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Solid-Phase Synthesis of Disulfide Heterodimers of Peptides

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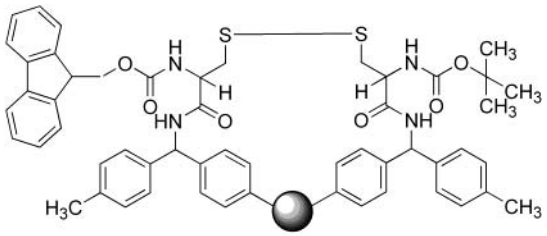
ABSTRACT



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The synthesis of the orthogonal disulfide template 1 and its use to synthesize unsymmetrical intermolecular disulfide bond peptides on a solid support are described. Application of template 1 to synthesize bioconjugates of cell permeable moieties based on the disulfide bond is demonstrated.

The search for new methods to achieve selective and complete heterodisulfide formation is an important area of research.[1] One strategy involves activation of the thiol function of one of the peptides, followed by the addition of a second peptide in its free thiol form. Aromatic sulfenyl protecting/activating groups[2] such as 3-nitro-2-pyridinesulfenyl (Npys) have been widely used for solution-phase syntheses of disulfide heterodimers.[3] The application of this reaction can be found in the synthesis of a variety of bioconjugates of peptides,[4] oligonucleotides,[5] peptide nucleic acids,[6] and polymers.[7] However, this seemingly facile method of disulfide heterodimerization has disadvantages of its own. The reaction is heavily dependent on the pH of the medium, the peptide sequence, and the solvent used. The reaction is much slower under acidic conditions, while disulfide exchange and the instability of disulfide bonds are main concerns under basic conditions.[8] Under equilibrium conditions, methods directed toward formation of intermolecular disulfide bridges are further limited by disproportionation of the desired unsymmetric heterodimers to symmetric homodimer species.[9] Practical difficulties associated with this reaction include tendency of homodimerization while activating one of the cysteine residues,[10] difficulty of incorporation of Npys in Fmoc-based solid-phase peptide synthesis,[11] conformation-dependent side reactions in interstrand disulfide

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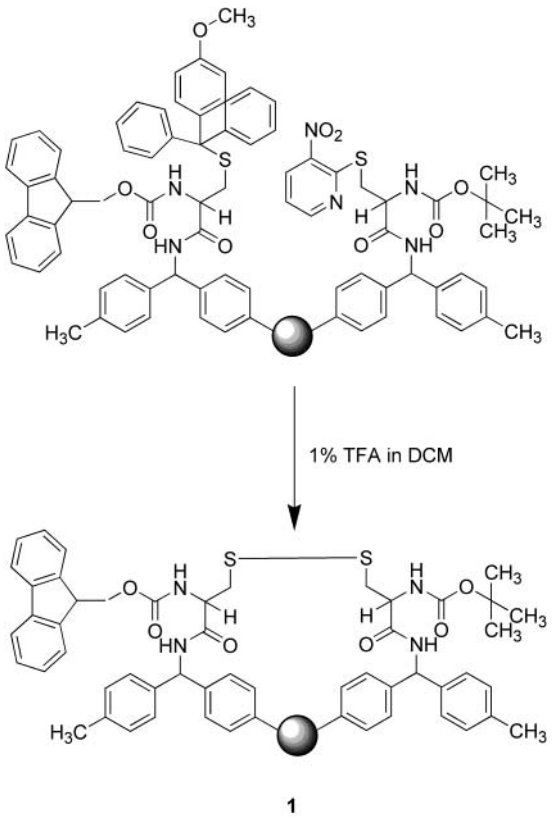
bridging,[12] and difficulties with conjugation due to solubility problems.[13] The latter can be particularly problematic in the reversible lipidization of peptides where adequate solubility of both a cysteine-linked fatty acid and a thiol peptide in the same solvent required for disulfide bond formation is unlikely.

