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Studies on Peptides. XXXI.[1,2] Synthesis of Two Model Pentapeptides related to Clostridial Ferredoxin

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As model compounds, related to ferredoxin, two pentapeptides, H-Ser-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH and H-Ser-Ser-Val-Ser-Ser-OH were synthesized.

It is well documented that ferredoxin, one of the non-heme iron proteins, possesses labile sulfur which can be liberated from the molecule as hydrogen sulfide in acidic conditions or by treatment with various iron-chelating reagents.[4] The characteristic feature of this labile sulfur seems to associate closely with the electron transferring property of this protein.[5]

Previously, Bayer, *et al.*[6] postulated the idea that the source of this labile sulfur was of amino acid origin which was released from cysteinyl groups. They demonstrated that from a cysteine methyl ester Fe (II) complex, hydrogen sulfide could be released by acid under certain conditions. However their *beta*-elimination theory from cysteine to dehydroalanine seems unlikely, since the resulting dehydroalanine as well as pyruvic acid, were not well characterized in the hydrolysate of this acid treated protein.[7] Recently, Gersonde, *et al.*[8] expressed the idea that iron-catalyzed substitution of cysteine and serine may be a possible explanation of the source of the labile sulfur of ferredoxin.

Recently, the entire amino acid sequence of ferredoxin from *Clostridium pasteurianum* has been elucidated,[9] in which, the presence of 8 cysteinyl residues was established. Lovenberg, *et al.*[10] observed that crystalline clostridial ferredoxin contains two distinct classes of sulfur: the acid labile sulfide in amounts approximately equimolar with the non-heme iron (*ca.* 8 atoms of sulfur) and the sulfur in the cysteinyl residues of the polypeptide. They demonstrated that ferredoxin could only be reconstituted from apoprotein, so-called apoferredoxin, by the use of inorganic sulfide, *i.e.*, sodium sulfide, in addifon to ferrous ammonium sulfate and mercaptoethanol. Quite recently, these results were further confirmed in ^{35}(S)-labeled ferredoxin.[11] They have shown that the labile sulfur is of the inorganic sulfide origin bound originally to the native protein.

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2) Amino acids, peptides and their derivatives mentioned in this communication are of the L-configuration and their abbreviated designations are those recommended by IUPAC-IUB Commission for Biological Nomenclature. *Biochemistry*, **5**, 1445, 2485 (1966); *ibid.*, **6**, 362 (1967).
3) Location: *Sakyo-ku, Kyoto.*
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The inorganic sulfur content of clostridial ferredoxin was reexamined by Bayer, *et al.*[12] in 1969. They concluded that in ferredoxin there are 5 to 6 additional sulfurs besides the 8 sulfurs from cysteinyl moieties and a model of multi-center three dimentional iron–sulfur cluster in ferredoxin was proposed rather than a pseudo-linear array assinged early by Phillips, *et al.*[13] However the exact number of sulfur and iron in the molecule as well as their mode of combination remain to be established.

Some attempts were made to prepare the artificial non-heme iron proteins for the better understanding of the nature of sulfur-iron binding by treating serum albumin[14] or soybean trypsin inhibitor[15] with chemical reagents, such as mercaptoethanol, Na_2S and Fe (II).

In consideration of the above current observations, we undertook the synthesis of a model pentapeptide, H-Ser-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH(A). The sequence of which corresponds positions 7 to 11 of clostridial ferredoxin (a serine and cysteine-rich portion) in order to examine a possibility of formation of cysteine-iron-sulfur complex in one hand and to investigate the substitution reaction of cysteine to serine on the other (with collaboration of Dr. Gersonde). In latter aspect, a model pentapeptide, H-Ser-Ser-Val-Ser-Ser-OH (B) was also synthesized. Synthetic outlines of these model pentapeptides are recorded in this paper.

