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Matrix Metalloproteinase Inhibition by Heterotrimeric Triple-Helical Peptide Transition State Analogs

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Abstract

Matrix metalloproteinases (MMPs) have been implicated in numerous pathologies. An overall lack of selectivity has rendered active site targeted MMP inhibitors problematic. The present study describes MMP inhibitors that function by binding both secondary binding sites (exosites) and the active site. Heterotrimeric triple-helical peptide transition-state analog inhibitors (THPIs) were assembled utilizing click chemistry. Three different heterotrimers were constructed, allowing for the inhibitory phosphinate moiety to be present uniquely in the leading, middle, or trailing strand of the triple-helix. All heterotrimeric constructs had sufficient thermally stability to warrant analysis as inhibitors. The heterotrimeric THPIs were effective against MMP-13 and MT1-MMP, with K_i spanning 100–400 nM. Unlike homotrimeric THPIs, the heterotrimeric THPIs offered complete selectivity between MT1-MMP and MMP-1. Exosite-based approaches are providing inhibitors with desired MMP selectivities.

Keywords

matrix metalloproteinase; collagen; transition state analog; heterotrimer; click chemistry

Introduction

Matrix metalloproteinases (MMPs) are the family of Zn^{2+} dependent endopeptidases that constitute a specialized group of proteolytic enzymes secreted by resident cells of various tissues. One of the roles ascribed to MMPs is the degradation of extracellular matrix (ECM) components, including collagens.^[1] While degradation of collagen is required for many physiological processes including wound healing, cell migration, tissue remodeling, and

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organ morphogenesis, accelerated breakdown is associated with numerous human pathologies (tumor angiogenesis and metastasis, osteoarthritis, post-myocardial infarction remodeling, neurodegenerative diseases, and neuropsychiatric disorders).^[2] Although MMP inhibitors have been developed since the 1980s, there have been no successful clinical trials. MMP inhibitory compounds either showed no significant therapeutic advantage or had considerable side effects, such as musculoskeletal syndrome (MSS).^[3] The lack of selectivity of MMP inhibitors may well have contributed to MSS.^[4] In addition, some MMPs have host beneficial functions, making them anti-targets.^[5]

To obtain selective MMP substrates and inhibitors, one can examine the abilities of MMP family members to catalyze collagen hydrolysis. Collagen is the most abundant protein in the ECM and the most abundant protein in the human body.^[6] Collagen is composed of three polypeptide chains each having the conformation of a left handed helix twisted together in a right handed fashion to form a triple-helix super-secondary structure. Each polypeptide strand in the triple-helix is composed of the repeating motif Gly-Xxx-Yyy, where Xxx and Yyy are often (2*S*)-proline and (2*S*,4*R*)-4-hydroxyproline (Hyp), respectively. Triple-helical structure offers exceptional mechanical strength to collagens and their resistance towards proteolytic enzymes.

There are 28 different types of collagen known in humans, many with multiple subtypes.^[6] Collagens may be homotrimeric or heterotrimeric. Two α 1(I) chains and one α 2(I) chain are present in the most abundant collagen, type I. In contrast, types II and III collagens have three α 1(II) and α 1(III) chains, respectively. The large structure of collagen has made direct methods for analyzing its biophysical and biological properties difficult. Thus, numerous peptide models of heterotrimeric and homotrimeric collagens have been developed to facilitate studies of its properties.

Our laboratory has taken the approach of utilizing collagen-model triple-helical peptides to develop MMP inhibitors.^[7] The strategy to the design of MMP inhibitors encompasses replacing a trigonal planar amide bond of a substrate with a hydrolytically stable functional group of tetrahedral geometry in the original carbonyl position. This substitution mimics the intermediate formed during enzyme-catalyzed hydrolysis. Our prior inhibitors were homotrimeric triple-helical peptides incorporating phosphinate transition state analogs.^[7–8] The phosphinate functional group with its pair of electronically equivalent anionic oxygens has emerged as an effective tetrahedral intermediate mimic in peptides leading to potent and selective transition state inhibitors of MMPs. As some MMPs favor heterotrimeric collagens (type I) to homotrimeric collagens (types II and III),^[9] heterotrimeric triple-helical peptide inhibitors may offer even greater selectivity than homotrimeric inhibitors within the MMP family. Presently, heterotrimeric triple-helical peptides incorporating a phosphinate in one strand and leaving the other two strands as native sequences were envisioned. The inhibitory sequence was derived from type I collagen α 1(I)769–783, which is hydrolyzed at the Gly-Ile bond by MMP-1, MMP-8, MMP-13, and MT1-MMP (MMP-14).^[1] The P₁-P₁' subsites were replaced by a Gly{PO₂H-CH₂}Ile transition state analog.