A partial solution to these problems can be presented if the unsymmetric intermolecular disulfide bridges can be formed exclusively on the solid support. A solid-phase system has been considered to be ideal for intramolecular disulfide bond formation. It is believed that rigid fixation of molecules on the solid-phase restricts their mobility and decreases intermolecular contacts, which ensures predominant occurrence of intramolecular reactions.[14] This concept of pseudodilution has been validated by successful solid-phase synthesis of several single-chain disulfide-bridged peptides.[15,16] Site−site interactions occurring within the solid support also have been exploited[17] to synthesize disulfide homodimers of deamino-oxytocin.[18] Solid-phase-mediated heterodisulfide formation has been achieved by incorporating an Npys moiety on Bio-Gel-type resin and reacting it with the thiol containing peptide in aqueous solution.[19] However, there are no reports of on-resin disulfide heterodimerizations. Herein we report the synthesis of the orthogonal template **1**, its use to achieve unsymmetrical intermolecular disulfide bond formation on a solid support, and its application for synthesizing reversible bioconjugates of cell permeable moieties based on the disulfide bond.

Template **1** was constructed from Boc-Cys(Npys)-OH,[20] Fmoc-Cys(Mmt)-OH,[21] and 4-methylbenzhydrylamine (MBHA)[22] resin. A mixture of these protected amino acids in a molar ratio of 1:1.3 was loaded onto MBHA resin (substitution level of 1.2 mmol/g) using DCC and HOBt as coupling reagents to achieve equimolar loading.[23] The 4-methoxytrityl (Mmt) moiety is considerably acid labile, while the 3-nitro-2-pyridinesulfenyl (Npys) moiety is stable even under strongly acidic conditions. Hence, the Mmt side chain protecting group was selectively cleaved from the Fmoc-protected cysteine by treating the resin with 1% TFA in DCM by a continuous flow method.[24] This unleashed the free thiol for its subsequent nucleophilic attack on the adjacent Npys protecting group of the Boc-protected cysteine to form intermolecular and unsymmetrical disulfide bonds while maintaining the orthogonal Boc and Fmoc protection of the amines (Scheme 1). This modified MBHA support with a built-in intermolecular disulfide bridge can be used as an orthogonal template for the synthesis of disulfide heterodimers of peptides or other organic molecules.

Scheme 1



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Peptides represent a class of therapeutic candidates that often suffer from cellular uptake problems. Covalent attachment of cell permeable moieties (CPMs) to bioactive peptides is essential for the cellular transport of the latter. Reversible conjugation of CPMs via a disulfide linkage is a strategy for peptides targeted for an intracellular milieu, where the highly reducing environment within a cell reduces the intermolecular disulfide bridge to liberate the bioactive peptide.[25] However, the formation of an unsymmetric, interstrand disulfide bridge can be synthetically challenging.

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(24) MBHA resin loaded with the two cysteine derivatives was preswelled in DCM and packed into a reaction column. Next, 1% TFA in DCM was pumped (2 mL/min) through the resin. The nucleophilic attack is immediate and can be monitored by measuring the absorbance of the column eluant. When the reaction was complete, as indicated by the return of the absorbance baseline, the resin was washed five times with DCM and five times with methanol and kept swollen overnight in DCM. Generally, no further removal of Npys moiety was observed after overnight swelling.
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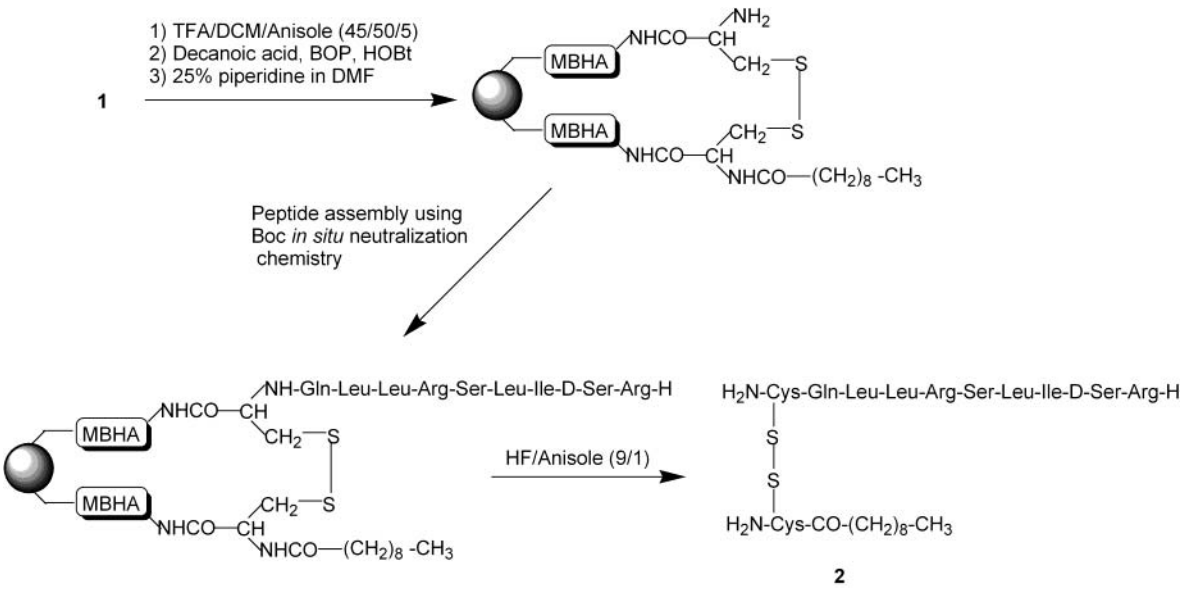
Scheme 2



