Investigation of the Dimethylsulfoxide–Trifluoroacetic Acid Oxidation System for the Synthesis of Cystine-Containing Peptides[1)]

Takaki KOIDE, Akira OTAKA, and Nobutaka FUJII*

Faculty of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606–01, Japan. Received January 5, 1993

Disulfide bonds of peptides were effectively established between *S*-protected cysteine residues as well as free cysteine residues by the action of dimethylsulfoxide in trifluoroacetic acid. Oxytocin and α-human calcitonin gene-related peptide were synthesized using this oxidation system. The feasibility of this method for the formation of two disulfide bridges of apamin was also examined.

Keywords cystine-containing peptide; disulfide bond-formation; dimethylsulfoxide; oxytocin; calcitonin gene-related peptide; apamin

To date, many kinds of cystine-containing peptides have been isolated from various natural sources and characterized, *e.g.*, oxytocin, calcitonin, endothelins, and growth factors. Their disulfide bridges play important roles in the expression of their biological activities by stabilizing their three-dimensional structures. In the synthesis of cystine-containing peptides, the disulfide bond-forming reaction is one of the most important steps. Therefore our research interest has been focused on methodological improvements in the chemical synthesis of cystine-containing peptides.

For disulfide bond-formation, conventional air oxidation[2)] or $K_3Fe(CN)_6$[3)] oxidation in aqueous media is generally employed. In terms of efficient and unambiguous synthesis, however, these methods are still less than satisfactory. In the case of basic or hydrophobic peptides, we often encounter problems of solubility of the peptide in aqueous media, even if the peptide backbone has been successfully constructed and deprotection/cleavage has been well conducted. To solve this problem, we have developed two disulfide bond-forming systems; one utilizes thallium(III) oxidation[4)] and the other relies on an acid-catalyzed reaction involving *S*-protected cysteine sulfoxides.[5)] Both methods can be conducted in TFA, which freely dissolves most peptides, even protected peptides, and both were successfully applied to the synthesis of several biologically active cystine-containing peptides. By the combination of these two systems in TFA, regioselective disulfide bond-formation of two disulfide bridges of apamin has been accomplished.[6)]

In a recent preliminary report,[7)] we have demonstrated another disulfide bond-forming reaction which proceeded in TFA, using DMSO as an oxidant. By employing this system, cysteine and some *S*-protected cysteine derivatives [Cys(Trt), Cys(MBzl), Cys(Dbs), and Cys(Bzh)] have been shown to be effectively converted to cystine. In this paper, we wish to report further investigations and experimental details of the DMSO–TFA oxidation system for the synthesis of several cystine-containing peptides. We also with to discuss the character of TFA as a solvent for disulfide bond-forming reactions.

Results and Discussion

Disulfide Bond Formation between Unprotected Cysteine Residues In order to evaluate the efficiency of the DMSO–TFA oxidation for intra-chain disulfide bond-formation between free cysteine residues of peptides, two model peptides were synthesized.

The first attempt was made using a small cystine-containing peptide amide, oxytocin; H–Cys–Tyr–Ile–Gln–Asn–Cys–Pro–Leu–Gly–NH_2 (disulfide bond between Cys^1 and Cys^6). The peptidyl resin for oxytocin was synthesized by an Fmoc-based solid-phase method[8)] on a PAL™-resin. For the protection of the two sulfhydryl groups of the Cys residues, the MBzl group[9)] was employed. After deprotection of all protective groups and simultaneous cleavage of the peptide from the resin by using the 1 M TMSBr–*m*-cresol–thioanisole–EDT–TFA system,[10)] the resulting SH-peptide was submitted to 10% (v/v) DMSO–TFA oxidation. The disulfide bond-forming reaction was complete within 1 h at room temperature, and the desired disulfide product was efficiently generated. No significant side products were detected by HPLC analysis (Fig. 1).

Next, the larger peptide, α-human calcitonin gene-related peptide (α-hCGRP) was synthesized using this cyclization method. The protected 37-residue peptide amide constructed by solution-phase methods[11)] was treated with the 1 M TMSOTf–*m*-cresol–thioanisole–EDT–TFA system[12)] to remove all protective groups (Ad from Cys, Mts from Arg, Bzl from Ser, and Z from Lys). The resulting SH-peptide was then treated with 10% (v/v) DMSO–TFA at room temperature for 1 h. The crude product was purified by HPLC to give homogeneous oxidized α-hCGRP in 17% yield based on the protected peptide (Chart 1, Fig. 2).