The synthesis of heterotrimeric triple-helical peptides is not straightforward. To date, the vast majority of collagen-model heterotrimers have been prepared either by using disulfide-

mediated cross-linking or electrostatic-assisted self-assembly methods.^[10] However, neither procedure is facile due to their inherent limitations. Isolation of the heterotrimeric triple-helices is the major problem in the self-assembly method, whereas exchange of disulfide bonds followed by strand exchange could occur in the disulfide-mediated cross-linking procedure.^[10–11] Recently Byrne *et al.* reported a modular synthetic strategy for the synthesis of homotrimeric and heterotrimeric triple-helical peptides by chemically stapling the C-terminal ends utilizing the “2 + 1 strand click synthesis” approach.^[11] In following with that scheme, we synthesized double-stranded peptides (DSPs) branched at the C-terminus through a Lys side chain. DSPs contained a C-terminal ϵ -azido Lys (or, more properly, 6-azidonorleucine), clickable to a single-stranded peptide (SSP) bearing a propargyl moiety at the C-terminus. The click chemistry approach was utilized to design and synthesize three heterotrimeric triple-helical peptide inhibitors (THPIs) bearing the inhibitory phosphinate moiety in one strand while keeping the other two strands as native sequences (Figure 1). Initially, we constructed a heterotrimeric triple-helical substrate that incorporated the $\alpha 1(I-III)769-783$ collagen sequence. This peptide was tested with MMP-1 to evaluate the effect of the triazole moiety introduced by click chemistry on enzyme catalysis. We subsequently assembled and examined the inhibitory properties of three THPIs with MMP-1, MMP-8, MMP-13, and MT1-MMP. Finally, we compared inhibition results between heterotrimeric and homotrimeric THPIs for MMP-1 and MT1-MMP.

Results and Discussion

Preparation of Heterotrimeric, Triple-Helical Model Clicked Peptides (THCP6 and THCP7)

Two models of the MMP cleavage site in types I-III collagen were constructed to evaluate (a) the efficiency of the methodology for heterotrimer assembly, (b) the stability of the triple-helix, and (c) the possible effect of the click-generated triazole on MMP recognition of the substrate. The model sequences were (Gly-Pro-Hyp)₅-Gly-Pro-Gln-Gly~Leu-Ala-Gly-Gln-Arg-Gly-Ile-Arg-(Gly-Pro-Hyp)₅ and (Gly-Pro-Hyp)₆-Gly-Pro-Gln-Gly~Leu-Ala-Gly-Gln-Arg-Gly-Ile-Arg-(Gly-Pro-Hyp)₅, analogs of which we have previously demonstrated to be MMP-1 substrates when present in branched or self-assembled THPs.^[12] SSP1 (Scheme 1) was synthesized by first incorporating Fmoc-(2*S*)-propargylglycine (Fmoc-Pra-OH), which possesses an alkyne side chain that is amenable to click chemistry, followed by assembly of the rest of the peptide. The retention time of SSP1 was 6.55 min, and MALDI-TOF MS analysis provided $[M+H]^+ = 3977.5$ Da (theoretical 3978.3 Da).

For the synthesis of DSP3 and DSP4 (Scheme 1), the resin was Fmoc-deprotected and *N*-acylated with Fmoc-*N*^ε-1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl-L-lysine [Fmoc-Lys(ivDde)-OH] followed by attachment of Fmoc- ϵ -azido-L-lysine [Fmoc-Lys(N₃)-OH]. The incorporation of Lys(ivDde) allows for controlled branching of the peptide, as the ivDde group is stable to Fmoc removal conditions but labile to hydrazine.^[13] The resin-bound dipeptide was then fully deprotected (removal of Fmoc and ivDde) to provide a polymer-supported active intermediate, and the simultaneous stepwise assembly of two α strands of identical sequence proceeded. The retention time of DSP3 was 6.90 min. The peptide was digested with immobilized trypsin and MS analysis indicated $[M+H]^+ = 2220.7$ Da (theoretical 2220.0 Da for fragment (Gly-Pro-Hyp)₅-Gly-Pro-Gln-Gly-Leu-Ala-

Gly-Gln-Arg-OH). The retention time of DSP4 was 6.90 min. The peptide was digested with immobilized trypsin and MS analysis indicated $[M+H]^+ = 2487.9$ Da (theoretical 2487.0 Da for fragment (Gly-Pro-Hyp)₆-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-OH). DSP3 and DSP4 were assessed to be sufficiently pure for subsequent click reactions.

