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Synthesis of a Tetradecapeptide corresponding to Sequence 90—103 of Bovine Adrenodoxin[1]

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Two tetradecapeptides, Z–Leu–Gly–Cys(Bzl)–Gln–Ile–Cys(Bzl)–Leu–Thr–Lys(Z)–Ala–Met–Asp(OBzl)–Asn–Met–OH (I) and its benzyl ester (VIII) were synthesized. I was synthesized by the condensation of two heptapeptides [Z–Leu–Gly–Cys(Bzl)–Gln–Ile–Cys(Bzl)–Leu–$NHNH_2$ (II) and Boc–Thr–Lys(Z)–Ala–Met–Asp(OBzl)–Asn–Met–OH (III)], while VIII was synthesized stepwise by the condensation of 4 fragments [Z–Leu–Gly–Cys(Bzl)–Gln–OH (XI), Boc–Ile–Cys(Bzl)–Leu–OH (X), Boc–Thr–Lys(Z)–Ala–$NHNH_2$ (IV) and Boc–Met–Asp(OBzl)–Asn–Met–OBzl (IX)]. Deblocked I and deblocked VIII each formed a chelate with iron and sulfur.

Keywords——synthesis of a tetradecapeptide; partial sequence of bovine adrenodoxin; chelate formation of a synthetic peptide with iron and sulfur; absorption spectrum of a chelate; peptide synthesis by fragment condensation

Bovine adrenodoxin,[3] which is a component of the electron transfer systems in adrenal cortex, is an iron-sulfur protein and its amino acid sequence was determined by Tanaka *et al.*[4] They pointed out two characteristic sequences of the form –Cys–X–Y–Cys– (X, Y: other amino acids) at positions 52—55 (–Cys–Ser–Thr–Cys–) and 92—95 (–Cys–Gln–Ile–Cys–), and suggested that the mercapto groups of these four cysteine residues were chelated to iron and inorganic sulfur as shown in Fig. 1.

Sequences of the form –Cys–X–Y–Cys– are common in iron-sulfur proteins, which contain a chelate of cysteine residues with iron and inorganic sulfur. We were interested in this characteristic sequence and synthesized partial sequences of bovine adrenodoxin containing the –Cys–X–Y–Cys– sequence in order to investigate their chelate formation with iron and sulfur. In previous papers, we reported the synthesis of two heptapeptides corresponding to sequences 50—56 and 90—96,[5] and the synthesis of a tetradecapeptide corresponding to sequence 50—63.[6] Here we describe the synthesis of tetradecapeptides corresponding to sequence 90—103, Z–Leu–Gly–Cys(Bzl)–Gln–Ile–Cys(Bzl)–Leu–Thr–Lys(Z)–Ala–Met–Asp(OBzl)–Asn–Met–OH(I) and its benzyl ester (VIII), and chelate formation of the deblocked products with iron and sulfur. An outline of the synthesis of I is given in Fig. 2.

I was synthesized by condensation of the amino-terminal heptapeptide, Z–Leu–Gly–Cys(Bzl)–Gln–Ile–Cys(Bzl)–Leu–$NHNH_2$ (II, sequence 90—96), with the carboxyl-terminal

1) Amino acids and 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, OBzl=benzyl ester, ONp=*p*-nitrophenyl ester, Boc=*tert*-butoxycarbonyl. Other abbreviations used in this paper are: DCC=dicyclohexylcarbodiimide, DMF=dimethylformamide, DMSO=dimethylsulfoxide, THF=tetrahydrofuran, TFA=trifluoroacetic acid, HOBt=N^1-hydroxybenzotriazole.

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

3) K. Suzuki and T. Kimura, *Biochem. Biophys. Res. Commun.*, **19**, 340 (1965). Y. Omura, D.Y. Cooper, O. Rosenthal, and R.W. Estabrook, "Non-Heme Iron Protein," ed. by A. San Pietro, Antioch Press, Yellow Springs, Ohio, 1965, p. 401.

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5) Y. Okada, C. Kawasaki, M. Okinaka, and K. Kawasaki, *Chem. Pharm. Bull.*, **27**, 1360 (1979).

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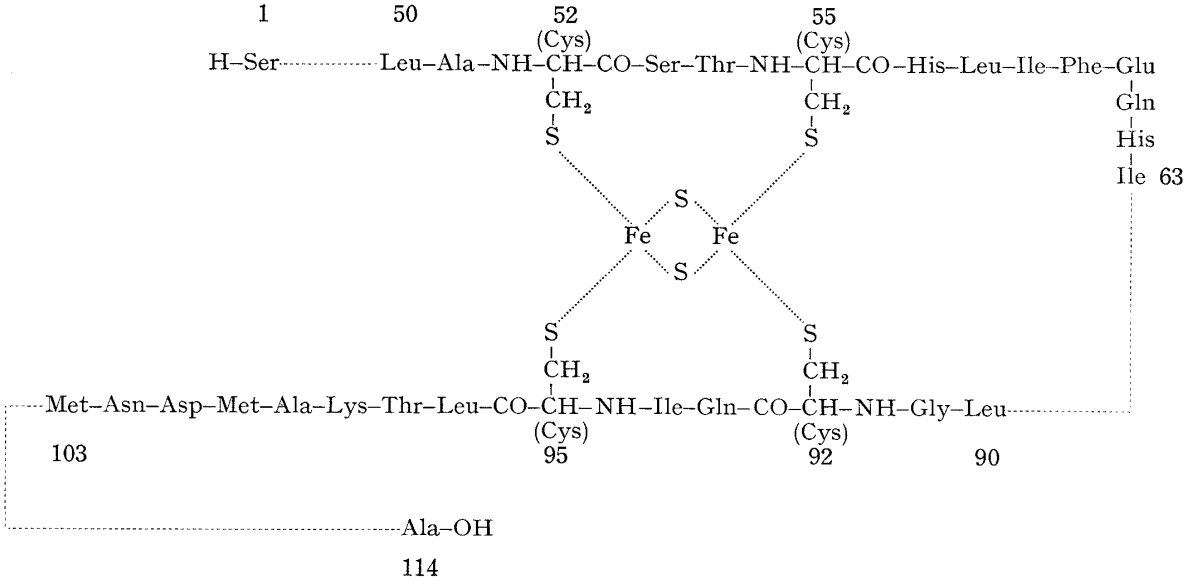

Fig. 1. Possible Chelate Structure of Adrenodoxin[4]

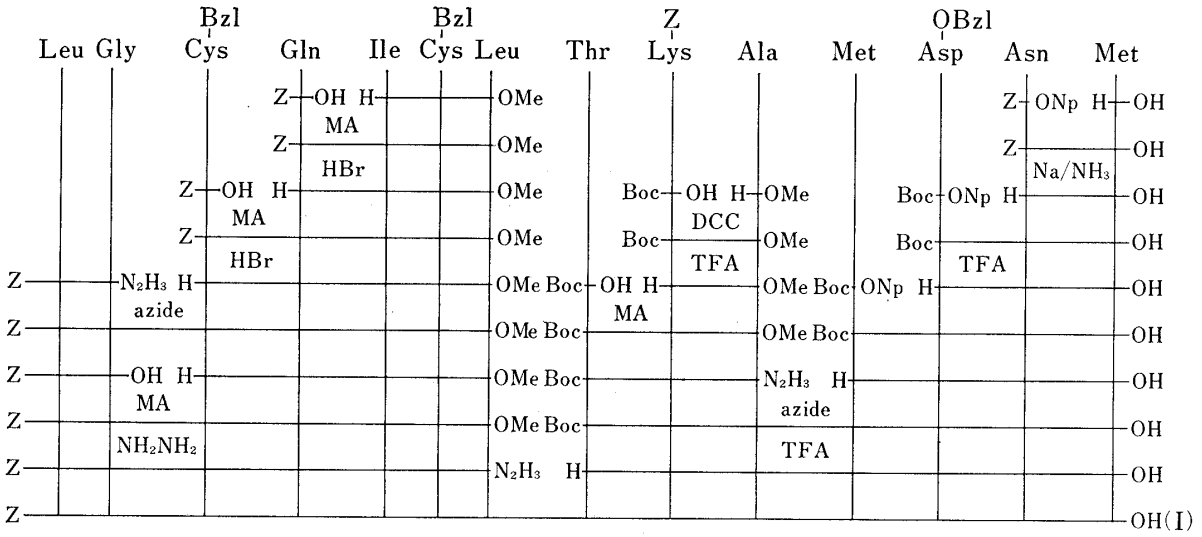

Fig. 2. Synthetic Scheme for the Tetradecapeptide (I)

MA: mixed anhydride method.

heptapeptide, Boc–Thr–Lys(Z)–Ala–Met–Asp(OBzl)–Asn–Met–OH(III, sequence 97—103).

