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Design of Stable β-Hairpin Mimetics through Backbone Disulfide Bonds

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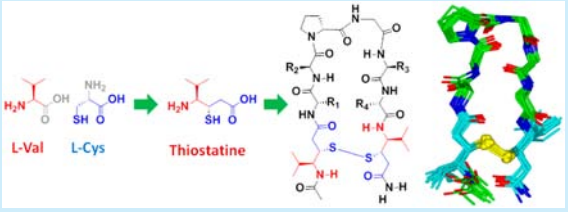
Supporting Information

ABSTRACT: The synthesis and utilization of novel thiostatines (β-SH-substituted γ-amino acids) in the design of backbone-disulfide-stabilized β-hairpin mimetics, solution conformations of hybrid β-hairpins and Cys-disulfide-stabilized α-peptide analogue, their thiol exchange, and proteolytic stability are investigated. The results suggest that thiostatines can be used to design proteolytically stable water-soluble β-hairpin mimetics without deviating from overall β-hairpin conformation.



The design of stable protein secondary structure (α-helix and β-sheet) mimetics is recognized as a promising approach for structure- and mechanism-based drug discovery. In this context, a variety of strategies including lactam,[1] heterocyclic,[2] and disulfide bridges,[3] hydrocarbon linkers,[4] and utilization of C−C covalent bonds as intramolecular H-bond surrogates[5] have been developed to mimic a short and stable α-helix. In contrast to the α-helix, little is learned regarding the design of covalently bridged stable β-hairpin mimetics other than cysteine disulfides. Besides stabilizing the protein structures, β-hairpins often constitute binding epitopes of proteins and play a crucial role in biomolecular recognition.[6] In addition, the β-hairpin structural motif has been found within the large family of cationic antimicrobial peptides,[7] mammalian defensins,[8] protegrins,[9] tachyplesins,[10] etc. Along with their exploitation as protein epitope mimetics,[11] antibiotics,[12] and biomaterials,[13] β-hairpins have also been used to dissolve the Aβ-amyloidal aggregations.[14] The β-hairpin consists of two antiparallel β-strands and a reverse turn. The β-hairpin structure is generally stabilized by intramolecular H-bonds existing between the two β-strands and van der Waals interactions between the side chains. The stability of antiparallel β-strands can be further improved by aromatic−aromatic interactions,[15] ionic interactions,[16] as well as cation−π interactions.[17] Nature often utilizes covalent disulfide bonds to stabilize β-hairpin conformations; however, studies also reveal that the contribution of disulfide bridges[7−10] to the β-hairpin stability is context-dependent.[18] In addition, investigations also suggest that replacement of the −S−S− disulfide bond with $-CH_2-S-$ or $-CH_2-CH_2-$ destabilizes the β-hairpin registry and leads to the poor biological activity of the peptides.[19] Besides stabilizing protein conformations, disulfides have been extensively used in the cyclization of biologically active linear peptides to improve their pharmacological properties.[20] In addition to the cysteine disulfides, efforts have also been made to introduce a sulfur atom directly on the α-carbon;[21] however, Erlenmeyer's rule dictates that this kind of heteroatom substitution at the α-amino acids would lead to a hydrolytically labile N−C^{α} bond.[22] Thus, only disulfides of cysteine residues have been used to covalently stabilize β-hairpin conformations. The widespread applications of β-hairpin scaffolds motivated us to investigate the possibilities to design stable β-hairpin motifs through β-strand stapling.