The click reaction for THCP6 was performed with 1:1 ratio of SSP1 and DSP3 (0.5 μ mol of each) using 5 equiv of CuSO₄•5H₂O and 20 equiv of sodium ascorbate at room temperature (Scheme 1). The use of a lower equivalent of CuSO₄ (1 equiv) and sodium ascorbate (6 equiv) left the reaction incomplete even after >72 h. Ultimately, the desired THCP6 (Scheme 1) was prepared in 74% yield.

The retention time of THCP6 was 6.75 min, in between that of SSP1 and DSP3. CD spectroscopic analysis of THCP6 indicated triple-helical character characterized by a positive maximum at $\lambda = 225$ nm and negative maximum at $\lambda = 200$ nm (Figure 2). CD spectroscopic analysis of the thermal transition (Figure 3) revealed a T_m value of 46.3 °C.

To examine the ability of MMP-1 to catalyze the hydrolysis of THCP6, a 50 μ mol solution of the peptide was mixed with 10 nM activated recombinant full-length MMP-1 and hydrolysis was monitored for 24 h by analytical RP-HPLC. After 24 h, the reaction mixture was quenched with 2 M EDTA, filtered, and a mass spectrum recorded. The spectrum showed a peak at 1695.9 Da, which corresponded to the fragment (Gly-Pro-Hyp)₅-Gly-Pro-Gln-Gly-OH (theoretical 1693.0 Da). MMP-1 hydrolyzed THCP6 at the Gly~Leu bond, releasing (Gly-Pro-Hyp)₅-Gly-Pro-Gln-Gly-OH as expected based on the sequence specificity of MMP-1.^[1] Thus, the presence of the click triazole did not impede MMP-1 activity.

MALDI-TOF MS analysis of the intact THCP6 was unsuccessful, as the peptide did not ionize due to the large molecular mass and branched structure. This is a common problem for branched, collagen-model peptides.^[11, 14] The peptide was digested with immobilized trypsin and the MS analysis indicated $[M+H]^+ = 2220.3$ Da (theoretical 2220.0 Da for fragment (Gly-Pro-Hyp)₅-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-OH). It was not conclusive as to whether this fragment was from SSP1 and/or DSP3 of THCP6, as both contain 5 Gly-Pro-Hyp repeats at the *N*-terminus.

The click reaction for THCP7 was performed with 0.5 μ mol each of SSP1 and DSP4 (Scheme 1). The retention time of THCP7 was 6.70 min, in between those of SSP1 and DSP4, and less than that of THCP6. The incorporation of the additional Gly-Pro-Hyp repeats would be expected to result in THCP7 being more hydrophilic than THCP6 and having a lower RP-HPLC retention time. THCP7 was digested with immobilized trypsin and the mass spectrum indicated $[M+H]^+ = 2221.4$ Da (theoretical 2220.0 Da for (Gly-Pro-Hyp)₅-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-OH) and $[M+H]^+ = 2488.7$ Da (theoretical 2487.0 Da for (Gly-Pro-Hyp)₆-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-OH). These fragments could only be obtained if THCP7 was assembled from SSP1 and DSP4, respectively, via the click reaction. Thus, the combined studies with THCP6 and THCP7 indicated that the click approach could be utilized to assemble triple-helical heterotrimers of reasonable thermal stability that functioned as MMP substrates.

Design of Heterotrimeric, Triple-Helical Peptide Transition State Analog Inhibitors

When constructing homotrimeric, THP transition state analog inhibitors, the phosphinate dipeptide was inserted in all three strands.^[7–8] For heterotrimeric constructs, the phosphinate dipeptide is in only one strand. At present, the most favorable interaction of the triple-helical register of collagen chains with MMPs is unknown, and therefore we do not know which strand (leading, middle, or trailing) would be the desirable one to incorporate the transition state analog. Thus, there were three variants of the heterotrimeric, triple-helical peptide transition state analog to be synthesized (Figure 1). These analogs contain the phosphinate dipeptide within the chain originating from the Lys47 ϵ -azido group (designated THCP8), the Lys48 ϵ -azido group (designated THCP9), or the Lys47 α -amino group (designated THCP12). However, one cannot assign which are the leading, middle, or trailing strands.

Preparation and Characterization of Heterotrimeric, Triple-Helical Peptide Transition State Analog Inhibitors

The synthesis of THCP8 (Scheme 2) required DSP4 (described above) and SSP2. The retention time of SSP2 was 6.47 min. The peptide was digested with immobilized trypsin and the MS analysis indicated $[M+H]^+ = 2256$ Da (theoretical 2255.0 Da for (Gly-Pro-Hyp)₅-Gly-Pro-Gln-Gly{PO₂H-CH₂}Ile-Ala-Gly-Gln-Arg-OH) and $[M+H]^+ = 1506.8$ Da (theoretical 1505.0 Da for (Gly-Pro-Hyp)₅-Gly-Pra-NH₂).

