1986 © *1989 The Chemical Society of Japan* *Bull. Chem. Soc. Jpn.*, *62*, *1986—1994 (1989)* [Vol. 62, No. 6

A New Method for Determination of Disulfide Pairing in Peptides[†]

Yuji Hidaka and Yasutsugu Shimonishi*
Institute for Protein Research, Osaka University, Yamada-oka 3-2, Suita, Osaka 565

(Received December 21, 1988)

A new method was developed for determination of disulfide pairings in peptides. This method consisted in the synthesis of peptides with deuteriocysteine residues at given positions and the analysis of deuteriocystine generated in the acid hydrolyzates of the synthetic peptides by fast atom bombardment mass spectrometry. The availability of this method was confirmed by its application for determining the positions of disulfide linkages in a heat-stable enterotoxin of enterotoxigenic *Escherichia coli.*

Many biologically interesting peptides contain several Cys residues joined intramolecularly through disulfide bonds.[1-3] The roles of these disulfide linkages in the biological effects of these peptides are not yet clear, but these linkages are known to be important for stabilizing the native conformations of the peptides essential for expression of their biological activity.[4] The mismatching of disulfide pairs during construction of the spatial structure may change the native conformations of peptides and result in marked reduction or loss of their biological activities. Therefore, information on the disulfide pairing in peptides is very useful for understanding of the bioactive conformations of peptides, the mechanisms of a peptide-to-peptide or peptide-to-protein interaction, etc.

In general, the disulfide pairing of cystine residues in peptides is determined by isolation and sequencing of peptides containing cystine, which are generated by either partial acid or enzymatic hydrolysis of the original peptides.[1] Recently a method was reported for measurement of the molecular weights of peptides containing cystine, by direct analysis of the enzymatic digest of peptides by FAB mass spectrometry, resulting in determination of disulfide pairing in the original peptides.[5] These methods are useful for determination of disulfide pairing in peptides that contain Cys residues at interspersed positions in the peptide chain and that release peptides containing cystine with one disulfide linkage, but they are not suitable for peptides in which Cys residues are located in vicinal positions in the peptide chain and from which peptides containing cystines with multiple-disulfide linkages are generated by hydrolysis, because of the difficulty in cleaving peptide bonds between vicinal Cys residues. Another method to determine disulfide pairing in peptides is synthesis of peptides with disulfide linkages at given positions using selectively removable protecting groups at Cys residues and then comparison of the synthetic peptides with the natural one.[6,7] However, this method is very tedious and time-consuming, because peptides with all possible disulfide pairings must be synthesized for comparison with the natural peptide. Furthermore disulfide exchange reaction may occur under the experimental conditions used.

In this paper, we report a new method for determining disulfide pairing in a peptide by synthesizing a peptide with the same sequence as that of the natural peptide but with deuterio-Cys residues and then analyzing hydrolyzates of the peptide by FAB mass spec-

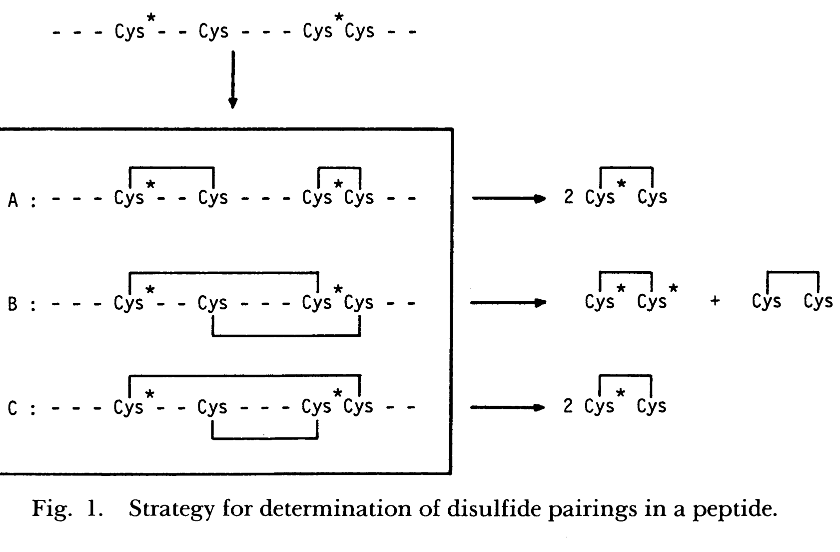

Fig. 1. Strategy for determination of disulfide pairings in a peptide.

[†] We dedicate this paper to Professor Haruaki Yajima on the occation of his retirement from Kyoto University.

trometry. The application of this method to confirm the positions of disulfide bonds that were previously determined by chemical synthesis[7] in a heat-stable enterotoxin produced by enterotoxigenic *Escherichia coli*[8] is also described.

Method

The strategy of the present method is as follows: A protected peptide with deuterio-Cys residues (denoted as Cys* in this paper) at given positions is first synthesized by a solid-phase or solution method, and after removal of the protecting groups, the resulting peptide is oxidized to form intramolecular disulfide linkages between Cys residues, as shown in Fig. 1. The oxidized peptide is then purified by HPLC to isolate the fraction with the same retention time on HPLC as that of its corresponding natural or standard peptide, which has one of the possible disulfide pairings shown in Fig. 1. The purified peptide is then hydrolyzed to constituent amino acids including cystine in acidic medium. If no disulfide-exchange reaction occurs during this hydrolysis, the hydrolyzate contains one kind of cystine (Cys*,Cys) from peptide A or C and two kinds of cystines ($(\mathrm{Cys})_2$ and $(\mathrm{Cys}^*)_2$) from peptide B. In the case of deuterio-Cys (Cys*) in which two hydrogens are replaced by two deuteriums, the three kinds of cystines should have the theoretical mass spectra shown in Fig. 2. The hydrolyzate from peptide B, consisting of two kinds of cystines ($(\mathrm{Cys})_2$ and $(\mathrm{Cys}^*)_2$) should show the mass spectrum of a mixture of equal amounts of these two cystines, whereas the hydrolyzates of peptides A or C should