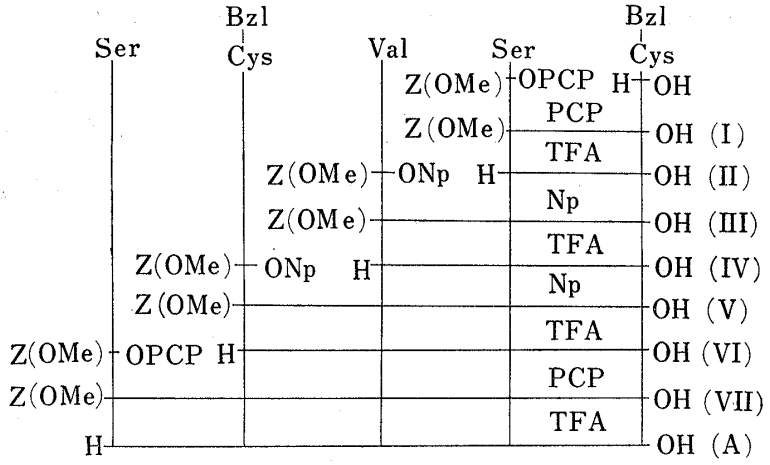

Fig. 1. Synthetic Route to H-Ser-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH

The synthetic scheme of A is illustrated in Fig. 1. The benzyl group removable by hydrogen fluoride[16] was selected as the protecting group for the sulfhydryl group of cysteine and *p*-methoxybenzyloxycarbonyl[Z(OMe)][17] removable by trifluoroacetic acid (TFA) was used extensively as the α-amino portecting group of amino acids employed. By means of the active ester procedure, elongation of the peptide chain was performed in the stepwise manner starting from H-Cys(Bzl)-OH. All of the protected intermediates which contain Cys(Bzl) were less soluble in ethyl acetate, therefore purification of these peptides by extraction pro-

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13) W.H. Phillips, E. Knight, Jr. and D.C. Blomstrom, "Non-heme Iron Protein," Role in Energy Conversion, San Pietro Ed., Antioch Press Yellow Springs, Ohio, 1965, p. 69; H. Brintzinger, G. Palmer and R.H. Sando, *Proc. Natl. Acad. Sci., U.S.*, **55**, 387 (1966).
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17) F. Weygand and K. Hunger, *Ber.*, **95**, 1, 7 (1962); F. Weygand and E. Nintz, *Z. Naturforsch.*, **20b**, 429 (1965).

cedure was difficult. In order to purify every protected peptide, each crude product was washed bachwise with a solution of citric acid and water and recrystallized from appropriate solvents.

First, Z(OMe)-Ser-OH[17] was condensed with the triethylammonium salt of H-Cys(Bzl)-OH by means of pentachlorophenyl (PCP) ester method[18] to give Z(OMe)-Ser-Cys(Bzl)-OH (I). The protecting group of which was removed by TFA in the presence of anisole to give H-Ser-Cys(Bzl)-OH(II). The dipeptide (II) was then condensed with Z(OMe)-Val-OH by the *p*-nitrophenyl (Np) ester method[19] to afford Z(OMe)-Val-Ser-Cys(Bzl)-OH (III). Treatment of this protected tripeptide (III) with TFA in the usual manner gave H-Val-Ser-Cys(Bzl)-OH(IV) in a crystalline form. Addition of Z(OMe)-Cys(Bzl)-OH to (IV) was performed by the Np ester method to give Z(OMe)-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (V). After repeating the similar deblocking procedure, the resulting partially protected tetrapeptide, H-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (VI) was condensed with Z(OMe)-Ser-OH by the PCP ester method to give Z(OMe)-Ser-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (VII). Removal of Z(OMe) group of VII was performed by TFA in the usual manner and the analytically pure partially protected pentapeptide, H-Ser-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (A), was obtained.

