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Disulfide Bond Formation in *S*-Acetamidomethyl Cysteine-Containing Peptides by the Combination of Silver Trifluoromethanesulfonate and Dimethylsulfoxide / Aqueous HCl

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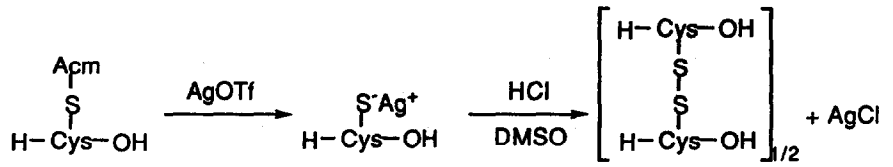
Abstract: *S*-Acetamidomethyl (Acm) cysteine was found to be converted quantitatively to cystine by deprotection of the Acm group with silver trifluoromethanesulfonate (AgOTf) followed by dimethylsulfoxide (DMSO) / aqueous hydrochloric acid (HCl) treatment. No significant side reactions were observed with oxidation-sensitive amino acids such as Met, Tyr, and Trp under these reaction conditions. This method has been applied successfully to the syntheses of oxytocin and a Trp-containing peptide, urotensin II.

The disulfide bond-forming reaction is one of the most important steps during the syntheses of cystine-containing peptides. Thus we have developed several disulfide bond-forming reactions[1,2]. Recently, the conversion of some *S*-protected cysteins and cysteine to cystine has been demonstrated using DMSO / trifluoroacetic acid (TFA)[3] or some sulfoxide-silyl compound / TFA[4,5]. One potential limitation with the use of these sulfoxide mediated reactions for peptide synthesis is the modification of Trp. This side reaction is analogous to that seen with the iodine oxidation used conventionally for the conversion of *S*-Acm cysteinyl residues [Cys(Acm)][6] to a cystinyl residue[7]. One of our research interests has therefore been the development of a facile and side reaction-free method for disulfide bond formation in Trp-containing peptides. Addition of DMSO to aqueous acidic or neutral media containing a cysteine-peptide was found to yield the corresponding cystine-peptide without side reactions[8,9]. Increasing the acidity of the reaction media may be partially responsible for inducing sulfoxide-mediated side reactions on Trp[10].

Previously, we reported that the AgOTf / TFA-mediated deprotection of a Cys(Acm)-peptide gave an S-Ag cysteine [Cys(Ag)]-peptide, which led to the generation of a cysteine-peptide by thiol treatment without side reactions on Met, Trp, or Tyr[11]. Treatment of H-Cys(Ag)-OH with HCl was also reported to give cysteine and AgCl[12]. However, the resulting cysteine derivative must be subjected to an oxidation step (*e.g.*, air oxidation) to obtain the corresponding cystine derivative.

Based on the above findings, we attempted to convert Cys(Acm) residues to a cystinyl residue by deprotection of the Acm group with AgOTf followed by DMSO / aqueous HCl treatment (Scheme 1). In the present paper, we report this two-step conversion of H-Cys(Acm)-OH to cystine and its practical application

to the synthesis of cystine-containing peptides.



Scheme 1. Formation of Cystine by the Combination of AgOTf and DMSO / Aqueous HCl

In this study, 10 % DMSO / 1 N HCl or 50 % DMSO / 1 N HCl was adopted as an oxidant, so that the lower acidity as compared with DMSO / TFA might circumvent side reactions. First, HCl·H-Cys(Acm)-OH was treated with AgOTf in TFA containing anisole for 1 h. Treatment of the resulting H-Cys(Ag)-OH with 10 % DMSO / 1 N HCl or 50 % DMSO / 1 N HCl gave cystine along with AgCl. The generation of cystine was monitored using an amino acid analyzer[13] (Fig. 1). Nearly quantitative conversion of H-Cys(Ag)-OH to cystine was accomplished with 10 % DMSO / 1 N HCl or 50 % DMSO / 1 N HCl. Oxidation-sensitive amino acids remained intact under reaction conditions of 50 % DMSO / 1 N HCl; recoveries of Met, Tyr, and Trp were 92, 99, and 102% (20 h), respectively.

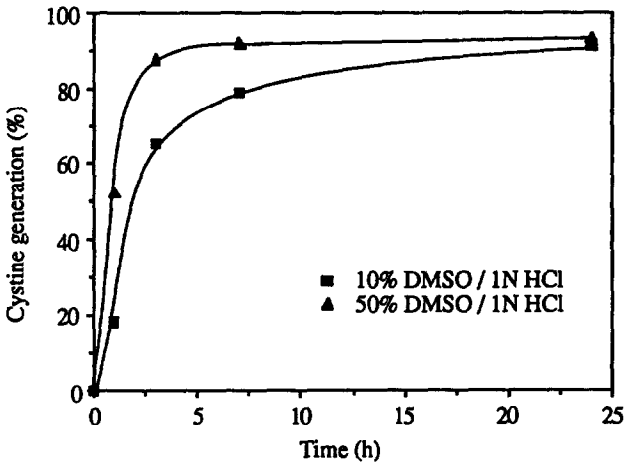


Fig. 1. Generation of Cystine (%) after Treatment with 10 % DMSO / 1 N HCl or 50 % DMSO / 1 N HCl