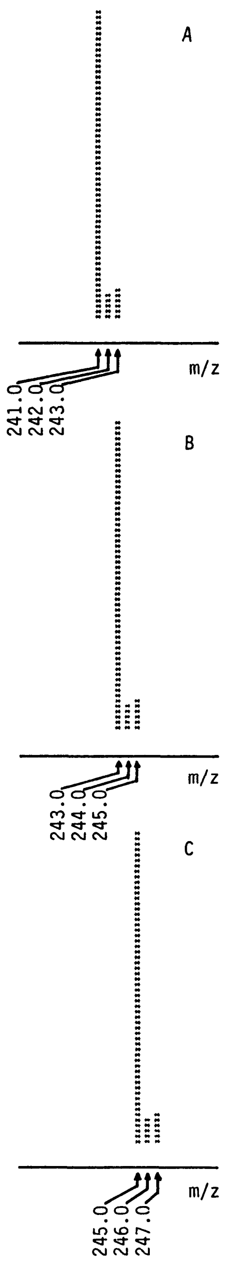

Fig. 2. Theoretical molecular ion distribution of A) $(\mathrm{Cys})_2$, B) (Cys*, Cys), and C) $(\mathrm{Cys}^*)_2$ (two hydrogens are replaced by two deuteriums in Cys*).

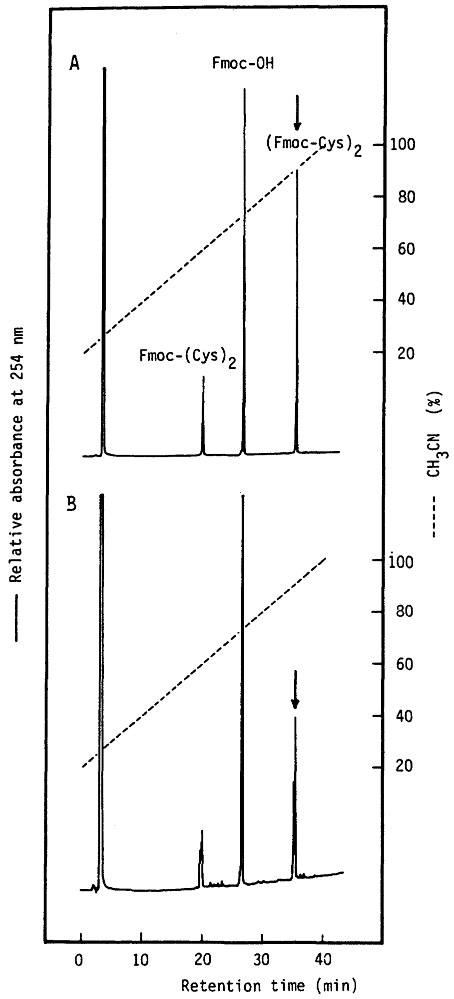

Fig. 3. HPLC profiles of the reaction products of cystine with Fmoc-Cl on a reversed-phase column (Cosmosil C_{18}) with a linear gradient of $\mathrm{CH_3CN}$ in 0.05% TFA with increase of 2% min^{-1} at 30 °C: A) without previous treatment and B) after treatment with 5.7 M HCl at 110 °C for 24 h.

exhibit mass spectra consisting of Cys and Cys*. Thus, if the mass spectrum of the hydrolyzate can be measured with similar accuracy to that of the theoretical mass spectrum, the types of cystine present in the hydrolyzate, and so the disulfide pairing in a peptide can be determined. This method has the characteristics that only one kind of the protecting group is used for cysteine residues and disulfide linkages are formed simultaneously after removal of the protecting group but not stepwisely, whereas intermediates with fewer numbers of disulfide linkages than those of peptides should be purified and identified in the method for determination of disulfide pairings using selectively removable protecting groups.[6,7] This theoretical treatment can be extended to complex peptides containing more cystine residues than those in Fig. 1.

Results and Discussion

Detection of Cystine by Fast Atom Bombardment (FAB) Mass Spectrometry: For this strategy, we tried to find suitable conditions for analysis of cystine in hydrolyzates of peptides by FAB mass spectrometry. First we examined a mixture of amino acids including cystine instead of an acid hydrolyzate of a peptide by FAB mass spectrometry, because we considered that direct analysis of an acid hydrolyzate of a peptide was simple and time-saving. However, we could not detect signal of cystine with a satisfactory signal to noise ratio by mass spectrometry, because the signal intensity of cystine was much smaller than those of other amino acids.[9] Consistent with these findings, the hydrophobicity indices of amino acids[10] suggest that ionization of cystine is difficult in an amino acid mixture including amino acids with hydrophobic side chains. Therefore, we tried to compensate for the hydrophilic character of cystine by adding a substituent with a hydrophobic character to it. For this we treated the amino acid mixture with Fmoc-Cl, and then examined it by FAB mass spectrometry. However, we could not detect the signal of the reaction product of cystine in the FAB mass spectrum due to the intense noise signals derived from the excess reagents. Then, we tried to isolate the reaction product of cystine ((Fmoc-Cys)$_2$) from the reaction mixture and examined it alone by FAB mass spectrometry. Fmoc-amino acids are known to be easily