THCP8 assembly (Scheme 2) was achieved by click reaction of SSP2 with DSP4 (0.6 μ mol of each peptide). The retention time of THCP8 was 6.80 min, in between those of SSP2 and DSP4. THCP8 was digested with immobilized trypsin and the mass spectrum indicated $[M+H]^+ = 2491.5$ Da (theoretical 2487.0 Da for (Gly-Pro-Hyp)₆-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-OH) and $[M+H]^+ = 2257.8$ Da (theoretical 2255.0 Da for (Gly-Pro-Hyp)₅-Gly-Pro-Gln-Gly{PO₂H-CH₂}Ile-Ala-Gly-Gln-Arg-OH), fragments derived from the DSP4 and SSP2, respectively.

The synthesis of THCP9 (Scheme 3) required SSP2 (described above) and DSP5. The retention time of DSP5 was 6.84 min. The peptide was digested with immobilized trypsin and the MS analysis indicated $[M+H]^+ = 2482.9$ Da (theoretical 2487.0 Da for fragment (Gly-Pro-Hyp)₆-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-OH).

THCP9 assembly (Scheme 3) was achieved by click reaction of SSP2 with DSP5 (2.5 μ mol of each peptide). The retention time of THCP9 was 6.73 min, in between those of SSP2 and DSP5. THCP9 was digested with immobilized trypsin and the mass spectrum indicated $[M+H]^+ = 2490.6$ Da (theoretical 2487.0 Da for (Gly-Pro-Hyp)₆-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-OH) and $[M+H]^+ = 2257.9$ Da (theoretical 2255.0 Da for (Gly-Pro-Hyp)₅-Gly-Pro-Gln-Gly{PO₂H-CH₂}Ile-Ala-Gly-Gln-Arg-OH), fragments derived from the DSP5 and SSP2, respectively.

The synthesis of THCP12 (Scheme 4) required SSP10 and SSP11. The retention time of SSP10 was 7.58 min. SSP10 was digested with immobilized trypsin and the MS analysis indicated $[M+H]^+ = 1717.5$ Da (theoretical 1719.8 Da for fragment (Gly-Pro-Hyp)₅-Gly-Lys(N₃)-Lys(N₃)-NH₂) and $[M+H]^+ = 2519.2$ Da (theoretical 2522.6 Da for fragment (Gly-Pro-Hyp)₆-Gly-Pro-Gln-Gly{PO₂H-CH₂}Ile-Ala-Gly-Gln-Arg-OH). The retention time of

SSP11 was 6.56 min, and MS analysis indicated $[M+H]^+ = 4032.3$ Da (theoretical 4034.3 Da).

THCP12 assembly was achieved by the double click coupling of 2.2 μ mol of SSP10 and 4.4 μ mol of SSP11 (Scheme 4). The retention time of THCP12 was 6.77 min, in between those of SSP10 and SSP11. THCP12 was digested with immobilized trypsin and MS analysis indicated $[M+H]^+ = 2520.2$ Da (theoretical 2522.6 Da for (Gly-Pro-Hyp)₆-Gly-Pro-Gln-Gly{PO₂H-CH₂}Ile-Ala-Gly-Gln-Arg-OH) and $[M+H]^+ = 2220.8$ Da (theoretical 2220.0 Da for (Gly-Pro-Hyp)₅-Gly-Pro-Gln-Gly-Leu-Ala-Gly-Gln-Arg-OH), fragments derived from the two SSPs (SSP10 and SSP11, respectively) used in the assembly of THCP12.

The CD spectra of THCPs (Figure 2) indicated triple-helix formation. All THCP melts exhibited cooperative transitions (Figure 3), with T_m values of 41.4, 37.7, and 38.2 °C for THCP8, THCP9, and THCP12, respectively. The relative stabilities of the THCPs was consistent with the stringent conditions required for trypsin digestion (37–43°C, up to 12 h), in similar fashion to those required for trypsin processing of collagen triple-helices.^[15] By comparison to THCP6, there was an observed decrease in triple-helix stability due to incorporation of the phosphinate of 4.9–8.6 °C. This is considerably less than when the phosphinate is incorporated into all three chains, where T_m values decreased by 24.5 °C.^[7] However, the homotrimer constructs were not stapled at the C-terminus, and the stapling entropically enhances triple-helical stability, shifting the equilibrium from unfolded to folded states.^[11, 16] Also, less destabilization due to one phosphinate compared with three is consistent with prior reports of interruptions in one strand of the triple-helix being less destabilizing than interruptions in all three strands.^[17]