Direct Disulfide Bond Formation between Protected Cysteine Residues Direct disulfide bond-formation between side chain-protected cysteine residues seems to be

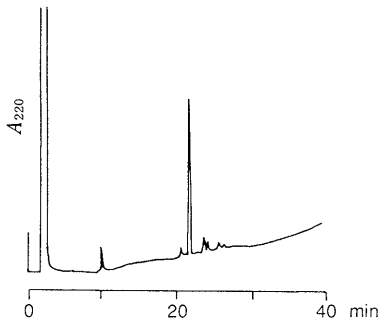

Fig. 1. HPLC of Crude Oxytocin

Column, Cosmosil C18-AR (0.46 × 15 cm); elution, linear gradient with MeCN (10—40% in 30 min) in 0.1% aqueous TFA at a flow rate of 1 ml/min; detection, 220 nm.

more useful than between free cysteine residues, since free SH-peptides are generally difficult to handle under aerobic conditions, and particularly during the synthesis of large peptides, the *S*-protected peptides are often initially purified before disulfide bond-formation to facilitate purification of the final product. Therefore we investigated the applicability of the DMSO–TFA oxidation to direct disulfide bond-formation between *S*-protected cysteine residues using oxytocin as a model peptide. The *S*-protective MBzl[9] and Acm groups[13] which have generally been used in recent peptide syntheses were employed. An oxytocin derivative containing Cys(Acm) and free cysteine residues in the molecule was also synthesized and submitted to the DMSO–TFA oxidation in the expectation of preferential disulfide bond-formation between the free cysteine residues.

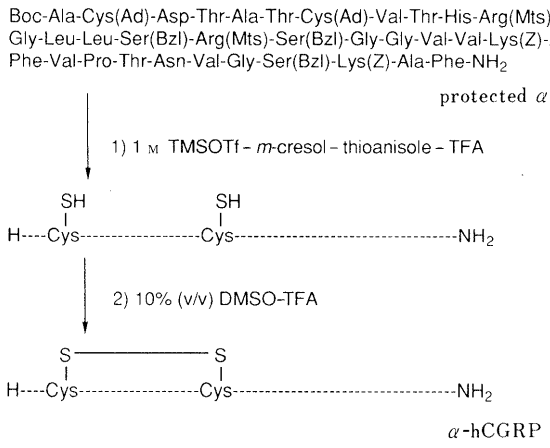

Chart 1. Synthetic Scheme for α-hCGRP

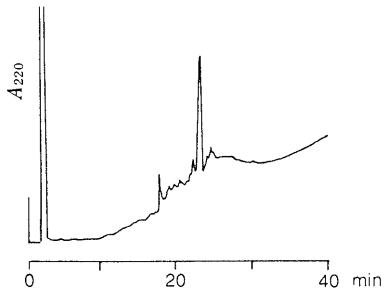

Fig. 2. HPLC of Crude α-hCGRP

Column, μBondasphere 5 μC-18-100 Å (0.39 × 15 cm); elution, linear gradient with MeCN (20—50% in 30 min) in 0.1% aqueous TFA at a flow rate of 1 ml/min; detection: 220 nm.

Three resin-bound peptides for disulfide bond-forming reactions; [Cys(MBzl)^{1,6}]-oxytocin, [Cys (Acm)^{1,6}]-oxytocin, and [Cys(Acm)^{1}, Cys(SH)^{6}]-oxytocin, were constructed by the Fmoc-based solid-phase method. Each *S*-protected oxytocin derivative was obtained by deprotection/cleavage using TFA–*m*-cresol–thioanisole–EDT–water (80 : 5 : 5 : 5 : 5, v/v)[14] followed by HPLC purification. For disulfide bond-formation, each purified *S*-protected oxytocin derivative was treated with 10% (v/v) DMSO–TFA at room temperature (Chart 2), and the progress of the reaction was monitored by analytical HPLC. As shown in Table I, disulfide bond-formation between Cys(MBzl) residues was completed within 2 h and the desired oxytocin was formed in good yield without significant side reactions. On the other hand, [Cys(Acm)^{1,6}]-oxytocin survived nearly intact even after a 12-h treatment. But to our surprise, in the case of [Cys(Acm)^{1}, Cys(SH)^{6}]-oxytocin, oxytocin was formed as a main product in a much shorter reaction time (1 h) than in the

Table I. Generation of Oxytocin from *S*-Protected Oxytocin Derivatives by DMSO–TFA Oxidation

Starting material	Generation of oxytocin (%)[a]		
	1 h	2 h	12 h
[Cys(MBzl)^{1,6}]-oxytocin	64 (21)	86 (0)	
[Cys(Acm)^{1,6}]-oxytocin	<5 (93)	<5 (92)	<5 (92)
[Cys(Acm)^{1},Cys(SH)^{6}]-oxytocin	69 (0)[b]		

a) Yields are calculated from HPLC peak areas. Recovery (%) of the starting material is shown in parentheses. *b*) The peak of the dimeric product of [Cys(Acm)^{1}, Cys^{6}]-oxytocin was observed.