III was synthesized as follows. Because of the presence of two methionine residues in the sequence, the α-amino groups of acylating amino acids were protected with the Boc group[7] which was removable by mild acidolysis. The Z group[8] is not suitable for use as a protecting group in a methionine-containing peptide because the conditions of its removal, such as HBr treatment,[9] hydrogenolysis[8] and sodium treatment in liq. ammonia,[10] may cause difficulty. Careful treatment of methionine-containing peptide with sodium in liq. ammonia may be applicable, and in fact this procedure has been adopted successfully in the

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9) A. Berger, *J. Biol. Chem.*, **17**, 1564 (1952); D. Ben-Ishai, *J. Org. Chem.*, **19**, 62 (1954); R.A. Boissonnas, G. Guttmann, P.A. Jaquenoud, and J.P. Waller, *Helv. Chim. Acta*, **38**, 1491 (1955).
10) R.H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).

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synthesis of several biological peptides, such as α-MSH[11] and ACTH fragment.[12] We also adopted the Z group for protection of the α-amino group of asparagine at position 102, because the yield of Z–Asn–Met–OH was better than the yield of Boc–Asn–Met–OH. The ε-amino group of lysine, which had to be protected until the last step, was also protected with the Z group. The β-carboxyl group of aspartic acid was protected as benzyl ester[13] which was stable to TFA treatment and was removable with sodium in liq. ammonia. III was synthesized by the condensation of the amino-terminal tripeptide, Boc–Thr–Lys(Z)–Ala–$NHNH_2$ (IV), with the carboxyl-terminal tetrapeptide, Boc–Met–Asp(OBzl)–Asn–Met–OH (V) which was constructed by stepwise elongation from carboxyl-terminal methionine by the *p*-nitrophenyl ester method.[14] Z–Asn–ONp[15] was coupled with methionine to afford Z–Asn–Met–OH in 25% yield. Boc–Asn–ONp[16] was also coupled with methionine, but the yield was low and the product was not analytically pure. A mixed anhydride[17] of Boc–Asn–OH[18] was also coupled with methionine, but the product could not be isolated. Z–Asn–Met–OH was treated with sodium in liq. ammonia to remove the Z group. The resulting deblocked material was coupled with Boc–Asp(OBzl)–ONp[19] to afford Boc–Asp(OBzl)–Asn–Met–OH, followed by TFA treatment to remove the Boc group. Boc–Met–ONp[20] was coupled with the deblocked tripeptide to afford V followed by TFA treatment to remove the Boc group. IV was derived from the corresponding tripeptide methyl ester, Boc–Thr–Lys(Z)–Ala–OMe, by hydrazinolysis. The tripeptide methyl ester was synthesized by the mixed anhydride method[17] by coupling Boc–Thr–OH[21] with H–Lys(Z)–Ala–OMe, which was obtained by the DCC method[22] by coupling Boc–Lys(Z)–OH[23] with H–Ala–OMe[24] followed by TFA treatment. The synthesis of Boc–Lys(Z)–Ala–OMe has already been reported by another group,[25] but the material was not characterized well. We crystallized and characterized it. IV was coupled by the azide method[26] with deblocked V to afford the heptapeptide (III).

The amino-terminal heptapeptide (II) was synthesized as follows. The mercapto group of cysteine was protected with the benzyl group,[27] which was stable to TFA and HBr treatment and removable by exposure to sodium in liq. ammonia. α-Amino groups of acylating amino acids were protected with the Z group, which was removed by HBr treatment. The amino-terminal heptapeptide methyl ester, Z–Leu–Gly–Cys(Bzl)–Gln–Ile–Cys(Bzl)–Leu–OMe (VI), was synthesized by the azide coupling of Z–Leu–Gly–$NHNH_2$[5], or by the mixed anhydride coupling of Z–Leu–Gly–OH,[28] with H–Cys(Bzl)–Gln–Ile–Cys(Bzl)–Leu–OMe,

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which was itself constructed by stepwise elongation by the mixed anhydride method from H–Ile–Cys(Bzl)–Leu–OMe. H–Ile–Cys(Bzl)–Leu–OMe, prepared from the corresponding Z derivative[5] by HBr treatment, and Z–Gln–OH were coupled by the mixed anhydride method to afford Z–Gln–Ile–Cys(Bzl)–Leu–OMe, followed by HBr treatment to remove the Z group. Z–Cys(Bzl)–OH[27] was linked to the deblocked tetrapeptide methyl ester by the mixed anhydride method to afford Z–Cys(Bzl)–Gln–Ile–Cys(Bzl)–Leu–OMe (VII), followed by HBr treatment to remove the Z group. Z–Leu–Gly–$NHNH_2$ was coupled by the azide method with the deblocked VII to afford the heptapeptide methyl ester (VI). VI was also synthesized by the coupling of Z–Leu–Gly–OH with deblocked VII by the mixed anhydride method. The coupling yield of VI was 63% by the azide method and 72% by the mixed anhydride method. VI was converted to the corresponding hydrazide (II), but this reaction was slow even with large excesses of hydrazine. The bulky side chain of leucine residue might hinder the hydrazinolysis. Camble[29] reported that the hydrazinolysis of a synthetic intermediate of gastric inhibitory polypeptide, Z–Asp–Phe–Val–Asn–Trp–Leu–OMe, was slow, and that the use of a mixture of DMF and *n*-butanol as a solvent accelerated the rate of hydrazinolysis of that peptide compared with the use of DMF alone. The hydrazinolysis of VI was slow even in a mixture of *n*-butanol and DMF, and the yield was only 12%.

