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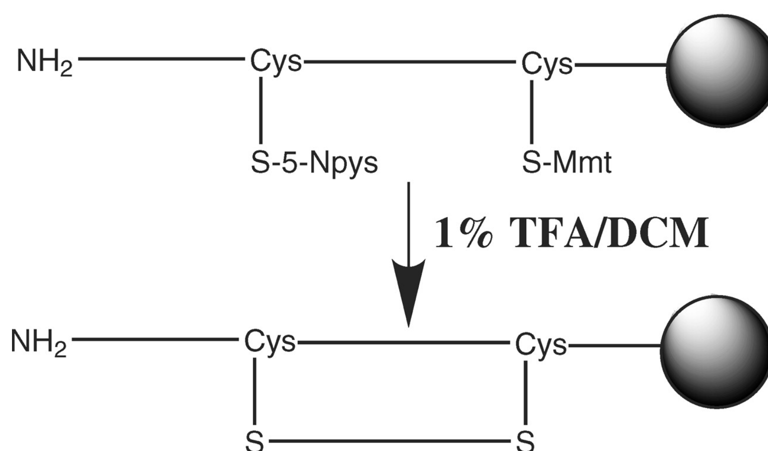

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An Effective Method of On-Resin Disulfide Bond Formation in Peptides

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Since its conception in the early 1960s, solid-phase methods have been used extensively in organic synthesis.[1] In the area of peptide science, a variety of chemical transformations have been accomplished on solid support, including the formation of intramolecular disulfide bridges.[2] Disulfide bridges play a pivotal role in maintaining biologically active conformations of several natural and synthetic peptides. Conventional solution-phase techniques for intramolecular disulfide bond formation typically involve oxidation of free thiol precursors at high dilution. Similarly, the oxidation on solid support utilizes pseudodilution, a kinetic phenomenon expected to favor facile intramolecular interactions, in the microporous resins.[3] Methods to achieve on-resin disulfide bond formation for Fmoc-based peptide synthesis are limited. A few Fmoc-compatible thiol protecting groups, such as Cys(triphenylmethyl), Cys(fluorenylmethyl), Cys(acetamidomethy),[2] Cys(2,4,6-trimethoxybenzyl),[4] Cys(*tert*-butylthio),[5] and Cys(xanthenyl),[6] have been tested toward making disulfide bridges on the solid support. Typically, the disulfide bond formation is accomplished using either an oxidizing agent or mild basic conditions. The current methods of on-resin disulfide bond formation have several limitations, including side reactions of oxidation sensitive residues, such as methionine and tyrosine; use of hazardous oxidants containing thallium and mercury and difficulties in their postoxidation removal;[7] iodine-mediated scission of indole ring of tryptophan with subsequent peptide cleavage; lower peptide yields due to the formation of dimeric products in base-catalyzed oxidations and subsequent problematic peptide purification; and lower yields because of the slow or incomplete oxidation on solid support.[8] Here, we describe a facile, non-oxidative solid-phase method of disulfide bond formation that circumvents the above-mentioned limitations.

To demonstrate the wide applicability of this new approach, we have synthesized five disulfide-bridged peptides (Figure 1). Schemes1 and 2 illustrate the steps involved for the synthesis of peptides **2** and **5**, respectively. Peptides **1**, **2**, **4**, and **5** were assembled using standard Fmoc chemistry on Wang resin (Novabiochem, 0.65 mmol/g). *tert*-Butylthio (S-*t*-Bu) and 4-methoxytrityl (Mmt) were used as thiol protecting groups of the cysteines undergoing disulfide bond formation. In the first step, S-*t*-Bu was removed by reduction to liberate free thiol by treating the resin with 20% mercaptoethanol in dimethylformamide for 3 h. The resin was then reacted with a 10-fold excess of 2,2′-dithiobis(5-nitropyridine) (DTNB) in dichloromethane for 1 h, and the free thiol was thus reprotected and activated with the 5-nitropyridin-