Table II. Treatment of H–Cys(Acm)–OH with 10% (v/v) DMSO–TFA in the Presence or Absence of 2-ME

2-ME (eq)	Reaction time (min)	Yield (% of theoretical)[a]		
		Cys(Acm)	Mixed SS[b]	Cystine
None	15	89		10
	30	82		18
	60	70		29
1	15	59	30	<2
	30	0	90	8

a) Yields were estimated by amino acid analysis. *b*) Mixed SS, *S*-2-hydroxyethylthio cysteine.

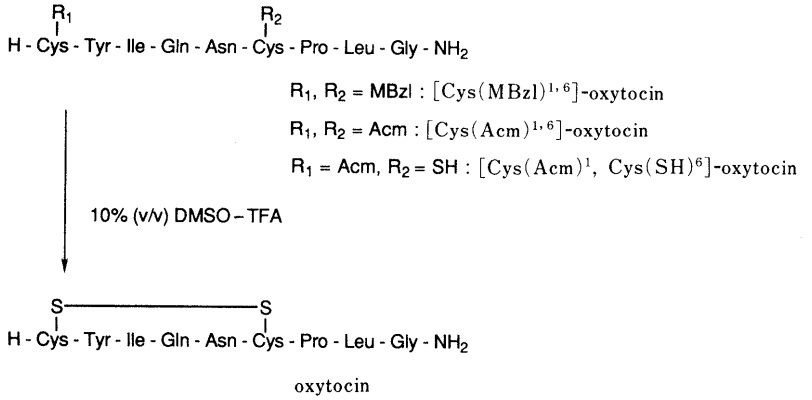

Chart 2. Disulfide Bond Formation from *S*-Protected Oxytocin Derivatives

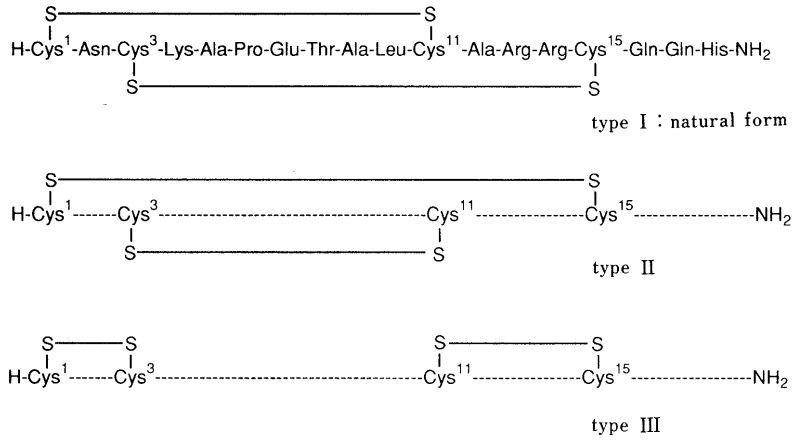

Chart 3. Structures of Apamin and Its Disulfide Isomers

case of [Cys(Acm)^{1,6}]-oxytocin. Here the formation of the homodimer of [Cys(Acm)^1, Cys^6]-oxytocin was also observed.

These results revealed that Cys(MBzl) residues in the peptide were effectively and directly converted to the cystine residue, and that the *S*-Acm group, which is itself considerably resistant to DMSO–TFA oxidation, was readily cleaved in the presence of the accessible thiol. In fact, in the model experiment using H–Cys(Acm)–OH, the cleavage of the *S*-Acm group was promoted by addition of 1 eq of 2-ME, and formation of the mixed disulfide, *S*-2-hydroxyethylthio cysteine, was observed on amino acid analysis (Table II).

Attempts at Disulfide Bond-Formation of Apamin Next, in order to examine the feasibility of using this oxidation system in the synthesis of peptides containing two or more disulfide bridges in the molecule, disulfide bond-formation to form apamin, an 18-residue bee venom peptide amide, was attempted. For the disulfide bond-formation, fully reduced apamin, obtained by the reduction of synthetic apamin,[6] was submitted to the DMSO–TFA oxidation. The crude product was then analyzed by HPLC in comparison with authentic samples of three types of disulfide isomer of apamin (Chart 3). The type I (natural form), the type II and the type III isomers, all synthesized by using regioselective disulfide bond formation (see Experimental), showed different retention times on analytical HPLC. Unexpectedly, the retention time of the main product after treatment of the fully reduced apamin with 10% (v/v) DMSO–TFA was identical to that of the type III disulfide isomer, which contains two disulfide bridges between Cys^1 and Cys^3, and between Cys^{11} and Cys^{15} (Fig. 3). This main product was purified by HPLC and its identity was confirmed by FAB-MS analysis.