Next, the synthetic route to B is illustrated in Fig. 2. H-Ser-Ser-OH[20] was condensed with Z-Val-OH by means of the mixed anhydride procedure to give Z-Val-Ser-Ser-OH (VIII). Z-Val-OPCP failed to form the desired amide bond with H-Ser-Ser-OH. This protected tripeptide (VIII) was partially soluble in water and therefore extraction of this compound with ethyl acetate was difficult. After the reaction mixture was condensed *in vacuo*, the residue was acidified with dilute HCl and stored in cold. The gelatinous mass thus obtained was recrystallized from methanol and ethyl acetate. H-Val-Ser-Ser-OH (IX) obtained after hydrogenation of (VIII) was then condensed with Z-Ser-Ser-OH by the azide procedure[21] to give Z-pentapeptide, Z-Ser-Ser-Val-Ser-Ser-OH (X). This compound was also obtained as the gelatinous mass from the acidified condense of the reaction mixture. After hydrogenation of (X), the serine rich pentapeptide, H-Ser-Ser-Val-Ser-Ser-OH (B), was obtained in a crystalline form.

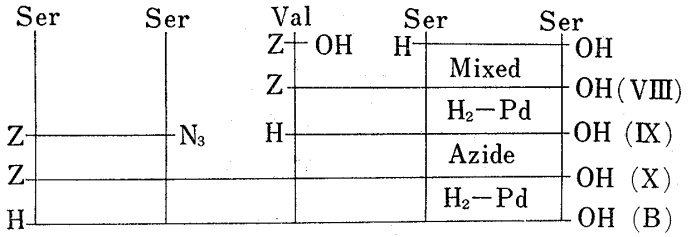

Fig. 2. Synthetic Route to B

Examination of these synthetic peptides as a model of ferredoxin will be reported in the future.

Experimental

General experimental methods employed are essentially the same as described in the Part XXII[22] of this series. Thin layer chromatography was performed on silica gel (Kiesel: gel G. Merck). *Rf* values refer to the following solvent systems: Rf_1: $CHCl_3$–MeOH–H_2O (40:15:5), Rf_2: *n*-BuOH–AcOH–pyridine–H_2O (4:1:1:2), Rf_3: *n*-BuOH–AcOH–H_2O (4:1:5). In order to detect Z(OMe)-peptides on thin layer chromatography, ninhydrin and 0.1N HCl in acetone were sprayed and the plate was heated in an oven (80°) for 10 min.

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22) H. Yajima, Y. Okada, H. Kawatani and N. Mizokami, *Chem. Pharm. Bull.* (Tokyo), **17**, 1229 (1969).

Z(OMe)-Ser-Cys(Bzl)-OH (I)——Z(OMe)-Ser-OPCP[23] (39.00 g) in a mixture of DMF (70 ml) and dioxane (40 ml) was added to a solution of H-Cys(Bzl)-OH (10.55 g) and triethylamine (13.9 ml) in H_2O (50 ml). The solution was stirred at room temperature for 72 hr. The solvent was evaporated *in vacuo*. AcOEt and 15% citric acid was added to the residue and the resulting solid was collected by filtration and recrystallized from MeOH; yield 11.45 g (64%), mp 149—150°, $[\alpha]_D^{25}$ −15.9° (c=1.0, DMF). Rf_1: 0.34. *Anal.* Calcd. for $C_{22}H_{26}O_7N_2S$: C, 57.1; H, 5.7; N, 6.0. Found: C, 56.9; H, 5.8; N, 6.0.

H-Ser-Cys(Bzl)-OH (II)——Z(OMe)-Ser-Cys(Bzl)-OH (18.22 g) was treated with TFA (30 ml) in the presence of anisole (11 ml) at room temperature for 1 hr. Dry ether (300 ml) was added and the resulting solid was recrystallized from MeOH in the presence of triethylamine; yield 13.35 g (88%), $[\alpha]_D^{23}$ −43.2°, mp 140° (decomp). Rf_1: 0.08, Rf_2. 0.60. *Anal.* Calcd. for $C_{13}H_{18}O_4N_2S$: C, 52.3; H, 6.0; N, 9.4. Found: C, 52.3; H, 6.0; N, 9.4.