The carboxyl-terminal heptapeptide (III) was treated with TFA to remove the Boc group and coupled by the azide method with the amino-terminal heptapeptide (II) to afford Z–Leu–Gly–Cys(Bzl)–Gln–Ile–Cys(Bzl)–Leu–Thr–Lys(Z)–Ala–Met–Asp(OBzl)–Asn–Met–OH (I). I was purified by Dowex 50 (H^+ form) treatment to remove the unreacted amino component, followed by Sephadex LH-20 column chromatography to remove the unreacted carboxyl component. I was treated with sodium in liq. ammonia to remove all protecting groups, and the resulting peptide was completely digested with aminopeptidase M.[30]

Reconsidering the synthetic route to I, the poor yield in the initial coupling of Z–Asn–OH with methionine and the slow hydrazinolysis of the amino-terminal heptapeptide methyl

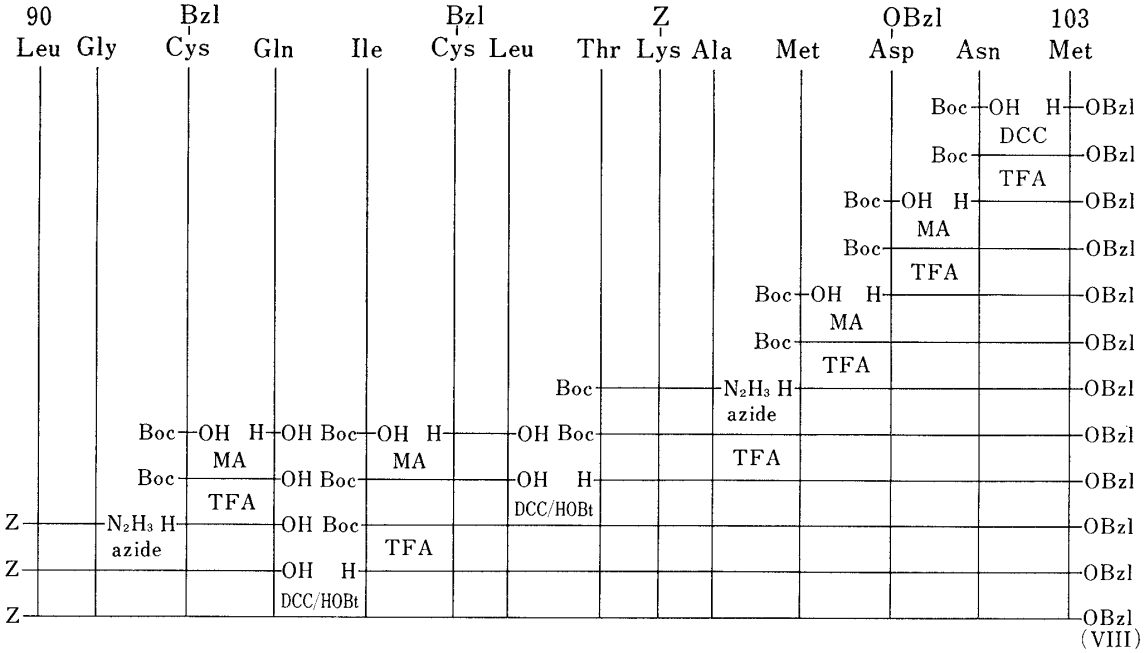

Fig. 3. Synthetic Scheme for the Tetradecapeptide (VIII)

MA: Mixed anhydride method.

29) R. Camble, "Chemistry and Biology of Peptides," ed. by J. Meienhofer, Ann Arbor Science Publishers, 1972, p. 281.

30) K. Hofmann, F.M. Finn, F. Limetti, J. Montibeller, and G. Zanetti, *J. Am. Chem. Soc.*, **88**, 3633 (1966).

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ester are disadvantageous. In order to overcome these disadvantages, we also synthesized a tetradecapeptide corresponding to the benzyl ester of I, Z–Leu–Gly–Cys(Bzl)–Gln–Ile–Cys-(Bzl)–Leu–Thr–Lys(Z)–Ala–Met–Asp(OBzl)–Asn–Met–OBzl (VIII), by another route. The synthetic scheme for VIII is illustrated in Fig. 3.

To construct the tetradecapeptide benzyl ester (VIII), stepwise fragment condensations from the carboxyl-terminal fragment, Boc–Met–Asp(OBzl)–Asn–Met–OBzl (IX), were employed. Boc–Thr–Lys(Z)–Ala–$NHNH_2$ (IV), Boc–Ile–Cys(Bzl)–Leu–OH (X) and Z–Leu–Gly–Cys(Bzl)–Gln–OH (XI) were linked one by one to fragment IX by the azide method or the DCC/HOBt procedure[31] to extend the peptide chain.

The carbonyl-terminal tetrapeptide (IX) was synthesized as follows. Boc–Asn–OH[18] was coupled by the DCC method with methionine benzyl ester, which was prepared according to the procedure reported by Izumiya *et al.*,[32] to afford Boc–Asn–Met–OBzl in 45% coupling yield. The coupling yield was not good, but it was much better than the 25% coupling yield for Z–Asn–Met–OH prepared by coupling Z–Asn–ONp with methionine in the former synthetic route. After removal of the Boc group with TFA, the dipeptide benzyl ester was coupled by the mixed anhydride method with Boc–Asp(OBzl)–OH[19] to afford Boc–Asp(OBzl)–Asn–Met–OBzl, followed by TFA treatment to remove the Boc group. The deblocked tripeptide benzyl ester was coupled by the mixed anhydride method with Boc-Met-OH[23] to afford Boc–Met–Asp(OBzl)–Asn–Met–OBzl (IX).

The fragment IX was treated with TFA to remove the Boc group and the resulting peptide was coupled by the azide method with Boc–Thr–Lys(Z)–Ala–$NHNH_2$ (IV) to afford Boc–Thr–Lys(Z)–Ala–Met–Asp(OBzl)–Asn–Met–OBzl (XII), followed by TFA treatment to remove the Boc group.

The fragment X was synthesized by coupling Boc–Ile–OH[23] and H–Cys(Bzl)–Leu–OH[33] by the mixed anhydride method, and was purified by silica gel column chromatography. X was condensed by the DCC/HOBt procedure with deblocked XII to afford Boc–Ile–Cys(Bzl)–Leu–Thr–Lys(Z)–Ala–Met–Asp(OBzl)–Asn–Met–OBzl (XIII), which was purified by Sephadex LH-20 column chromatography. XIII was treated with TFA to remove the Boc group.

The amino-terminal tetrapeptide (XI) was synthesized as follows. Boc–Cys(Bzl)–OH[23] was coupled by the mixed anhydride method with glutamine to afford Boc–Cys(Bzl)–Gln–OH, followed by TFA treatment to remove the Boc group. This deblocked dipeptide was coupled by the azide method with Z–Leu–Gly–$NHNH_2$[5] to afford Z–Leu–Gly–Cys(Bzl)–Gln–OH (XI).

The amino-terminal tetrapeptide (XI) was condensed by the DCC/HOBt procedure with the deblocked carboxyl-terminal decapeptide (XIII) to afford the tetradecapeptide, Z–Leu–Gly–Cys(Bzl)–Gln–Ile–Cys(Bzl)–Leu–Thr–Lys(Z)–Ala–Met–Asp(OBzl)–Asn–Met–OBzl (VIII), which was purified by Sephadex LH-20 column chromatography. VIII was treated with sodium in liq. ammonia to remove all protecting groups and the resulting peptide was completely digested with aminopeptidase M. Compared with the former synthetic route to I, this route offers a better coupling yield and an easy purification procedure for the tetradecapeptide (VIII).

Chelate formation of the deblocked I or deblocked VIII with iron and sulfur was carried out according to a procedure reported by Taniguchi *et al.*[34] for the reconstruction of modified adrenodoxins from modified apoadrenodoxins. The deblocked I (or VIII) was treated with 2-mercaptoethanol in Tris buffer (pH 7.5) and the mixture was combined with sodium sulfide and ferric chloride. The resulting precipitate was removed by centrifugation and the absorption spectrum of the brown-colored supernatant was measured. The spectrum is shown in

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32) N. Izumiya and S. Makisumi, *Nippon Kagaku Zasshi*, **78**, 662, 1768 (1957).
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34) T. Taniguchi and T. Kitamura, *Biochem.*, **14**, 5573 (1975).