In order to clarify the reason why exclusive formation of the unnatural type III isomer occurred upon DMSO–TFA treatment, the fully reduced apamin was exposed to various additional oxidation systems including Tl(TFA)$_3$–TFA oxidation,[4] 10% (v/v) DMSO–water oxidation,[15] and air oxidation at pH 8. After each treatment, the ratio of individual disulfide isomers was estimated by measuring peak areas on analytical HPLC. The results are summarized in Table III. The Tl(TFA)$_3$–TFA oxidation gave the same result as the DMSO–TFA oxidation. On the other hand, the air oxidation produced only the natural disulfide isomer (type I). When DMSO in water was used, all three disulfide isomers were formed. From these results, it was concluded that the exclusive formation of the type III disulfide isomer

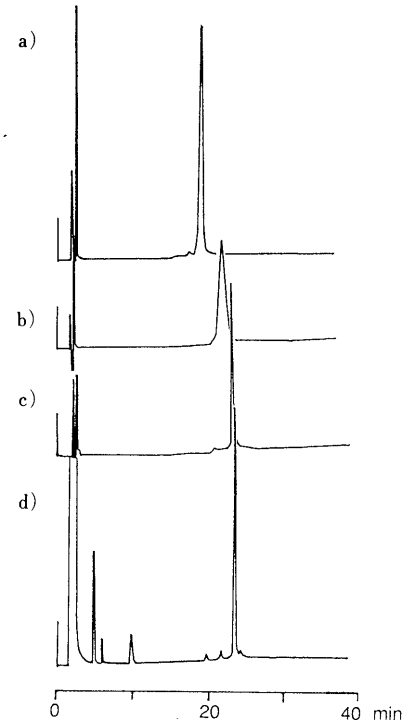

Fig. 3. HPLC of Apamin Isomers and Crude Product after DMSO–TFA Oxidation

Column, Cosmosil C18-AR (0.46 × 15 cm); elution, linear gradient with MeCN (10—20% in 30 min) in 0.1% aqueous TFA at a flow rate of 1 ml/min; detection, 220 nm. a) type I; b) type II; c) type III; d) crude product after DMSO–TFA treatment.

Table III. Disulfide Bond Formation of Apamin by Various Oxidation Systems

Oxidation system	Relative peak areas of the three disulfide isomers (%)		
	Type I	Type II	Type III
10% (v/v) DMSO–TFA	0	0	100
Tl (TFA)$_3$ (5 eq)–TFA	0	0	100
10% (v/v) DMSO–water	58	33	9
Air oxidation (pH 8)	100	0	0

by DMSO–TFA oxidation was not due to the character of the oxidant, but rather to conformational differences of the peptide in the different solvents. One of the most important factors influencing the conformation of peptides is thought to be the solvent polarity. At 20 °C, values of the dielectric constants of water, DMSO, and TFA are 80.1, 48.9, and 8.55, respectively. It is reasonable to consider that in a much less polar solvent such as TFA, the peptide chain would take nearly unfolded structures, which could be account for the exclusive formation of the type III disulfide isomer in this solvent. The formation of the type II and III isomers in the 10% (v/v) DMSO–water system could also be explained by conformational effects resulting from the decreased solvent polarity due to addition of DMSO.

Conclusion

In this study, we have shown the usefulness of the DMSO–TFA oxidation system for the practical synthesis of cystine-containing peptides. By using this method, disulfide bridges of peptides are effectively established not only between free cysteine residues but also between Cys(MBzl) residues in short reaction times without serious side reactions. We also found that the *S*-Acm group, which is itself fairly stable to DMSO–TFA oxidation, was readily cleaved in the presence of the free sulfhydryl group, leading to the formation of the disulfide bond. In recent papers,[16] direct disulfide bond-formtion in TFA between various *S*-protected cysteine residues, including Cys(Acm), was reported using combinations of sulfoxides and silyl compounds. These methods, which utilize the oxidizing ability of sulfoxides, do not require the use of hazardous heavy metals such as mercury[13,17] and thallium.[4] The use of TFA as a solvent also eliminates problems of peptide solubility. Here it should be noted that in the synthesis of tryptophan and/or methionine-containing peptides, suppression of the oxidative modification of these residues[7] is a problem remaining to be solved. In addition, results obtained from the experiments using apamin provided information about the properties of TFA as a solvent for disulfide bond-forming reactions, and reconfirmed the importance of peptide conformation for the correct pairing of disulfide bridges. We consider that the present oxidation method, combined with other disulfide bond-forming techniques, represents a useful advance in the stepwise and regioselective disulfide bond-forming technology for the synthesis of multi cystine-containing peptides.