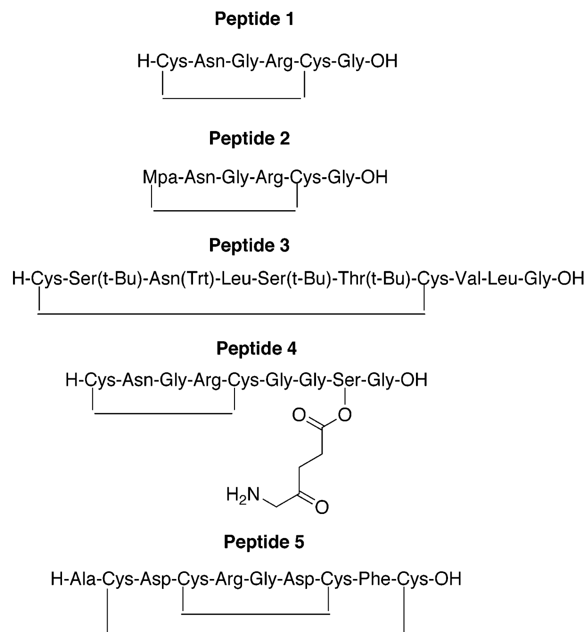
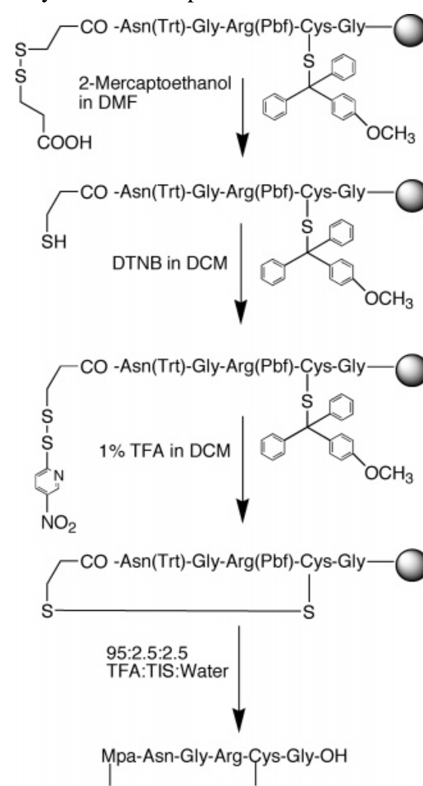


Figure 1. Cyclic peptides synthesized using non-oxidative disulfide bond formation. Mpa: mercaptopropionic acid.

Scheme 1. Synthesis of Peptide **2**



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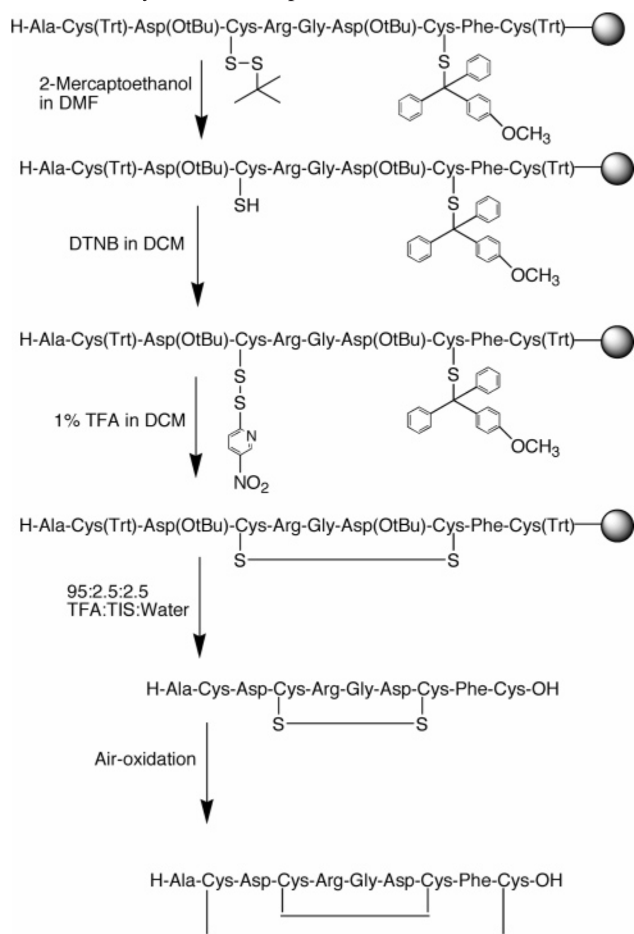
Scheme 2. Synthesis of Peptide **5**



sufenyl (5-Npys) group. This was followed by the cyclization step in which the resin was treated with 1% trifuoroacetic acid (TFA) in dichloromethane (DCM) in the presence of triisopropylsilane (TIS) as the scavenger. The reaction was monitored by measuring the absorbance of 5-nitropyridine-2-thione at 386 nm. All cyclizations were completed in <20 min.

