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Efficient synthesis of trypsin inhibitor SFTI-1 via intramolecular ligation of peptide hydrazide

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ABSTRACT

Cyclic peptide trypsin inhibitor **SFTI-1** was synthesized via intramolecular ligation of a linear peptide hydrazide with high yield. This cyclization strategy did not cause epimerization at the C-terminal Arg residue. CD spectrum and NMR spectroscopy analysis demonstrated that well-folded **SFTI-1** could be obtained via standard oxidative folding process. Thus, we present a simple and cost-efficient strategy for the synthesis of **SFTI-1**.

Cyclic peptides, which are generally referred to peptides with an end-to-end or head-to-tail linkage of the peptide backbone through an amide bond, have been considered as useful tools in pharmaceutical science and chemical biology.[1] Compared to their linear counterparts, the cyclization of peptide has generated conformational constrained analogues with improved bioactivity, selectivity, bioavailability, and higher receptor-binding affinities.[2,3] For the preparation of cyclic peptides, a classical approach relies on protected linear precursors which are lactamized in organic solvents, either at high dilution or using pseudo-dilution on a solid support.[4] A major drawback of this approach is the necessity of high enthalpic activation for the acylating moiety during the lactamization process, which often results in epimerization of the C-terminal amino acid residue and oligomerization to dimers and trimers.[5]

To overcome aforementioned limitations, many groups developed alternative methods to cyclize fully unprotected peptides and proteins in aqueous solution mainly based on the native chemical ligation (NCL).[6] Moreover, Staudinger ligation, thiazolidine-forming ligation, and an imine-induced ring-closing/contraction strategy used for peptide cyclization have also been reported with own advantages such as the moderately high concentrations for cyclization.[7] Recently, peptide hydrazides were reported to be a thioester equivalent reagent in NCL.[8] The hydrazide based ligation has been used for protein total and semi-synthesis, protein modification, and the synthesis of cyclic peptides and cyclic proteins.[9] One of the main advantages is that peptide hydrazides can be easily prepared by using Fmoc solid-phase peptide synthesis, and therefore various cyclic peptides could be synthesized with high efficiency in an epimerization-free manner.[10] Another advantage is that good yield can be obtained for moderately high concentrations of linear reactants.

Sunflower trypsin inhibitor (**SFTI-1**) is a naturally occurring cyclic peptide with only 14 residues, which is regarded as one of the smallest and most potent trypsin inhibitor as known.[11] Since its discovery, **SFTI-1** has attracted much interest because of its small size and high potency, which make it an ideal candidate as a platform for the design of novel small proteinase inhibitors.[12] However, the biosynthesis and peptide cyclization mechanisms for **SFTI-1** are still beyond complete understanding, which has restricted their production by genetic approaches.[13] Based on this, many groups have reported the synthesis of **SFTI-1** using solid-phase Fmoc chemistry with the latter cyclization of **SFTI-1** in the solution or an on-resin cyclization approach.[13] Recently, Goransson et al. first synthesized **SFTI-1** under microwave-accelerated solid phase peptide synthesis and native chemical ligation with improved efficiency (45% isolated yield).[14] Moreover, Tam and co-workers reported the synthesis of **SFTI-1** using a C-terminal

