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Regioselective Double Disulfide Formation using Silylchloride-Sulfoxide System

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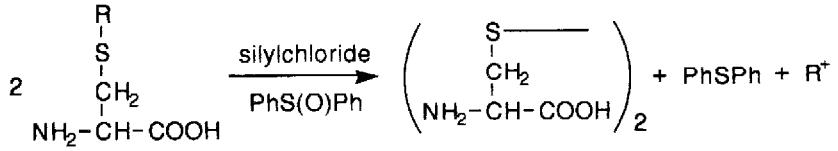
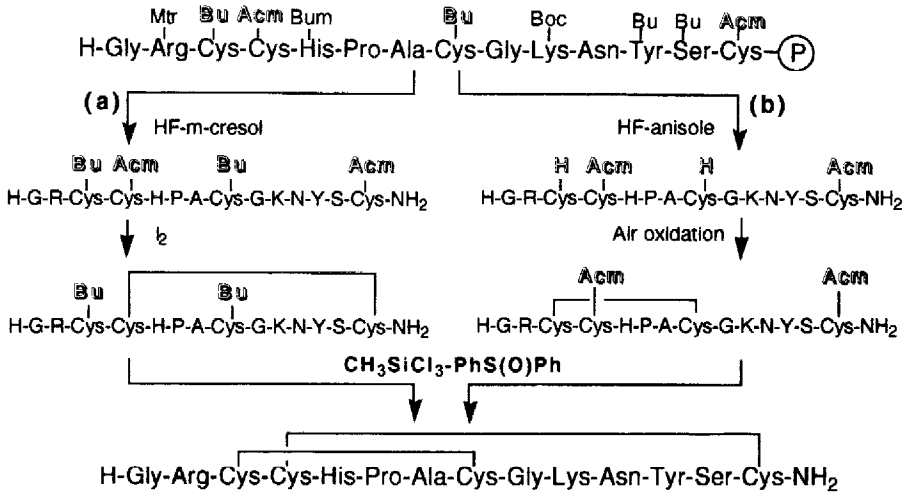
Abstract: *Two intermolecular or intramolecular disulfide bonds are formed regioselectively by the combination of silylchloride-sulfoxide method with conventional oxidation method. This stepwise method for double disulfide formation is successfully applied to the syntheses of conotoxin M1 and β-human atrial natriuretic peptide.*

For the synthesis of multiple disulfide-containing peptides, spontaneous air-oxidation is still less satisfactory. The yield is often low because of the formation of isomers or polymers[1]. Regiospecific disulfide bond formation can not be achieved by the air-oxidation method. For this regiospecific purpose, usually, successive deprotection-oxidation of two orthogonal cysteine S-protecting groups (e.g., HF-stable Acm and HF-labile MeBzl) are carried out[2]. For the unambiguous synthesis of complex peptide containing several cystine residues, however, the combination of orthogonal S-protecting groups and the deprotection-oxidation method is very restricted[3]. Recently, we have developed a new disulfide-bond forming reaction using silylchloride-sulfoxide system[4]. This reagent system can cleave various S-protecting groups of cysteine to form cystine directly within 10 to 30 min (Scheme 1). In this paper, we wish to report the regioselective disulfide bond formation by the combination of our new reagent system and conventional oxidation methods.

First of all, we have examined the combination of silylchloride-sulfoxide system with iodine-oxidation[5]. As two orthogonal S-protecting groups, Acm and t-Bu groups were selected. Acm group is stable to strong acid such as HF[6], but cleavable with iodine to form cystine[7]. In contrast, t-Bu group can be kept intact by iodine oxidation[8]. Concerning the stability of Cys(t-Bu) to acid, it has been reported briefly that the t-Bu group is resistant to usual HF treatment but cleavable with relatively high concentration of HF in the presence of large excess of anisole[9]. However, the behavior of the S-t-Bu group in a peptide derivative has not been well known. We have treated [Cys(t-Bu)^{1,6}]-oxytocin[4] with HF and found that the S-t-Bu group in the peptide derivative can be kept intact by the treatment with



