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Design and Synthesis of Redox Stable Analogues of Sunflower Trypsin Inhibitors (SFTI-1) on Solid Support, Potent Inhibitors of Matriptase

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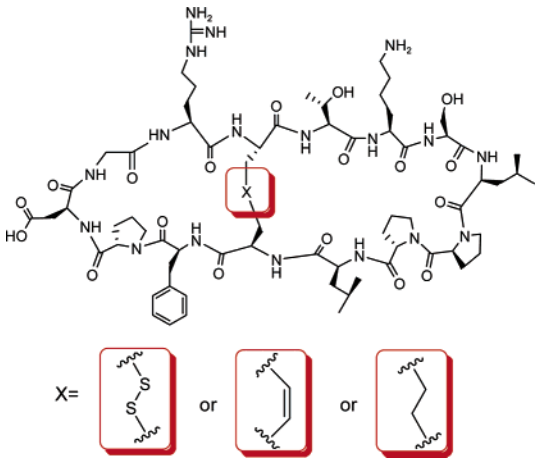
ABSTRACT



Matriptase is a member of the emerging class of type II transmembrane serine proteases. It was found that the sunflower trypsin inhibitor (SFTI-1), isolated from sunflower seeds, inhibits matriptase with a subnanomolar K_i of 0.92 nM. On the basis of this result, we designed and synthesized its proteolytically stable analogues, SFTI-2 and SFTI-3. SFTI-3 exhibited very good binding affinity to matriptase, and it was metabolically stable.

Proteinases are ubiquitously distributed, having key roles in a diverse range of physiological biological processes including blood coagulation, the complement cascade, and peptide hormone processing pathways.[1] Protease inhibitors are widely distributed in nature; their main role is to regulate the activity of proteolytic enzymes.[2,3] These inhibitors can be classified into a number of families on the basis of their active-site structures and their ability to inhibit the cleavage of specific peptide sequences within proteins. Among these, inhibitors of serine proteases are being studied most extensively. One of the well-known serine protease inhibitory agents, the

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[#] In memory of Dr Robert B. Dickson

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Bowman−Birk inhibitor (BBI), found in seeds of legumes and other plants, belongs to the Bowman−Birk inhibitor family of small proteins with a MW range of 6000−8000.[2]

Matriptase is a type II transmembrane serine protease that is found on the surfaces of epithelial cells and certain other cell types.[4−6] Matriptase may function to degrade the extracellular matrix as well as several cellular regulatory proteins. Matriptase is found as a large transmembrane protein in most cancer cells, including human breast cancer cells.[4,5,7] Inhibition of the matriptase enzyme is a logical target for intervention because it was demonstrated that matriptase can activate key prometastatic substrates, and it may function in epithelial cell migration, cancer invasion, and metastasis.[8]

Our group and three other groups published the synthesis of the sunflower trypsin inhibitor (**SFTI-1**) that was originally isolated from sunflower seeds around the same time.[9−12] **SFTI-1** is not only the smallest naturally occurring peptidic protease inhibitor isolated to date, with only 14 residues, but also the most potent of all BBI proteins, combining size reduction and retention of biological properties. **SFTI-1** inhibited β-trypsin with a subnanomolar K_i of 0.1 nM, and it inhibited cathepsin G with a comparable K_i.[13] The small size and high trypsin inhibitory activity of **SFTI-1** make that inhibitor an attractive template for the design of new protease inhibitors with the potential to be used as therapeutic agents. In earlier studies, it was found that **SFTI-1** inhibited matriptase (K_i = 0.92 nM), with comparable effectiveness to trypsin.[9] **SFTI-1** was partially characterized by classical techniques, and its structure was confirmed on the basis of the electron density map of the inhibitor cocrystallized with bovine-β-trypsin.[10] The constrained, conformationally rigid structure of **SFTI-1** provides a promising template toward further development of more specific inhibitors of extracellular matrix serine proteases, such as matriptase. The disadvantage of the disulfide bridge of **SFTI-1** is that it is sensitive to reduction. During the past decades, great efforts have been made to mimic the intramolecular disulfide bridges with other appropriate linkages to enhance the metabolic stabilities without losing biological activities and selectivities.[14] Mimicking the intramolecular disulfide bond with a dicarba linker has been proven to be one of the most effective approaches. These dicarba analogues are more metabolically stable. In addition, these mimics usually preserve the conformation of parent compounds, which is important to prevent dramatic activity and specificity changes. Upon the basis of these considerations, we designed two bicyclic compounds, **SFTI-2** and **SFTI-3** (Figure 1), for more thorough evaluation.