MMP Inhibition by Heterotrimeric THCPs

THCP8, THCP9, and THCP12 were initially tested against MT1-MMP at 25 °C. THCP8, THCP9, and THCP12 effectively inhibited MT1-MMP with $K_i = 382$, 210, and 320 nM, respectively (Table 1). To evaluate selectivity, THCP8, THCP9, and THCP12 were studied against other collagenolytic MMPs (MMP-1 and MMP-13), along with MMP-3 serving as a non-collagenolytic MMP control. MMP-13 was also inhibited by all three THCPs with K_i in the 100 nM range (136, 106, and 180 nM, respectively; Table 1). There was little or no activity of any of the THCPs against MMP-1 and MMP-3. Thus, all three THCPs were very selective towards MMP-13 and MT1-MMP compared with MMP-1 and MMP-3. The lack of MMP-1 activity was not simply due to the presence of the triazole group, as THCP6 was a substrate for MMP-1. The selectivity away from MMP-3 is important as this enzyme has been identified as an antitarget based on its role in immunological responses.^[5a]

The K_i values for THCP9 were slightly lower in comparison to the other two inhibitors, THCP8 and THCP12. Unfortunately, the chain register within the heterotrimer is unknown, and thus the optimal position of the inhibitor in the leading, middle, or trailing strand cannot be stated presently. Overall, the differences in K_i values were within 2–3 fold, indicating that the activity of THPI was not unduly influenced by the presence of the inhibitor moiety in one specific strand of the triple-helix.

Activity of Homotrimeric THPI

We previously described a homotrimeric THPI, $\alpha 1(\text{I-III})\text{Gly}\Psi\{\text{PO}_2\text{H-CH}_2\}\text{Leu THPI}$,^[8a, 8b] that is analogous to our heterotrimeric THPI. We tested this THPI with MMP-1 and MT1-MMP. K_i values were 7.8 and 120 nM for MMP-1 and MT1-MMP, respectively (Table 1). Compared to the homotrimeric THPI, the heterotrimeric THPI bearing the phosphinate only in one strand was highly selective for MT1-MMP versus MMP-1.

Selectivity and Utility of THPIs

THPIs offer selectivity within the MMP family, in that only collagenolytic MMPs are effectively inhibited.^[7–8] Manipulation of sequence can offer further selectivity of THPIs within the collagenolytic MMPs.^[18] The heterotrimeric THPIs developed herein have demonstrated that the presence of the inhibitor moiety in all three chains is not critical for inhibitory activity towards some collagenolytic MMPs. In the case of MT1-MMP, the analogous homotrimeric THPI was three times more active than any of the heterotrimeric THPIs (Table 1). However, the selectivity between MMP-1 and MT1-MMP was lost when the inhibitor moiety was in all three chains of the triple-helix. This remarkable selectivity between MMP-1 and MT1-MMP has not been observed with prior homotrimeric THPIs (M. Bhowmick, D. Tokmina-Roszyk, T. Robichaud, K. Harmon, H. Wong, M.L. Lindsey, B. Steffensen, and G.B. Fields, manuscript in preparation). Homotrimeric THPIs are more “destabilized” at the site of phosphinate incorporation than heterotrimeric THPIs, based on the differences in T_m values between THPIs and their analogous THP substrates (see earlier discussion). This may be due to charge repulsion between the three phosphinates in homotrimers. Prior studies demonstrated that, as triple-helical sequences are made more stable, MMP-1 and MT1-MMP activity is reduced.^[19] However, MMP-1 is much more negatively impacted by stabilization of triple-helices than MT1-MMP.^[19] Thus, the selectivity observed for MMP-1 and MT1-MMP may be due to the single inhibitor chain within the heterotrimeric THPI being maintained within a triple-helical structure, where the MMP-1 CAT domain does not access the chain.

The selectivity is also consistent with the proposed different mechanisms of action of MMP-1 and MT1-MMP towards collagen.^[11] More specifically, recent structural studies indicate that the alignment of the triple-helix is different in the MMP-1 and MT1-MMP hemopexin-like domains (C. Luchinat, L. Cerofolini, M. Fragai, S. Amar, S. Pahwa, and G.B. Fields, manuscript in preparation).^[20]

Conclusions

Heterotrimeric THPIs, assembled utilizing click chemistry, were found to have sufficient thermal stability and to be effective against MMP-13 and MT1-MMP, with K_i values spanning 100–400 nM. However, unlike homotrimeric THPIs, the heterotrimeric THPIs offered complete selectivity between MT1-MMP and MMP-1. Further manipulation of the triple-helical sequence within heterotrimeric THPIs may yield even more selective inhibitors of MT1-MMP. MT1-MMP is prevalent drug target, particularly in cancer initiation and progression,^[21] and probes based on triple-helical structure offer great promise due to their stability, pharmacokinetics, and efficacy *in vivo*.^[8c, 22] Overall, exosite-based approaches

utilizing triple-helical constructs are providing inhibitors with desired selectivities within the MMP family.