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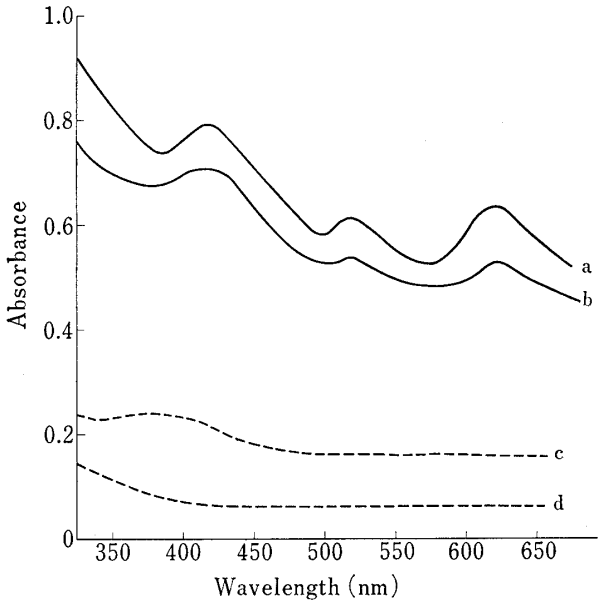

Fig. 4. Absorption Spectra of the Synthetic Peptides with Iron and Sulfur

a) Deblocked VIII (4.58×10^{-8} mol/ml) with $FeCl_3$ (2.8×10^{-7} mol/ml), Na_2S (3.3×10^{-7} mol/ml) and $HSCH_2CH_2OH$ (5.5×10^{-6} mol/ml) in Tris buffer.
b) Deblocked I (4.8×10^{-8} mol/ml) with $FeCl_3$, Na_2S and $HSCH_2CH_2OH$ in Tris buffer.
c) A mixture of $FeCl_3$ (1.4×10^{-5} mol/ml), Na_2S (1.6×10^{-5} mol/ml) and $HSCH_2CH_2OH$ (2.76×10^{-4} mol/ml) in Tris buffer.
d) Deblocked I or deblocked VIII (2.3×10^{-6} mol/ml) in Tris buffer.

Fig. 4. Iron-sulfur proteins have absorption maxima at 300—600 nm arising from chelate formation of the protein with iron and sulfur. For example, spinach ferredoxin has absorption maxima at 325 nm, 420 nm and 465 nm,[35] while Bacillus ferredoxin has an absorption maximum at 420 nm.[35] Kimura *et al.*[36] reported that oxidized native adrenodoxin had absorption maxima at 414 nm and 455 nm. In our experiment, the brown-colored solution exhibited absorption maxima at 422 nm, 520 nm and 620 nm. Neither the deblocked peptide in Tris buffer nor a mixture of the reagents (2-mercaptoethanol, sodium sulfide and ferric chloride) in Tris buffer showed any absorption peak in the range of 350—600 nm. Thus, the absorption maxima of the brown-colored solution at 422 nm, 520 nm and 620 nm did indicate chelate formation of the synthetic peptide with iron and sulfur. In a previous paper,[6] we reported chelate formation of a synthetic tetradecapeptide corresponding to sequence 50—63 of bovine adrenodoxin with iron and sulfur. In that experiment, we observed an absorption maximum at 415 nm. Thus, the absorption spectra due to chelate formations of the two tetradecapeptides corresponding to sequence 50—63 and 90—103 are different. The reason for this is not clear, but the amino acid sequences around cysteine residues are interesting. Fig. 5 compares the constituent amino acids in sequences 50—63 and 90—103. Comparing amino acids which are in the same positions with the respect to cysteine residues in the two different sequences, hydrophobic amino acids are present at positions 94 and 96, and hydrophilic amino acids are present at positions 97 and 98 in the sequence 90—103, while hydrophilic amino acids are present at positions 54 and 56, and hydrophobic amino acids are present at positions 57 and 58 in the sequence 50—63. In addition, two methionine residues are present in the sequence 90—103 while two histidine residues are present in the sequence 50—63. These amino acids, methionine and histidine, can both act as a ligand in a chelate with metal. It is possible that such differences of constituent amino acids in the sequences 50—63 and 90—103 affect the mode of chelate formation of the peptides with iron and sulfur. We are carrying

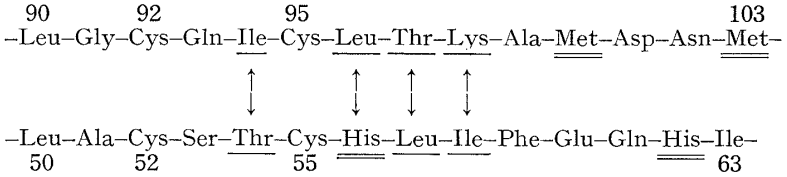

Fig. 5. Comparison of the Amino Acids in Residues 50—63 and 90—103 of Adrenodoxin

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36) T. Kimura and K. Suzuki, *J. Biol. Chem.*, **242**, 485 (1967).

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out further studies on chelate formation with the synthetic peptides, and the results will be published elsewhere.

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 aminopeptidase digests were determined with a JEOL JLC-6AH amino acid analyzer. 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).

Z-Asn-Met-OH——A solution of Z-Asn-ONp[15] (8.3 g) in dioxane (70 ml) was added to a solution of H-Met-OH (3.2 g) in a mixture of H_2O (60 ml), Et_3N (2.94 ml) and pyridine (5 ml). The mixture was stirred at room temperature for 2 days and the solvent was evaporated off. The residue was dissolved in H_2O (30 ml) and the solution was washed with AcOEt. The solution was then diluted to 100 ml with EtOH and treated with Dowex 50 (H^+). The resin was removed by filtration and the solvent was evaporated off. The residue was recrystallized from EtOH; yield 2.12 g (25%), mp 179°, $[\alpha]_D^{21}$ −11.4° (c=1.1, MeOH), Rf^1 0.18. *Anal.* Calcd for $C_{17}H_{23}N_3O_6S$: C, 51.3; H, 5.8; N, 10.6. Found: C, 51.4; H, 5.6; N, 10.7.

Boc-Asp(OBzl)-Asn-Met-OH——A solution of Z-Asn-Met-OH (1.8 g) in liq. ammonia (1000 ml) was treated with sodium 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 dissolved in H_2O and the solution was washed with AcOEt. The solution was then adjusted to pH 3 with AcOH and concentrated. EtOH was added and the resulting precipitate was collected by filtration, washed with EtOH and dried; 1.1 g (93%), Rf^1 0.36, single spot by ninhydrin test and H_2PtCl_6-KI test. This powder was used for the next reaction without further purification. The powder was dissolved in a mixture of H_2O (3 ml), dioxane (9 ml) and Et_3N (0.63 ml), and Boc-Asp(OBzl)-ONp[19] (3.55 g) was added to the solution. The mixture was stirred at room temperature for 6 hr, then the solvent was evaporated off. The residue was dissolved in 1% $NaHCO_3$ and the solution was washed with AcOEt. The solution was then acidified with citric acid and the resulting precipitate was extracted with AcOEt. The AcOEt layer was washed with H_2O and dried over Na_2SO_4. The solvent was evaporated off and the residue was precipitated from AcOEt/petro. ether; yield 1.13 g (48%), mp 175—178°, $[\alpha]_D^{21}$ −22.6° (c=0.5, MeOH), Rf^1 0.85, Rf^3 0.78. *Anal.* Calcd for $C_{25}H_{36}N_4O_9S$: C, 52.8; H, 6.4; N, 9.9. Found: C, 52.9; H, 6.4; N, 9.9.

