized from AcOEt and ether; yield 8.9 g (69.6%), mp 145—146°, $[\alpha]_D^{23}$ −28.8° (c=1.0, MeOH). *Anal.* Calcd. for $C_{24}H_{30}N_2O_5$: C, 67.6; H, 7.09; N, 6.6. Found: C, 67.8; H, 7.38; N, 6.8.

Z-Leu-Ile-Phe-OMe (VII)——A cold solution of H–Ile–Phe–OMe (VIa) (prepared from 2.13 g of VI by hydrogenolysis over a palladium catalyst) and Z–Leu–OH (1.35 g) in DMF (30 ml) was treated with DCC (1.24 g). The reaction mixture was stirred at room temperature overnight. Urea derivative was filtered off and the filtrate was condensed. AcOEt and water were added to the residue to form a precipitate, which was collected by filtration and washed with AcOEt; yield 2.1 g (77.8%), mp 178—180°, $[\alpha]_D^{23}$ −22.5° (c=1.0, MeOH). *Anal.* Calcd. for $C_{30}H_{41}N_3O_6$: C, 66.8; H, 7.66; N, 7.8. Found: C, 66.6; H, 7.95; N, 8.1.

Z-Leu-Ile-Phe-NHNH$_2$ (VIII)——Hydrazine hydrate (80%, 2.2 ml) was added to a solution of VII (2.0 g) in MeOH (10 ml) and DMF (10 ml). The solution was kept at room temperature overnight. After removal of the solvent, MeOH was added to form a white crystalline material, which was collected by filtration and washed with MeOH; yield 1.5 g (75%), mp 227—228°, $[\alpha]_D^{23}$ −21.0° (c=1.0, DMF). *Anal.* Calcd. for $C_{29}H_{41}N_5O_5$: C, 64.5; H, 7.66; N, 13.0. Found: C, 64.1; H, 7.59; N, 12.7, Amino acid ratios in an acid hydrolysate: Leu 1.1; Ile 1.0; Phe 1.0 (average recovery 90%).

Z-Leu-Ile-Phe-Glu(OBzl)-Gln-His-Ile-OH (IX)——A solution of *t*-Boc–Glu(OBzl)–Gln–His–Ile–OBu^t (V) (1.54 g) in trifluoroacetic acid (TFA) (10 ml) containing 1 ml of anisole was stored at room temperature for 1 hr. Addition of ether gave a white precipitate (Va) (Rf^1 0.10), which was collected by filtration, washed with ether and dried over KOH pellets. Va was dissolved in DMF (10 ml) and the pH of the solution was adjusted to 8 with triethylamine (0.3 ml). Z–Leu–Ile–Phe–N_3, prepared as follows, was added to this cold solution. A solution of VIII (1.08 g) in DMF (4 ml) and DMSO (4 ml) cooled to −15° was treated with 1 N HCl in DMF (4 ml), followed by isoamylnitrite (0.27 ml). After 5 min, triethylamine (0.6 ml) was added. The solution was mixed with the solution of Va prepared above. The reaction mixture was stirred at 4° for 48 hr and the solvent was removed by evaporation. AcOEt was added to the residue to give a precipitate, which was collected by filtration, washed with AcOEt, 1% AcOH and water and dried; yield 1.5 g (67.4%),

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mp 238—240° (dec.), $[\alpha]_D^{23}$ −14.6° (c=1.0, DMF), Rf^1 0.68. *Anal.* Calcd. for $C_{58}H_{78}N_{10}O_{13}\cdot 3H_2O$: C, 59.2; H, 7.19; N, 11.9. Found: C, 59.3; H, 6.82; N, 11.9, Amino acid ratios in an acid hydrolysate: Leu 1.0; Ile 2.0; Phe 1.0; Glu 1.9; His 0.9 (average recovery 84%).

H-Leu-Ile-Phe-Glu-Gln-His-Ile-OH (X)——IX (4.0 g) in 20% AcOH (40 ml) was hydrogenated over a palladium catalyst. After removal of palladium and the solvent, the residue was dissolved in water and lyophilized to give a fluffy powder; yield 3.2 g (100%), $[\alpha]_D^{23}$ −28.2° (c=1.0, 50% AcOH), Rf^1 0.46, Rf^2 0.69. *Anal.* Calcd. for $C_{53}H_{66}N_{10}O_{11}\cdot 4H_2O$: C, 53.2; H, 7.67; N, 14.4. Found: C, 53.3; H, 7.67; N, 14.0, Amino acid ratios in an acid hydrolysate: Leu 1.0; Ile 2.2; Phe 1.0; Glu 2.2; His 0.9 (average recovery 87%).

