Structural Determination of Ancovenin, a Peptide Inhibitor of Angiotensin I Converting Enzyme

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The structure of ancovenin, a new peptide inhibitor of angiotensin I converting enzyme was determined chemically. Ancovenin is composed of 16 amino acid residues including three lanthionine residues which form a complex tricyclic structure bridged by sulfide bonds. Moreover, this peptide includes dehydroalanine as one of its unusual amino acids. In the present study, plausible mechanisms of the Edman degradation of dehydroalanine and lanthionine-containing peptides were also elucidated.

A new lanthionine-containing peptide named ancovenin (Fig. 1) was isolated from a culture broth filtrate of *Streptomyces* sp. No. A647P-2 in a soil sample collected at Hachioji, Tokyo.[1] Ancovenin showed an inhibitory activity against angiotensin I converting enzyme (ACE). The amino acid composition of ancovenin was already determined by amino acid analyses of the intact molecule as well as the peptide obtained by partial hydrogenation.[1] Three kinds of unusual amino acids were found in the molecule, i.e., dehydroalanine (Dha), *meso*-lanthionine (*meso*-Lan), and *threo*-methyllanthionine (*threo*-Melan). Although ancovenin is composed of 16 amino acid residues, it is conveniently regarded as nonadecapeptide, since lanthionine residue is formally represented as two Ala residues which are linked by a sulfide bond to each other, i.e., Ala Ala, and similarly methyllanthionine as Ala Abu. The exact molecular weight of ancovenin was given to be 1960 by measurement of fast atom bombardment mass spectrum.[2,3] The value thus obtained indicated that two of four carboxyl groups related to three acidic amino acids and *C*-terminal one are present in amide form.

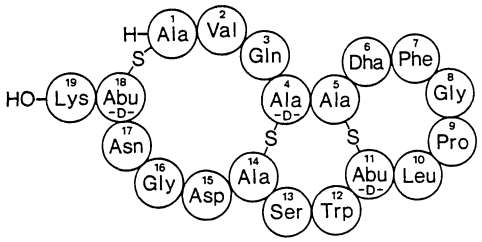

Fig. 1. Structure of ancovenin.

The sequence analysis of ancovenin was carried out as summarized in Fig. 2. Through the sequencing study, we realized that the Edman degradation stopped at the position of Dha residue due to a formation of pyruvoyl peptide as shown in Fig. 3. On the other hand, Lan or Melan residue was cleavable as shown in Fig. 4. However, we could not detect phenyl(thiohydantoin) (Pth) derivatives of these amino acids, since a thiazolinone part formed at the first cycle might be decomposed via β-elimination under basic conditions during several reaction steps. Taking account of these facts, the presence of Dha residue near *N*-terminal region was first assumed, since the Edman degradation of ancovenin did not produce any Pth derivative after the fourth cycle of the reaction. In fact, the position of Dha[6] was confirmed as Ala[6] in

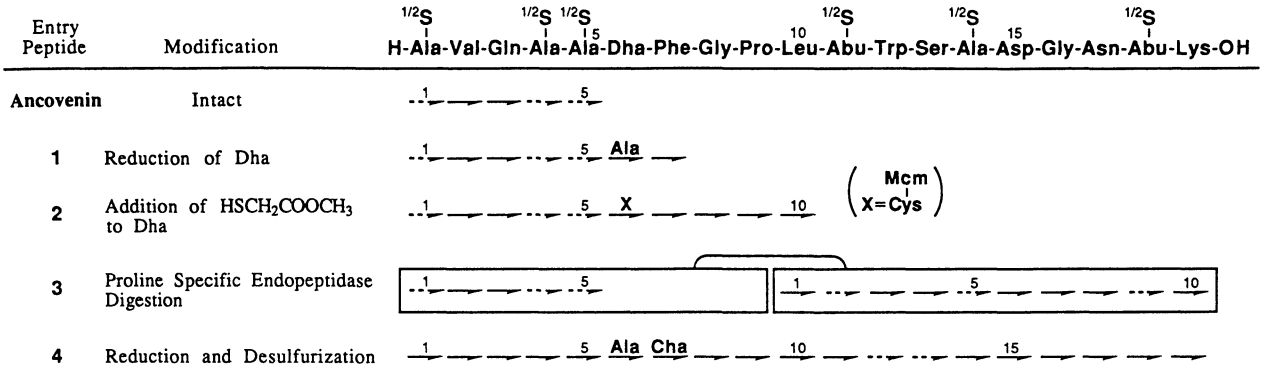

Fig. 2. Sequence analysis of intact and modified ancovenin.
——→: Edman degradation (------→: Pth derivative was not detected).