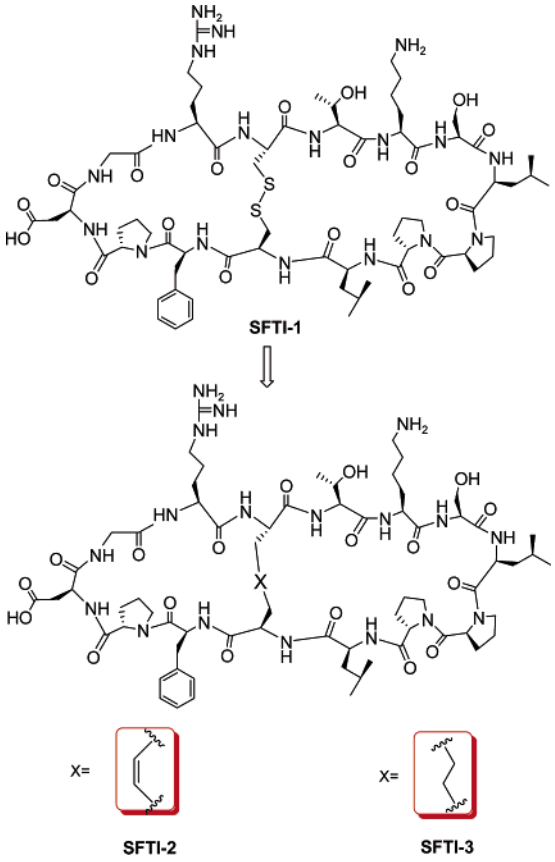

Figure 1. Structures of **SFTI-1**, **SFTI-2**, and **SFTI-3**.

In the retrosynthetic analysis of macrocyclic peptides, ring disconnection is so strategically important that it can ultimately determine the success of a synthesis. Poor disconnections can lead to slow cyclization rates, facilitating side reactions such as oligomerization and/or epimerization of the C-terminal residue.[15] Analyzing the sequence of the cagelike molecules, we chose achiral glycine as the C-terminal end to avoid racemization during resin anchoring and subsequent backbone macrocyclization. In addition, such disconnection makes the two turn-inducing proline amino acid residues located in the middle of the linear peptide

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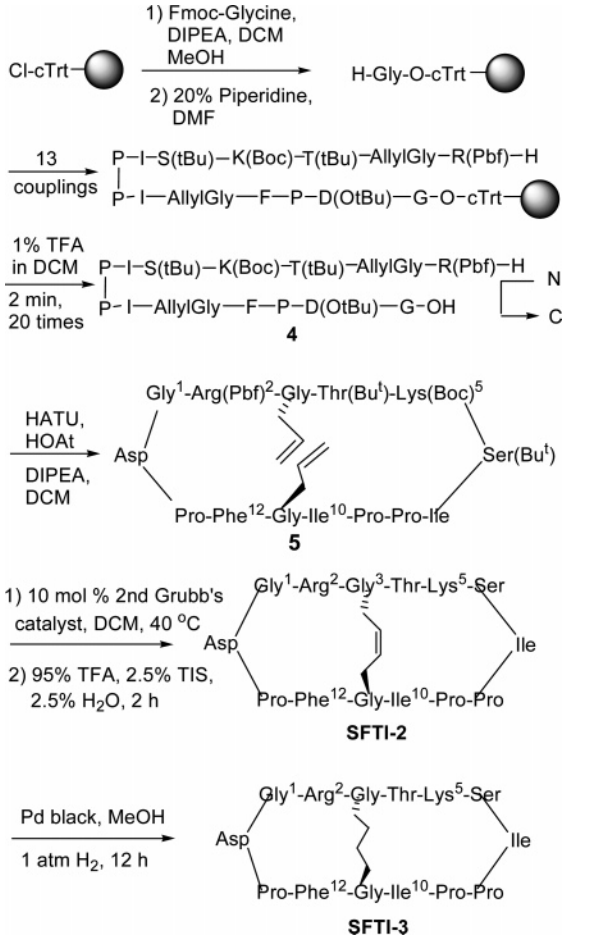
precursor, which will result in the N- and C-termini approaching each other to make the head−tail macrocyclization more robust.