Z(OMe)-Val-ONp——According to Bodanszky, *et al.*,[19] the title compound was prepared and recrystallized from EtOH and petroleum ether, yield 78%. mp 76—78°, *Anal.* Calcd. for $C_{20}H_{22}O_7N_2$: C, 59.7; H, 5.5; N, 7.0. Found: C, 59.8; H, 5.6; N, 7.0.

Z(OMe)-Val-Ser-Cys(Bzl)-OH (III)——Z(OMe)-Val-ONp (24.14 g) in dioxane (70 ml) was added to a well stirred solution of H-Ser-Cys(Bzl)-OH (5.97 g) and triethylamine (5.6 ml) in H_2O (30 ml). After stirring for 48 hr, the solution was condensed and H_2O was added to the residue. The aqueous phase was washed with AcOEt and then acidified with 10% citric acid. The resulting solid was collected by filtration, washed with H_2O and recrystallized from MeOH; yield 7.47 g (67%), mp 182—185°. Rf_1: 0.31, Rf_2: 0.78. $[\alpha]_D^{28}$ −5.0° (c=1.0, DMF). *Anal.* Calcd. for $C_{27}H_{35}O_8N_3S$: C, 57.7; H, 6.3; N, 7.5. Found: C, 57.7; H, 6.4; N, 7.3.

H-Val-Ser-Cys(Bzl)-OH (IV)——In the usual manner, Z(OMe)-Val-Ser-Cys(Bzl)-OH (14.88 g) was treated with TFA (20 ml) in the presence of anisole (7.6 ml) for 1 hr. Dry ether was added and the resulting precipitate was collected by filtration. The trifluoroacetate thus obtained was converted to the free peptide by crystallization from MeOH in the presence of triethylamine: yield 11.63 g (87%), mp 216—217°, $[\alpha]_D^{30}$ −19.5° (c=1.0, AcOH). Rf_1: 0.44, Rf_2: 0.84. *Anal.* Calcd. for $C_{18}H_{27}O_5N_3S\cdot H_2O$: C, 52.0; H, 7.0; N, 10.1. Found: C, 52.5; H, 6.9; N, 10.3.

Z(OMe)-Cys(Bzl)-ONp——According to Bodanszky, *et al.*,[19] the title compound was prepared and recrystallized from EtOH and petroleum ether, yield 94%, mp 86—88°. *Anal.* Calcd. for $C_{25}H_{24}O_7N_2S$: C, 60.5; H, 4.9; N, 5.6. Found: C, 60.6; H, 4.7; N, 5.5.

Z(OMe)-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (V)——Z(OMe)-Cys(Bzl)-ONp (7.45 g) in DMF (55 ml) was added to a solution of H-Val-Ser-Cys(Bzl)-OH (5.12 g) and triethylamine (4.2 ml) in H_2O (30 ml) and after the solution was stirred at room temperature for 72 hr, the solvent was evaporated. To the residue, 15% citric acid was added and the resulting precipitate was collected, washed with H_2O and recrystallized from MeOH; yield 8.01 g (80%), mp 179—181°, $[\alpha]_D^{30}$ −4.0° (c=1.0, DMF), Rf_2: 0.76. *Anal.* Calcd. for $C_{37}H_{46}O_9N_4S_2\cdot H_2O$: C, 57.5; H, 6.3; N, 7.3. Found: C, 57.3; H, 6.1; N, 7.4.

H-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (VI)——Z(OMe)-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (5.00 g) was treated with TFA (6.6 ml) in the presence of anisole (2 ml). The trifluoroacetate was isolated in the usual manner and converted to the free peptide by recrystallization from MeOH in the presence of triethylamine; yield 3.70 g (92%), mp 210° (decomp.), $[\alpha]_D^{22}$ −19.8° (c=0.5, AcOH). Rf_2: 0.8. *Anal.* Calcd. for $C_{28}H_{38}O_6N_4S_2\cdot 1/2H_2O$: C, 56.1; H, 6.6; N, 9.3. Found: C, 55.9; H, 6.6; N, 9.5.