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peptide **1** prepared by mild hydrogenation of ancovenin and also as *S*-(methoxycarbonylmethyl)-cysteine[6], i.e., Cys(Mcm)[6], in peptide **2** prepared by an addition of $HSCH_2COOCH_3$ to ancovenin. Although we could not determine the sequence beyond 11th amino acid residue of peptide **2**, Pro[9] residue was observed in the peptide. Based on this information, we carried out a proline specific endopeptidase digestion[4] to cleave ancovenin molecule in a middle part of the molecule. Interestingly, the enzymic digestion produced peptide **3** as a sole product. This fact indicated that Pro residue must be located in one of the sulfide rings. The Edman degradation of peptide **3** possessing two amino terminals in the molecule was then carried out. In principle, each degradation should give two Pth derivatives arising from two chains. However at the first step, the residue 1 did not give Pth derivative as known in the degradation of the intact ancovenin. At the second step, only Pth derivative of Val which can be assigned at the residue 2 was observed indicating that the residue 11 in another chain may be involved in a moiety of either lanthionine or methyllanthionine. On the other hand, at the third step, both of Pth derivatives of Gln and Trp were obtained. Since Gln can be assigned as the residue 3, Trp must be now determined as the residue 12 (see **3** in Fig. 2).

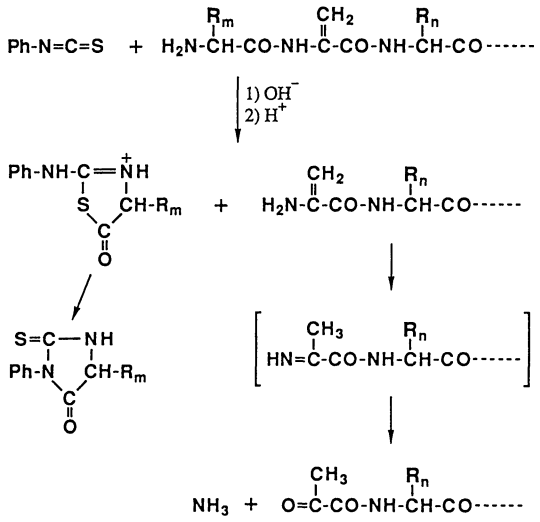


Fig. 3. Plausible mechanism of the Edman degradation of dehydroalanine-containing peptide.

On the basis of the results obtained in this way, we revealed a partial sequence of ancovenin shown as **3** in Fig. 2. In order to obtain more informations about three sulfide amino acid residues, ancovenin was strongly hydrogenated under increased pressure at 50 °C to prepare a desulfurized peptide **4**. In this compound, several amino acid residues as well as sulfide amino acids were present in modified forms, i.e., Dha for Ala, *meso*-Lan for two Ala, *threo*-Melan for Ala and α-aminobutyric acid (Abu), and Phe for 3-cyclohexylalanine (Cha). However, we could not detect Trp as 3-(octahydroindolyl)alanine (Oha) in amino acid analysis, since Oha was not eluted from the column under analytical conditions so far used. The Edman degradation of peptide **4** proceeded completely until the last cycle. Although we failed to detect Oha and Ser as Pth derivatives at 12th and 13th cycles because of decompositions, these positions were already assigned as Trp[12] and Ser[13] from the sequence analysis of the peptide **3**. Consequently, the whole sequence of ancovenin was given as depicted at the