Z-Leu-Ala-Cys(Bzl)-Ser-Thr-Cys(Bzl)-His-NHNH$_2$ (XI)——Z–Leu–Ala–Cys(Bzl)–Ser–Thr–Cys(Bzl)–His–OH[4] (100 mg) was dissolved in DMF (4 ml), and an ethereal solution of diazomethane (prepared from 1 g of nitrosomethylurea) was added. This reaction mixture was stored at 0° for 2 hr. After removal of excess diazomethane and the solvent, the residue was dissolved in DMF (2 ml) and hydrazine hydrate (80%, 0.2 ml) was added. This solution was allowed to stand at room temperature overnight. After removal of the solvent, water was added and the resulting powder was collected by filtration, washed with water and dried; yield 60 mg (60%), mp 210—220° (dec.), $[\alpha]_D^{23}$ −20.7° (c=1.0, DMSO), Rf^1 0.74. *Anal.* Calcd. for $C_{50}H_{67}N_{11}O_{11}S_2\cdot H_2O$: C, 55.6; H, 6.44; N, 14.3. Found: C, 55.6; H, 6.25; N, 14.2, Amino acid ratios in an acid hydrolysate: Leu 1.1; Ala 1.1; Ser 1.0; Thr 0.9; His 0.9 (average recovery 80%).

Z-Cys(Bzl)-His-OMe——(a) Mixed Anhydride: A cold solution of H–His–OMe (prepared from 6.8 g of H–His–OMe·2HCl and 7.84 ml of triethylamine) in DMF (30 ml) was reacted with a mixed anhydride prepared from 9.5 g of Z–Cys(Bzl)–OH with 3.92 ml of triethylamine and 2.69 ml of ethyl chloroformate at −10° in THF (40 ml). The mixture was stirred at 0° for 3 hr. After removal of the solvent, the residue was dissolved in AcOEt (30 ml) and 5% Na_2CO_3 (30 ml). The solution was stored overnight at 0° to form a gelatinous solid, which was collected by filtration and recrystallized from AcOEt and ether; yield 7.0 g (50.3%), mp 118—119°, $[\alpha]_D^{23}$ −30.1° (c=1.0, MeOH), Rf^1 0.69, Rf^2 0.80. *Anal.* Calcd. for $C_{25}H_{28}N_4O_5S$: C, 60.5; H, 5.68; N, 11.3. Found: C, 60.3; H, 5.64; N, 10.9.

(b) N,N′-Dicyclohexylcarbodiimide: Z–Cys(Bzl)–OH (3.5 g) and H–His–OMe (prepared from 2.42 g of H–His–OMe·2HCl and 2.8 ml of triethylamine) were dissolved in DMF (20 ml) and the solution was cooled to −10°. DCC (2.47 g) was added and the resulting mixture was stirred overnight, while slowly warming it to room temperature. Urea derivative was filtered off and the filtrate was condensed. The residue was dissolved in AcOEt, then the solution was washed with 5% Na_2CO_3, 5% AcOH and water, dried over Na_2SO_4 and concentrated. Ether was added to the residue to give a solid material, which was collected by filtration and recrystallized from AcOEt and ether; yield 2.5 g (50%), mp 120—121°, $[\alpha]_D^{23}$ −31.8° (c=1.0, MeOH), Rf^1 0.69, Rf^2 0.80. *Anal.* Calcd. for $C_{25}H_{28}N_4O_5S$: C, 60.5; H, 5.68; N, 11.3. Found: C, 61.0; H, 5.71; N, 11.0.

Z-Cys(Bzl)-His-NHNH$_2$ (XII)——Hydrazine hydrate (80%, 5.0 ml) was added to a solution of Z–Cys(Bzl)–His-OMe (4.3 g) in MeOH (30 ml). The solution was kept at room temperature overnight, and the resulting white crystalline material was collected by filtration, washed with MeOH and recrystallized from MeOH; yield 3.8 g (83.7%), mp 175—177°, $[\alpha]_D^{23}$ −28.7° (c=1.0, DMF). *Anal.* Calcd. for $C_{24}H_{28}N_6O_4S$: C, 58.1; H, 5.68; N, 16.9. Found: C, 58.0; H, 5.66; N, 16.9.