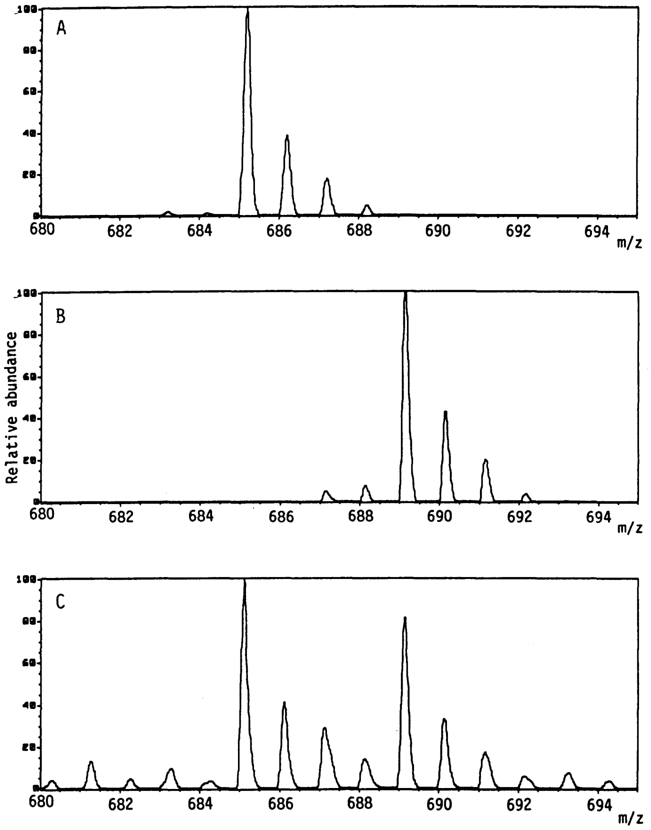

Fig. 4. FAB mass spectra in the molecular ion region of A) (Fmoc-Cys)$_2$, B) (Fmoc-Cys*)$_2$, C) a mixture of A) and B).

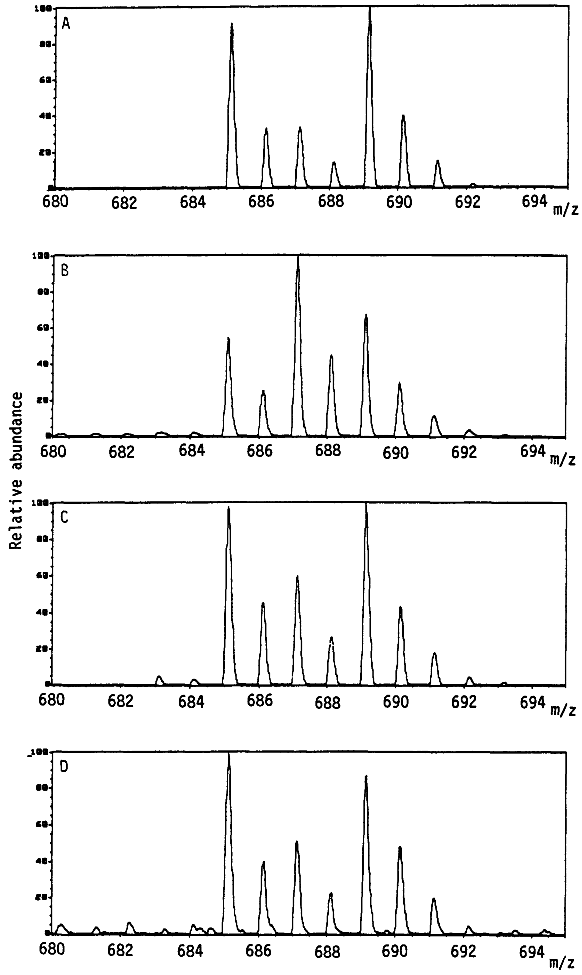

Fig. 5. FAB mass spectra in the molecular ion region of (Fmoc-Cys)$_2$ fractions separated from the reaction products of a mixture of equal amounts of cystine and cystine* with Fmoc-Cl: A) without previous treatment, and after incubation at 110 °C for 24 h in B) 5.7 HCl, C) 4M CH_3SO_3H, and D) 4M TFA.

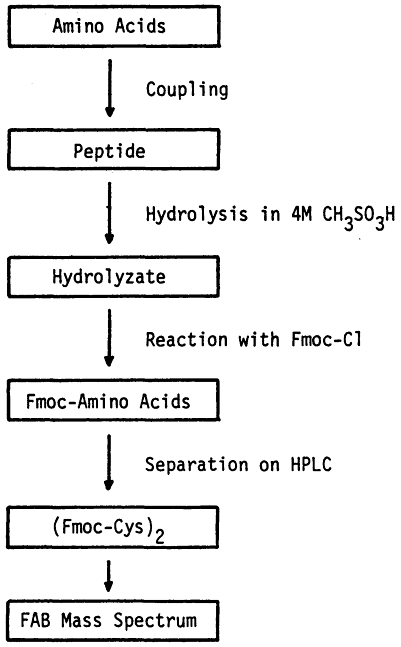

Fig. 6. Flow sheet for detection of cystine in a peptide.

separated by HPLC with a mixture of organic solvents and phosphate buffer as a mobile phase.[11] However, as solvent containing an inorganic salt is not suitable for mass measurement of amino acids and peptides,[12] we used volatile CH_3CN containing 0.05% aq TFA instead of the above mixture as a mobile phase for separation of (Fmoc-Cys)$_2$ from other Fmoc-amino acids by HPLC, as shown in Fig. 3. We found that the (Fmoc-Cys)$_2$ separated in this way could easily be detected in the FAB mass spectrum, as shown in Fig. 4A. These results indicated that cystine could be detected by FAB mass spectrometry of an acid hydrolyzate of a peptide that had been treated by the procedures as shown in Fig. 6.

Conditions for Reaction of Cystine with Fmoc-Cl: As shown in Fig. 4A, (Fmoc-Cys)$_2$ could easily be detected by FAB mass spectrometry. Then we examined the conditions for generating (Fmoc-Cys)$_2$ from cystine in satisfactory yield as a model of the reaction of the hydrolyzate of a peptide with Fmoc-Cl. The reaction was not completed at pH 6.8, but proceeded well at pH 7.8 in 1% aqueous ammonium hydrogen carbonate and (Fmoc-Cys)$_2$ was obtained mainly, as shown in Fig. 3A.