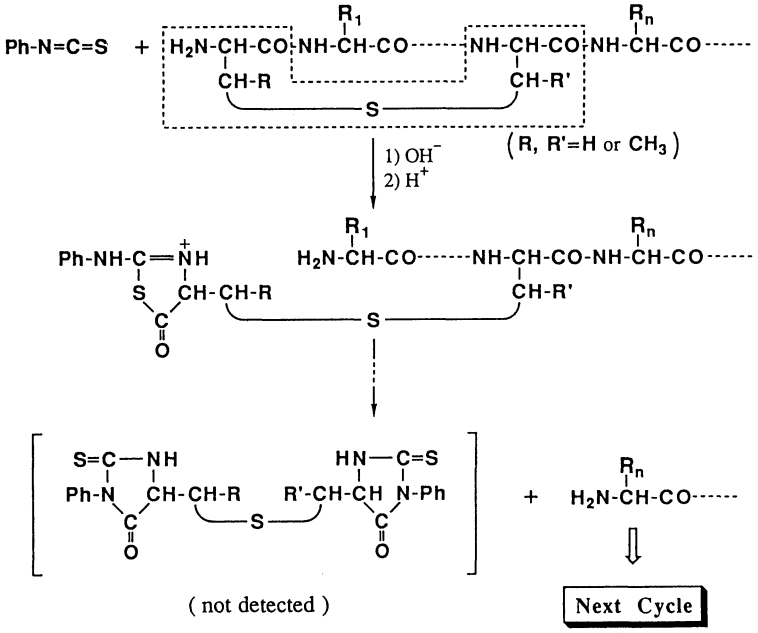


Fig. 4. Plausible mechanism of the Edman degradation of lanthionine-containing peptide.

upper line in Fig. 2. At this stage, six positions belonging to three sulfide amino acid residues were shown as Ala or Abu, since any informations about mode of sulfide bridges were not yet obtained.

The assignment of the position of sulfide bridge was first focused on three Ala residues, i.e., Ala[1], Ala[4], and Ala[5], belonging to sulfide amino acids by application of the Edman-dansyl (Dns) method. For this purpose, three authentic Dns derivatives of the sulfide amino acids, i.e., Dns-D-Ala L-Ala (**A**), Dns-L-Ala D-Abu (*threo*) (**B**), and Dns-D-Ala D-Abu (*threo*) (**C**) were prepared. These compounds were effectively separated in HPLC as shown in Fig. 5. Thus, each Dns derivative originating from Ala[1], Ala[4], and Ala[5] was clearly assigned to be **B**, **A**, and **B**, respectively. (Fig. 6) Since the enantiomers of the above three authentic samples also give the same pattern in HPLC, the absolute stereochemistry of each derivative was not yet decided at this stage. However, this result unequivocally indicated that Ala[14] must be the counterpart of *meso*-Lan residue together with Ala[4] giving us an assignment of the first sulfide bridge between Ala[4] and Ala[14].

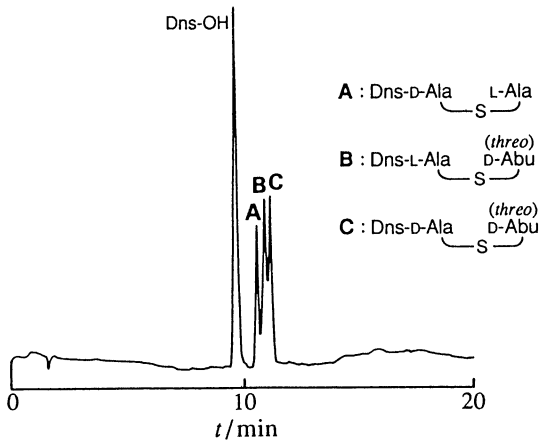
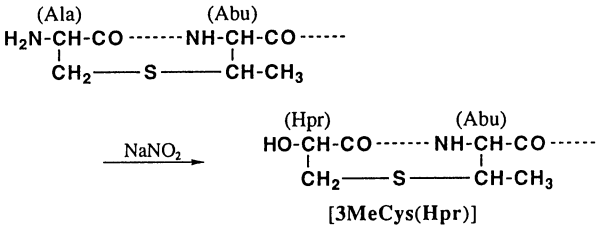


Fig. 5. HPLC profile of mono-Dns derivatives of *meso*-lanthionine and *threo*-methyllanthionine.

In order to determine the positions of other two sulfide bridges, we then applied the subtractive-Edman method to the peptide **3** obtained above (Fig. 7a). Two cycles of the Edman degradation were carried out showing a stepwise disappearance of *threo*-Melan residue after each cycle of the reaction indicating that Ala[1] was not a partner of Abu[11], because otherwise stepwise disappearance of *threo*-Melan can not be explained (Table 1). In order to confirm this result, one of *threo*-Melan residues was attempted to be differentiated as *S*-(2,3-dihydroxy-3-oxopropyl)-3-methylcysteine [3MeCys(Hpr), Hpr Abu]. Namely, Ala[1] residue was converted into 2-hydroxypropionyl (Hpr) residue by deamination with sodium nitrite as shown below.[5] The modified peptide was then digested



with proline specific endopeptidase to obtain peptide **5** in Fig. 7. The subtractive Edman degradation of peptide **5** (Fig. 7b) gave a result that *threo*-Melan still remained in 1st cycle but disappeared in 2nd cycle, whereas 3MeCys(Hpr) remained even at the second cycle of the reaction as shown in Table 2. These facts suggested that the second sulfide bond must be located between Ala[1] and Abu[18] and the third one between Ala[5] and Abu[11]. (Fig. 7c)

