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A rapid and facile method for the preparation of peptide disulfides

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Abstract: A selective and efficient method for disulfide bond formation in peptides utilizing carbon tetrachloride in dichloromethane in the presence of tetrabutylammonium fluoride (TBAF) is described. The reaction proceeded rapidly and no side reaction was observed with nucleophilic amino acids such as Met, His, Tyr or Trp. This method has been applied to three model peptides using solution and on-the-resin disulfide formation.

Disulfide bonds between cysteine residues are an important means for stabilizing protein and peptide conformations.[1] Numerous peptide hormones containing disulfide-linked cyclic peptide analogs with a well-defined, comparatively rigid conformation have been used for the study of the structure-activity relationship and conformational properties of peptides. Due to the complex functionality and reactivity of the individual amino acid side chains, it is important to have a range of reagents available for disulfide bond formation. As a result of efforts to obtain cyclized peptides, various oxidation methods are available now, but synthetic methods for selective disulfide bond formation in peptides are still limited.[2,3,4,5]

We now wish to report a combination of CCl_4 and TBAF for the synthesis of peptide disulfides. Oxidation reaction of thiol to disulfide employing CCl_4 and triethylamine was first described by Wenschuh *et al.*[6] Carbon tetrachloride is considered to act as an oxidant giving chloroform and disulfide, but the reaction was very slow and the resin-bound oxidation of α-conotoxin S1 sequence with CCl_4 and triethylamine did not succeed.[7] And more recently, Annis *et al.* reported an oxidation by a combination of tertially amine and air without CCl_4.[2] On the other hand, TBAF acts as a unique base and is applied to a variety of base-assisted reactions.[8] We have already reported the utility of TBAF as a deprotecting agent of 9-fluorenylmethoxycarbonyl (Fmoc)[9] or phenacyl ester,[10] and the preparation of a cyclic peptide containing methylenedithioether structure from Cys residues utilizing TBAF in dichloromethane (CH_2Cl_2).[11]

In order to examine the usefulness of CCl_4/TBAF for the intramolecular disulfide bond formation, three model peptides, DPDPE(Tyr-[DPen-Gly-Phe-DPen]-OH), HBP51(Tyr-[DPen-Gly-Phe(*p*-F)-Pen]-Phe-OH)[12] and [Arg[8]]-Vasopressin([Cys-Tyr-Phe-Gln-Asn-Cys]-Pro-Arg-Gly-NH_2) were prepared by solution-phase or on-the-resin disulfide bond formation.

The linear peptides were synthesized by the Boc or Fmoc solid-phase technique. The amino acid coupling reactions were performed by using the 2-(1*H*-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *in situ* neutralization method for the Boc synthesis.[13]

Disulfide bond formation in solution phase: As shown in Scheme 1-a) for the preparation of DPDPE, the linear peptides were cleaved from the resin and the protecting groups were removed by treatment with liquid hydrogen fluoride / *m*-cresol/ 1,2-ethanedithiol at -1℃ for 1 hour. Hydrogen fluoride was removed by evaporation and the resulting materials were washed with ether, dissolved in acetic acid, and precipitated in ether to obtain crude peptides. For the preparation of

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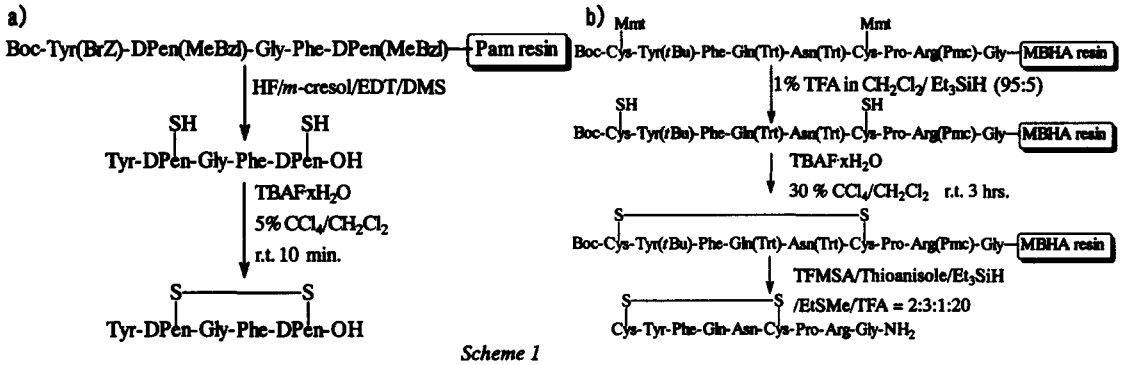