We predict that strands in the β-hairpin can be stapled within the proximity of H-bond distances without much deviation from the overall folding of the β-hairpin through the disulfide bonds from β-SH-substituted γ-amino acids. Inspired by the natural occurrence and biological activity of β-hydroxy γ-amino acids (statine),[22,23] we sought to investigate the stereochemically pure synthesis of analogous β-sulfhydryl γ-amino acids and their utility as templates in the β-hairpin design. As sulfhydryl groups are attached to the β-carbon atom similar to the statines, they may not be governed by Erlenmeyer's rule. We anticipate that reactive sulfhydryl groups can be introduced though the Michael addition on α,β-unsaturated γ-amino esters. Another interesting feature of these β-thiol-substituted γ-amino acids is that the functional groups of cysteine (−SH) and any other amino acids side chain can be incorporated into a single unit. Herein, we report the synthesis of orthogonally protected novel thiostatines, utility of thiostatines in the design of water-soluble β-hairpins, conformational analysis of a backbone-disulfide-stabilized β-hairpin (**P1**, Ac-γVal(β-S-)-Lys-Val-^{D}Pro-Gly-Leu-Lys-γVal(β-S-)-$CONH_2$, Scheme 1), comparison of novel β-hairpin with control peptide (**P2**, Ac-Cys(S-)-Val-Lys-Val-^{D}Pro-Gly-Leu-Lys-Val-Cys(S-)-$CONH_2$, Scheme 1), disulfide exchange reactions, and proteolytic stability of **P1** and **P2**.

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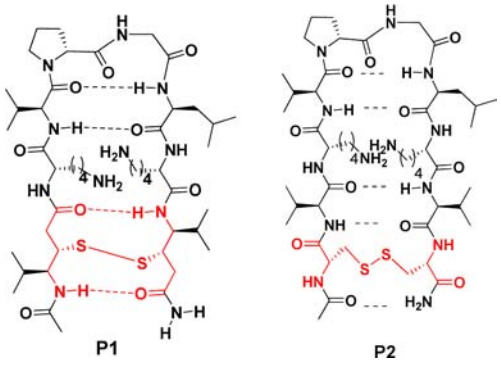
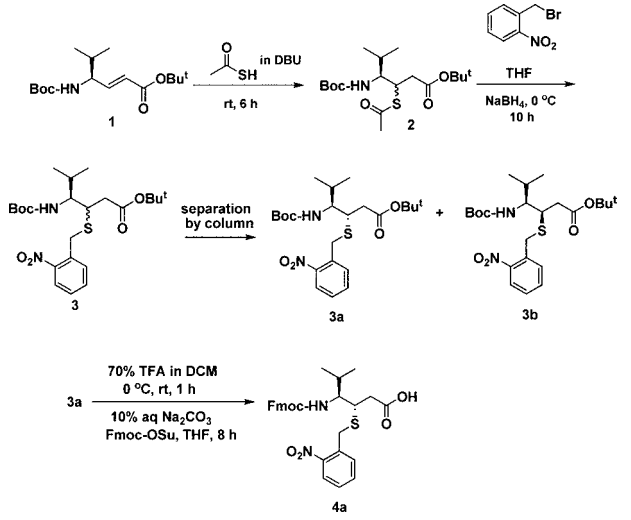
Scheme 1. Designed β-Hairpins Constituted with Backbone (P1) and Side-Chain Disulfide Bonds (P2)



As we have been interested in utilizing naturally occurring nonribosomal γ-amino acids in the design of peptidomimetics,[24] we expected that β-SH-substituted γ-amino acids can be accessed through conjugate addition of thiols to *E*-vinylogous amino esters.[25] The synthesis of orthogonally protected β-thiol-substituted γ-amino acids (thiostatines) is shown in Scheme 2.

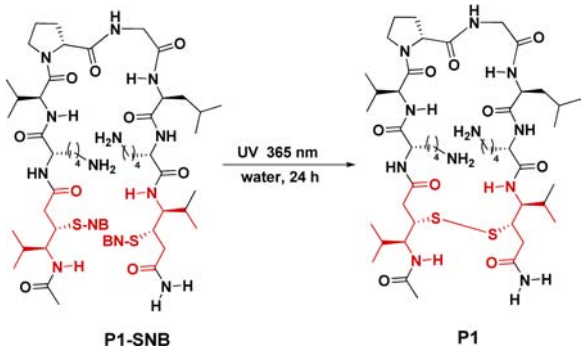
Scheme 2. Schematic Representation of the Synthesis of Orthogonally Protected Thiostatine (Fmoc-γVal(β-SNB)-OH)