Differentiation of Cystine and Deuteriocystine by Mass Spectrometry: To determine whether cystine could be distinguished from deuteriocystine by mass spectrometry, we examined (Fmoc-Cys)$_2$ and its deuterio derivative ((Fmoc-Cys*)$_2$), synthesized by the procedure described above, and their mixture by FAB mass spectrometry. In this experiment, we used 3,3,3′,3′-tetradeuteriocystine (denoted as cystine*), which was synthesized as a mixture of the (*S*,*S*)-, (*R*,*R*)-, and (*R*,*S*)-isomers by the procedure described below, as a deuteriocystine. Figures 4A—4C show the FAB mass spectra of (Fmoc-Cys)$_2$, (Fmoc-Cys*)$_2$, and their mixture. Their mass distributions were very similar to the theoretical molecular ion distributions of these compounds (data not shown), indicating that cystine* can be distinguished from cystine and implying that (Fmoc-Cys, Fmoc-Cys*), which consists of half-cystine and half-deuteriocystine, gives signals between the mass values of Fmoc derivatives of cystine and cystine* and so can easily be distinguished from these cystines.

Conditions for Acid Hydrolysis of Peptides: Another important premise for the present approach was to find conditions for hydrolysis of a peptide without accompanying disproportionation of disulfide linkages. If a marked disulfide-exchange reaction takes place in this hydrolysis, determination of disulfide pairing in a peptide is impossible. For examination of this possibility, we used a model system in which cystine, cystine*, and their mixture were each treated under the usual conditions for acid hydrolysis of a peptide and allowed to react with Fmoc-Cl as described above. Cystine or cystine* treated with 5.7 M[†] HCl, 4 M CH_3SO_3H, or 4 M TFA at 110 °C for 24 h gave the same HPLC profile as that shown in Fig. 3B, in which the two peak fractions, indicated by arrows, contained (Fmoc-Cys)$_2$ and (Fmoc-Cys*)$_2$, respectively, while the reaction product of cystine with Fmoc-Cl gave a single peak without prior acid treatment (Fig. 3A). The reason why after this treatment (Fmoc-Cys)$_2$ gave two peak fractions will be discussed later. These two peak fractions were isolated together and examined by FAB mass spectrometry. The reaction products from cystine and cystine* exhibited mass spectra with signals of monoisotope masses at m/z=685 and 689, respectively, with signals of isotope masses that were identical with those of cystine and cystine* without acid treatment, as shown in Figs. 4A and 4B, respectively.

On the other hand, when a mixture of equimolar amounts of cystine and cystine* was treated directly with Fmoc-Cl without previous acid treatment, it gave the mass spectrum shown in Fig. 5A, which was slightly different from that seen in Fig. 4C: the peak height of the ion signal at m/z=687 was slightly increased. The mass value of this ion signal was 2 amu more than that of (Fmoc-Cys)$_2$ (m/z=685) and 2 amu less than that of (Fmoc-Cys*)$_2$ (m/z=689). This finding suggested that this ion signal was derived from (Fmoc-Cys, Fmoc-Cys*), which was formed by rearrangement of disulfide linkages of cystine and cystine* during the reaction with Fmoc-Cl and consisted of half-cystine and half-cystine*. The degree of this disulfide-exchange reaction was calculated by subtraction of the peak height of the isotope

---

[†] 1 M=1 mol dm^{-3}.

mass of $(\text{Fmoc-Cys})_2$ at $m/z=687$ from the observed peak height at this mass value, as shown in Table 1.

When a mixture of equimolar amounts of cystine and cystine* was treated in 5.7 M HCl, 4 M CH_3SO_3H or 4 M TFA at 110 °C for 24 h and then with Fmoc-Cl, it showed the FAB mass spectra depicted in Figs. 5B—5D, respectively. The mass spectrum (Fig. 5B) of the mixture of cystine and cystine* treated with 5.7 M HCl differed from that seen in Fig. 5A in showing decreased intensities of the ion signals at $m/z=685$ and 689 and increased intensity of the ion signal at $m/z=687$. As most of the ion signal at $m/z=687$ was considered to be due to Fmoc-half-cystine and Fmoc-half-cystine* as described above, treatment of cystine with 5.7 M HCl was concluded to cause considerable rearrangement of its disulfide linkages. The degree of this disulfide-exchange reaction was estimated to be about 60% by a similar calculation to that described above (Table 1). On the contrary, treatment of the mixture of cystine and cystine* with 4 M CH_3SO_3H or 4 M TFA gave the similar mass spectra (Figs. 5C and 5D, respectively) to that without acid treatment seen in Fig. 5A. The amounts of the disulfide-exchange reaction during these treatments were estimated to be as shown in Table 1, suggesting that the disulfide pairings of cystine residues in a peptide were rearranged slightly, but not significantly, during treatment with 4 M CH_3SO_3H or 4 M TFA. The recoveries of amino acids from the hydrolyzate of a peptide with 4M CH_3SO_3H were excellent, but rather low with 4 M TFA (data not shown), indicating that the hydrolysis of a peptide with 4 M CH_3SO_3H is better than that with 4 M TFA. These results show that cystine can be recovered with slight disulfide disproportionation by hydrolysis of a peptide with 4 M CH_3SO_3H at 110 °C for 24 h, a treatment that is generally used for peptide hydrolysis.[13]

Racemization of Cystine in Acid Hydrolysis: When cystine was kept under the conditions for acid hydrolysis of a peptide and then treated with Fmoc-Cl, the reaction product gave two peak fractions, which were eluted very close to each other on HPLC, as shown in Fig. 3B, while a mixture of cystine with Fmoc-Cl gave only one peak fraction, as shown in Fig. 3A. Coelution of the reaction product $(\text{Fmoc-Cys})_2$ in Fig. 3A with these two peak fractions indicated that the second of these two peak fractions was that of $(\text{Fmoc-Cys})_2$. Moreover, these two peak fractions had the same molecular weight as that of $(\text{Fmoc- Cys})_2$, as shown in Figs. 7A and 7B. These results suggested that cystine was partially racemized under the conditions for acid hydrolysis of a peptide, and that the first of these two peaks was that of the meso-isomer of cystine and that the second was that of the D- and L-isomers. This was confirmed by keeping cystine under the same conditions as those described above but in 5.7 M DCl in D_2O and then treating it with 1 M HCl to remove deuterium attached to amino and carboxyl groups. The resulting cystine gave a similar HPLC profile to that in Fig. 3B, but its mass spectrum showed increments of ion signals at mass values by 1 and 2 amu more than that of cystine at $m/z=685$, as shown in Fig. 7C. This finding indicates that cystine was deuterated at its α,α'-positions during incubation in acidic conditions; that is, cystine was racemized by replacement of protons on its 2,2′-carbons by deuterium from D_2O under the conditions for acid hydrolysis of a peptide. The degree of racemization was calculated to be about 40% from the mass distributions as shown in Figs. 7A and 7C.