Scheme 1

DPDPE, 20 mg of crude linear (SH) peptide was suspended in 20 ml of 5% CCl_4 / CH_2Cl_2 at room temperature and 130 µl of 1 M-TBAF in THF was added in one portion to the vigorously stirred suspension. The insoluble suspension turned clear at the moment that TBAF was added, and the disulfide formation completed at the time that the suspension turned homogeneous, which usually is less than one minute. Oxidation reaction was quenched after 10 minutes by adding 100 µl of acetic acid and the solvent was removed by nitrogen blowing or by evaporation. The crude peptide was dissolved in acetic acid and purified by reversed-phase HPLC. Cyclic peptide was obtained as shown in Figure1, and 13 mg (65%) of cyclic DPDPE was obtained after HPLC purification. ESI-MS: m/z = 644 $(M-H)^-$.

Oxidation on resin (Scheme 1-b): Side-chain protected peptide resin containing Cys(Mmt)[14] was prepared by the Fmoc strategy, treated with 1% TFA in CH_2Cl_2 / triethylsilane (Et_3SiH) (95:5) and moderately stirred at room temperature for 30 minutes. The resultant linear peptide resin was washed with CH_2Cl_2 several times, dried and then treated with TBAF in 30% CCl_4/CH_2Cl_2 at room temperature for 3 hours. The resin-bound peptide was then cleaved according to the previously reported procedure.[15] A portion of 100 mg (0.45 mmol/g) of the obtained resin was treated with 6 ml of TFMSA/thioanisole/Et_3SiH/ethylmethylsulfide/TFA (2:3:3:1:20). The mixture was gently stirred for 1.5 hours at room temperature and then filtrated. The residual resin was washed with a small volume of TFA and the combined filtrate was dropped into a vigorously stirred diethyl ether. The ether was removed by filtration and the precipitate was washed with ether followed by HPLC purification to obtain 37mg (76%) of cyclic [Arg^8]-Vasopressin. FABMS: m/z = 1084 $(M+H)^+$.

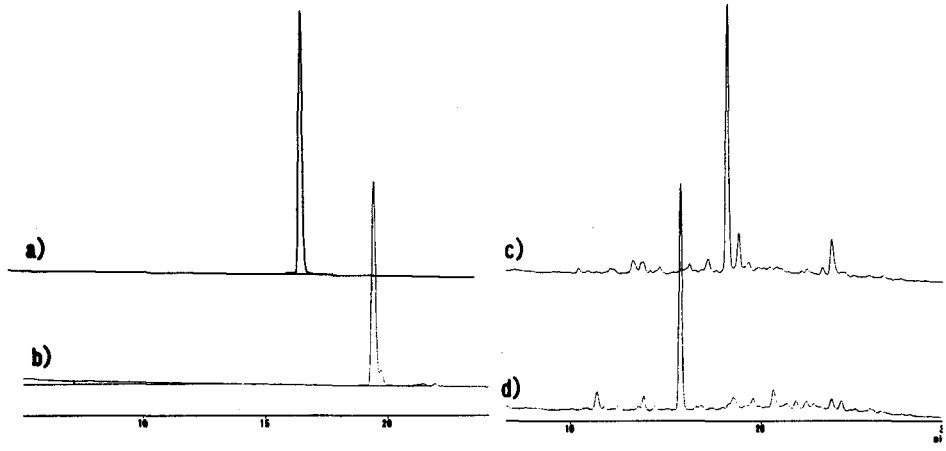

Figure 1. Analytical HPLC of HBP51 and DPDPE before and after treatment with CCl_4/TBAF in CH_2Cl_2 in solution. a) purified linear HBP51; b) reaction mixture after the treatment of a); c)crude linear DPDPE ; d) reaction mixture after the treatment of c) . A=0.1% TFA (aq.), B= CH_3CN Gradients:5% B to 65% B over 30 min. Eluate was monitored by UV measurement at 220 nm.