The starting material, *tert*-butyl ester of α,β-unsaturated γ-Val (**1**), was synthesized using Wittig reaction and subjected to thioacetic acid Michael addition in the presence of DBU. Due to the self-dimerization of free thiols, we are encouraged to utilize less reactive thioacetic acid as a source of thiol in the conjugate addition reaction. In addition, the acetyl group in thioacetic acid acts as a temporary protecting group, and it can be removed through the mild $NaBH_4$ reduction. The diastereomeric mixture of thioacetate conjugate addition products (**2**) were purified and subjected to the $NaBH_4$ reduction in the presence of *o*-nitrobenzyl bromide to derive photochemically labile protecting group for free −SH (**3**).[26] In the absence of *o*-nitrobenzyl bromide, we isolated disulfide-bridged β-SH-substituted amino acid dimers quantitatively (data not shown). In contrast to the thioacetic acid conjugated addition products (**2**), the *o*-nitro-*S*-benzyl *anti* (**3a**) and *syn* (**3b**) diastereoisomers (with respect to amino acid side chain) of thiostatines were separated in 60:40 ratio through column chromatography. We utilized major *anti* diastereoisomer **3a** for the synthesis of **P1**. The stereochemistry of **3a** was determined from the single crystal structure of α,γ-hybrid peptide (see Supporting Information). The Boc- and *tert*-butyl-protecting groups were removed using TFA in DCM, and the free amine was further protected using the Fmoc group to derive **4a** and utilized in the solid-phase peptide synthesis. As we encountered the elimination products during the saponification of ethyl ester of *S*-(NB)thiostatines, we only used acid-labile *tert*-butyl esters.

The model peptide **P1** was synthesized using a solid-phase method by standard Fmoc chemistry on Knorr amide resin at 0.2 mmol scale. All coupling reactions were performed using standard HBTU/HOBt coupling conditions. The thiostatines were incorporated at 1 and 8 positions of the octapeptide β-hairpin. To induce the chain reversal in the peptide, we used ^{D}Pro-Gly as the turn segment.[27] The hydrophilic residue Lys was incorporated at positions 2 and 7 of the antiparallel β-strands to promote solubility of the peptide in aqueous medium. After the synthesis, the peptide **P1**-SNB (NB, *o*-nitrobenzyl group) was released from the resin and purified using reverse-phase HPLC. The pure **P1**-SNB was further subjected to the UV-light irradiation at 365 nm in water to deprotect the *o*-nitro-*S*-benzyl group.[26] The completion of the reaction was monitored using MALDI-TOF/TOF and reverse-phase HPLC. Deprotection of the *o*-nitrobenzyl group and concomitant disulfide formation through the aerial oxidation of free SH were achieved in a single step, as illustrated in Scheme 3.

Scheme 3. Deprotection of Photolabile *o*-Nitro-*S*-benzyl Groups and Concomitant Oxidation of Thiols to Disulfide



The control β-hairpin, **P2**, was designed based on the fact that the Cys disulfides give stability to the β-hairpin when they are at non-H-bonding positions in the antiparallel β-strands.[18] The cysteine residues were incorporated at positions 1 and 10 (Scheme 1) of the **P2** and synthesized using a solid-phase method similar that for **P1**. To achieve the disulfide formation between the Cys residues, the crude peptide was dissolved in ammonium carbonate solution (20 mM, pH 7.4), stirred overnight, and purified by reverse-phase HPLC. The purified disulfide-bridged peptides were then subjected to 2D NMR and CD analysis to establish their solution conformation.

The wide dispersion of amide NHs and $C^{\alpha}H/C^{\gamma}H$ chemical shifts in ^{1}H NMR signaled well-defined structures of **P1** and **P2** in water. However, it was apparent that greater shift dispersion, presumably reflecting a higher β-hairpin fold population, was found for peptide **P2**. Fully annotated ^{1}H NMR spectra are given in the Supporting Information. The high $^{3}J_{NH\text{-}C^{\alpha}H}$ values of β-strand residues suggest that both peptides adopt