Experimental

General HPLC was performed on a Waters single pump system equipped with a 600E system controller, a 484 tunable absorbance detector and a Hitachi D-2500 chromato-integrator. The solvents for HPLC were water and MeCN, both containing 0.1% (v/v) TFA, and both were degassed before use. For analytical HPLC, Cosmosil C18-AR (0.46 × 15 cm) or μBondasphere 5 μC18-100 Å (0.39 × 15 cm) was used at a flow rate of 1 ml/min. Preparative HPLC was performed on a YMC Pack D-ODS-5 S-5 100A at a flow rate of 8 ml/min or a μBondasphere 5μC18-100 Å (1.9 × 15 cm) at a flow rate of 17 ml/min. SIMS spectra were obtained with a Hitachi M-90 mass spectrometer. FAB-mass spectra were recorded on a VG Analytical ZAB-SE instrument. Acid hydrolysis of peptides was performed with 6 N HCl containing 1% (v/v) phenol at 110 °C for 20 h. Amino acid analysis was performed with a Hitachi 835 amino acid analyzer.

Solid-Phase Synthesis Oxytocin Derivatives All peptidyl resins for oxytocin derivatives were constructed manually by standard Fmoc-based solid-phase methods[8] on a PAL™-resin (Millipore, 0.33 mmol/g, 0.1 mmol scale). The *tert*-Bu group was used for side chain protection of the Tyr residue. For the *S*-protection of two Cys residues, three different protecting schemes were employed; resin A: MBzl groups for both Cys^1 and Cys^6, resin B: Acm groups for both Cys^1 and Cys^6, resin C: an Acm group for Cys^1, and a Trt group for Cys^6.

Oxytocin from Its Reduced Form Resin A (23.0 mg, 5.3 μmol) was treated with *m*-cresol (61 μl)–thioanisole (150 μl)–EDT (25 μl)–TFA (940 μl)–TMSBr (165 μl) in an ice bath for 1 h. After removal of resulting HBr gas by evaporation, the resin was separated by filtration. Ice-chilled ether was then added to the filtrate, and the resulting powder of reduced oxytocin was collected by centrifugation and washed thoroughly with ether. The precipitate of reduced oxytocin was treated with 10% (v/v) DMSO–TFA containing 1% anisole (2.0 ml) at room temperature. After 1 h, ice-chilled ether was added to form a powder. The crude product was then purified by HPLC and the solvent was removed by lyophilization to give the title compound as a white powder; yield 3.2 mg (60% based on the C-terminal amino acid). Amino acid ratios in a 6 N HCl hydrolysate (values in parentheses are theoretical): Asx 0.75 (1), Glx 0.91 (1), Pro 1.25 (1), Gly 1.05 (1), cystine N.D. (1), Ile 1.00 (1), Leu 1.06 (1), Tyr 0.89 (1). SIMS *m/z*: Found 1007 $(M+H)^+$; Calcd for $C_{43}H_{67}N_{12}O_{12}S_2$; 1007.44.

α-hCGRP Protected α-hCGRP[11] (20.5 mg, 4.05 μmol) was treated with *m*-cresol (244 μl)–thioanisole (600 μl)–TFA (3.45 ml)–TMSOTf (970 μl) in an ice-bath for 2 h, then cold ether was added to precipitate the crude reduced α-hCGRP. The powder was treated with 10% (v/v) DMSO–TFA containing 1% anisole (5.0 ml) at room temperature for 1 h. The crude product precipitated with ether was collected by centrifugation, then washed thoroughly with ether and purified by HPLC; yield 2.7 mg (17% based on the protected peptide). Amino acid ratios in a 6 N HCl hydrolysate (values in parentheses are theoretical): Asx 4.08 (4), Thr 3.71 (4), Ser 2.73 (3), Pro 1.02 (1), Gly 4.07 (4), Ala 4.25 (4), cystine N.D. (1), Val 4.45 (5), Leu 3.13 (3), Phe 2.00 (2), Lys 1.68 (2), His 0.97 (1), Arg 2.03 (2). SIMS *m/z*: Found 3788 $(M+H)^+$; Calcd for $C_{163}H_{268}N_{51}O_{49}S_2$; 3787.95.