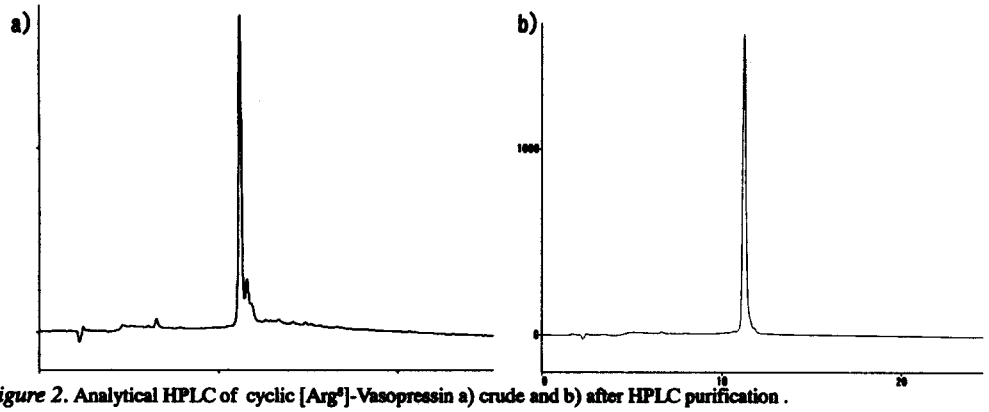

Figure 2. Analytical HPLC of cyclic [Arg^8]-Vasopressin a) crude and b) after HPLC purification. HPLC conditions are given in Figure 1.

The oxidation reaction proceeded quite rapidly and was found to be completed in less than one minute when solution-phase oxidation was monitored by HPLC. Oxygen was not necessary for the disulfide-bond formation and the oxidation proceeded under nitrogen. Oxidation reaction did not occur without TBAF. Carbon tetrachloride obviously played an important role and without CCl_4, no disulfide bond formation occurred in CH_2Cl_2, which gave methylenedithioether quantitatively instead of disulfide peptides.[11] Although one drawback to the use of TBAF is its hygroscopicity, a small amount of water did not affect the reaction and the oxidation proceeded in THF with 1% water (THF:CCl_4:H_2O=94:5:1). Usual disulfide-bond formations in solution require high-dilution conditions to optimize the ratio of internal disulfide formation, but this oxidation system gave satisfactory results for even high concentration (5mg/ml) conditions, presumably because the reaction starts from an insoluble suspension and the rate of the oxidation is higher than the rate at which peptides dissolve in the solvent by the aid of TBAF. When triethylamine was used instead of TBAF, the oxidation was so slow that only a mixture of polymerized peptide was obtained after an overnight reaction.

Possible side products during oxidation of thiol to disulfide were cleared in the case of Met, His, Tyr and Trp in unprotected form. As shown in Fig. 3, model peptides containing amino acids which are susceptible to oxidative side reactions were treated in the oxidation procedure using CCl_4/TBAF. Some side products from Trp, and His were observed after overnight treatment, but the disulfide formation was more dominant and these oxidation-sensitive side chains remained intact after the treatment with CCl_4/TBAF for 10 minutes, which is long enough for completion of the disulfide bond