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characteristic extended conformations. The CD analyses reveal that both peptides displayed a strong negative band at 218 nm in aqueous medium, suggesting a β-hairpin conformation in solution. Using TOCSY and ROESY spectra, the amino acid types and the sequential connectivity of the residues were established. The characteristic strong sequential NH↔C$^{\alpha}$H NOEs and long-range NOEs between the residues Lys2 C$^{\alpha}$H and NH of γVal 8 (C$^{\alpha}$H↔HN) and NH↔NH NOEs between the residues Val3 and Leu6 of **P1** in the ROESY spectrum reveal a well-folded antiparallel β-hairpin character in aqueous medium. The strong NOEs between the δ protons of ^{D}Pro4 and Val3 C$^{\alpha}$H confirm the *trans*-imide bond at proline. The fully assigned partial ROESY spectrum highlighting NH↔C$^{\alpha}$H, cross-strand NH↔NH, and C$^{\alpha}$H↔C$^{\gamma}$H regions are given in the Supporting Information. However, we did not observe any characteristic cross-strand NH↔NH and CH↔CH interactions between the terminal thiostatine residues. Further, the 2D NMR analysis of the control peptide, **P2**, also reveals the strong sequential NH↔C$^{\alpha}$H as well as NH↔NH interactions between Val4 and Leu8 residues, evidencing a well-folded β-hairpin conformation. Unlike **P1**, a strong C$^{\alpha}$H↔C$^{\alpha}$H NOE between residues 1 and 10 has been observed in **P2** along with a strong NOE between C-terminal amide NH and the N-terminal Ac group, revealing well-registered strands even at the terminals.

The intramolecular H-bonds between the antiparallel β-strands are further examined using temperature-dependent ^{1}H NMR. It has been established that if the values of temperature coefficient are less negative than −4.5 ppb K^{-1}, such amide NHs are considered to be less solvent exposed and are involved in the intramolecular H-bonds.[28] Analysis reveals that, except for γVal8 NH, all other amide NHs of **P1** showed upfield chemical shifts with increasing temperature (from 283 to 333 K). The observed dδ/dt values were found to be within −4.5 ppb K^{-1} for all residues that are involved in the cross-strand intramolecular H-bonds. The residues which are exposed to the solvent exhibited a dδ/dt value above −6.5 ppb K^{-1}. Interestingly, amide NH of γVal8 showed the temperature coefficient +3.8 ppb K^{-1}, which indicates that γVal8 has better intramolecular H-bond registry with increasing temperature. The unexpected −4.6 ppb K^{-1} observed for Gly indicates its probable involvement in the strong intermolecular H-bond with other β-hairpins or solvent molecules. The control peptide **P2** also displayed a similar trend revealed from the dδ/dt values of backbone amide protons. The chemical shifts and temperature coefficients of both the peptides are tabulated in the Supporting Information. The variable temperature CD analysis also reveals that there is no incremental change in the CD minima at 218 nm with increasing temperature, suggesting the stable β-hairpin conformations of peptides even at elevated temperature. Overall, the NMR and CD analyses imply that both peptides adopted well-folded β-hairpins in aqueous medium. Based on the experimentally deduced NOEs at room temperature, the NMR structure calculations of **P1** and **P2** were performed using distance-restrained molecular dynamic simulations (Insight II 2005, Accelrys Inc.).[29] The overlay of 10 low-energy conformers of NMR-calculated individual structures of **P1** and **P2** is shown in Figure 1. The NMR model reveals that **P1** and **P2** adopt well-folded β-hairpins through backbone disulfides and side-chain disulfides, respectively. Thus, the novel thiostatines provide a unique opportunity to covalently connect two β-strands within the proximity of H-bond distances. In addition, it is worth noting that the side-chain functionalities of cysteine and valine residues are embedded in a single thiostatine residue.

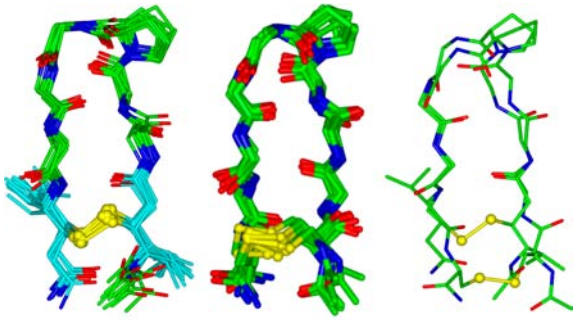

Figure 1. Solution structures of **P1** and **P2** derived from 2D NMR data. Superposition of **P1** and **P2**.