The disulfide bond formation is based on the sequence of two disulfide exchange reactions, both of which do not require an oxidizing agent and can be carried out in the inert atmosphere. The first reaction involves nucleophilic attack of free thiol, obtained following the deprotection of Cys(S-*t*-Bu), on the activated disulfide of DTNB. Because of the polymer-bound nature of the thiol, byproduct 5-nitropyridine-2-thione can be easily washed away along with the excess of DTNB. The cyclization is the second disulfide exchange reaction that involves another nucleophilic attack of free thiol, obtained following the deprotection of Cys(Mmt), on the polymer-bound 5-Npys-activated disulfide. Solution-phase counterparts of such disulfide exchange reactions require acidic conditions and inert atmosphere to avoid cross-linking and dimeric products.[9] Such stringent conditions were not required in the case of our disulfide bond formation.

Peptide **1** is a small hexapeptide analogue of aminopeptidase-N-targeting peptide CNGRC,[10] whereas peptide **2** is a mercaptopropionic (Mpa) analogue of peptide **1**. Facile synthesis of peptide **2** documents that this method can also be utilized to synthesize Mpa analogues of disulfide-bridged peptides. Commercially available 3,3′-dithiopropionic acid was used as a Fmoc-Cys(S-*t*-Bu) counterpart in the synthesis of peptide **2** (Scheme 1). The cyclization was achieved using 1% TFA in DCM. Such mild acidic conditions are routinely used for the cleavage of peptide chains from the Trt-type solid supports. This prompted us to synthesize fully protected peptide **3** using the 2-chlorotrityl resin, where the peptide cleavage and the cyclization were achieved in a single step. Peptide **3** is the 1−10 protected fragment of the salmon calcitonin peptide.[11]

Other than Cys(Mmt), mild acidic conditions are also used for the selective deprotection of side chains of several other amino acids, such as Lys(methyltrityl), Ser(trityl), and Thr-(2-chlorotrityl). Taking advantage of this selective deprotection, it is possible to unmask the side chains of such amino acids under the mild acidic condition of disulfide bond formation. In addition, this strategy can be utilized to make conjugates or prodrugs of disulfide-bridged peptides on the solid support. Accordingly, we synthesized peptide **4**, which is a peptide prodrug of a photodynamic therapy agent 5-aminolevulinic acid.[12] Along with the above-mentioned aminopeptidase targeting cyclic peptide, we incorporated Ser(trityl) in the peptide chain (peptide **4**); under the mild acidic conditions, the hydroxyl group of serine was unmasked and was made available for further conjugation of Boc-protected 5-aminolevulinic acid on the solid-support. Cleavage of the peptide chain under anhydrous conditions yielded peptide **4**.

Next, we synthesized peptide **5**, a bicyclic RGD-4C peptide ligand for $\alpha_v\beta_3$ integrin.[13] Because of its four cysteine residues, this peptide has three possible disulfide-bonded forms, and while only two of these forms are present in detectable quantities in the spontaneously cyclized peptide, only one form, which is shown as peptide **5** (Scheme 2), strongly binds the integrin.[14] Traditional synthesis of such bicyclic peptides is often challenging and requires an additional dimension of orthogonality on the solid support. Facile synthesis of peptide **5** using the non-oxidative cyclization method demonstrates the utility of this procedure toward the synthesis of bicyclic peptides. The internal i, $i + 4$ disulfide bond formation was carried out on the resin using the non-oxidative method, whereas the second disulfide bridge was formed in solution after deprotection of the Trt groups of the remaining cysteines following the peptide cleavage. Thus, in the case of peptide **5**, we have used two trityl(Trt)-based protecting groups for the cysteines, Cys-(Trt) and Cys(Mmt). It is well-known that trityl-based functional groups can be selectively cleaved under different acidic conditions. In fact, it has been reported that Mmt can be selectively cleaved in the presence of the Trt group under mild acidic conditions of 0.5−1% TFA in DCM.[15] In addition, in the case of peptide **5**, we indeed observed a clean reaction of Mmt deprotection in the presence of the Trt groups because only the desired isomer of RGD-4C was observed in the crude peptide product.

Matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrograms of the crude peptides did not indicate any amounts of peptides containing cysteines protected with 5-Npys, S-*t*-Bu, or Mmt (Supporting Informa-

Table 1. Product Characterization of the Peptides **1**−**5**

peptide	M + 1[a]	M + 1[b]	RT[c]	yield[d], %	yield[e], %
1	607.20	608.40	7.84	84.4	>99
2	592.19	592.91	10.95	77.0	>99
3	1404.72	1404.06	27.41	66.7	>99
4	920.32	920.90	6.05	78.0	>99
5	1088.30	1091.29	17.15	71.3	>99

[a] Calculated molecular weight. [b] Molecular weight found using MALDI-TOF mass spectrometer. [c] Retention time; RP-HPLC conditions: Vydac C18 column; solvent gradient; buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in acetonitrile; flow rate, 1 mL/min over the range of 0−100% B in 50 min; UV detection, 220 nm. [d] Peptide synthesis. [e] Cyclization.

tion). This confirmed the quantitative yields of the nonoxidative cyclization reaction. The product characterization only showed traced amount of dimeric or cross-linked products in the crude peptides **1** and **2**. Dimeric or cross-linked products of peptides **3**, **4**, and **5** were not seen, Table 1 summarizes the yields of cyclization and peptide synthesis. Peptide synthesis yields of crude peptides are based on the initial substitution level of the resin. Crude peptides were further characterized by performing reversed-phase high-pressure liquid chromatography (RP-HPLC). Oxidation yields were determined by the detection of free thiols on the solid support[16] as well as in the peptide chain after cleavage using the Ellman's reagent.[17]

Presence of only monomeric cyclic peptides in the product underscores the fact that the phenomenon of pseudodilution is critical for the success of this reaction.[18] It can be envisioned that depending on the loading of the resin, interpeptide disulfide bonds could be formed between the neighboring free and activated thiol groups, yielding dimeric products. To evaluate the contribution of on-resin pseudodilution for this reaction, we attempted the cyclization on a chlorotrityl resin with the substitution level of 1.6 mmol/g, and as expected, we observed large amounts of dimeric peptides along with the monomeric cyclic peptides. However, the intramolecular bridge formation was selectively obtained when the resins with the substitution level of 0.65 mmol/g or lower were used. It suggested that the same chemistry could be utilized for making intramolecular as well as intermolecular disulfide bonds, depending on the substitution level of the resin. Indeed, the synthesis of disulfide-bond-linked heterodimeric peptides has been demonstrated previously using a high-loading MBHA resin (1.2 mmol/g).[19]

Owing to its high solubility in organic solvents, such as DCM, and its high reactivity toward free thiols, we also attempted to use 2,2′-dithiodipyridine in the place of DTNB for this cyclization. Similar to DTNB, 2,2′-dithiodipyridine has been extensively used in activating free thiol groups toward disulfide exchange reactions.[20] However, surprisingly, 2,2′-dithiodipyridine did not react with the polymer-bound thiol groups. Recently, the use of CLEAR-OX, which is based on the solid-supported Ellman's reagent, has been reported for the synthesis of disulfide-bridged peptides;[21] however, we observed that Ellman's reagent could not quantitatively derivatize/activate thiol bound to the polystyrene-type solid supports. The reagent also has poor solubility in the organic solvents.

In conclusion, we have developed a novel and facile method of nonoxidative, on-resin intramolecular cyclization. The novelty of this methodology is 4-fold: (1) The procedure reports quantitative and site-specific derivatization of polymer-bound thiol with DTNB. Boc-Cys(3-Npys)-OH is commercially available and has been used for obtaining disulfide-bridged conjugates of peptides;[22] however, Fmoc-Cys(3-Npys)-OH cannot be utilized in Fmoc solid-phase peptide synthesis due to the instability of the Npys group to piperidine.[23] Hence, the Cys(Npys) residue can be introduced only at the N terminus of the peptide using Boc-Cys(3-Npys)-OH, making non-N-terminus derivatization tedious.[24] Our approach of cyclization utilizing Cys(S-*t*-Bu) makes it possible to quantitatively derivatize the polymer-bound thiol with the Npys moiety at any position in the peptide chain. (2) DTNB has been used for Fmoc-based chemistry for activating free thiol groups by adding this reagent in the peptide cleavage mixture.[25] This approach is nonselective because all the thiol groups get activated after the peptide is cleaved from the resin. Our approach allows selective activation of the resin bound thiol. (3) Because the nonoxidative method does not utilize conventional oxidants, such as iodine, mercury(II) acetate, thallium(III) trifluoroacetate, or even the basic conditions, it eliminates all the possible limitations associated with the oxidative-type cyclization reactions, and the facile nature of the reaction ensures quantitative yields for cyclization. (4) Because of the mild reaction conditions for cyclization, this method can be used for synthesizing protected cyclic peptide fragments. It is compatible with other methods of disulfide bond formation, and the methodology can easily be extended to Boc-based peptide synthesis.

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Supporting Information Available. Supporting Information as cited in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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