Thus the whole structure of ancovenin was now obtained as mentioned above except for stereochemistry. The configurations of usual amino acids were determined by gas chromatography based on the separation of enantiomer of each amino acid. Acid hydrolyzate of ancovenin was treated with HCl in isopropyl alcohol and then with $(CF_3CO)_2O$.[6] The mixture of *N*-(trifluoroacetyl) amino acid isopropyl

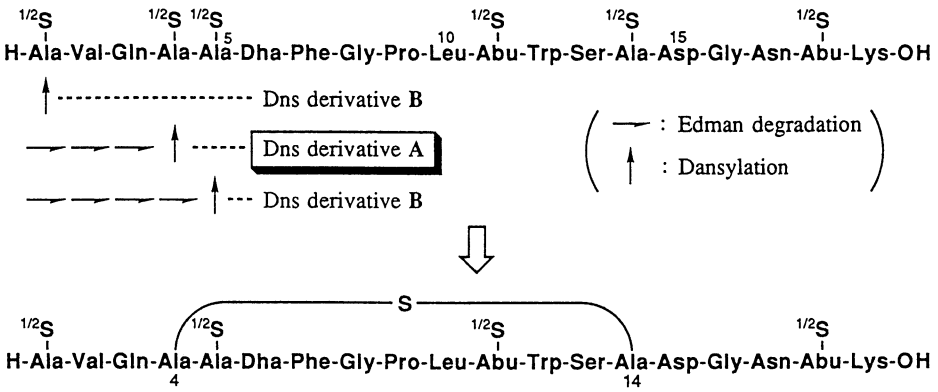


Fig. 6. Assignment of sulfide bridge belonging to *meso*-lanthionine.

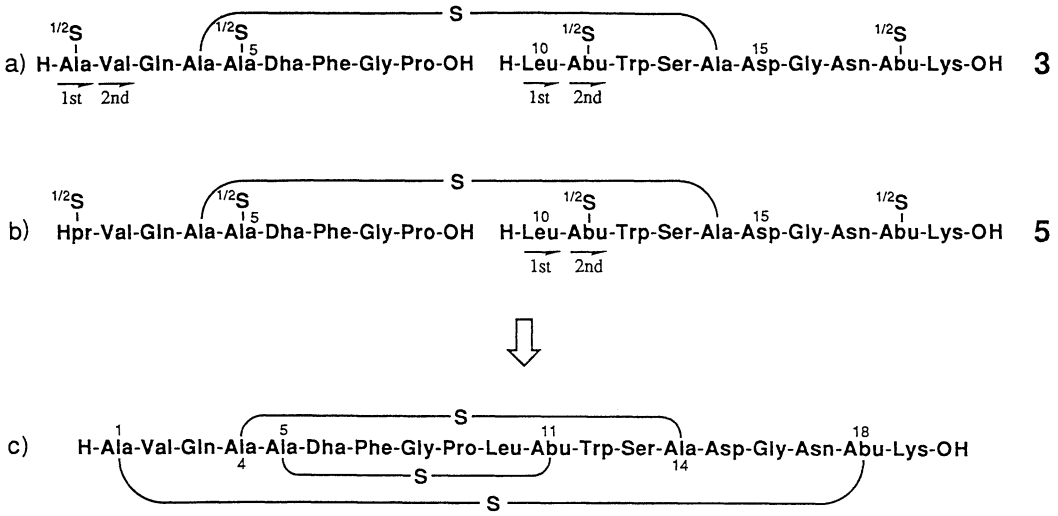


Fig. 7. Assignment of sulfide bridge belonging to *threo*-methyllanthionine on the basis of the subtractive Edman degradation of peptides **3** and **5**.