Experimental Section

Materials

All chemicals and solvents were peptide synthesis or molecular biology grade and used without further purification. Reagents for peptides synthesis and purification of crude products were purchased respectively: standard *N*^α-(9-fluorenylmethoxycarbonyl)- (Fmoc)-amino acids and 2-(6-chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU) were from AGTC Bioproducts (Wilmington, MA); Fmoc-Pra from Advanced ChemTech (Louisville, KY); N-hydroxybenzotriazole•H₂O (HOBt•H₂O), Fmoc-*N*^ε-(7-methoxycoumarin-4-yl)-acetyl-L-lysine [Fmoc-Lys(Mca)-OH], Fmoc-Lys(N₃)-OH, Fmoc-*N*^ε-(2,4-dinitrophenyl)-L-lysine [Fmoc-Lys(Dnp)-OH], and Fmoc-Lys(ivDde)-OH from Anaspec (Fremont, CA); thioanisole from Fluka; α-cyano-4-hydroxycinnamic acid, sinapinic acid, 2,5-dihydroxybenzoic acid, and trifluoroacetic acid (TFA) from Thermofisher Scientific; NovaPEG Rink amide resin (substitution level = 0.49 mmol/g) from NovaBiochem; TentaGel S Ram resin (substitution level = 0.26 mmol/g) from CreoSalus (Louisville, Kentucky); piperazine from Acros Organics; *N,N*-diisopropylethylamine (DIPEA), *N*-methylmorpholine (NMM), *N,N*-dimethylformamide (DMF, ACS reagent 99.8%), acetonitrile (HPLC grade), *N,N'*-diisopropylcarbodiimide (DIC), hydrazine hydrate, and H₂O (HPLC grade) from Sigma Aldrich; methyl *tert*-butyl ether (MTBE) from J.T. Baker; and Fmoc-4*R*-fluoro-L-proline (Fmoc-Flp-OH) from Bachem (Torrance, CA). Milli-Q water was used for chemical reactions. Fmoc-4*R*-methyl-L-proline (Fmoc-mep-OH), Knight SSP [Lys(Mca)-Pro-Leu-Gly-Leu-Lys(Dnp)-Ala-Arg-NH₂], fluorogenic triple-helical peptide (fTHP)-15 [(Gly-Pro-Hyp)₅-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)₅-NH₂], and α1(I-III)GlyΨ{PO₂H-CH₂}Leu THPI [C₆-Gly-Pro-Flp-(Gly-Pro-Hyp)₄-Gly-Pro-Gln-GlyΨ{PO₂H-CH₂}(*R,S*)Leu-Ala-Gly-Gln-Arg-Gly-Ile-Arg-(Gly-Pro-Hyp)₄-Gly-Pro-Flp-NH₂] were synthesized by methods described previously.^[8a, 23] Phosphinate pseudodipeptide Fmoc-GlyΨ{P(OAd)(OH)-CH₂}Ile-OH [O-adamantyl-P-(9-fluorenylmethyloxycarbonyl-aminomethyl)-P-{2-(2-butyl)propionic acid-3-yl)-phosphinate] was synthesized as described.^[24]

Human recombinant, full-length proMMP-1, proMMP-3, proMMP-13, and proMT1-MMP were purchased from R&D Systems (Minneapolis, MN) and activated prior to use. Trypsin-3 and 4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride (AEBSF) were also purchased from R&D Systems. *p*-Aminophenylmercuric acetate was purchased from EMD Biosciences (San Diego, CA) and trypsin (TPCK-treated) from Worthington Biochemical Corporation (Lakewood, NJ).

General Peptide Synthesis Procedure

Peptides were synthesized by solid-phase methodology with Fmoc chemistry using the Liberty Microwave Peptide Synthesizer (CEM Corporation, Mathews, NC). The coupling of Fmoc-amino acids was performed with 5 equiv of each amino acid, 4.9 equiv of HCTU/

HOBt, and 10 equiv of NMM for 5 min at 50 °C and 25W. The Fmoc group was removed with 10% piperazine in DMF, where the initial deprotection was for 30 sec and followed by a second deprotection for 180 sec. A maximum of 75 °C with microwave power of 35W was set for both deprotection steps. Exceptions to these protocols are described below for selected amino acids from each peptide.