Oxytocin from Its *S*-Protected Derivatives Three *S*-protected oxytocin derivatives, [$Cys(MBzl)^{1,6}$]-oxytocin, [$Cys(Acm)^{1,6}$]-oxytocin, and [$Cys(Acm)^1$, $Cys(SH)^6$]-oxytocin were obtained from resins A, B, and C, respectively, by the following procedure. Each peptidyl-resin (120 mg) was treated with TFA–*m*-cresol–thioanisole–EDT–water (80 : 5 : 5 : 5 : 5, v/v, 2.5 ml) at room temperature for 1 h. After removal of the resin by filtration, cold ether was added to give a precipitate. The crude product was then purified by HPLC; yield [$Cys(MBzl)^{1,6}$]-oxytocin: 23 mg (67%), [$Cys(Acm)^{1,6}$]-oxytocin: 29 mg (88%), [$Cys(Acm)^1$, $Cys(SH)^6$]-oxytocin: 30 mg (79%). Amino acid ratios in 6 N HCl hydrolysates (values in parentheses are theoretical): [$Cys(MBzl)^{1,6}$]-oxytocin: Asx 0.93 (1), Glx 1.02 (1), Pro 1.29 (1), Gly 1.08 (1), cysteine N.D. (2), Ile 1.00 (1), Leu 1.05 (1), Tyr 1.03 (1), [$Cys(Acm)^{1,6}$]-oxytocin: Asx 0.95 (1), Glx 1.03 (1), Pro 1.29 (1), Gly 1.06 (1), cysteine N.D. (2), Ile 1.00 (1), Leu 1.05 (1), Tyr 0.97 (1), [$Cys(Acm)^1$, $Cys(SH)^6$]-oxytocin: Asx 0.94 (1), Glx 1.03 (1), Pro 1.04 (1), Gly 1.07 (1), cysteine N.D. (2), Ile 1.00 (1), Leu 1.05 (1), Tyr 0.98 (1). SIMS, *m/z*: [$Cys(MBzl)^{1,6}$]-oxytocin: Found 1249 $(M+H)^+$; Calcd for $C_{59}H_{85}N_{12}O_{14}S_2$; 1249.57, [$Cys(Acm)^{1,6}$]-oxytocin: Found 1151 $(M+H)^+$; Calcd for $C_{49}H_{79}N_{14}O_{14}S_2$; 1151.53, [$Cys(Acm)^1$, $Cys(SH)^6$]-oxytocin: Found 1080 $(M+H)^+$; Calcd for $C_{46}H_{74}N_{13}O_{13}S_2$; 1080.49.

Each purified *S*-protected oxytocin derivative (5.0 μmol) was treated with 10% (v/v) DMSO–TFA containing 1% (v/v) anisole (2.0 ml). The progress of the reaction was monitored by HPLC and the yield of the desired product and the amount of the remaining starting material were determined by comparing the relative peak areas to those of standard peptide solutions of known concentrations.

Dimerization of [$Cys(Acm)^1$, $Cys(SH)^6$]-Oxytocin [$Cys(Acm)^1$, $Cys(SH)^6$]-oxytocin (2.4 mg) was dissolved in 0.05 M Tris·HCl buffer (pH 8, 240 μl) and allowed to stand for 2 d. Acetic acid was then added to the reaction mixture, and the main product was purified by HPLC; yield 1.6 mg (67%). SIMS *m/z*: Found 2158 $(M+H)^+$; Calcd for $C_{92}H_{145}N_{26}O_{26}S_4$; 2157.97.

***S*-2-Hydroxymethylthio Cysteine** 2-ME (22.3 μl, 0.32 mmol) was added to an ice-cold solution of *S*-acetamidomethyl cysteine sulfoxide (68 mg, 0.21 mmol) in TFA (1.36 ml). The reaction mixture was stirred at room temperature for 5 h. Ice-chilled ether was added to form a precipitate, which was collected by centrifugation. A portion of the crude product was then purified by HPLC and the fraction containing the main product was lyophilized to give a hygroscopic powder. SIMS *m/z*: Found 198 $(M+H)^+$; Calcd for $C_5H_{12}NO_3S_2$; 198.03.

Treatment of H–Cys(Acm)–OH with 10% (v/v) DMSO–TFA in the Presence or Absence of 2-ME H–Cys(Acm)–OH (20 μmol), with glycine

(20 μmol) as an internal standard, was treated at room temperature with 10% (v/v) DMSO–TFA in the presence or absence of 2-ME (20 μmol). At intervals, 50 μl aliquots were withdrawn from the reaction mixture and diluted with 10 ml of water, and the concentrations of H–Cys(Acm)–OH, *S*-2-hydroxyethylthio cysteine, and cystine in each sample were determined by amino acid analysis. Results are shown in Table II.

DMSO–TFA Oxidation of Fully Reduced Apamin Fully reduced apamin was obtained by treatment of synthetic apamin[6] with DTT (50 eq) in 0.05 M Tris·HCl buffer (pH 8) at 37°C for 1 h, followed by purification using a Sep-Pak® (Millipore). The lyophilized, reduced apamin (*ca.* 500 μg) was then treated with 10% (v/v) DMSO–TFA (500 μl) at room temperature for 2 h. Ice-chilled ether was added to the reaction mixture to give a precipitate. The main product was purified by HPLC, and the solvent was removed by lyophilization; FAB-MS *m/z*: Found 2027.26 $(M+H)^+$, Calcd for $C_{79}H_{132}N_{31}O_{24}S_4$; 2026.89. The disulfide array of the product was identified as type III by comparison of the product with authentic samples of apamin and its disulfide isomers by analytical HPLC (Fig. 3).