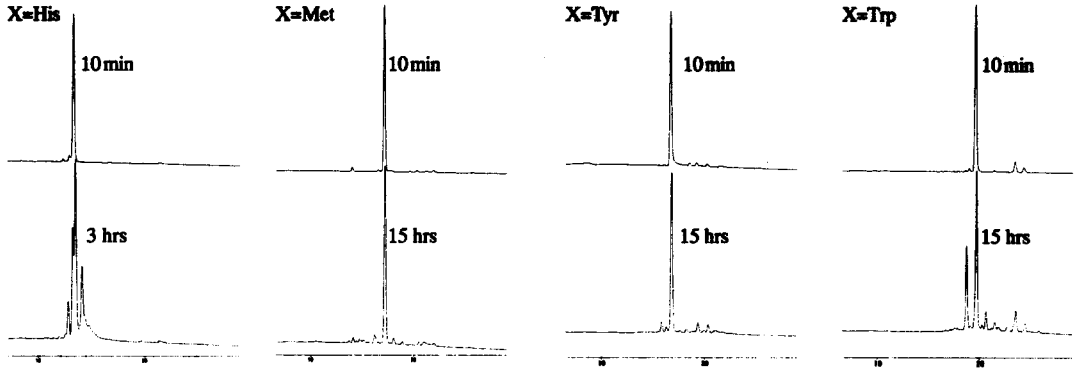

Figure 3. HPLC analysis of Ac-X-Cys-Gly-Phe-Cys-Gly-OH (X : side chain unprotected His, Met, Tyr or Trp) at several stages during oxidation in solution.

formation.

Solvents other than CH_2Cl_2 were tested for the solution-phase oxidation of DPDPE. We tested THF, acetonitrile, chloroform, DMF each containing 5% CCl_4 as well as 100% CCl_4 as the solvent. The oxidation reaction proceeded quantitatively in all the solvents except for chloroform. When chloroform was used as the solvent, no reaction occurred and the starting linear (SH) peptides were recovered.

In conclusion, oxidation of thiols to disulfides using CCl_4/TBAF as an oxidizing agent has been found to be a useful synthetic method in both on-the-resin and solution-phase. The disulfide formation reaction proceeded quite rapidly without using high-dilution conditions. The choice of solvents was wide allowing use of a variety of solvents. Additionally, this procedure circumvents the solubility problems of peptides in solution since TBAF worked for solvating all the peptides tested in this study in aprotic solvents such as CH_2Cl_2. Removal of the solvent was exceptionally easy because of the low boiling point of the solvent. This method makes solution-phase disulfide bond formation quite easy and is considered to be useful for hydrophobic peptides and protected peptide fragments as well as side-chain unprotected peptides.

References and Notes

[1] Thornton, J. M. J. Mol. Biol. **1981**, 151, 261

[2] Annis, I.; Hargittai, B.; Barany, G. Methods in Enzymology **1997**, 289, 198

[3] Eichler, J.; Houghten, R. A. Protein and Peptide Lett. **1997**, 4,157

[4] Akaji, K. Yakugaku Zasshi **1996**, 116, 441

[5] Misicka, A.; Hruby, V. J. Polish J. Chem. **1994**, 681, 893

[6] Wenschuh, E.; Heydenreich, M.; Runge, R.; Fischer, S. Sulfur Lett. **1989**, 8, 251

[7] Munson, M. C.; Barany, G. J. Am. Chem. Soc. **1993**, 115, 10203

[8] Clark, J. H. Chem. Rev. **1980**, 80, 429

[9] Ueki, M.; Amemiya, M. Tetrahedron Lett. **1987**, 28, 6617

[10] Ueki, M.; Aoki, H.; Katoh, T. Tetrahedron Lett. **1993**, 34, 2783

[11] Ueki, M.; Ikeo, T.; Hokari, K.; Nakamura, K.; Saeki, A.; Komatsu, H. Bull. Chem. Soc. Jpn. in press.

[12] Hruby, V. J.; Bartosz-Bechowski, H.; Davis, P.; Slaninova, J.; Zalewska, T.; Stropova, D.; Porreca, F.; Yamamura, H. J. Med. Chem. **1997**, 40, 3957

[13] Bogusky, M. J.; Naylor, A. M.; Pitzenverger, S. M.; Nutt, R. F.; Brady, S. F.; Colton, C. D.; Sisko, J. T.; Anderson, P. S.; Veber, D. F. Int. J. Pept. Protein Res. **1992**, 40, 180

[14] Barlos, K.; Gatos, D.; Hatzi, O.; Koch, N.; Greece, P.; Int. J. Pept. Protein Res. **1996**, 47, 148

[15] Limal, D.; Briand, J. -P.; Dalbon, P.; Jolivet, M. J. Peptide Res. **1998**, 52, 121