Z(OMe)-Ser-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (VII)——To a solution of H-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (1.00 g) and triethylamine (0.47 ml) in H_2O (30 ml), there was added a solution of Z(OMe)-Ser-OPCP (2.62 g) in DMF (60 ml) and the mixture was stirred at room temperature for 48 hr. An additional active ester (0.80 g) was added and the pH of the solution was adjusted to *ca.* 8 with triethylamine. Stirring was further continued for 24 hr and the solvent was evaporated *in vacuo*. AcOEt was added to the residue. The resulting solid was collected by filtration, washed with 10% citric acid and H_2O and recrystallized from MeOH; yield 1.07 g (74%), mp 190—193°, $[\alpha]_D^{26}$ −20.0° (c=1.0, DMF). Rf_2: 0.75, Rf_3: 0.87. *Anal.* Calcd. for $C_{40}H_{51}O_{11}N_5S_2\cdot H_2O$: C, 55.9; H, 6.2; N, 8.1. Found: C, 55.7; H, 5.9; N, 8.1.

H-Ser-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (A)——Z(OMe)-Ser-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (1.90 g) was treated in the usual manner with TFA (2.3 ml) and anisole (0.7 ml). The resulting gelatinous trifluoroacetate was converted to the corresponding free peptide by triethylamine as described above. Yield 1.28 g (86%), mp 229—231°, $[\alpha]_D^{27}$ +24.0° (c=0.5, dimethylsulfoxide). Rf_2: 0.76. Amino acid ratios in acid hydrolysate $Ser_{1.74}$ $Val_{1.00}$ Cys(Bzl)+cystine$_{1.95}$ (average recovery 95%). *Anal.* Calcd. for $C_{31}H_{43}O_8N_5S_2\cdot H_2O$: C, 53.5; H, 6.5; N, 10.1. Found: C, 53.5; H, 6.6; N, 10.2.

Treatment of H-Ser-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (A) with HF——H-Ser-Cys(Bzl)-Val-Ser-Cys(Bzl)-OH (30.5 mg) was treated with anhydrous HF (approximately 3 ml) in the presence of anisole (0.3 ml) at 22° for 60 min. The container was evacuated and the residue was placed over KOH pellets *in vacuo* overnight and then dissolved in H_2O (10 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 5 g) for 30 min. The solution was filtered and the filtrate, after washing with ether, was

23) E. Klieger, *Ann.*, **724**, 204 (1969).

lyophilized to give fluffy powder; yield 12.2 mg (64%). Rf_2: 0.47 with a minor impurity Rf_2: 0.30. Amino acid ratios in an acid hydrolysate $Ser_{1.93}$ $Val_{1.00}$.

According to Hirs,[24] the sample (5.05 mg) was dissolved in 90% formic acid (2 ml) and 28% H_2O_2 (0.1 ml) was added. After 30 min, the solution was treated with PtO_2 until the iodine test became negative. The solution was filtered and the solvent was evaporated. The residue was submitted to the enzymatic hydrolysis according to Hofmann, *et al.*[25] amino acid ratios in an AP-M[26] digest: $Ser_{1.86}$ Cysteic $acid_{1.88}$ $Val_{1.00}$ (recovery 71%).

Z-Val-Ser-Ser-OH (VIII)——A mixed anhydride, prepared from Z-Val-OH (21.00 g) with triethylamine (11.6 ml) and ethyl chloroformate (8.0 ml) in dry THF (150 ml), was added to a solution of H-Ser-Ser-OH[20] (16.00 g) and triethylamine (11.6 ml) in H_2O (150 ml) and the solution was stirred in an ice–bath for 2 hr. The solvent was evaporated and the residue was acidified to pH 3 with 2N HCl. The resulting gelatinous mass, after standing overnight in a refrigerator, was collected by filtration, washed with AcOEt and H_2O and recrystallized from MeOH and AcOEt; yield 16.55 g (47%), mp 176—178°, $[\alpha]_D^{23}$ −25.6° (c=1.0, MeOH). *Anal.* Calcd. for $C_{19}H_{27}O_8N_3 \cdot 1/2H_2O$: C, 52.5; H, 6.5; N, 9.7. Found: C, 52.9; H, 6.4; N, 9.5.