Peptides were cleaved from the resin by treatment with TFA H₂O thioanisole (90:5:5) (1.5 mL/100 mg of resin-bound peptide), unless otherwise noted, for 3–4 h under inert atmosphere (Ar). Crude peptides were precipitated with cold MTBE and centrifuged, and the resulting pellets dissolved in H₂O and lyophilized to obtain peptides sufficiently pure for click reactions.

General Click Reaction Procedure

A solution of CuSO₄•5H₂O (5 equiv) in 5% DMF in H₂O (30 µL/µmol of CuSO₄•5H₂O) was added to a solution of SSP (1 equiv) and DSP (1 equiv) in H₂O (300 µL/µmol of total peptide, SSP + DSP). The reaction mixture was vortexed and sodium ascorbate (20 equiv) was added. The resulting solution was mixed for 1–2 min and kept at room temperature with occasional mixing. After 12 h, the reaction mixture was diluted with H₂O to 2 mL, filtered through a Titan 2, 30 mm filter green 0.45 µm Nylon membrane, and purified by RP-HPLC (see below). The pure fractions were combined and lyophilized to afford a white solid.

General Procedures for Peptide Analysis and Purification

Analytical chromatographic separations were performed on a Vydac C18 column (5 µm, 300Å, 150 × 4.6 mm) utilizing an Agilent 1260 Infinity series HPLC with a diode array detector. Solvent A was 0.1% TFA H₂O, solvent B was 0.1% TFA acetonitrile, a linear gradient of 2–98% solvent B over 20 min was applied, the flow rate was 1.0 mL/min, and UV detection was at λ = 220 nm. Preparative chromatographic separations were performed on a Vydac C18 column (15–20 µm, 300Å, 250 × 22 mm) utilizing the same instrument with a multi-wavelength detector (Santa Clara, CA). Clicked peptides were purified using a flow rate of 10.0 mL/min, a linear gradient of 5–60% solvent B over 60 min, and UV detection at λ = 220 nm. Fractions were collected in the 20–50 min range and analyzed via RP-HPLC. Pure fractions were pooled, frozen, lyophilized, and stored at -20 °C in amber vials until use.

Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectra were obtained on an Applied Biosystems Voyager DE^{TM}-PRO Biospectrometry workstation (Carlsbad, CA). The matrix was sinapinic acid, α-cyano-4-hydroxycinnamic acid, or 2,5-dihydroxybenzoic acid.

Trypsin Digestion Procedure

0.5 mg of desired peptide was dissolved in 250 µL of 0.1 M NaHCO₃ solution. 85 µL of immobilized, TPCK-treated trypsin resin solution was measured into a 2 mL centrifuge tube and washed with 250 µL of 0.1 M NaHCO₃ solution three times. The peptide solution in NaHCO₃ was then added to the washed trypsin and incubated at 37–43 °C for 4–12 h. The tube was centrifuged to remove the trypsin. The pH of the solution was adjusted to 4 with 0.1 M HCl. The solution was applied to a pre moist C18 ZipTip, washed with solvent A to

remove salt, and finally washed with solvent B to collect peptide fragments. The filtrate was used to record MALDI-TOF mass spectra.

Synthesis of Single-Stranded Peptide 1 (SSP1)

The synthesis of SSP1 (Scheme 1) was performed in 0.1 mmol scale on NovaPEG Rink amide resin (0.48 mmol/g). The coupling of Fmoc-Pra was carried out at 45 °C for 45 min using 25W microwave power. The coupling of Pro14 was repeated.

Synthesis of Single-Stranded Peptide 2 (SSP2)

The synthesis of SSP2 (Schemes 2 and 3) was performed on Tentagel resin (0.26 mmol/g). The coupling of Fmoc-Pra was as for SSP1. Residues Arg24 and Arg27 were double coupled, where the first coupling was for 25 min at 25 °C using no power followed by 5 min at 25 °C using 25W power, and the second coupling was for 5 min at 50 °C with microwave power of 25W. Fmoc removal at Ala21 was achieved by initial treatment for 30 sec and final treatment for 3 min at 75 °C with microwave power of 35W. Coupling of the Fmoc-GlyΨ{P(OAd)(OH)-CH₂}Ile-OH dipeptide (3 equiv) was performed using DIC/HOBt/DIPEA (3 equiv/6 equiv/3 equiv) for 80 min at 40 °C with microwave power of 25W and then left overnight at ambient temperature. The removal of the Fmoc group, immediately after incorporation of the dipeptide into the sequence and for the rest of the synthesis, was performed in two stages; initial and final treatments at 50 °C without microwave power but with increased reaction time of 500 sec and 800 sec, respectively. The coupling of the rest of the amino acids after dipeptide incorporation was performed with increased reaction time of 20 min, with double coupling of Pro14 and Gln18. Cleavage of the peptide from the resin and side chain deprotection was carried out using 7 mL of triisopropylsilane phenol H₂O TFA (2:5:5:88) under inert atmosphere (Ar) for approximately 4 h.