Table 1. Interchange Reaction of the Disulfide Linkage in Cystine under Various Conditions for Acid Hydrolysis of Peptides

Acid	Reaction with Fmoc-Cl	Disulfide-exchange reaction (%)
–	+	4.8
5.7 M HCl	+	62.4
4 M CH_3SO_3H	+	14.0
4 M TFA	+	9.8

Preparation of a Derivative of 3,3-Dideuterio-cysteine for Peptide Synthesis: As described above, the protons on the 2,2′-carbons of cystine are replaced

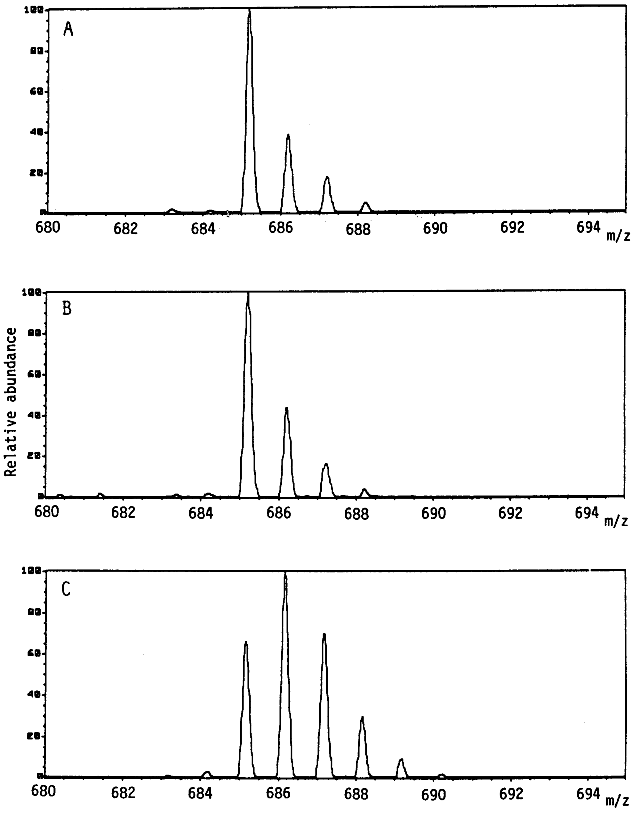

Fig. 7. FAB mass spectra: A) and B) peak fractions indicated by arrows in Figs. 3A and 3B, respectively, and C) $(\text{Fmoc-Cys})_2$ prepared by treatment of cystine with 5.7 M DCl in D_2O at 110 °C for 24 h and with Fmoc-Cl.

by protons derived from a solvent during acid treatment, indicating that a deuteriocysteine with a deuterium substitution on its 2-carbon is not suitable for peptide synthesis by the present method. Moreover, with increase in the mass difference between cystine and deuteriocystine, differentiation of their mass spectra becomes easier. Therefore, we synthesized a cysteine derivative in which two deuteriums were incorporated at the 3,3-positions. The synthesis and use in a peptide of this compound have been reported,[14,15] but we synthesized a 3,3-dideuteriocysteine derivative, *N*-Boc-*S*-(4-methylbenzyl)-3,3-dideuterio-DL-cysteine by the scheme shown in Fig. 8, because this is a rapid and simple procedure for synthesis of this cysteine derivative. Moreover, as separation of the L-isomer from the synthetic racemic compound did not seem necessary, we used the DL-cysteine derivative for peptide synthesis, as described below.

Use of the Method to Establish the Disulfide Pairings in a Heat-Stable Enterotoxin: A heat-stable enterotoxin produced by a porcine strain of enterotoxigenic *Escherichia coli* has the amino acid sequence shown in Fig. 9.[8] The positions of the three intramolecular disulfide pairings were recently determined by chemical synthesis of the enterotoxin using selectively removable protecting groups on Cys residues.[7] To confirm the validity of the present method, we used this method to establish the positions of the disulfide linkages of the enterotoxin and compared them with those determined previously. Thus, we synthesized protected peptides with the sequence from position 5 to 17 of the enterotoxin with Cys* at two positions, which had been determined to be linked or not to be linked in the enterotoxin previously;[7] that is 5 and 10 or 6 and 14 or 5 and 14 or 9 and 17. These peptides were synthesized by a solid-phase method, oxidized by air to form intramolecular disulfide linkages after removal of the protecting groups, and purified by HPLC. The HPLC profile of the synthetic peptide with two Cys* at positions 6 and 14 is

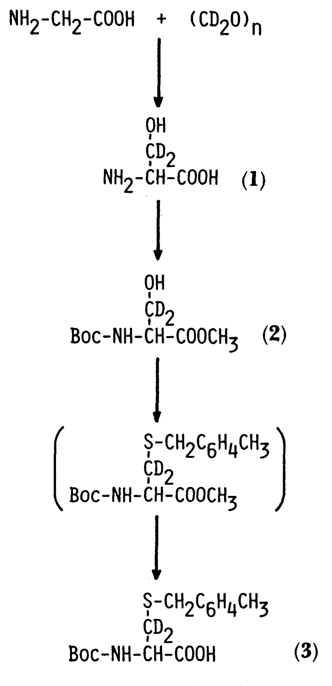

Fig. 8. Scheme for synthesis of *N*-Boc-*S*-(4-methylbenzyl)-3,3-dideuterio-DL-cysteine.