In order to demonstrate the usefulness of this method for the syntheses of cystine-containing peptides, oxytocin[14] and a Trp-containing peptide, urotensin II[15] (Fig. 2), were synthesized. Manual Fmoc-based solid-phase techniques[16], followed by deprotection with TFA - *m*-cresol - EDT - thioanisole - H_2O (80 : 5 : 5

: 5 : 5, v / v)[3], were used for the preparation of Cys(Acm)-oxytocin and Cys(Acm)-urotensin II[17]. Treatment of these Cys(Acm)-peptides with AgOTf / TFA-anisole, followed by the addition of ether, yielded Cys(Ag)-peptides. Reaction of the resulting Cys(Ag)-peptides with 50 % DMSO / 1 N HCl for 7 h gave solutions containing the corresponding cystine peptides and AgCl, which were subjected to filtration to remove AgCl. HPLC purification of crude peptides yielded pure products in good yields[18]. The HPLC retention time of each purified peptide was identical with that of an authentic sample of oxytocin or urotensin II.

oxytocin : H-Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2

urotensin II : H-Ala-Gly-Thr-Ala-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH

Fig. 2. Structures of Oxytocin and Urotensin II

In conclusion, this procedure has proven to be a useful synthetic methodology for the preparation of cystine-peptides, particularly when Trp residues are present. Additionally, the solvent systems used in this procedure (TFA and DMSO / 1 N HCl) circumvent the solubility problems of hydrophobic or basic peptides, since these solvents can dissolve most peptides. Finally, should it be desired, this procedure may be utilized for stepwise regioselective disulfide bond formation when used in a combination with other disulfide bond-forming method.

General procedure for the disulfide bond formation in Cys(Acm)-peptide using AgOTf and DMSO / 1 N HCl (the preparation of urotensin II). Cys(Acm)-urotensin II (2.6 μmol) was treated with AgOTf (27 mg, 40 eq) in TFA (500 μl) in the presence of anisole (10 μl) at 4 °C for 1.5 h. Ice-chilled dry ether (1 ml) was added to the reaction mixture and the resulting powder was collected by centrifugation. After being washed twice with ice-chilled dry ether (2 ml), the product was treated with 50% DMSO / 1 N HCl (4 ml) at room temperature for 7 h. Resulting AgCl was removed by filtration, and the total volume of the filtrate was brought to 10 ml with H_2O. The crude peptide in filtrate was purified by HPLC on a Cosmosil packed column (5μC18-100Å, 20 x 250 mm) using a linear gradient of MeCN (29 to 32 %, for 30 min) in 0.1 % TFA aq. at a flow rate 8.0 ml / min. The solvent was removed by lyophilization to give a white fluffy powder. The yield of the disulfide bond formation was 80 % [calculated from the HPLC[19] peak area of urotensin II (retention time 18.7 min) using authentic standard]. FAB-MS *m / z*: 1383.7 $(M+Na)^+$ (calcd 1383.5).

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Abbreviations: Fmoc = 9-fluorenylmethyloxycarbonyl, FAB-MS = fast atom bombardment-mass spectrometry.

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13. HCl · H-Cys(Acm)-OH (4.6 mg, 20 μmol) and H-Gly-OH (internal standard, *ca.* 10 μmol) were treated with AgOTf (26 mg, 100 μmol) in TFA (500 μl) in the presence of anisole (4.8 μl, 40 μmol) at 4 °C. After 1h, ice-chilled dry ether was added to the reaction mixture to afford a powder. The product was then treated with 10 % DMSO / 1 N HCl or 50 % DMSO / 1 N HCl (2 ml each) at room temperature. At intervals (1, 3, 7, 24 h), an aliquot (500 μl each) was removed and generation (%) of cystine from Cys(Acm) was quantitated by an amino acid analyzer.
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17. The structures of Cys(Acm)-oxytocin and Cys(Acm)-urotensin II are H-Cys(Acm)-Tyr-Ile-Gln-Asn-Cys(Acm)-Pro-Leu-Gly-NH_2 and H-Ala-Gly-Thr-Ala-Asp-Cys(Acm)-Phe-Trp-Lys-Tyr-Cys(Acm)-Val-OH, respectively.
18. Purified oxytocin: yield, 72 %; FAB-MS *m / z*, 1029.6 $(M+Na)^+$ (calcd 1029.4).
19. HPLC conditions : μ-Bondasphere 5μC18-100Å (3.9 x 150 mm) column with a gradient elution of MeCN (25-30 %, for 30 min) in 0.1 % TFA aq. at a flow rate of 1.0 ml / min.

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