Boc-Met-Asp(OBzl)-Asn-Met-OH (V)——Boc-Asp(OBzl)-Asn-Met-OH (920 mg) was dissolved in a mixture of TFA (6 ml) and anisole (0.2 ml), and the solution was stirred at room temperature for 40 min. Ether was added and the resulting precipitate was collected by filtration, washed with ether and dried. This deblocked tripeptide was dissolved in DMF (2 ml) and the pH of the solution was adjusted to 8 with Et_3N. Boc-Met-ONp[24] (1.1 g) was added to the solution and the mixture was stirred at room temperature for 24 hr. The solvent was evaporated off and the residue was dissolved in 1% Na_2CO_3. The solution was washed with ether[37] and acidified with 5% citric acid. The resulting precipitate was extracted with AcOEt, and the AcOEt layer was washed with H_2O and dried over Na_2SO_4. The solvent was evaporated off and the residue was precipitated from AcOEt/petro. ether, and washed with ether in a mortar; yield 490 mg (51%), mp 159°, $[\alpha]_D^{21}$ −31.5° (c=0.5, MeOH), Rf^1 0.87, Rf^3 0.83. *Anal.* Calcd for $C_{30}H_{45}N_5O_{10}S_2$: C, 51.2; H, 6.5; N, 9.9. Found: C, 51.2; H, 6.3; N, 9.9. Amino acid ratio in an acid hydrolysate (6 N HCl, 24 hr): $Met_{1.81}Asp_{2.00}$ (average recovery 88%).

Boc-Lys(Z)-Ala-OMe——H-Ala-OMe·HCl[24] (2.8 g) was added to a mixture of acetonitrile (10 ml) and Et_3N (2.7 ml), and the mixture was combined with a solution of Boc-Lys(Z)-OH[23] (6.4 g) in AcOEt (15 ml). A solution of DCC (3.5 g) in AcOEt (10 ml) was added to the mixture at −10° and the whole was stirred for 24 hr in a cold room. The resulting precipitate was removed by filtration and the solvent was evaporated off. The residue was dissolved in ether and the ether layer was washed successively with 5% Na_2CO_3, H_2O, 5% citric acid and H_2O, then dried over Na_2SO_4. The solvent was evaporated off and the residue was crystallized from ether/petro. ether; yield 6.9 g (88%), mp 68—70°, $[\alpha]_D^{21}$ −26.4° (c=1.0, MeOH), Rf^1 0.89, Rf^3 0.85 [lit.:[29] *Rf* 0.94 on Chromar (Mallinckrodt) in $CHCl_3$/MeOH (95/5)]. *Anal.* Calcd for $C_{23}H_{35}N_3O_7$: C, 59.3; H, 7.6; N, 9.0. Found: C, 59.4; H, 7.7; N, 9.1.

Boc-Thr-Lys(Z)-Ala-NHNH$_2$ (IV)——Boc-Lys(Z)-Ala-OMe (3.6 g) was treated with TFA (7 ml) in the presence of anisole (0.4 ml) at room temperature for 30 min and the product was precipitated with petro. ether. The precipitate was washed with petro. ether 3 times by decantation and dried. This material was dissolved in THF (5 ml) and the solution was adjusted to pH 8 with Et_3N. A mixed anhydride, prepared from Boc-Thr-OH[21] (3.4 g) with Et_3N (2.14 ml) and ethylchloroformate (1.48 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

37) Ether used for methionine-containing peptides was distilled over $FeSO_4$ and stored over $FeSO_4$.

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in AcOEt. The AcOEt layer was washed successively with 5% citric acid, H_2O, 5% $NaHCO_3$ and H_2O, and dried over Na_2SO_4. The solvent was evaporated off and the residue was dissolved in EtOH. The EtOH was evaporated off and the residue was dissolved in EtOH again. This procedure, evaporation and dissolving in EtOH, was repeated 3 times to remove AcOEt. The oily residue was dissolved in EtOH (20 ml) and hydrazine hydrate (1 ml) was added to the solution. The mixture was left at room temperature for 12 hr then concentrated to half the initial volume. H_2O was added to the solution and the resulting precipitate was collected by filtration and recrystallized from EtOH; yield 3.45 g (79%), mp 179°, $[\alpha]_D^{21}$ −25.0° (c=1.0, MeOH), Rf^1 0.77, Rf^3 0.68. *Anal.* Calcd for $C_{46}H_{42}N_8O_7$: C, 55.1; H, 7.5; N, 14.8. Found: C, 55.3; H, 7.4; N, 14.9.

Boc-Thr-Lys(Z)-Ala-Met-Asp(OBzl)-Asn-Met-OH (III)——Boc–Met–Asp(OBzl)–Asn–Met–OH (V, 700 mg) was deblocked with TFA in the presence of anisole in the usual manner. The deblocked material was dissolved in DMF (5 ml) and the solution was adjusted to pH 8 with Et_3N.

Next, 7 N HCl/dioxane (0.51 ml) and *t*-butyl nitrite (0.14 ml) were added successively to a solution of Boc–Thr–Lys(Z)–Ala–$NHNH_2$ (679 mg) in DMF (3 ml) at −20° and the mixture was stirred for 10 min. The mixture was then adjusted to pH 8 with Et_3N and combined with the deblocked tetrapeptide solution 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 AcOEt, 5% citric acid and H_2O in a mortar, and dried. The material was precipitated from DMF/ether; yield 370 mg (33%), mp 117—119°, $[\alpha]_D^{25}$ −17.0° (c=0.4, DMF), Rf^1 0.80, Rf^3 0.48. *Anal.* Calcd for $C_{51}H_{75}N_9O_{16}S_4$: C, 54.0; H, 6.7; N, 11.1. Found: C, 54.3; H, 6.7; N, 11.4. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Thr_{0.87}Lys_{1.03}Ala_{1.00}Met_{1.78}Asp_{2.09}$ (average recovery 86%).

Z-Gln-Ile-Cys(Bzl)-Leu-OMe——Z–Ile–Cys(Bzl)–Leu–OMe[5] (5 g) was dissolved in a mixture of anisole (0.5 ml) and 25% HBr/AcOH (10.7 ml), and the solution was stirred at room temperature for 30 min. Dry ether was added and the resulting precipitate was collected by filtration, washed with ether and dried. This deblocked material was dissolved in DMF (30 ml) and the solution was adjusted to pH 8 with Et_3N. A mixed anhydride, prepared from Z–Gln–OH (4.6 g) with Et_3N (1.6 ml) and ethylchloroformate (2.3 ml) in DMF (20 ml) at −10°, was added to the solution and the mixture was stirred in an ice-bath for 5 hr. The solvent was evaporated off and the residue was washed with 5% Na_2CO_3, 5% AcOH and H_2O in a mortar. Recrystallized from MeOH; yield 5.1 g (86%), mp 231°, $[\alpha]_D^{25}$ −34.7° (c=0.9, DMF), Rf^1 0.86. *Anal.* Calcd for $C_{36}H_{51}N_5O_8S$: C, 60.6; H, 7.2; N, 9.8. Found: C, 60.3; H, 7.2; N, 9.6.

Z-Cys(Bzl)-Gln-Ile-Cys(Bzl)-Leu-OMe (VII)——Z–Gln–Ile–Cys(Bzl)–Leu–OMe (1 g) was deblocked with 25% HBr/AcOH (1.36 ml) in the presence of anisole (0.07 ml) in the usual manner. The deblocked material was dissolved in DMF (7 ml) and the solution was adjusted to pH 8 with Et_3N. A mixed anhydride, prepared from Z–Cys(Bzl)–OH[27] (0.97 g) with Et_3N (0.27 ml) and ethylchloroformate (0.39 ml) at −10°, was added to the solution and the mixture was stirred for 5 hr. The solvent was evaporated off and the residue was precipitated from DMF/AcOEt. The material was washed with H_2O and dried. This product was dissolved in a mixture of DMF, MeOH and H_2O (12: 4: 1) and the solution was treated with Dowex 50 (H^+). The resin was removed by filtration and the solvent was evaporated off. The residue was washed successively with 5% AcOH, 5% Na_2CO_3 and H_2O in a mortar, and precipitated from DMF/AcOEt; yield 706 mg (56%), mp 252—255°, $[\alpha]_D^{25}$ −60.6° (c=0.8, DMF), Rf^1 0.89, Rf^3 0.67. *Anal.* Calcd for $C_{46}H_{62}N_6O_9S_3$: C, 60.9; H, 6.9; N, 9.3. Found: C, 60.9; H, 6.9; N, 9.2.