Table 1. Result of Subtractive Edman Degradation of Peptide **3**

Amino acid	Amino acid composition		
	Intact	1st cycle	2nd cycle
Asp	2.09	2.22	2.16
Ser	0.94	1.07	1.27
Glu	1.02	0.99	1.12
***threo*-Melan**	**2.01**	**1.16**	**0.55**
Pro	0.84	1.02	1.51
meso-Lan	1.06	1.01	1.07
Gly	2.00	2.00	2.00
Val	0.72	0.78	0.04
Leu	0.99	0.15	0.07
Phe	0.90	0.91	0.97
Lys	0.97	0.50[a)]	0.58[a)]
Trp[b)]	—	—	—

a) A low recovery of Lys is due to an incomplete hydrolysis of N^{ε}-phenylthiocarbamoyl group. b) Not detected.

Table 2. Result of Subtractive Edman Degradation of Peptide **5**

Amino acid	Amino acid composition		
	Intact	1st cycle	2nd cycle
3MeCys(Hpr)	**1.00**	**0.98**	**0.93**
Asp	2.09	2.10	2.06
Ser	1.02	1.03	0.99
Glu	0.97	0.99	0.94
***threo*-Melan**	**1.14**	**1.02**	**0.39**
Pro	0.94	1.18	1.00
meso-Lan	1.08	1.16	1.02
Gly	2.00	2.00	2.00
Val	0.99	0.99	1.06
Leu	1.03	0.14	0.12
Phe	1.04	1.10	1.01
Lys	1.14	0.69	0.63
Trp	—	—	—

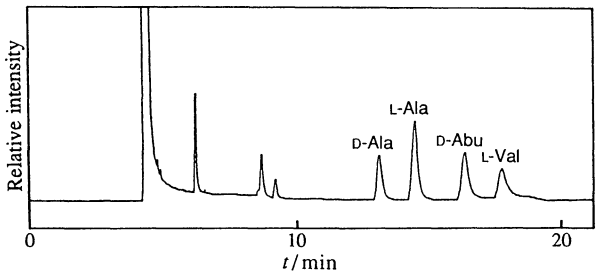


Fig. 8. Gas chromatographic analysis for configurations of Ala and Abu residues in peptide **4**.

esters thus prepared was gas-chromatographed on a glass capillary column coated with chiral stationary phase.[7] From the result in this experiment, all usual amino acids were determined to be of L-form.[2,3]

Configurations of amino acids in the desulfurized peptide **4** in Fig. 2 were next analyzed in a similar manner. In this case, the analysis was carried out isothermally at 95 °C in order to focus on the separation of enantiomers of Ala and Abu residues. The gas chromatogram indicated that the configuration of α-carbon atom of Abu part in two *threo*-Melan residues was undoubtedly of D-form. (Fig. 8) On the other hand, we observed D-Ala and L-Ala in a ratio of 1.65 to 3.35. This result indicated the presences of 3 mol of DL-Ala and 2 mol of L-Ala residues in peptide **4** suggesting the formation of DL-Ala from 1 mol of Dha and 1 mol of *meso*-Lan under reductive conditions for desulfurization. Therefore, the remaining 2 mol of L-Ala residues were assumed to be attributed from Ala counterpart in *threo*-Melan residues and thus the absolute structure of this thiobis[amino acid] was concluded to be L-Ala—S—(*threo*) D-Abu. Indeed, the specific rotation of *threo*-Melan isolated from the hydrolyzate of ancovenin was quite identical with that of authentic

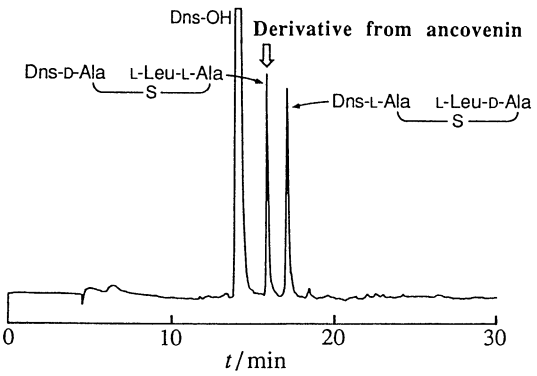


Fig. 9. HPLC profile of diastereoisomeric derivatives of *meso*-lanthionine.

(*threo*)
L-Ala D-Abu synthesized in our previous work.[8]
S

Finally we had to determine the configurations of Ala[4] and Ala[14] belonging to *meso*-lanthionine. For this purpose, we attempted to prepare a suitable diastereoisomer of this amino acid. Mono-Dns-*meso*-Lan obtained by the Edman-dansyl method of ancovenin (Fig. 6) was coupled with *N*-[*N*-(*t*-butoxycarbonyl)-L-leucyloxy]succinimide (Boc-L-Leu-OSu) whose *t*-butoxycarbonyl (Boc) group was then removed. Dns-Ala[4] L-Leu-Ala[14] thus obtained was completely identical with authentic Dns-D-Ala L-Leu-L-Ala in HPLC as shown in Fig. 9. As a result, the stereochemical arrangement of *meso*-lanthionine was assigned to be D-Ala[4] L-Ala[14]. Consequently, we now propose the total structure of ancovenin as shown in Fig. 1.