Scheme 1. Cystine formation by silylchloride-sulfoxide system



Scheme 2. Synthesis of Conotoxin M1

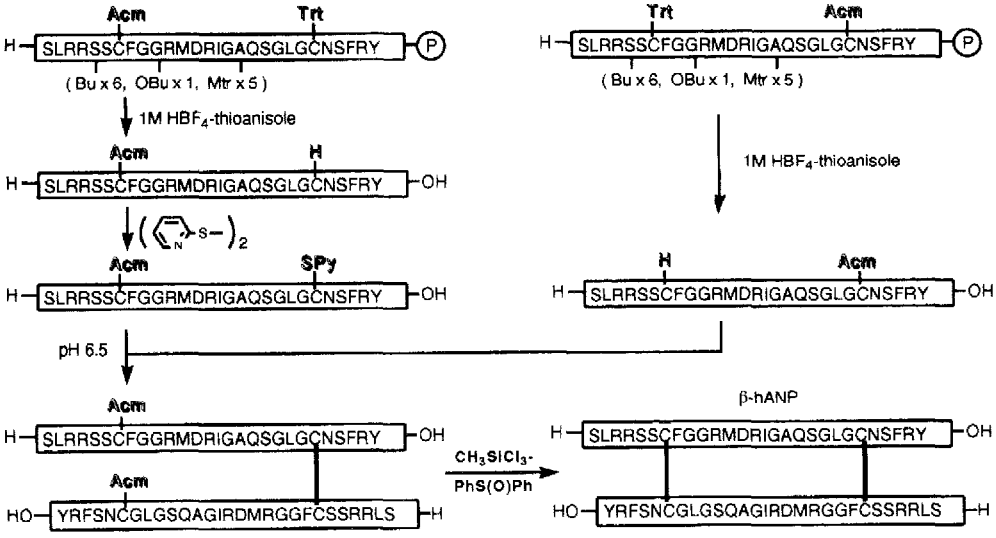
HF-m-cresol but cleavable with HF-anisole. Both Acm and t-Bu groups can be cleaved by the treatment with silylchloride-sulfoxide to form cystine. From these properties of the Acm and t-Bu groups, it appears feasible to construct two disulfide bonds by successive treatment with iodine and silylchloride-sulfoxide of the peptide carrying no further protecting groups other than these two kinds of S-protecting groups. To examine the feasibility of this scheme, we have synthesized conotoxin M1[10], a peptide neurotoxin consisting of 14 amino acid residues and containing two disulfide bonds in a molecule [Scheme 2, path (a)].

Construction of the peptide chain was carried out by Fmoc-based solid-phase synthesis starting from 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin[11]. The fully protected peptide resin was treated with HF-m-cresol (4°C, 20 min) to afford [Cys(t-Bu)^{3,8}, Cys(Acm)^{4,14}]-conotoxin M1. After FPLC (Pharmacia) purification[12], the S-protected conotoxin M1 in CH_3OH-H_2O=8:2 (peptide concentration; 1μmol/ml) was treated with 20 % I_2 in CH_3OH (20 eq.) at 25°C for 15 min to construct the first disulfide bond between the cysteine residues having Acm groups. The crude product was adsorbed on Diaion HP-20 and then eluted with 80% CH_3CN in 0.1% aq.TFA. The isolated peptide in TFA (peptide concentration; 1μmol/ml) was then treated with CH_3SiCl_3 (150 eq.) in the presence of PhS(O)Ph (10 eq.) at 25°C for 10 min to construct the second disulfide bond. The product was isolated by the adsorption to Diaion HP-20 and purified by FPLC[12] (10 % yield based on the starting C-terminal resin). The homogeneous peptide[13] possessed the same elution time based on analytical HPLC as that of an authentic conotoxin M1 purchased from Peptide Institue Inc., Osaka.

We then examined the combination of silylchloride-sulfoxide method with air-oxidation similarly using the same model peptide [Scheme 2, path (b)]. By the treatment of the above fully protected conotoxin M1-resin with HF-anisole (4°C, 60 min), the partially protected dihydropeptide, [Cys^{3,8}, Cys(Acm)^{4,14}]-conotoxin M1, was prepared. After FPLC purification[12], the product in aqueous solution (pH 8.0, peptide concentration; ca 0.2 μmol/ml) was air-oxidized at 25°C for 22h to construct the first disulfide bridge between the unmasked cysteine residues. The second disulfide bridge was constructed by the treatment of the product in TFA (peptide concentration; 1 μmol/ml) with CH_3SiCl_3

(150 eq.)-PhS(O)Ph (10 eq.) at 25°C for 10 min. The crude product was purified as described above. The purified conotoxin M1 was obtained in 27 % yield based on the starting C-terminal resin. The homogeneous peptide[13] had the same physicochemical properties as those of the sample obtained above. Thus, two intramolecular disulfide bonds in conotoxin M1 are formed regioselectively. In this scheme, the order of the stepwise disulfide formation also can be controlled by choosing the first deprotection method of the same protected peptide resin.