Z-Cys(Bzl)-His-Leu-Ile-Phe-Glu-Gln-His-Ile-OH (XIII)——Z–Cys(Bzl)–His–N_3 prepared as follows was added to a cold solution of X (320 mg) in 50% aqueous DMF (4 ml) containing 0.1 ml of triethylamine. A cold solution of XII (370 mg) in DMF (6 ml) cooled to −15° was treated with 5.5 N HCl in dioxane (0.26 ml), followed by isoamylnitrite (0.10 ml). After 5 min, triethylamine (0.20 ml) was added. This solution was added to a cold solution of X prepared as described above. This reaction mixture was stirred at 4° for 48 hr. After neutralization of the mixture with AcOH, the solvent was removed by evaporation. AcOEt was added to the residue to form a precipitate, which was collected by filtration, washed with AcOEt and water and dried; yield 280 mg (57%), mp 215—220° (dec.), $[\alpha]_D^{23}$ −24.9° (c=1.0, DMF), Rf^1 0.72, Rf^2 0.83. *Anal.* Calcd. for $C_{67}H_{90}N_{14}O_{15}S\cdot 4H_2O$: C, 56.1; H, 6.88; N, 13.7. Found: C, 56.0; H, 6.56; N, 13.5, Amino acid ratios in an acid hydrolysate: His 1.7; Leu 0.8; Ile 2.0; Phe 0.8; Glu 2.1 (average recovery 75%).

***t*-Boc-Cys(Bzl)-Ser-Thr-Cys(Bzl)-His-Leu-Ile-Phe-Glu-Gln-His-Ile-OH (XV)**——XIII (930 mg) was dissolved in AcOH (10 ml), and 20 ml of 25% HBr/AcOH was added. This solution was kept at room temperature for 1 hr and ether was added. The resulting white powder was collected by filtration, washed with ether and dried over KOH pellets *in vacuo*. This solid material was dissolved in DMF (10 ml) and the pH of the solution was adjusted to 8 with triethylamine. Tripeptide azide prepared as follows was added to this cold solution. A solution of *t*-Boc–Cys(Bzl)–Ser–Thr–NHNH$_2$ (XIV)[4] (1.0 g) in DMF (10 ml) cooled to −15° was treated with 6.0 N HCl in dioxane (0.67 ml), followed by isoamylnitrite (0.27 ml). After 5 min, 0.56 ml of triethylamine was added. This solution was combined with the solution of XIIIa in DMF prepared above. This reaction mixture was stirred at 4° for 48 hr. After removal of the solvent, AcOEt and 5% AcOH were added to the residue to form a solid material, which was collected by filtration, washed with AcOEt and water and dried; yield 1.03 g (88.5%), mp 225—228° (dec.), $[\alpha]_D^{23}$ −16.9° (c=1.0, DMF), Rf^1 0.68. *Anal.* Calcd. for $C_{81}H_{115}N_{17}O_{20}S_2\cdot 3H_2O$: C, 55.1; H, 6.91; N, 13.5. Found: C, 55.1; H, 6.97; N, 13.0, Amino acid ratios in an acid hydrolysate: Ser 1.0; Thr 0.9; His 1.7; Leu 0.9; Ile 1.9; Phe 0.9; Glu 2.0 (average recovery 78%).

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Z-Leu-Ala-Cys(Bzl)-Ser-Thr-Cys(Bzl)-His-Leu-Ile-Phe-Glu-Gln-His-Ile-OH (A)——(a) C-Terminal heptapeptide (X) (72 mg) was dissolved in 40% aqueous DMF (1.5 ml) containing triethylamine (0.03 ml). N-Terminal heptapeptide azide prepared as follows was added to this solution. XI (84 mg) was dissolved in DMSO (1 ml) and DMF (2 ml). This solution was cooled to −15°, and 6 N HCl in dioxane (0.027 ml) followed by isoamylnitrite (0.01 ml) was added. After 5 min, triethylamine (0.023 ml) was added. This solution was mixed with a solution of X in aqueous DMF prepared as described above. This reaction mixture was stirred at 4° for 48 hr. The solvent was removed by evaporation and AcOEt and 5% AcOH were added to the residue to give a white precipitate, which was collected by filtration and washed with ether. This material in DMF (1 ml) was applied to a column of Sephadex LH-20 (1×170 cm) equilibrated with DMF. The column was eluted with DMF and fractions (3 g each) were collected. Removal of the solvent from fraction 1 (tube Nos. 29—34), fraction 2 (35—39) and fraction 3 (40—56) and addition of ether gave solid materials. Amino acid ratios in their acid hydrolysates were as follows: fraction 1: Thr 1.1; Ser 1.0; Glu 1.9; Ala 1.2; Leu 2.0; Ile 1.8; Phe 0.9; His 1.7, fraction 2; Thr 1.0; Ser 0.8; Glu 0.6; Ala 1.0; Ile 0.6; Leu 1.2; Phe 0.3; His 1.3, fraction 3; Glu 2.0; Ile 2.0; Leu 1.2; Phe 1.0; His 0.9. Fraction 1 was rechromatographed as described above to give purified tetradecapeptide (A); yield 30 mg (19%), mp 230—240°(dec.), $[\alpha]_D^{23}$ −17.2° (c=1.0, DMSO), Rf^1 0.75, Rf^2 0.84. *Anal.* Calcd. for $C_{93}H_{129}N_{19}O_{22}S_2 \cdot 9H_2O$: C, 53.4; H, 7.03; N, 12.7. Found: C, 53.4; H, 6.75; N, 12.5, Amino acid ratios in an acid hydrolysate: Leu 2.0; Ala1.2;Ser 0.9; Thr 1.0; His 1.8; Ile 2.0; Phe 0.9; Glu 2.1; Cys was not determined (average recovery 80%).