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Experimental

General Procedure of Amino Acid Analysis of Peptide. Each 25 nmol of peptide was hydrolyzed with 6 M HCl (100 μl; 1 M=1 mol dm^{-3}) in the presence of thioglycolic acid (2-mercaptoacetic acid) (4 μl) at 110 °C for 24 h in a sealed tube after sufficient evacuation. The hydrolyzate was concentrated in vacuo at 60 °C and the residue was dissolved in 0.2 M sodium citrate buffer (pH 2.20, 500 μl) for amino acid analysis. The analysis was carried out with a Hitachi KLA-5 analyzer under following conditions—column: packed with a Hitachi #2618 resin (0.9×25 cm) and warmed at 55 °C; buffer: sodium citrate buffers of 0.2 M (pH 3.00, 80 min)-0.2 M (pH 4.25, 40 min)-0.8 M (pH 6.60, 110 min).

Mild Hydrogenation of Ancovenin—Preparation of Peptide 1. Ancovenin (11 mg, 5.7 μmol) was dissolved in a mixture of water (10 ml), methanol (2 ml), and acetic acid (0.2 ml). Hydrogen was introduced into the solution in the presence of Pd black (170 mg). The hydrogenation was continued until starting material disappeared in HPLC analysis. The reaction mixture was filtered to remove the catalyst and the filtrate was lyophilized. The residue was purified by preparative HPLC (Cosmosil 5C$_{18}$, 4×125 mm, 31% aqueous acetonitrile containing 0.1% of TFA, 1.0 ml min^{-1}) to obtain peptide **1** (0.59 mg, 5.3%).

Addition of Methyl Thioglycolate to Dehydroalanine Residue—Preparation of Peptide 2. Ancovenin (0.20 mg, 0.10 μmol) was dissolved in 1 M *N*-ethylmorpholine acetate buffer (pH 8.5, 10 μl) and ethanol (10 μl). To the solution was added methyl thioglycolate (5.0 μl, 55 μmol) and the mixture was allowed to stand for 5 d at room temperature. The reaction mixture was concentrated in vacuo to obtain an oily residue which was triturated with ether several times and then dissolved in a small amount of water. The solution was lyophilized to obtain peptide **2** (0.14 mg, 70%) as a white powder.

Proline Specific Endopeptidase Digestion of Ancovenin—Preparation of Peptide 3.[4] To a solution of ancovenin (1.4 mg, 0.71 μmol) in 70 μl of 20 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.0) was added proline specific endopeptidase solution (20 μl, enzyme/substrate=1/40 (w/w)) purchased from Seikagaku Kogyo Co., Ltd., Japan. The digestion was carried out for 17 h at room temperature. The reaction mixture was directly purified by preparative HPLC (Cosmosil 5C$_{18}$, 6×250 mm, 31% CH_3CN in 0.1% aqueous TFA, 2.0 ml min^{-1}). The fraction including peptide **3** was lyophilized to obtain a pure material as white powder, yield 1.1 mg (78 %).

Reductive Desulfurization of Ancovenin—Preparation of Peptide 4. Ancovenin (0.98 mg, 0.50 μmol) dissolved in water (2.5 ml) and acetic acid (40 μl) was hydrogenated with Pd black catalyst (50 mg) in a pressure bottle under 10 kg cm^{-2} for 2 d at 50 °C. The reaction mixture was centrifuged and the supernatant was lyophilized to obtain peptide **4** (0.32 mg, 34 %) as white powder.

Preparation of 3-Cyclohexylalanine (Cha) and 3-(Octahydroindolyl)alanine (Oha). L-Phenylalanine (0.10 g, 0.61 mmol) in water (20 ml) and acetic acid (0.4 ml) was hydrogenated in the presence of Pd black (50 mg) under increased pressure (10 kg cm^{-2}) for 20 h at 50 °C. The reaction mixture was centrifuged and the supernatant was lyophilized. The residue was purified by column chromatography on HP-20. Water eluate containing Cha was lyophilized to obtain pure compound, yield 25 mg (24%); retention time in amino acid analysis: 147.9 min.