Both of the cysteines were replaced with L-allylglycine to construct the olefin bridge by ring-closure metathesis (RCM). In general, an intramolecular RCM reaction only occurs when the molecule adopts a favorable conformation and the two olefins are in proximity to each other. Therefore, we chose to perform macrocyclization at first and then proceeded with the RCM reaction.

The syntheses of the linear peptide precursor of **SFTI-2** and **SFTI-3** were achieved on the solid phase with Fmoc chemistry. To allow macrocyclization and subsequent RCM reactions to proceed smoothly and selectively, amino acid residues have to be in properly protected form after cleavage from the resin. In the next step, the extremely acid-sensitive 2-chlorotrityl chloride resin was selected.[16,17] The anchoring of Fmoc-glycine was performed in a low-density manner, and the remaining resin chloride functions were capped with MeOH to avoid the formation of deletion sequences caused by sterical hindrance and aggregation during peptide chain elongation. On completion of the peptide sequence, the side chain protected peptide was cleaved from the resin with 1% TFA in DCM to afford the cyclization precursor, H−Arg-(Pbf)−AllylGly-Thr(^{t}Bu)−Lys(Boc)−Ser(^{t}Bu)−Ile-Pro-Pro-Ile−AllylGly-Phe-Pro-Asp(O^{t}Bu)−Gly−OH. The macrolactamization was performed with HATU/HOAt/DIPEA in anhydrous DCM by virtue of the high efficiency of coupling reagent HATU in the peptide macrocyclization.[18] The formation of the olefin bridge was successfully achieved using the second-generation Grubbs catalyst [(PCy_3)(Im(Mes)$_2$)Ru=CHPh][19] at a concentration of 0.6×10^{-3} M. The obtained bicyclic intermediate was subsequently deprotected with TFA containing 2.5% triisopropyl silane and 2.5% water. After HPLC purification, product **SFTI-2** was obtained in a 9% overall yield. Interestingly, this olefin-bridged product **SFTI-2** is specifically in cis configuration, and no trans product was detected during HPLC separation. The olefin geometry assignment was based upon the coupling constant of the olefinic protons (10.0 Hz).[20] This cis-specific olefin bond formation may be attributed to the conformation of the two allylglycine side chains and was locked by the cyclized backbone and intramolecular hydrogen bond network. After hydrogenolysis with a catalytic amount of Pd-black, **SFTI-3** was obtained in a quantitative yield from **SFTI-2** (Scheme 1). Notably, we lost half of the product if we chose the commonly used 10% Pd-C as the catalyst, most likely because the compound is bound to charcoal and cannot be easily washed off with organic solvents at room temperature.

Scheme 1. Synthesis of Cyclopeptides **SFTI-2** and **SFTI-3**



Encouraged by the successful RCM reaction in solution, we further explored solid-phase RCM macrocyclization with the intention to develop a more concise and efficient synthetic method for rapidly synthesizing new dicarba-bridged **SFTI-1** analogues. As shown in Scheme 2, we performed RCM macrocyclization on the solid phase right after peptide chain elongation. The resin containing a fully protected linear peptide precursor was treated with Grubb's catalyst [(PCy_3)-(Im(Mes)$_2$)Ru=CHPh] in dry degassed CH_2Cl_2 at 40 °C for 48 h to afford the olefin-containing macrocycle. To prevent aggregation of the resin-bound peptide, LiCl was added during the metathesis reaction. After RCM reaction, the resin was washed with dimethyl sulfoxide using the procedure developed by Vederas's group,[21] to remove organometallic byproducts and to facilitate purification. After removal of the N-terminal Fmoc with piperidine and acidic cleavage from the resin, a protected olefin-containing cyclopeptide was obtained in a 40% overall yield and HPLC analysis of this crude intermediate indicated a purity of 55%. The crude