H-Val-Ser-Ser-OH (IX)——Z-Val-Ser-Ser-OH (8.00 g) in 98% MeOH (500 ml) containing AcOH (4 ml) was hydrogenated over a Pd catalyst in the usual manner for 4 hr. Bulk of the solvent was evaporated and the resulting crystalline product was dissolved in hot H_2O. The solution was filtered to remove the catalyst and the filtrate was condensed *in vacuo*. Addition of EtOH to the residue gave fine crystalls; yield 5.08 g (94%), mp 215—216°, $[\alpha]_D^{25}$ +9.9° (c=0.5, H_2O), single ninhydrin positive spot Rf_2: 0.37. *Anal.* Calcd. for $C_{11}H_{21}O_6N_3 \cdot 1/2H_2O$: C, 44.0; H, 7.4; N, 14.0. Found: C, 44.0; H, 7.6; N, 13.9.

Z-Ser-Ser-Val-Ser-Ser-OH (X)——Z-Ser-Ser-$NHNH_2$ (6.59 g) was converted to the corresponding azide according to Hofmann, *et al.*[21] The solid azide thus obtained was added to a solution of H-Val-Ser-Ser-OH (2.91 g) and triethylamine (2.8 ml) in a mixture of DMF (70 ml) and H_2O (40 ml). The solution was stirred at 4° for 48 hr. The solvent was evaporated and the residue was dissolved in 3% NH_4OH, which after washing with AcOEt, was neutralized with 6N HCl. The solution was condensed to one fourth of the original volume and acidified to pH 3 with 6N HCl. The gelatinous mass, after standing overnight in a refrigerator, was collected, washed with H_2O, and recrystallized from MeOH; yield 2.41 g (40%), mp 189—191°, $[\alpha]_D^{19}$ −3.9° (c=0.5, DMF). *Anal.* Calcd. for $C_{25}H_{37}O_{12}N_5 \cdot 2.5H_2O$: C, 46.6; H, 6.6; N, 10.9. Found: C, 46.7; H, 6.5; N, 10.8.

H-Ser-Ser-Val-Ser-Ser-OH (B)——Z-Ser-Ser-Val-Ser-Ser-OH (2.15 g) in 98% MeOH (150 ml) containing AcOH (2 ml) was hydrogenated in the usual manner. The catalyst was removed by filtration and the filtrate was condensed *in vacuo*. EtOH was added to the residue to form solid, which was recrystallized from H_2O and EtOH; yield 1.60 g (95%), mp 194—196°. $[\alpha]_D^{22}$ −57.8° (c=0.5, H_2O), Rf_3: 0.26, single ninhydrin positive spot. Amino acid ratios in acid hydrolysate: $Ser_{3.64}$ $Val_{1.00}$ (average recovery 91%). Amino acid ratios in AP-M digest[26]: $Ser_{4.40}$ $Val_{1.00}$ (average recovery 74%). *Anal.* Calcd. for $C_{17}H_{31}O_{10}N_5$: C, 43.9; H, 6.7; N, 15.1. Found: C, 43.6; H, 6.8; N, 14.8.

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26) G. Pfleiderer and P.G. Celliers, *Biochem. Z.*, **339**, 186 (1963); G. Pfleiderer, P.G. Celliers, M. Stanulovic, E.D. Washmuth, H. Determan and G. Braunitzer, *ibid.*, **340**, 552 (1964). Röhm & Haas (Darmstadt) preparation was used.