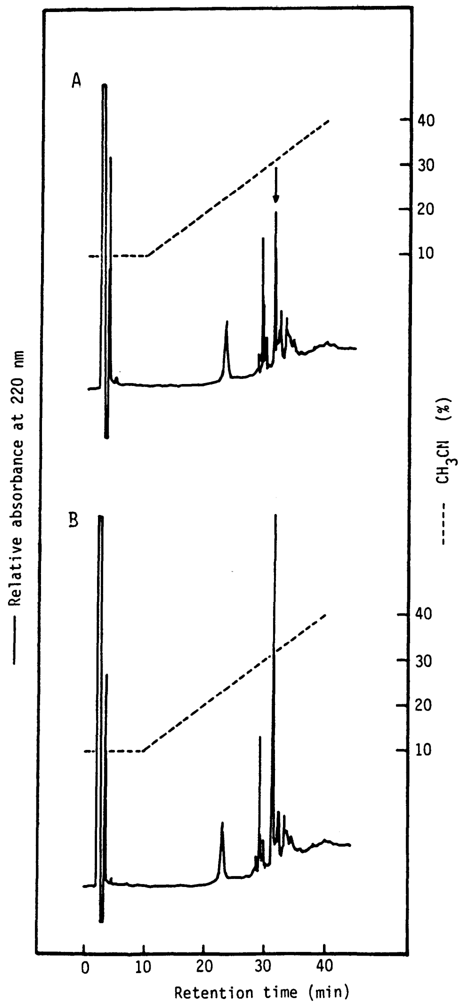

Fig. 10. HPLC profiles of A) a crude synthetic heat-stable enterotoxin with the sequence from position 5 to 17 shown in Fig. 9 and with two Cys* at positions 6 and 14 and B) a mixture of A) and a standard peptide.[16c)]

```
1               5                   10                  15            18
Asn-Thr-Phe-Tyr-Cys-Cys-Glu-Leu-Cys-Cys-Asn-Pro-Ala-Cys-Ala-Gly-Cys-Tyr
```

Fig. 9. Amino acid sequence of a heat-stable enterotoxin (ST_P) produced by a porcine strain of enterotoxigenic *Escherichia coli.*[8]

shown in Fig. 10. The peak fraction, which had the same retention time as that of the standard peptide shown by an arrow, was isolated. The purified peptide had almost the same biological activity as that of the standard peptide. Analytical data of synthetic peptides are summarized in Table 2. The peptide with Cys* at positions 6 and 14 was hydrolyzed under the conditions described above and treated with Fmoc-Cl, and the (Fmoc-Cys)$_2$ fractions were isolated by HPLC and analyzed by FAB mass spectrometry. The mass spectrum (Fig. 11A) was similar to the theoretical mass spectrum of a mixture of (Fmoc-Cys)$_2$ and (Fmoc-Cys*)$_2$ (w/w, 2/1), as shown in Fig. 11C. The mass spectra from peptides with Cys* at positions 5 and 10, and 9 and 17 were also quite similar to that in Fig. 11A. However, the spectrum of the peptide with Cys* at positions 5 and 14 was quite different from the theoretical spectrum in Fig. 11C, but almost the same as the theoretical mass spectrum of a mixture of (Fmoc-Cys)$_2$ and (Fmoc-Cys, Fmoc-Cys*) (w/w, 1/2), as shown in Figs. 11B and 11D, respectively. These results indicated that the disulfide pairings of the enterotoxin were present between positions 5 and 10, 6 and 14, and 9 and 17, but not between positions 5 and 14. These findings were compatible with the positions of the disulfide linkages of the enterotoxin determined previously.[7] Thus it was demonstrated that this method is applicable for determination of disulfide pairings in peptides.

Table 2. Amino Acid Compositions,[a] Mass Values,[b] and Yields of Synthetic Peptides

	$Cys^{*5,10}$	$Cys^{*6,14}$	$Cys^{*9,17}$	$Cys^{*5,14}$	Theoretical value
Asp	1.01	1.04	1.38	1.22	1
Glu	1.04	0.99	1.02	1.00	1
Gly	0.99	1.02	1.02	1.07	1
Ala	2	2	2	2	2
Cys	5.50	3.79	4.57	4.18	6
Leu	0.95	0.81	0.96	0.84	1
Pro	0.97	1.05	1.09	1.04	1
$[M+H]^+$	1287.1	1286.9	1287.0	1286.7	1287.3
Yield (%)	6.0	7.5	6.9	6.9	

a) Values were calculated as mol/mol of Ala. b) $[M+H]^+$, mass value of quasi-molecular ion.

In summary, we developed a new method for determining disulfide pairings in peptides. This method involves the following procedures: i) synthesis of a peptide with the same sequence as that of the native or a standard peptide and with deuterio-Cys residues at given positions, ii) hydrolysis of the peptide in 4 M CH_3SO_3H at 110 °C for 24 h, and reaction of the hydrolyzate with Fmoc-Cl, and iii) HPLC separation and FAB mass analysis of the (Fmoc-Cys)$_2$ fraction including deuteriocystine. The method was applied to confirm the positions of the three intramolecular disulfide linkages in a heat-stable enterotoxin.

Experimental

The general and analytical methods used were as described previously.[7,16] All chemicals used for synthetic experiments were of reagent grade, while those used for analysis were of guaranteed grade. All amino acids except glycine and deuteriocysteine were of the L-configuration and Boc-amino acid derivatives were purchased from the Peptide Institute Inc. (Minoh, Osaka) unless otherwise mentioned. A reversed-phase column (Cosmosil C_{18}, 4X250 mm) was obtained from Nacalai Tesque Inc. (Kyoto). ^{1}H NMR spectra were recorded with a JEOL JNM-GX-400 spectrometer. Mass spectra were taken with an FAB mode on a JEOL HX-100 mass spectrometer. The abbreviations used in this paper are those recommended by the IUPAC-IUB [*J. Biol. Chem.*, **261**, 1 (1986)]. Additional abbreviations are: FAB, fast atom bombardment; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-Cl, 9-fluorenylmethyl chloroformate; HPLC, high-performance liquid chromatography;