Z-Leu-Gly-Cys(Bzl)-Gln-Ile-Cys(Bzl)-Leu-OMe (VI)——A) By the Azide Method: Z–Cys(Bzl)–Gln–Ile–Cys(Bzl)–Leu–OMe (VII, 910 mg) was deblocked with 25% HBr/AcOH (1 ml) containing 5% anisole in the usual manner. The deblocked material was dissolved in DMF (8 ml) and the solution was adjusted to pH 8 with Et_3N.

Next, 6 N HCl/dioxane (0.37 ml) and *tert*-butyl nitrite (0.24 ml) were added successively to a solution of Z–Leu–Gly–$NHNH_2$[5] (674 mg) in DMF (4 ml) at −10°, and the mixture was stirred for 10 min. The mixture was then adjusted to pH 8 with Et_3N and combined with the solution of deblocked pentapeptide methyl ester described above. The reaction mixture was stirred for 48 hr in a cold room, then the solvent was evaporated off. The residue was washed with 5% AcOH and H_2O in a mortar and dissolved in DMF (15 ml). The solution was treated with Dowex 50 (H^+) and the resin was removed by filtration. The solvent was evaporated off and the residue was precipitated from DMF/AcOEt; yield 680 mg (63%), mp 268—271°, $[\alpha]_D^{25}$ −42.1° (c=1.1, DMF), Rf^1 0.96, Rf^3 0.57. *Anal.* Calcd for $C_{54}H_{76}N_8O_{11}S_2$: C, 60.2; H, 7.1; N, 10.4. Found: C, 60.2; H, 6.9; N, 10.1. Amino acid ratios in an acid hydrolysate (6N HCl, 48 hr): $Leu_{1.98}Gly_{1.00}Glu_{1.00}Ile_{0.91}$ (average recovery, excluding S-benzylcysteine, 81%).

B) By the Mixed Anhydride Method: VII (500 mg) was deblocked with HBr in the manner described above. The deblocked material was dissolved in DMF (5 ml) and the solution was adjusted to pH 8 with Et_3N. A mixed anhydride, prepared from Z–Leu–Gly–OH[28] (1.1 g) with Et_3N (0.46 ml) and ethyl chloroformate (0.32 ml) in DMF (10 ml) at −10°, was added to the solution and the mixture was stirred for 5 hr. The solvent was evaporated off and the residue was washed with 5% AcOH, H_2O and AcOEt in a mortar. The material was precipitated from DMF/AcOEt; yield 426 mg (72%), mp 265—269°, Rf^1 0.96, Rf^3 0.57. Amino acid ratios in an acid hydrolysate (6 N HCl, 48 hr): $Glu_{1.00}Gly_{1.00}Ile_{0.90}Leu_{2.20}$ (average recovery, excluding S-benzylcysteine, 83%).

Z-Leu-Gly-Cys(Bzl)-Gln-Ile-Cys(Bzl)-Leu-$NHNH_2$ (II)——Hydrazine hydrate (0.1 ml) was added to a

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solution of VI (216 mg) in a mixture of DMF (6 ml) and *n*-BuOH (2 ml) and the solution was stirred at room temperature for 4 days. The resulting precipitate was collected by centrifugation, washed with cold DMF and H_2O, and dried. The material was precipitated from DMSO/AcOEt; yield 48 mg (12%), mp 280—284°, $[\alpha]_D^{22}$ −36.6° (c=0.6, DMSO), Rf^1 0.92, Rf^3 0.49. *Anal.* Calcd for $C_{53}H_{76}N_{10}O_{10}S_2$: C, 59.1; H, 7.1; N, 13.0. Found: C, 59.2; H, 7.0; N, 12.8.

Z-Leu-Gly-Cys(Bzl)-Gln-Ile-Cys(Bzl)-Leu-Thr-Lys(Z)-Ala-Met-Asp(OBzl)-Asn-Met-OH (I)——Boc–Thr–Lys(Z)–Ala–Met–Asp(OBzl)–Asn–Met–OH (III, 90 mg) was treated with TFA (0.3 ml) containing 5% of anisole in the usual manner. The deblocked material was dissolved in DMF (1.2 ml) and the solution was adjusted to pH 8 with 10% Et_3N in DMF. Next, a 10% solution of 5.5 N HCl/dioxane in DMF (0.55 ml) and 10% *t*-butylnitrite in DMF (0.12 ml) were added successively to a solution of Z–Leu–Gly–Cys(Bzl)–Gln–Ile–Cys(Bzl)–Leu–$NHNH_2$ (II, 108 mg) in a mixture of DMSO (0.5 ml) and DMF (1 ml) at −10° and the mixture was stirred for 15 min. The mixture was adjusted to pH 8 with Et_3N and added to the solution of the deblocked III. The reaction mixture was stirred for 48 hr in a cold room and the solvent was evaporated off. The residue was washed with 10% AcOH and H_2O in a mortar, and dried. This material was dissolved in DMF (25 ml) and the solution was treated with Dowex 50 (H^+) at −20°. The resin was removed by filtration and the filtrate was concentrated to 2 ml. The solution was applied to a Sephadex LH-20 column (1.5×165 cm) and the column was developed with DMF at a flow rate of 1 ml/5 min. Fractions of 3 ml were collected and each fraction was checked by thin-layer chromatography using the H_2PtCl_6-KI test. Fractions 42—48 which had Rf^1 0.81 were pooled and the solvent was evaporated off. The residue was precipitated from DMF/AcOEt; yield 46 mg (28%), mp 244—248°, $[\alpha]_D^{25}$ −28.4° (c=0.4, DMF), Rf^1 0.84, Rf^2 0.89. *Anal.* Calcd for $C_{99}H_{139}N_{17}O_{24}S_4$: C, 57.2; H, 6.7; N, 11.5. Found: C, 57.0; H, 6.9; N, 11.4. Amino acid ratios in an acid hydrolysate (6 N HCl, 48 hr): $Leu_{1.94}Gly_{1.00}Glu_{1.07}Ile_{0.93}Thr_{0.84}Lys_{0.98}Ala_{1.02}Met_{1.69}Asp_{1.97}$ (average recovery, excluding S-benzylcysteine, 80%).

Aminopeptidase M Digestion of the Deblocked I——I (20 mg) was treated with sodium in liq. ammonia (350 ml) until the solution remained blue for 5 sec. The blue color was discharged with ammonium chloride and the ammonia was evaporated off under a nitrogen stream. The residue was dissolved in water and the solution was lyophilized; hygroscopic powder, 40 mg. A portion (4 mg) of the crude deblocked I was digested with aminopeptidase M for 48 hr according to a procedure reported by Hofmann *et al.*:[30] $Leu_{2.09}Gly_{1.01}$-$Cys_{1.78}(X+Thr)_{2.11}$[38]$Ile_{1.02}Lys_{1.11}Ala_{1.00}Met_{1.78}Asp_{0.80}$ (average recovery, 73%).