Next, the application of silylchloride-sulfoxide system for the stepwise formation of two intermolecular disulfide bonds was examined. For this object, we have synthesized β-human atrial natriuretic peptide (hANP) which is an antiparallel dimer of α-hANP[14] (Scheme 3). Two fully protected peptide resins having the same amino acid sequence of α-hANP but different combination of S-protecting groups (Acm and Trt) were prepared by Fmoc-based solid-phase synthesis starting from 4-benzyloxybenzyl alcohol resin. Each protected peptide resin was treated with 1M HBF_4-thioanisole[15] (4°C, 90 min) and the product was purified by FPLC[12] to afford partially protected α-hANP, *i.e.*, [Cys(Acm)^7,Cys^{23}]-α-hANP or [Cys^7,Cys(Acm)^{23}]-α-hANP. [Cys(Acm)^7, Cys^{23}]-α-hANP in 2-propanol-2N AcOH was allowed to react with 2, 2'-dithiodipyridine (PySSPy)[16] (3 eq.) and the product was purified by gel-filtration on Sephadex G-15. The resulting [Cys(Acm)^7, Cys(S-Py)^{23}]-α-hANP (1 eq.) was mixed with [Cys^7, Cys(Acm)^{23}]-α-hANP (1 eq.) in $AcONH_4$ (peptide concentration; 0.9 μmol/ml) at pH 6.5 for 30 min to form the first disulfide bridge. After FPLC purification[12], the product in TFA (peptide concentration; 1.5 μmol/ml) was treated with CH_3SiCl_3 (200 eq.)-PhS(O)Ph (10 eq.) at 25°C for 30 min to construct the second disulfide bond between the cysteine residues having Acm group. The crude product was purified by preparative HPLC[17]. The purified β-hANP was obtained in 9 % yield based on [Cys(Acm)^7, Cys^{23}]-α-hANP. The homogeneous peptide[18] had the same elution time based on analytical HPLC as that of an authentic sample (Peptide Institue).



Scheme 3. Synthesis of β-hANP

In conclusion, double disulfide structure has been prepared unambiguously by the combination of silylchloride-sulfoxide method with conventional oxidation methods. This regioselective stepwise method has been shown to be effective for the formation of both intermolecular and intramolecular disulfide bonds.

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Abbreviation
Acm=acetamidomethyl, Trt=trityl, MeBzl=4-methylbenzyl, t-Bu=tert-butyl, TFA=trifluoroacetic acid.

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12. YMC ODS-AQ300 column (1.5 x 50 cm) eluted with a linear gradient of 60 % CH_3CN/0.1 % aq.TFA (0-100 %, 400 min) in 0.1 % aq.TFA at the flow rate of 3.0 ml/min.
13. HPLC [YMC AM302, 4.6 x 150 mm, CH_3CN (10-60 %, 30 min) in 0.1 % aq.TFA, 0.7 ml/min], retention time 10.54 min; Amino acid ratios after 6N HCl hydrolysis and LAP digestion (numbers in parentheses), Asp x 1, 1.02 (N.D.); Ser x 1, 0.88 (1.01); Pro x 1, 0.97 (0.81); Gly x 2, 2.00 (2.26); Ala x 1, 1.00 (0.91); Cystine x 2, 1.25 (1.60); Tyr x 1, 1.01 (1.04); Lys x 1, 0.98 (1.00); His x 1, 1.00 (0.82); Arg x 1, 0.96 (1.28).
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17. YMC D-ODS-5 column (20 x 250 mm) eluted with a linear gradient of CH_3CN (20-25 %, 80 min) in 0.1 % aq.TFA at the flow rate of 5.0 ml/min.
18. HPLC [YMC AM302, 4.6 x 150 mm, CH_3CN (20-25 %, 30 min) in 0.1 % aq.TFA, 0.7 ml/min], retention time 26.72 min; Amino acid ratios after 6N HCl hydrolysis and LAP digestion (numbers in parentheses), Asp x 4, 4.07 (2.02); Ser x 10, 8.59 (9.93); Glu x 2, 2.11 (N.D.); Gly x 10, 10.17 (10.06); Ala x 2, 2.09 (2.06); Cystine x 2, 0.45 (1.87); Met x 2, 1.94 (2.35); Ile x 2, 1.99 (2.01); Leu x 4, 4.09 (4.06); Tyr x 2, 2.05 (2.05); Phe x 4, 4.00 (4.00); Arg x 10, 9.42 (9.54).

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