(b) A solution of N-protected dodecapeptide (XV) (300 mg) in TFA (2 ml) containing anisole(0.5ml) was stored at room temperature for 1 hr. On addition of ether, a precipitate (XVa) was formed. This was collected by filtration, washed with ether and dried over KOH pellets. This material was dissolved in 10% aqueous DMF (5 ml) and the pH of the solution was adjusted to 8 with triethylamine. Z–Leu–Ala–N_3 prepared as follows was added. A solution of N-terminal dipeptide hydrazide (XVI)[4](130 mg) in DMF (5 ml) cooled to −15° was treated with 6 N HCl in dioxane (0.15 ml), followed by isoamylnitrite (0.054 ml). After 5 min, the pH of the solution was adjusted to 8 with triethylamine (0.12 ml) and the solution was added to the cold solution of XVa in DMF prepared above. This reaction mixture was stirred at 4° for 48 hr. After removal of the solvent, AcOEt was added to the residue. The resulting powder was collected by filtration, washed with AcOEt and 1% AcOH and dried; yield 280 mg (64.1%), mp 235—240° (dec.), $[\alpha]_D^{23}$ −17.8° (c=1.0, DMSO), −19.2° (c=0.5, DMF), Rf^1 0.75, Rf^2 0.87. *Anal.* Calcd. for $C_{93}H_{129}N_{19}O_{22}S_2 \cdot 6H_2O$: C, 54.8; H, 6.97; N, 13.1. Found: C, 54.7; H, 6.76; N, 12.8, Amino acid ratios in an acid hydrolysate: Leu 1.8; Ala 1.0; Ser 1.0; Thr 1.0; His 1.8; Ile 1.9; Phe 1.0; Glu 1.9; Cys was not determined (average recovery 77%).

To check the optical purity of the constituent amino acids of A, H–Leu–Ala–Cys(SO_3^-)–Ser–Thr–Cys(SO_3^-)–His–Leu–Ile–Phe–Glu–Gln–His–Ile–OH was derived as follows. Small pieces of Na were added to a solution of A (50 mg) in liquid ammonia (100 ml) at the boiling point of ammonia until the blue color remained for 30 sec. Ammonia was then removed completely, the residue was dissolved in 8 M guanidine hydrochloride (4 ml), and the pH of the solution was adjusted to 8 with dilute ammonia. Sodium sulfite (350 mg) and freshly prepared sodium tetrathionate (160 mg) were added to this solution. The reaction mixture was stirred at room temperature for 18 hr and applied to a column of Sephadex G-25 (2.5×140 cm) equilibrated with 5% AcOH. The column was eluted with 5% AcOH and fractions (3 ml each) were collected. Lyophilization of the effluent (tube Nos. 59—64) afforded the desired product, which was rechromatographed as described above to give the purified S-sulfonated tetradecapeptide; yield 16.5 mg (35.4%), $[\alpha]_D^{23}$ −49.5° (c=0.2, 20% AcOH), Rf^1 0.19, Rf^2 0.62 (ninhydrin and Pauly stain). Amino acid ratios in an acid hydrolysate : Leu 2.1; Ala 1.2; Ser 0.8; Thr 0.7; His 2.1; Ile 1.9; Phe 1.0; Glu 2.0; Cys was not determined (average recovery 75%), amino acid ratios in an APM digest: Leu 2.2; Ala 1.2; Ser 1.0; Thr+Gln 1.6 (calculated as Thr); His 1.5; Ile 1.8; Phe 0.9; Glu 1.0; S-sulfocysteine 1.8 (average recovery 70%).

Formation of the Iron-Sulfur Complex——Small pieces of Na were added to a solution of A (50 mg) in liquid ammonia (100 ml) at the boiling point of ammonia until the blue color remained for 10 sec. A small amount of ammonium chloride was added to the blue-colored solution then ammonia was removed completely. The residue was dissolved in 6 ml of 10 mM Tris buffer (pH 7.5) containing mercaptoethanol (41.3 mg), and this solution was allowed to stand at room temperature for 30 min. Na_2S ($Na_2S \cdot 9H_2O$, 47.6 mg) and $FeCl_3$ ($FeCl_3 \cdot 6H_2O$, 35.7 mg) were added to this solution. This reaction mixture was kept at 0° for 2 hr and then centrifuged. Absorption spectra of the brown-colored supernatant are shown in Fig. 4.

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