Methionine Benzyl Ester *p*-Toluenesulfonate——This compound was prepared according to a procedure reported by Izumiya *et al.*[32] A mixture of methionine (14 g), *p*-toluenesulfonic acid monohydrate (23 g), benzyl alcohol (50 ml) and benzene (100 ml) was refluxed for 5 hr. An azeotropic mixture was removed with Dean-Stark equipment during the reaction. The solution was concentrated and H_2O was added. The H_2O layer was washed 3 times with ether and adjusted to pH 10 with Na_2CO_3. The resulting precipitate was extracted with AcOEt and the AcOEt layer was washed with H_2O then dried over Na_2SO_4. The solvent was evaporated off and the resulting syrupy residue[39] was dissolved in ether. A saturated ethereal solution of *p*-toluenesulfonic acid was added and the resulting precipitate was collected by filtration, washed with ether and dried; yield 13.4 g (56%), mp 133—135°, $[\alpha]_D^{28}$ +1.0° (c=1.1, MeOH), Rf^1 0.82, Rf^2 0.92, Rf^3 0.80. *Anal.* Calcd for $C_{12}H_{17}NO_2S \cdot C_7H_8O_3S$: C, 55.5; H, 6.1; N, 3.4. Found: C, 55.5; H, 6.0; N, 3.3.

Boc-Asn-Met-OBzl——DCC (5 g) was added to a mixture of Boc–Asn–OH[18] (5.7 g) and methionine benzyl ester (5.8 g) in DMF (15 ml) at −30° and the mixture was stirred overnight in a cold room. The resulting precipitate was removed by filtration and the solvent was evaporated off. The residue was dissolved in AcOEt and the AcOEt layer was washed successively with 5% citric acid, H_2O, 10% Na_2CO_3 and H_2O, and dried over Na_2SO_4. The solvent was evaporated off and the residue was precipitated from AcOEt/ether; yield 4.98 g (45%), mp 142°, $[\alpha]_D^{25}$ −8.7° (c=1.0, MeOH), Rf^1 0.86, Rf^3 0.90. *Anal.* Calcd for $C_{21}H_{31}N_3O_6S$: C, 55.6; H, 6.8; N, 9.3. Found: C, 55.4; H, 7.0; N, 9.4.

Boc-Asp(OBzl)-Asn-Met-OBzl——Boc–Asn–Met–OBzl (2.5 g) was treated with TFA (4 ml) in the presence of anisole (0.4 ml) to remove the Boc group in the usual manner. The deblocked material was dissolved in DMF (8 ml) and the solution was adjusted to pH 8 with Et_3N. A mixed anhydride, prepared from Boc–Asp(OBzl)–OH[19] (2.1 g) with Et_3N (0.91 ml) and ethylchloroformate (0.63 ml) in DMF at −15°, was added to the solution and the mixture was stirred for 5 hr. The solvent was evaporated off and the residue was dissolved in AcOEt. The AcOEt layer was washed successively with 5% citric acid, H_2O, 10% Na_2CO_3 and H_2O, and dried over Na_2SO_4. The solution was concentrated and the resulting precipitate was collected by filtration and washed with ether; yield 2.36 g (65%), mp 135°, $[\alpha]_D^{26}$ −27.4° (c=0.8, MeOH), Rf^3 0.88. *Anal.* Calcd for $C_{32}H_{42}N_4O_9S$: C, 58.3; H, 6.4; N, 8.5. Found: C, 58.2; H, 6.5; N, 8.3.

Boc-Met-Asp(OBzl)-Asn-Met-OBzl (IX)——Boc–Asp(OBzl)–Asn–Met–OBzl (1.9 g) was treated with TFA to remove the Boc group in the usual manner. The deblocked material was dissolved in DMF (4 ml)

38) Calculated as Thr. This peak was asymmetric and had a shoulder. The height of the shoulder was about half that of the peak. Average recovery was calculated as [(X+Thr)+the shoulder peak=(X+Thr)×1.5].

39) This syrupy material was used for the coupling reaction.

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and the solution was adjusted to pH 8 with Et_3N. A mixed anhydride, prepared from Boc–Met–OH[33] (1.1 g) with Et_3N (0.6 ml) and ethylchloroformate (0.42 ml) in THF (11 ml) at −10°, was added to the solution and the reaction mixture was stirred for 5 hr. The solvent was evaporated off and the residue was dissolved in AcOEt. The AcOEt layer was washed successively with 5% citric acid, H_2O, 10% Na_2CO_3 and H_2O, and dried over Na_2SO_4. The solution was concentrated and ether was added. The resulting precipitate was collected by filtration, washed with ether and dried; yield 1.37 g (60%), mp 128°, $[\alpha]_D^{26}$ −36.3° (c=0.8, MeOH), Rf^3 0.78. *Anal.* Calcd for $C_{37}H_{51}N_5O_{10}S_2$: C, 56.3; H, 6.5; N, 8.9. Found: C, 56.1; H, 6.4; N, 8.9.

Boc-Thr-Lys(Z)-Ala-Met-Asp(OBzl)-Asn-Met-OBzl (XII)——The tetrapeptide (IX, 980 mg) was treated with TFA in the presence of anisole to remove the Boc group in the usual manner. The deblocked material was dissolved in DMF (3 ml) and the solution was adjusted to pH 8 with Et_3N.

Next, 4.5 N HCl/dioxane (1.24 ml) and *t*-butyl nitrite (0.21 ml) were added successively to a solution of Boc–Thr–Lys(Z)–Ala–$NHNH_2$ (IV, 1.05 g) in DMF (5 ml) at −20° and the mixture was stirred for 5 min. The mixture was then adjusted to pH 8 with Et_3N and combined with the solution of the deblocked tetrapeptide 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 3% citric acid and H_2O in a mortar. The material was precipitated from MeOH/ether; yield 960 mg (63%), mp 207°, $[\alpha]_D^{26}$ −20.8° (c=1.1, DMF), Rf^3 0.70. *Anal.* Calcd for $C_{58}H_{81}N_9O_{16}S_2$: C, 56.9; H, 6.7; N, 10.3. Found: C, 57.1; H, 6.8; N, 10.3. Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Thr_{0.94}Lys_{0.98}Ala_{1.00}Met_{1.88}Asp_{2.04}$ (average recovery 91%).

Boc-Ile-Cys(Bzl)-Leu-OH (X)——Z–Cys(Bzl)–Leu–OH[33] (6 g) was treated with 25% HBr/AcOH to remove the Z group in the usual manner. The resulting deblocked material was dissolved in DMF (15 ml) and the solution was adjusted to pH 8 with Et_3N. A mixed anhydride, prepared from Boc–Ile–OH[23] (3 g) with Et_3N (1.79 ml) and ethylchloroformate (1.25 ml) in DMF at −15°, was added to the solution and the mixture was stirred for 5 hr. The solvent was evaporated off and the residue was dissolved in a mixture of $CHCl_3$ and 5% citric acid. The $CHCl_3$ layer was washed with H_2O and dried over Na_2SO_4. The solution was concentrated and applied to a silica gel column (4×22 cm). The column was developed successively with $CHCl_3$ (0.5 l), 1% MeOH/$CHCl_3$ (4 l) and 3% MeOH/$CHCl_3$ (1 l). The desired material was eluted in the 1% MeOH/$CHCl_3$ fraction. The 1% MeOH/$CHCl_3$ fractions which showed Rf^3 0.58 were pooled and the solvent was evaporated off. The residue was precipitated from MeOH/petro. ether; yield 4.8 g (68%), amorphous powder, $[\alpha]_D^{25}$ −45.4° (c=1.0, MeOH), Rf^1 0.86, Rf^3 0.58. *Anal.* Calcd for $C_{27}H_{42}N_3O_6S$: C, 60.4; H, 7.9; N, 8.1. Found: C, 60.4; H, 8.1; N, 8.1. Amino acid ratios in an acid hydrolysate (6 N HCl, 36 hr): $Ile_{1.00}Leu_{1.02}$ (average recovery, excluding S-benzylcysteine, 92%).