L-Tryptophan (0.10 g, 0.49 mmol) in water (15 ml) and acetic acid (0.3 ml) was hydrogenated in the presence of Pd catalyst (150 mg) under the same conditions as mentioned above. The product was purified in a similar manner to the case of Cha, yield 112 mg (84%). Oha was not detected in amino acid analysis under our analytical conditions mentioned above.

Deamination[5] and Proline Specific Endopeptidase Digestion of Ancovenin—Preparation of Peptide 5. To a solution of ancovenin (0.59 mg, 0.30 μmol) in water (240 μl) was added 1 M aqueous acetic acid (60 μl) and 8.7 M sodium nitrite solution (120 μl, 1 mmol). The reaction mixture was allowed to stand for 10 min at 0 °C and the reaction was quenched by adjustment of pH to 8 with 1 M aqueous ammonia. A desalting was immediately carried out by preparative HPLC (Nucleosil 7C$_{18}$, 8×250 mm, linear gradient of 1—60% aqueous acetonitrile containing 0.1% TFA, 3.0 ml min^{-1}). To a solution of white powder obtained by lyophilization in 0.2 M ammonium acetate buffer (pH 8.0, 300 μl) was added proline specific endopeptidase solution (30 μl, enzyme/substrate=1/12 (w/w)) and the mixture was allowed to stand for 15 h at room temperature. The reaction mixture was purified by preparative HPLC (Cosmosil 5C$_{18}$, 4×125 mm, 31% aqueous acetonitrile containing 0.1%

TFA, 1.0 ml min^{-1}) to obtain peptide **5** as white powder, yield 0.14 mg (23%).

Preparation of *S*-(2,3-Dihydroxy-3-oxopropyl)-3-methylcysteine [3MeCys(Hpr)]. To a solution of (2*R*)-*S*-[*N*-benzyloxycarbonyl-(2*S*,3*S*)-3-methylalanin-3-yl]cysteine[8] (1.7 mg, 4.8 μmol) in water (1 ml) was added 1 M aqueous acetic acid (200 μl) and 8.7 M sodium nitrite solution (400 μl, 3.3 mmol) under cooling in an ice bath. The mixture was allowed to stand for 5 min at 0 °C and then extracted with ethyl acetate. The extract was concentrated in vacuo and a residue was hydrolyzed with 6 M HCl in a sealed tube for 12 h at 110 °C. The hydrolyzate was concentrated in vacuo to obtain 3MeCys(Hpr) as an oily substance which gave a single peak at 26.7 min in amino acid analysis.

General Procedure of Sequencing of Peptides. Each peptide (50 nmol) was subjected to the Edman degradation and the reaction was carried out according to Tarr's procedure.[9] Pth derivative of amino acid obtained was analyzed in HPLC under following conditions—column: Nucleosil 5C_{18} (4×250 mm); eluent: acetonitrile–0.01 M sodium acetate buffer of pH 4.5 (42 : 58 v/v); flow rate: 1.0 ml min^{-1}; detection: UV at 269 nm.

Preparation and Analysis of 5-Dimethylaminonaphthalene-1-sulfonyl [Dansyl (Dns)] Amino Acid from Peptide. A small amount (0.2—200 μmol) of ancovenin or peptide after the Edman degradation in 40 mM Li_2CO_3–HCl buffer (pH 9.5, 200 μl) was dansylated with Dns-Cl in acetonitrile (100 μl of 1.5 mg ml^{-1}=5.6 mM solution) according to Tapuhi's procedure.[10] Dansyl derivative of peptide was hydrolyzed with 6 M HCl (100 μl) and thioglycolic acid (4 μl) in a sealed tube for 12—18 h at 105 °C. Each Dns-amino acid was dissolved in 10 μl of 95% ethanol and a suitable amount of the solution was applied to HPLC analysis which was carried out under following conditions—column: Cosmosil 5C_{18} (4×125 mm); eluent: 5% to 50% CH_3CN in 0.05 M Tris-HCl (pH 7.75) (gradient elution); flow rate: 1.0 ml min^{-1}; detection: UV at 340 nm.