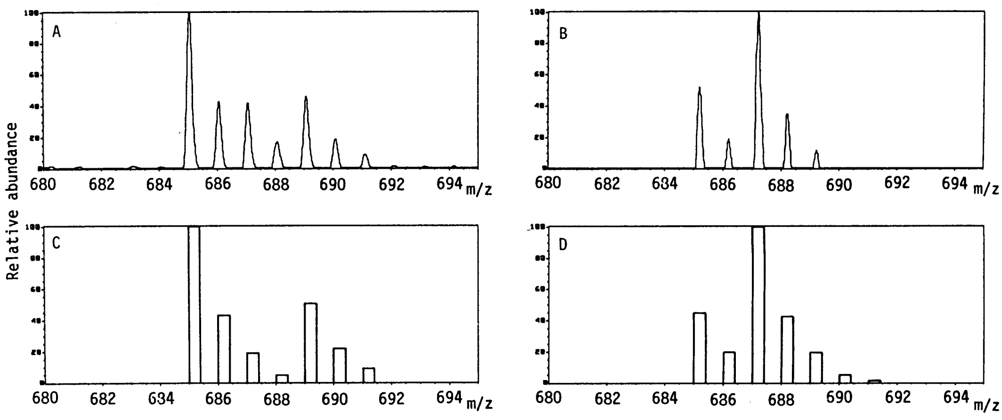

Fig. 11. A) and B), FAB mass spectra of (Fmoc-Cys,)$_2$ fractions isolated from hydrolyzates of heat-stable enterotoxin peptides with the sequence from position 5 to 17 and with two Cys* at positions 6 and 14 and 5 and 14, respectively. C) and D), theoretical molecular ion distributions of mixtures of (Fmoc-Cys)$_2$ and (Fmoc-Cys*)$_2$ (2/1) and (Fmoc-Cys)$_2$ and (Fmoc-Cys, Fmoc-Cys*) (1/2), respectively.

TFA, trifluoroacetic acid; amu, atomic mass unit.

3,3-Dideuterio-DL-serine (1). The title compound was synthesized from glycine (540 mg, 7.2 mmol) in 1 M Na_2CO_3 (30 ml), 0.2 M $CuSO_4$ (0.36 ml), and paraformaldehyde-d_2 (1.73 g, 36 mmol) by the procedure described in Ref. 17 with some modifications. Yield, 235 mg (31% based on the amount of glycine); MS m/z=108.0 (theoretical for $[M+H]^+$ 108.1); ^1H NMR (D_2O), δ=3.82 (1H, s, CH); Found: C, 33.52; H+D, 6.69; N, 13.00%. Calcd for $C_3H_5D_2NO_3$: C, 33.64; H+D, 6.59; N, 13.08%.

Boc-3,3-dideuterio-DL-Ser-OMe (2). Compound **1** (107 mg, 1 mmol) was dissolved in a mixture of dioxane and water (3 ml, v/v: 2/1), and mixed with Boc_2O (262 mg, 1.2 mmol) and triethylamine (140 μl, 1 mmol) under cooling in an ice-water bath. The mixture was stirred for 3 h at room temperature and then concentrated to a syrup under reduced pressure. The syrup was redissolved in MeOH (3 ml), treated with diazomethane, and concentrated to an oily residue under reduced pressure. The residue was dissolved in AcOEt, and washed with 0.2 M HCl, 5% aq $NaHCO_3$, and water. The washed solution was dried over Na_2SO_4 and concentrated to a syrup; 210 mg (95%); MS, m/z=222.0 (theoretical for $[M+H]^+$, 222.1); ^1H NMR ($CDCl_3$), δ=1.46 (9H, s, $(CH_3)_3C$), 3.79 (3H, s, OCH_3), 4.37 (1H, d, CH), 5.52 (1H, d, NH); Found: C, 48.38; H+D, 7.90; N, 6.17%. Calcd for $C_9H_{15}D_2O_5N$: C, 48.86; H+D, 7.74; N, 6.33%.

N-Boc-3,3-dideuterio-DL-Cys(MBzl)-OH (3). Compound **2** (800 mg, 3.6 mmol) was treated with 1.2 equiv each of Ph_3P, 4-methylphenylmethanethiol, and diethyl azodicarboxylate in dry toluene (18 ml) by the method described in Ref. 18. The reaction product was applied to a silica-gel column with ether as an eluent to remove the resulting Ph_3P=O. The eluate was concentrated to a solid under reduced pressure, redissolved in MeOH (36 ml), and saponified by mixing it with 1 M NaOH (4.3 ml). The solution was concentrated to dryness under reduced pressure, and the residue was dissolved in water (100 ml), adjusted to pH 2.0 by adding 1 M HCl, and extracted with AcOEt. The extract was washed with water, dried over anhydrous Na_2SO_4, and concentrated to a residue under reduced pressure. The residue was purified by chromatography on a silica-gel column with a mixture of $CHCl_3$, MeOH, and AcOH (v/v/v, 100/3/1) as eluent; Yield, 775 mg (66% based on the amount of compound **2**); mp 135 °C; MS, m/z=328.0 (theoretical for $[M+H]^+$ 328.1); ^1H NMR (DMSO-d_6/$CDCl_3$, v/v, 1/1), δ=1.46 (9H, s, $(CH_3)_3C$), 2.32 (3H, s, Ar-CH_3), 3.71 (2H, s, Ar-CH_2), 4.36 (1H, d, CH), 5.93 (1H, d, NH), 7.10—7.20 (4H, d,d, ArH); Found: C, 58.36; H+D, 7.20; N, 4.29; S, 9.59%. Calcd for $C_{16}H_{21}D_2O_4NS$: C, 58.69; H+D, 7.08; N, 4.28; S, 9.79%.

Synthesis of Peptides. Peptides were synthesized manually by the solid-phase method.[19,20] Briefly, Boc-Cys (MBzl)- or Boc-Cys* (MBzl)-polystyrene (2% divinylbenzene) (0.2—0.3 mmol of amino acid per 1 g of resin) was used as starting material. The γ-carboxyl group of glutamic acid was protected by Bzl. All couplings were carried out with 5-fold excess of Boc-amino acid and DCC except that of Boc-Cys*(MBzl)-OH (3 equiv). Boc-Asn-OH was coupled repeatedly twice with 2 equiv of 1-hydroxybenzotriazole. After coupling all the amino acid derivatives, peptides were liberated from the resin by treatment with anisole in anhydrous hydrogen fluoride at 0 °C for 1 h, extracted with 10% formic acid in an ice-water bath, and washed three times with hexane. The extract was diluted with water to a final peptide concentration of 5×10^{-5} M. The solution was adjusted to pH 8.0 and stood at room temperature until no free mercapto groups were detectable. The crude peptides were purified by high-performance liquid chromatography, as described below.