Boc-Ile-Cys(Bzl)-Leu-Thr-Lys(Z)-Ala-Met-Asp(OBzl)-Asn-Met-OBzl (XIII)——The carboxyl-terminal heptapeptide (XII, 366 mg) was treated with TFA in the presence of anisole to remove the Boc group in the usual manner. The deblocked heptapeptide was lyophilized from a mixture of 95% dioxane (10 ml) and 1 N HCl (0.5 ml) to convert the trifluoroacetate to the hydrochloride. The hydrochloride was dissolved in a mixture of DMF (5 ml) and 10% Et_3N/DMF (0.4 ml), and Boc–Ile–Cys(Bzl)–Leu–OH (X, 193 mg) and HOBt (68 mg) were added. The reaction mixture was stirred for 24 hr in a cold room and the resulting precipitate was removed by filtration. The filtrate was concentrated to 4 ml and applied to a Sephadex LH-20 column (1.5×165 cm). The column was developed with DMF at a flow rate of 1 ml/5 min. Fractions of 3 ml were collected and each fraction was tested with H_2PtCl_6–KI reagent. Fractions 47—52 were pooled and the solvent was evaporated off. The residue was precipitated from DMF/ether; yield 310 mg (62%), mp 218—233° (dec.), $[\alpha]_D^{26}$ −20.2° (c=0.5, DMF), Rf^1 0.80, Rf^3 0.41. *Anal.* Calcd for $C_{80}H_{114}N_{12}O_{19}S_3$: C, 58.4; H, 7.0; N, 10.2. Found: C, 58.2; H, 7.0; N, 10.1. Amino acid ratios in an acid hydrolysate (6 N HCl, 48 hr): $Ile_{1.00}$-$Leu_{1.10}Thr_{0.98}Lys_{0.96}Ala_{1.05}Asp_{2.04}Met_{1.76}$ (average recovery, excluding S-benzylcysteine, 93%).

Boc-Cys(Bzl)-Gln-OH——A mixed anhydride prepared from Boc–Cys(Bzl)–OH (3.1 g) with Et_3N (1.38 ml) and ethylchloroformate (0.96 ml) in THF (30 ml) at −10° was added to a solution of glutamine (1.46 g) in a mixture of H_2O (10 ml) and Et_3N (1.38 ml), and the reaction mixture was stirred for 5 hr. The solvent was evaporated off and the residue was dissolved in a mixture of AcOEt and 5% citric acid. The AcOEt layer was washed with H_2O and dried over Na_2SO_4. The solution was concentrated and the resulting precipitate was collected by filtration and washed with ether; yield 2.28 g (52%), mp 183—184°, $[\alpha]_D^{25}$ −34.1° (c=1.0, DMF), Rf^1 0.83, Rf^3 0.25. *Anal.* Calcd for $C_{20}H_{29}N_3O_6S$: C, 54.7; H, 6.7; N, 9.6. Found: C, 54.8; H, 6.8; N, 9.8.

Z-Leu-Gly-Cys(Bzl)-Gln-OH (XI)——Boc–Cys(Bzl)–Gln–OH (1 g) was treated with TFA in the presence of anisole in the usual manner. The deblocked dipeptide was dissolved in 90% DMF (7 ml) and the solution was adjusted to pH 8 with Et_3N.

Next, 4.5 N HCl/dioxane (3.3 ml) and *t*-butyl nitrile (0.59 ml) were added successively to a solution of Z–Leu–Gly–$NHNH_2$ (1.68 g) in DMF (10 ml) at −10° and the mixture was stirred for 5 min. The mixture was then adjusted to pH 8 with Et_3N and combined with the solution of the deblocked dipeptide described above. The reaction mixture was stirred for 24 hr in a cold room and the solvent was evaporated off. The residue was dissolved in 1% Na_2CO_3 and the solution was washed with AcOEt. The solution was acidified with 6 N HCl and the resulting precipitate was collected by filtration, washed with H_2O and dried. The material was precipitated from DMF/ether; yield 0.99 g (68%), mp 155—160°, $[\alpha]_D^{25}$ −34.0° (c=1.0, DMF), Rf^1 0.80, Rf^3 0.21. *Anal.* Calcd for $C_{31}H_{41}N_5O_8S$: C, 57.8; H, 6.4; N, 10.9. Found: C, 57.6; H, 6.4; N, 10.9.

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Amino acid ratios in an acid hydrolysate (6 N HCl, 24 hr): $Leu_{0.97}Gly_{1.00}Glu_{1.01}$ (average recovery, excluding S-benzylcysteine, 81%).

Z-Leu-Gly-Cys(Bzl)-Gln-Ile-Cys(Bzl)-Leu-Thr-Lys(Z)-Ala-Met-Asp(OBzl)-Asn-Met-OBzl (VIII)——The decapeptide benzyl ester (XIII, 211 mg) was treated with TFA in the presence of anisole to remove the Boc group in the usual manner. The deblocked decapeptide was lyophilized from a mixture of 80% dioxane (15 ml) and 1 N HCl (0.18 ml) to convert the trifluoroacetate to the hydrochloride. The hydrochloride was dissolved in a mixture of DMF (3 ml) and 10% Et_3N (0.18 ml) in DMF. Z–Leu–Gly–Cys(Bzl)–Gln–OH (XI, 116 mg) and HOBt (49 mg) were dissolved in this solution and then DCC (37 mg) was added at −20°. The reaction mixture was stirred for 48 hr in a cold room and the resulting precipitate was removed by filtration. The filtrate was concentrated to 3 ml and insoluble material was removed by centrifugation. The supernatant was applied to a Sephadex LH-20 column (1.5 × 165 cm) and the column was developed with DMF at a flow rate of 1 ml/5 min. Fractions of 3 ml were collected and each fraction was tested with H_2PtCl_6–KI reagent on TLC. Fractions 43—47 were pooled and concentrated. Ether was added and the resulting precipitate was collected by filtration, washed with ether and dried; yield 160 mg (58%), mp 245—250° (dec.), $[\alpha]_D^{26}$ −28.2° (c=0.5, DMF), Rf^1 0.84, Rf^3 0.51. *Anal.* Calcd for $C_{106}H_{145}N_{17}O_{24}S_4$: C, 58.7; H, 6.7; N, 11.0. Found: C, 58.4; H, 6.8; N, 10.7. Amino acid ratios in an acid hydrolysate (6 N HCl, 48 hr): $Leu_{2.20}Gly_{1.00}Glu_{1.04}Ile_{1.00}Thr_{0.90}Lys_{0.98}Ala_{1.10}Asp_{2.02}Met_{1.79}$ (average recovery, excluding S-benzylcysteine, 89%). Aminopeptidase M digestion of deblocked VIII was carried out according to the procedure described for the deblocked I: $Leu_{2.13}Gly_{1.00}Cys_{2.20}(Gln+Thr+Asn)_{2.79}$[40]$Ile_{1.28}Lys_{1.05}Ala_{1.13}Met_{1.87}Asp_{0.93}$ (average recovery 70%).

Chelate Formation of Deblocked VIII (or I) with Iron and Sulfur——VIII (or I, 30 mg) was treated with sodium in liq. ammonia to remove all the protecting groups in the manner described for I; hygroscopic powder, 60 mg. A part of the material (50 mg) was dissolved in a mixture of 10 mM Tris buffer (pH 7.5, 5 ml) and 2-mercaptoethanol (0.1 ml), and the solution was kept at room temperature for 30 min. Ferric chloride hexahydrate (19 mg) and sodium sulfide nonahydrate (20 mg) were added to the solution and the mixture was kept at 0° for 30 min. The resulting precipitate was removed by centrifugation and the brown-colored supernatant was diluted 50 times with 10 mM Tris buffer (pH 7.5). The absorption spectrum of this diluted solution was measured and absorption peaks were observed at 422 nm, 520 nm and 620 nm, as shown in Fig. 4.

40) Calculated as Thr.

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