To realize whether or not the buried disulfide bond in **P1** and exposed disulfide bond in **P2** can undergo disulfide exchange with thiols, we treated both the peptides with DTT,[30] and disulfide exchange was monitored using reverse-phase HPLC (see Supporting Information). Interestingly, both peptides undergo time-dependent disulfide exchange with DTT. However, both failed to undergo disulfide exchange with reduced glutathione (GSH). This is probably due to the better reducing property of DTT compared to that of the sterically hindered bulky GSH. These studies suggest that thiostatine disulfides can be accessed with sterically less hindered thiols. In addition, the ^{1}H and CD analysis of disulfide-reduced **P1(redu)** and **P2(redu)** showed relatively less ordered structures in aqueous medium.

Cyclization of linear peptides into disulfide-bridged cyclic peptides is generally practiced in the design of peptide-based drugs to increase their half-life.[31] The increased half-life of cyclic peptides is attributed to their increased stability against the proteases. In this context, we investigated the proteolytic stability of both **P1** and **P2** using trypsin protease, which cleaves at the C-terminals of basic residue Lys. Both peptides were incubated with trypsin in Tris buffer, and the peptide hydrolysis was monitored using reverse-phase HPLC. Strikingly, thiostatine disulfide-bridged β-hairpin is not susceptible to protease trypsin even after 24 h, whereas cysteine disulfide β-hairpin **P2** undergoes hydrolysis in a time-dependent manner (Figure 2). These results were further confirmed by the MALDI-TOF mass spectral analysis (see Supporting Information).

In conclusion, we have developed novel thiostatines with photolabile and solid-phase compatible protecting groups and examined potential utility in the design of water-soluble β-hairpin mimetics. Results suggests that, similar to the Cys disulfide **P2**, thiostatine-containing β-hairpin **P1** facilitates a well-registered H-bond between the antiparallel β-stands, which is dictated by the backbone disulfide bridge having appropriate geometry. The thiol exchange with DTT and GSH advocates that the disulfides of the hybrid β-hairpin are accessible with thiols having better reducing properties. Remarkably, the thiostatine-containing hybrid β-hairpin **P1** exhibits stability toward protease trypsin, which is in sharp contrast to the Cys-containing β-hairpin **P2**. Thus, thiostatines can be potentially used to design water-soluble and proteolytically stable hybrid β-hairpins without much deviation from the overall β-hairpin conformation. As statines have been extensively used as inhibitors of various aspartic acid and other proteases,[32] we hope that the novel thiostatines synthesized here may find

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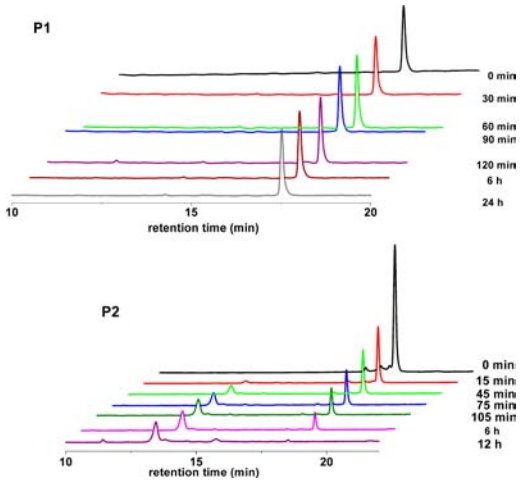

Figure 2. Proteolytic stability of **P1** and **P2** after the addition of trypsin protease.

applications in various fields of chemical biology, medicinal chemistry, and biomaterials.

■ ASSOCIATED CONTENT

Supporting Information

All experimental procedures, ^{1}H and 2D NMR spectra, structure calculation, thiol exchange, and proteolytic assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

§M.G.K. and S.M.M. contributed equally.

Notes

The authors declare no competing financial interest.

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