To test the utility of template **1** for preparing disulfide bond-linked CPMs, a lipidic moiety, decanoic acid,[26] and a hydrophilic cell-penetrating peptide, the arginine heptamer (R7),[27] were conjugated to peptide sequences. In each case, the bioactive portion of the conjugate consisted of the linear nonapeptide that contained the signature LXXLL motif of the nuclear receptor coactivator.[28] These peptides are targeted to disrupt the interaction between the estrogen receptor and the coactivator. Orthogonal template **1** was used to link the CPMs to this linear bioactive nonapeptide through an interstrand disulfide bridge.

In the first example, a dimer consisting of a lipid and a peptide component was constructed. The orthogonal template **1** was first Boc-deprotected and coupled with decanoic acid using in situ neutralization chemistry and BOP/HOBt as coupling reagents. Next, the Fmoc group was deprotected and the nonapeptide sequence assembled using Boc in situ neutralization chemistry (Scheme 2). HF cleavage yielded the lipopeptide **2** in which decanoic acid is conjugated via a disulfide linkage to the C-terminal cysteine of the bioactive peptide. The molecular weight of the peptide conjugate was determined by use of ESI-MS and MALDI-TOF MS and the peptide sequenced using amino acid analysis. Due to problems attributed to irreversible adsorption on a C18 reversed-phase column during the analytical HPLC run, the lipopeptide was analyzed after using a C8 solid-phase extraction column for removal of byproducts. The peptide was more than 95% pure and obtained in an overall yield of 63%.

In a second application of template **1** for bioconjugation, we attached a hepta-arginine segment, R7, a hydrophilic cell-penetrating peptide, to our LXXLL motif-containing peptide sequence. In this case, the Boc group was removed first; then, the hepta-arginine peptide was assembled on template **1** using Boc-Arg(Tosyl)-OH as the building block and BOP/HOBt as the coupling reagent, and the peptide chain was acetylated. The Fmoc protecting group was then removed from template **1** and the estrogen receptor targeting nonapeptide sequence assembled as before (Scheme 3). HF cleavage afforded a C-terminal disulfide heterodimer of the bioactive peptide conjugated via a disulfide bond to R7. Peptide **3** was characterized using ESI-MS, MALDI-TOF MS, amino acid analysis, and by reversed-phase (C18) HPLC. The crude peptide was about 95% pure as indicated by its HPLC profile and obtained in an overall yield of 45%.

Conjugation of both lipophilic and hydrophilic moieties to the targeting peptide demonstrates the versatility of template **1** for the synthesis of disulfide heterodimers. The robust nature of the built-in disulfide bridge is evident since a total of 16 amino acids were coupled manually on the template for the polyarginine compound **3** but no disulfide cleavage was observed in the final product after HF cleavage, as assessed by HPLC and mass spectrometry data. Along with manual synthesis, we have also successfully repeated the synthesis of peptide **3** on an automated synthesizer (APEX 396). Such an automation provides a decent prospect for further implementation of this bioconjugation reaction for making combinatorial disulfide libraries.[29] It appears that the basic conditions employed for Fmoc deprotection (20%