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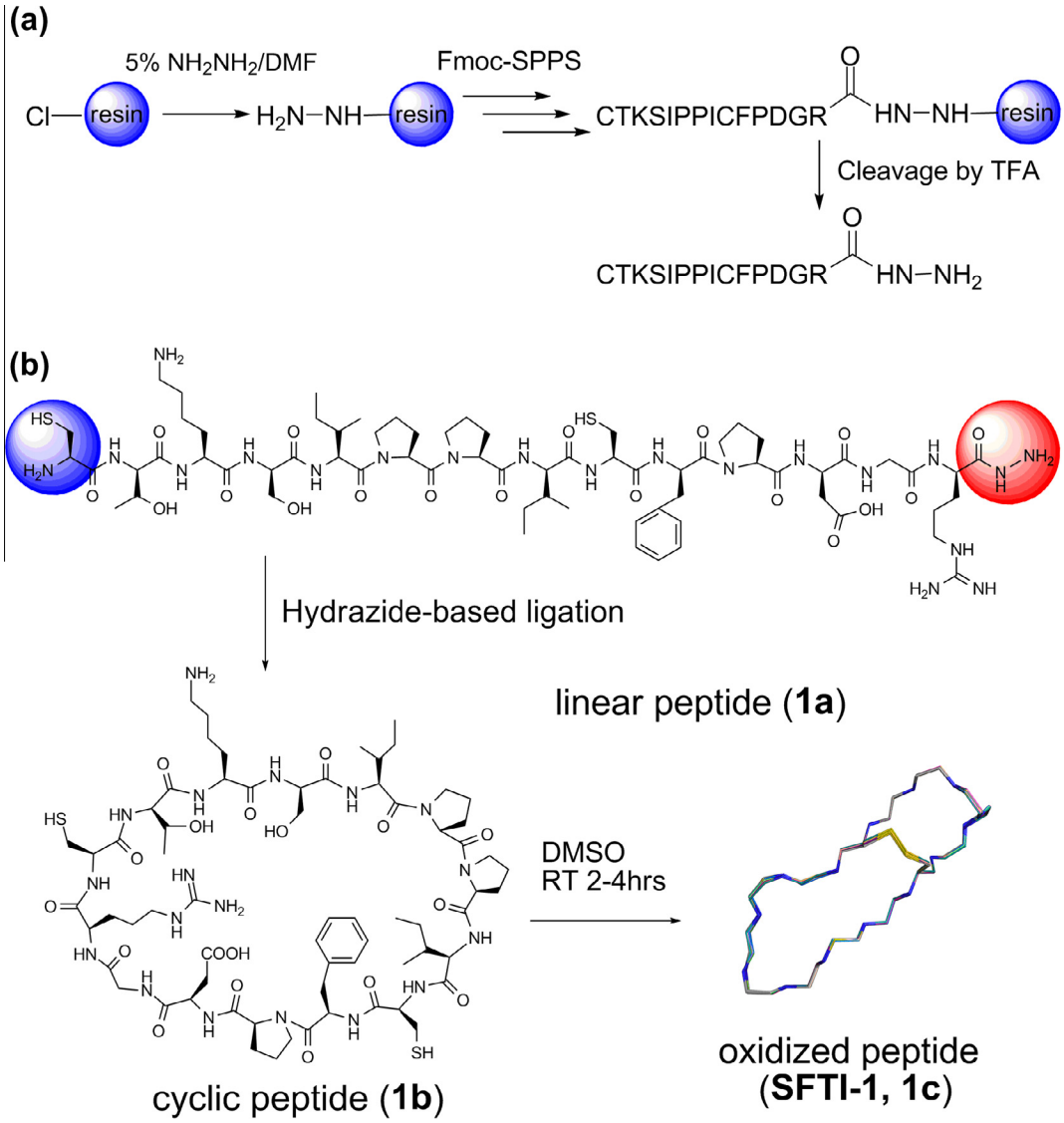
thioethylamido moiety (TEA) containing thioester surrogate (17% isolated yield).[15] In spite of these improvements, efforts are needed to overcome current constraints, for example, relatively long time (16–24 h), high temperature, and time-consuming steps for the synthesis of thioester and its surrogate for cyclization. Herein, we report a highly efficient and time-efficient method of the synthesis of **SFTI-1** via the ligation of peptide hydrazide. Take the advantage of this hydrazide based native chemical ligation, we found that **SFTI-1** can be synthesized with increased simplicity and lower costs (Scheme 1).

Our study began with the synthesis of linear peptide precursor **1a** by using the commercially available 2-Cl-(Trt)-Cl resin (Scheme 1). As previously reported, a hydrazine-Trt(2-Cl) resin was readily prepared as the key intermediate, and all the peptides were synthesized by the solid-phase method using standard Fmoc chemistry. After completing the synthesis, the peptides were cleaved from the resin simultaneously with the side chain deprotection in a one-step procedure, using a mixture of TFA/phenol/water/triisopropylsilane (88:5:5:2, v/v/v/v). The crude peptides were dissolved in CH_3CN/H_2O (1:1, v/v) and purified using RP-HPLC. The purity of linear peptide **1a** was confirmed by analytical HPLC and ESI-MS.

Then, we carried out the peptide cyclization in a one-pot fashion. Firstly, the linear peptides **1a** (1 mM) were dissolved in 800 μL of an aqueous phosphate buffer (100 mM) with 6.0 M guanidinium chloride and 10 equiv of oxidation solution $NaNO_2$ was added drop-wise to the reaction system. The reaction mixtures were held at −10 °C, pH 3.0, and stirred for 20 min. After that, we added 40 equiv of thiol additive MPAA (4-mercaptophenylacetic acid) to the reaction tubes. Solutions were then adjusted to pH 7.0, stirred for 2–4 h at room temperature. The reaction was monitored using HPLC and the cyclic peptide (**1b**) was identified by ESI-MS. The high yield (61% isolated yield) was obtained through aforementioned hydrazide based ligation. We next examined the correlation between the peptide concentration and the yield. We found that relatively high yield (57% isolated yield) can be obtained in low concentration (e.g., 60 μM) of the reactant while moderately high concentrations (e.g., 10 mM) also resulted in good yield (53% isolated yield). To further perform the intra-molecular disulfide bridge formation of **SFTI-1**, the oxidative folding was conducted using the solution of CH_3CN/H_2O (1:1, v/v). It has been reported that the disulfide bond is likely to reduce its conformational flexibility significantly in natural analogue.[11b] In detail, 1 mg **SFTI-1** was dissolved in 800 μL of 50% CH_3CN with



Scheme 1. (a) The concise scheme for linear peptide synthesis (SPPS); (b) The synthesis of **SFTI-1** via the ligation of peptide hydrazides.