Apamin and Its Disulfide Isomers Using Regioselective Disulfide Bond Formation The peptide chains of three disulfide isomers of apamin (type, I, II, and III) were assembled on DMBHA resins[18] using Fmoc-based solid-procedures. The following protective groups were used for the side chain protection; Boc for Lys and His, *tert*-Bu for Glu and Thr, and Mtr for Arg. For the type I, Cys(Trt) was incorporated at positions 1 and 11, and Cys(Acm) at positions 3 and 15. For the type II, Cys(Trt) was incorporated at positions 1 and 15, and Cys(Acm) at positions 3 and 11. For the type III, Cys(Trt) was incorporated at positions 1 and 3, and Cys(Acm) at positions 11 and 15. Each resin-bound protected peptide (50 mg) was treated with *m*-cresol (122 μl)–thioanisole (300 μl)–EDT (50 μl)–TFA (1.88 ml)–TMSBr (330 μl) in an ice-bath for 3 h, then the resulting HBr gas was removed by evapoation, and the resin was removed by filtration. Ice-chilled ether was added to the filtrate and the resulting powder was collected by centrifugation. The crude product was then applied to a column of Sephadex G-25 (3.5 × 50 cm), and eluted with 1 N AcOH. The fractions containing the fastest eluting main peak were collected and submitted to air oxidation at pH 7.5 at 4°C for 48 h (total volume *ca.* 200 ml). The reaction mixture was then lyophilized and desalted by gel filtration. Each crude Acm-peptide thus obtained was dissolved in MeOH–water (4 : 1, v/v, 10 ml), then added dropwise to MeOH–1 N HCl (4 : 1 v/v, 10 ml) containing iodine (46 mg). The mixture was allowed to stand at room temperature for 30 min, then the reaction was quenched by addition of ascorbic acid until the reaction mixture becomes colorless. MeOH was removed by evaporation, and the residue was applied to a column of Sephadex G-25, and eluted with 1 N AcOH. Fractions containing the main peak were collected and lyophilized to give a powder. The crude peptide was purified by HPLC, yielding type I: 1.1 mg (6%), type II: 1.8 mg (9%), type III: 1.9 mg (9% based on the C-terminal amino acid). FAB-MS *m/z*: type I: Found 2027.1, type II: Found 2026.7, type III: 2027.8 $(M+H)^+$; Calcd for $C_{79}H_{132}N_{31}O_{24}S_4$; 2026.89.

Disulfide Bond Formation of Apamin by Various Oxidation Systems Fully reduced apamin (*ca.* 300 μg) was treated with 200 μl of the following reaction systems; i) $Tl(TFA)_3$ (5 eq) in TFA at 4 °C for 5 min, ii) 10% (v/v) DMSO–water at room temperature for 45 h, iii) 0.05 M Tris–HCl buffer (pH 8) at room temperature for 6 h. Each reaction mixture was directly analyzed by HPLC, and the ratio of the three disulfide isomers was estimated from the corresponding peak areas on analytical HPLC. Results are shown in Table III.

Acknowledgement We are grateful to Mr. Tomoyuki Ohkawa and Dr. Yuji Ikenishi of Shionogi and Co., Ltd. for SIMS measurements. We thank Drs. Nobuharu Sigematsu and Hirokazu Tanaka of Fujisawa Pharmaceutical Co., Ltd., for FAB-mass spectral analysis. We also thank Dr. Terrence R. Bruke, Jr. of the Laboratory of Medicinal Chemistry, NCI, NIH, for helpful discussions during the preparation of this manuscript. Our thanks are extended to Prof. Toshiro Ibuka of the Faculty of Pharmaceutical Sciences, Kyoto University, for his encouragement.