Authentic samples of mono-Dns derivative of sulfide amino acids were prepared in a similar manner from *N*-benzyloxycarbonyl-(2*R*)-*S*-[(2*S*)-alanin-3-yl]cysteine (D-Ala–S–Z-L-Ala),[11] (2*R*)-*S*-[*N*-benzyloxycarbonyl-(2*S*,3*S*)-3-methyl-alanin-3-yl]cysteine (L-Ala–S–(*threo*)Z-D-Abu),[8] and (2*S*)-*S*-[*N*-benzyloxycarbonyl-(2*S*,3*S*)-3-methylalanin-3-yl]cysteine (D-Ala–S–(*threo*)Z-D-Abu),[8] respectively. Benzyloxycarbonyl group was finally removed by acid hydrolysis to obtain mono-Dns-*meso*-Lan or mono-Dns-*threo*-Melan.

Preparation of Diastereoisomeric Derivative of *meso*-Lanthionine. An aliquot of (2*R*)-*S*-[*N*-(5-dimethylaminonaphthalene-1-sulfonyl)-(2*S*)-alanin-3-yl]cysteine (Dns-D-Ala–S–L-Ala) prepared as mentioned above was dissolved in *N*,*N*-dimethylformamide (50 μl) and coupled with an excess of *N*-(*N*-*t*-butoxycarbonyl-L-leucyloxy)succinimide in the presence of triethylamine at 0 °C. The reaction mixture was allowed to stand overnight and concentrated in vacuo. A solution of the residue in trifluoroacetic acid (100 μl) was allowed to stand for 1 h and dried by blowing of nitrogen. The oily residue was dissolved in 95% ethanol and analyzed in HPLC under following conditions—column: μBondapak C_{18} (8×100 mm); eluent: 5% to 50% CH_3CN in 0.05 M Tris-HCl (pH 7.75) (gradient elution); flow rate: 1.0 ml min^{-1}; detection: UV at 340 nm. Diastereoisomer of the above compound was similarly prepared from Dns-L-Ala–S–D-Ala which was obtained as follows. Monobenzyloxycarbonyl derivative of *meso*-lanthionine (D-Ala–S–Z-L-Ala) (2—3 mg) was dissolved in a small amount of saturated sodium acetate solution (1 ml) and acetylated with acetic anhydride for 2 h at 0 °C. The reaction mixture was acidified with 1 M HCl and extracted with ethyl acetate. The extract was concentrated in vacuo and the residue was dissolved in 25% hydrogen bromide in acetic acid (200 μl) and anisole (200 μl). After 3 h at room temperature, ether and hexane were added to the solution. The precipitates were collected by centrifugation and washed with ether. The residue was dansylated and then hydrolyzed as mentioned above to obtain D-Ala–S–Dns-L-Ala.

Dns-*meso*-Lanthionine derivative obtained by the Edman-dansyl method of ancovenin (Fig. 5) was treated in a similar manner and the product was analyzed in HPLC.

Gas Chromatographic Analysis for Configuration of Amino Acids. Acid hydrolyzate of each peptide (0.2—1 μmol) was treated with saturated hydrogen chloride in isopropyl alcohol and then with trifluoroacetic anhydride.[6] The mixture of trifluoroacetyl amino acid isopropyl ester in acetone (0.3 ml) was gas-chromatographed on a glass capillary column coated with chiral stationary phase.[7] The analysis was carried out under following conditions—column length: 0.25 mm×30 m; temperature: 100 °C to 190 °C (3 °C min^{-1}) for 30 min and then kept at 190 °C for further 50 min (proline was reanalyzed at 120 °C); pressure: 1 kg cm^{-2} (nitrogen). Alanine and α-aminobutyric acid obtained from peptide **4** were analyzed at 95 °C for 20 min. In each case the D-isomer was eluted prior to the L-isomer.

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8) T. Wakamiya, Y. Oda, K. Fukase, and T. Shiba, *Bull. Chem. Soc. Jpn.*, **58**, 536 (1985). $[\alpha]_D^{23}$ of *threo*-Melan in 1M HCl: the product from ancovenin=−35.7° (*c* 0.230); synthetic L-Ala (*threo*) D-Abu=−35.5° (*c* 0.220).

9) G. E. Tarr, *Methods Enzymol.*, **47**, 335 (1977).

10) Y. Tapuhi, D. E. Schmidt, W. Lindner, and B. L. Karger, *Anal. Biochem.*, **115**, 123 (1981).

11) D-Ala Z-L-Ala was prepared from *N*-benzyloxycarbonyl-L-cysteine and D-β-chloroalanine in a similar manner to the preparation of D-Ala Z-L-Abu as mentioned in Ref. 8.