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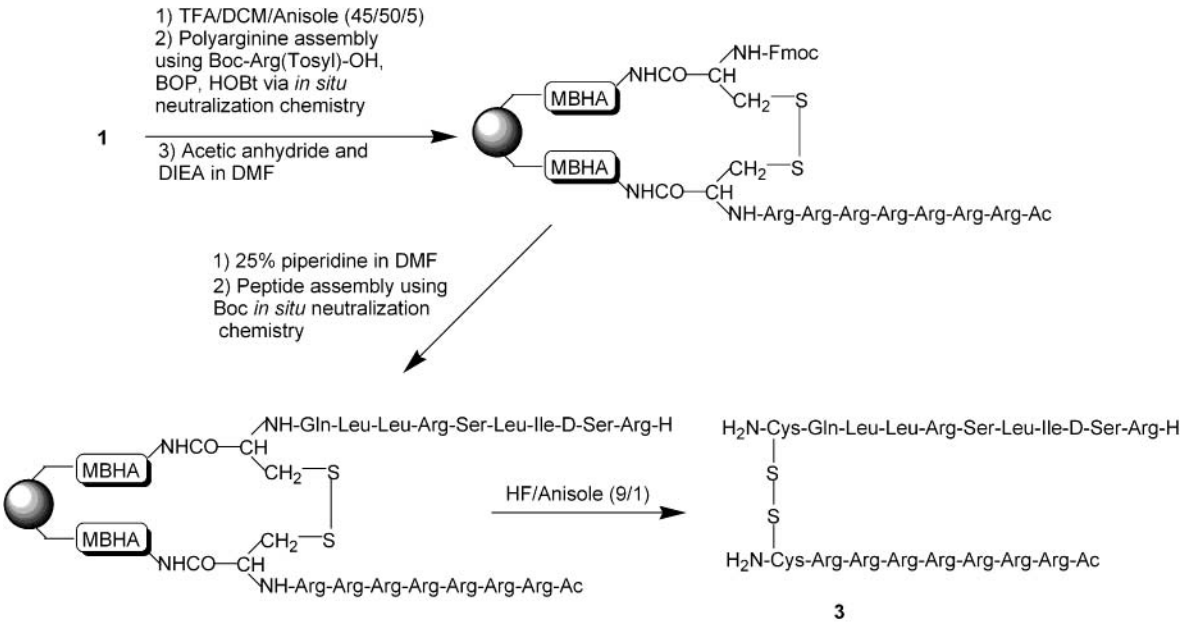
Scheme 3



piperidine in DMF for 20 min) in both syntheses did not affect the template disulfide moiety.

The resin exhibited reduced swelling in organic solvents after intermolecular disulfide bond formation in template **1**. This reduced swelling results from the fact that 1% DVB cross-linked polystyrene changes its microporous nature after disulfide cross-linking.[30,31] This reduced swelling, however, did not appear to have any negative effects on subsequent coupling efficiencies as monitored by the Kaiser test.[32] The ninhydrin test gave unequivocal results in all the peptide couplings with the exception of some ambiguity during the initial Boc deprotection of template **1**. Similar anomalies have been reported previously.[33] In a polystyrene solid support, a high degree of site separation (pseudodilution) can be achieved by using extensive cross-linking (>1% DVB) and a moderate substitution level (<0.5 mmol/g). However, site−site interactions occur if the supports are lightly cross-linked and highly substituted. The construction of template **1** using a 1% DVB cross-linked polystyrene with a substitution level of 1.2 mmol/g tends to further validate the dynamic nature of such lightly cross-linked and highly substituted microporous polymer networks[34] for effecting intermolecular contacts.

Although template **1** can be used for peptide assembly via either initial Boc or Fmoc deprotection, we have found that initial Boc deprotection virtually eliminates the monomeric and homodimeric byproducts, probably because it ensures complete Mmt deprotection and intermolecular disulfide coupling. Also, if the initial loadings of Boc-Cys(Npys)-OH and Fmoc-Cys(Mmt)-OH are not equimolar, monomeric thiol peptides and homodimeric products should be observed corresponding to the excess amino acid present. In the case of peptides **2** and **3** where the initial loadings were equimolar and where template **1** was initially Boc deprotected, exclusively unsymmetrical dimers were obtained as assessed by LC-MS and solid-phase extraction analysis. From the viewpoint of solid-phase peptide synthesis, template **1** provides a versatile method for the C-terminal disulfide conjugation, reduces the possibility of homodimerization, and demonstrates the utility of a solid support for the synthesis of disulfide heterodimers of macromolecules with incompatible solubilities.

Supporting Information Available: MALDI-TOF mass spectrograms and amino acid analysis data of peptides **2** and **3** and RP-HPLC profile of peptide **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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