References and Notes

1) All amino acids used here are of the L-configuration. The following abbreviations are used: TFA = trifluoroacetic acid, DMSO = dimethylsulfoxide, TMSBr = trimethylsilyl bromide, TMSOTf = trimethylsilyl trifluoromethanesulfonate, EDT = 1,2-ethanedithiol, Trt = triphenylmethyl, MBzl = 4-methoxybenzyl, Dbs = dibenzosuberyl, Bzh = benzhydryl, Acm = acetamidomethyl, Boc = *tert*-butoxycarbonyl, Fmoc = 9-fluorenylmethoxycarbonyl, Ad = 1-adamantyl, Mts = mesitylenesulfonyl, Mtr = 4-methoxy-2,3,6-trimethylbenzenesulfonyl, Z = benzyloxycarbonyl, $Tl(TFA)_3$ = thallic trifluoroacetate, 2-ME = 2-mercaptoethanol, DTT = dithiothreitol, MeCN = acetonitrile, SIMS = secondary ion mass spectrometry.
2) V. du Vigneaud, C. Ressler, J. M. Swan, C. W. Roberts, and P. G. Katsoyanis, *J. Am. Chem. Soc.*, **76**, 3115 (1954).
3) D. B. Hope, V. V. S. Murti, and V. du Vigneaud, *J. Biol. Chem.*, **237**, 1563 (1962).
4) N. Fujii, A. Otaka, S. Funakoshi, K. Bessho, and H. Yajima, *J. Chem. Soc., Chem. Communn.*, **1987**, 163; N. Fujii, A. Otaka, S. Funakoshi, K. Bessho, T. Watanabe, K. Akaji, and H. Yajima, *Chem. Pharm. Bull.*, **35**, 2339 (1987).
5) N. Fujii, A. Otaka, T. Watanabe, H. Arai, K. Bessho, and H. Yajima, *J. Chem. Soc., Chem. Commun.*, **1987**, 1676; N. Fujii, A. Otaka, S. Funakoshi. T. Watanabe, H. Arai, K. Bessho, and H. Yajima, *J. Protein Chem.*, **7**, 151 (1988).
6) T. Koide, A. Otaka, H. Arai, S. Funakoshi, N. Fujii, and H. Yajima, "Peptide Chemistry," ed. by N. Yanaihara, Protein Research Foundation, Osaka, 1989, p. 171.
7) A. Otaka, T. Koide, A. Shide, and N. Fujii, *Tetrahedron Lett.*, **132** 1223 (1991).
8) A. Dryland and R. C. Sheppard, *J. Chem. Soc., Perkin Trans. 1*, **1986**, 125; L. Cameron, M. Medal, and R. C. Sheppard, *J. Chem. Soc., Chem. Commun.*, **1987**, 270.
9) S. Akabori, S. Sakakibara, Y. Shimonishi, and Y. Nobuhara, *Bull. Chem. Soc., Jpn.*, **37**, 433 (1964).
10) N. Fujii, A. Otaka, N. Sugiyama, M. Hatano, and H. Yajima, *Chem. Pharm. Bull.*, **35**, 3880 (1987).
11) N. Fujii, A. Otaka, S. Funakoshi, M. Nomizu, K. Akaji, H. Yajima, I. Yamamoto, K. Torizuka, K. Kitagawa, T. Akita, K. Ando, T. Kawamoto, Y. Shimonishi, and T. Takao, *Chem. Pharm. Bull.*, **34**, 620 (1986).
12) N. Fujii, A. Otaka, O. Ikemura, K. Akaji, S. Funakoshi, Y. Hayashi, Y. Kuroda, and H. Yajima, *J. Chem. Soc., Chem. Commun.*, **1987**, 274.
13) D. F. Veber, D. J. Milkowski, S. L. Varga, R. G. Denkewalter, and R. Hirschmann, *J. Am. Chem. Soc.*, **94**, 5456 (1972).
14) T. Koide, H. Itoh, A. Otaka, H. Yasui, M. Kuroda, N. Esaki, K. Soda, and N. Fujii, *Chem. Pharm. Bull.*, **41**, 502 (1993).
15) N. Fujii, A. Otaka, A. Okamachi, T. Watanabe, H. Arai, H. Tamamura, S. Funakoshi, and H. Yajima, "Peptides," ed. by G. Jung and E. Bayer, Walter de Gruyter, Berlin, New York, 1988, p. 58; J. P. Tam, C. R. Wu, W. Liu, and J. W. Zhang, *J. Am. Chem. Soc.*, **113**, 6657 (1991).
16) K. Akaji, T. Tatsumi, M. Yoshida, T. Kimura, Y. Fujiwara, and Y. Kiso, *J. Chem. Soc., Chem. Commun.*, **1991**, 167; T. Koide, A. Otaka, H. Suzuki, and N. Fujii, *Synlett*, **1991**, 345; K. Akaji, T. Tatsumi, M. Yoshida, T. Kimura, Y. Fujiwara, and Y. Kiso, *J. Am. Chem. Soc.*, **114**, 4137 (1992).
17) L. Zervas and I. Photaki, *J. Am. Chem. Soc.*, **84**, 3887 (1962); M. Fujino and O. Nishimura, *J. Chem. Soc., Chem. Commun.*, **1976**, 988 and reference cited therein.
18) S. Funakoshi, E. Murayama, L. Guo, N. Fujii, and H. Yajima, *Coll. Czeck. Chem. Commun.*, **53**, 2791 (1988).