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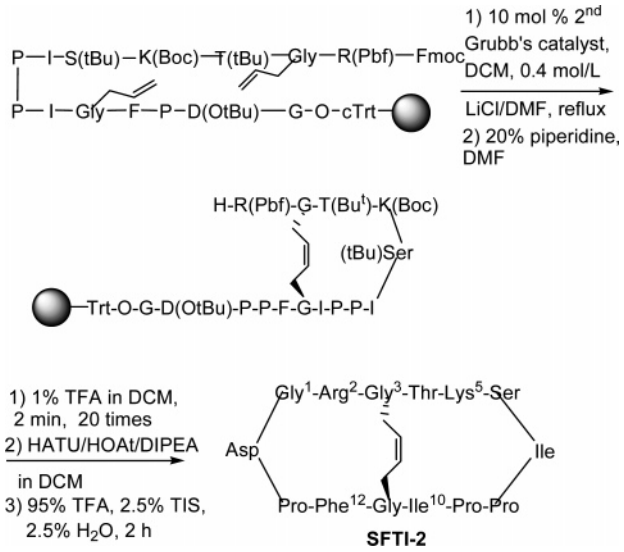
Scheme 2. Synthesis of Cyclopeptide **SFTI-2** Using RCM on the Solid Phase



peptide was then subjected to macrocyclization without purification using HATU as a coupling reagent followed by complete deprotection to give **SFTI-2** in a 5% overall yield and with an excellent purity of 96% determined by HPLC. No dimerization or intermolecular olefin metathesis byproducts were isolated during the synthesis, and the olefin bridge is in the cis configuration too. The success of this solid-phase RCM reaction might be attributed to the favorable conformation of the peptide precursor. An extensive intramolecular hydrogen bond network was observed in the X-ray analysis of **SFTI-1**.[13] The peptide precursor was probably already prealigned to the cyclized conformation by virtue of the two turn-inducing proline residues and intramolecular hydrogen bonds formed by the amide backbone.

The matriptase inhibitory activities of **SFTI-1**, **SFTI-2**, and **SFTI-3** are shown in Table 1. The olefin-bridged derivative **SFTI-2** is 25-fold less potent than the parent compound **SFTI-1**, and the olefin-reduced analogue **SFTI-3** exhibits inhibitory potency similar to that of **SFTI-1**.

Apparently, the disulfide bridge possesses quite different dihedral angle requirements relative to the olefin bridge that is in a cis geometry. Consequently, the incurred conformation change leads to the activity loss of **SFTI-2**. The reduction of olefin restores the conformation of the generated **SFTI-3**, and it results in a 10-fold activity increase. Considering the metabolic stability and inhibitory potency, **SFTI-3** is an attractive lead for further optimization.

Table 1. Relative Matriptase Inhibitory Activity of **SFTI-1**, **SFTI-2**, and **SFTI-3**[a]

compound	bridge type	relative matriptase inhibitory activity [K_i (nM)][b]
SFTI-1	disulfide bridge	1
SFTI-2	olefin bridge	25
SFTI-3	ethylene bridge	2.5

[a] The binding assay was performed on a Hitachi F4500 instrument by the method described in the Experimental Section. [b] The K_i values were determined by Dixon plots from two sets of data with different concentrations of substrate.

In summary, two potent proteolytically stable dicarba-bridged matriptase inhibitors were designed and synthesized upon the basis of the disulfide-bridged bicyclic peptide **SFTI-1**. Two synthetic routes were developed, featured with efficient solution-phase or solid-phase Grubbs ring-closing metathesis. The solid-phase RCM followed by in-solution macrocyclization approach is a quite robust method for rapid analogue synthesis during lead optimization. The dicarba-bridged **SFTI-3** not only inherits the potency of **SFTI-1** but also is metabolically stable, which makes this compound an attractive lead for further optimization. Various biological studies, including inhibitory studies of metastatic tumors in animal models, are currently in progress in our laboratory.

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Supporting Information Available: Experimental details and characterization data for **SFTI-2** and **SFTI-3** and detailed information about CD spectral analysis of **SFTI-1**, **SFTI-2**, and **SFTI-3**. ^{1}H copy of **SFTI-2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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