High-Performance Liquid Chromatography (HPLC). The synthetic peptide was purified on a reversed-phase column using a high-performance liquid chromatography system consisting of a Waters M600 multisolvent delivery system (Milford, MA) and Hitachi 655A variable wavelength UV monitor and D-2000 chromatointegrator (Tokyo). The peptide was eluted with a linear gradient of CH_3CN in 0.05% TFA or 0.01 M ammonium acetate (pH 5.7) with increase of 1% min^{-1} at a flow rate of 1 ml min^{-1}. Fractions of the eluate were monitored for absorbance at 220 nm.

Hydrolysis of Peptides. Amino acids or peptides were hydrolyzed at 110 °C for 24 h in a Waters pico-TAG™ workstation by either one of the following three methods: 1) The sample amino acid or peptide (ca. 2 nmol) was put into a vial and mixed with 5.7 M HCl (200 μl). 2) The sample (ca. 2 nmol) was put into a vial containing 4 M CH_3SO_3H (20 μl). 3) The sample (ca. 2 nmol) was put into a small test tube containing 4 M TFA (200 μl) and sealed in vacuo.

Reaction of Hydrolyzates of Peptides with Fmoc-Cl. The sample (ca. 50 nmol) was dissolved in 1% ammonium hydrogencarbonate (100 μl) at pH 7.8 and treated with 16 mM Fmoc-Cl in acetone (100 μl) at room temperature for 30 min. The solution was washed three times with hexane, insoluble materials were filtered off, and the filtrate was subjected to HPLC.

Amino Acid and Fast Atom Bombardment (FAB) Mass Spectrometric Analyses. The amino acid compositions of synthetic peptides were examined by amino acid analysis, and the molecular weights of synthetic peptides and deuteriocystine derivative were measured by FAB mass spectrometry, as described in Refs. 20 and 21, respectively.

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture (Scientific Research Grant No. 62470022). We deeply thank Drs. Tadao Harada and Hiroshi Ozaki of the Institute for Protein Research, Osaka University for helpful discussions. We are also indebted to Prof. Akira Tai (Basic Research Laboratory, Himeji Institute of Technology) for facilitating use of an NMR spectroscopy instrument, to Mrs. Aiko Kobatake for elemental analysis, and to Mrs. Aiko Kobatake and Miss Yumiko Kuhara for preparation of the manuscript.

References

1) A. P. Ryle, F. Sanger, L. F. Smith, and R. Kitai, *Biochem. J.*, **60**, 541 (1955).

2) W. R. Gray, A. Luque, B. M. Olivera, J. Barrett, and L. J. Cruz, *J. Biol. Chem.*, **256**, 4734 (1981).

3) S. Aimoto, T. Takao, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Eur. J. Biochem.*, **129**, 257 (1982).

4) R. Wetzel, *Trends in Biol. Sci.*, **12**, 478 (1987).

5) T. Takao, M. Yoshida, Y.-M. Hong, S. Aimoto, and

Y. Shimonishi, *Biomed. Mass Spectrom.*, **11**, 549 (1984).

6) Y. Nishiuchi and S. Sakakibara, *FEBS Lett.*, **148**, 260 (1982).

7) Y. Hidaka, H. Kubota, S. Yoshimura, H. Ito, Y. Takeda, and Y. Shimonishi, *Bull. Chem. Soc. Jpn.*, **61**, 1265 (1988).

8) T. Takao, T. Hitouji, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *FEBS Lett.*, **152**, 1 (1983).

9) A. Wada, Y. Hidaka, T. Takao, and Y. Shimonishi, unpublished data.

10) H. B. Bell and K. Breese, *Arch. Biochem. Biophys.*, **161**, 665 (1974).

11) S. Einarsson, B. Josefsson, and S. Lagerkvist, *J. Chromatogr.*, **282**, 609 (1983).

12) S. A. Martin, C. E. Costello, and K. Biemann, *Anal. Chem.*, **54**, 2362 (1982).

13) R. J. Simpson, M. R. Neuberger, and T.-Y. Liu, *J. Biol. Chem.*, **251**, 1936 (1976).

14) D. A. Upson and V. J. Hruby, *J. Org. Chem.*, **41**, 1353 (1976).

15) H. S. Beilan, D. T. Browne, and G. L. Kenyon, *Bioorg. Chem.*, **15**, 109 (1987).

16) a) S. Yoshimura, T. Takao, H. Ikemura, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Bull. Chem. Soc. Jpn.*, **57**, 1381 (1984); b) H. Ikemura, S. Yoshimura, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, Y. Takeda, and T. Miwatani, *Bull. Chem. Soc. Jpn.*, **57**, 2550 (1984); c) S. Yoshimura, H. Ikemura, H. Watanabe, S. Aimoto, Y. Shimonishi, S. Hara, T. Takeda, T. Miwatani, and Y. Takeda, *FEBS Lett.*, **181**, 138 (1985).

17) S. Akabori, T. T. Otani, R. Marshall, M. Winitz, and J. P. Greenstein, *Arch. Biochem. Biophys.*, **83**, 1 (1959).

18) H. Loibner and E. Zbiral, *Helv. Chim. Acta*, **59**, 2100 (1976).

19) R. B. Merrifield, *J. Am. Chem. Soc.*, **85**, 2149 (1963).

20) Y. Shimonishi, Y. Hidaka, M. Koizumi, M. Hane, S. Aimoto, T. Takeda, T. Miwatani, and Y. Takeda, *FEBS Lett.*, **215**, 165 (1987).

21) T. Takao, T. Hitouji, Y. Shimonishi, T. Tanabe, S. Inouye, and M. Inouye, *J. Biol. Chem.*, **259**, 6105 (1984).