Synthesis of Double-Stranded Peptide 3 (DSP3)

The synthesis of DSP3 (Scheme 1) was performed on NovaPEG Rink amide resin (0.6 mmol/g). Fmoc-Lys(ivDde)-OH was initially coupled to the resin followed by Fmoc-Lys(N₃)-OH. Both couplings were carried out using 3 equiv of amino acid, 2.7 equiv of HCTU, 2.8 equiv of HOBt, and 6 equiv of NMM at 50 °C for 20 min with microwave power of 25W. The resin-bound dipeptide was removed from the synthesizer, treated twice with 4 mL of 2% hydrazine hydrate in DMF for 1 h each time to remove the ivDde group, washed with DMF, and transferred back to the synthesizer reaction vessel. Coupling of the remaining residues was extended to 15 min and Pro14 was double coupled.

Synthesis of Double-Stranded Peptide 4 (DSP4)

The synthesis of DSP4 (Schemes 1 and 2) was carried out by adding one more Gly-Pro-Hyp repeat on resin-bound DSP3 applying the standard peptide synthesis protocol.

Synthesis of Double-Stranded Peptide 5 (DSP5)

The synthesis of DSP5 (Scheme 3) was performed on Tentagel resin (0.26 mmol/g) using the procedure outlined for DSP3 but first coupling Fmoc-Lys(N₃)-OH followed by Fmoc-Lys(ivDde)-OH.

Synthesis of Single-Stranded Peptide 10 (SSP10)

The synthesis of SSP10 (Scheme 4) was performed on Tentagel S Ram resin (0.26 mmol/g). Two consecutive Fmoc-Lys(N₃) residues were coupled to the resin as described for DSP3. Coupling up to Ala21 was as for SSP2. Arg24 and Arg27 were double coupled, with the coupling time extended to 10 min. Coupling of the Fmoc-GlyΨ{P(OAd)(OH)-CH₂}Ile-OH dipeptide was performed as described for SSP2. The coupling time of the remainder of the amino acids was increased to 20 min and Pro14 was double coupled. The removal of Fmoc groups after incorporation of the phosphinate moiety was carried out as described for SSP2.

Synthesis of Single-Stranded Peptide 11 (SSP11)

The synthesis of SSP11 (Scheme 4) was performed in 0.1 mmol scale on Tentagel S Ram resin (0.26 mmol/g) by following the procedure described for SSP1, except an extra Gly residue was included after the Pra.

Circular Dichroism (CD) Spectroscopy

CD spectra were recorded over the range $\lambda = 190\text{--}250$ nm on a JASCO J-810 spectropolarimeter using a 10 mm path length quartz cell. All peptides were dissolved in 0.1% aqueous acetic acid at a concentration of 15 μM and left overnight at 4 °C to allow for the formation of the triple-helix. The thermal transition curves were obtained by recording the molar ellipticity ($[\theta]$) at $\lambda = 225$ nm while the temperature was continuously increased in the range of 5–85 °C at a rate of 15 °C/h. The temperature was controlled using a JASCO PTC-348WI temperature control unit. For samples exhibiting sigmoidal melting curves, the inflection point in the transition region (first derivative) is defined as the melting temperature (T_m). Alternatively, T_m was evaluated from the midpoint of the transition. The spectra were normalized by designating the highest $[\theta]_{225\text{nm}}$ as 100% folded, and the lowest $[\theta]_{225\text{nm}}$ as 0% folded.^[25]

Metalloproteinase Activation

ProMMP-1, proMMP-3, and proMMP-13 were activated by mixing an equal volume of stock enzyme solution and activator (*p*-aminomercuric acetate to a final concentration of 1 mM) followed by 2–3 h incubation in a 37 °C water bath. ProMT1-MMP was activated by using trypsin-3 at a final concentration of 0.1 $\mu\text{g/mL}$ and incubating for 1 h at 37 °C. The reaction was stopped by addition of AEBSF (at a final concentration of 1 mM) and incubation for 15 min at room temperature. Activated MMPs were diluted to 20–100 nM in ice cold TSB*Zn buffer (50 mM Tris, 100 mM NaCl, 10 mM CaCl₂, 0.05% Brij-35, 0.02% NaN₃, 1 μM ZnCl₂pH 7.5) to prevent autoprolysis. Enzyme aliquots were kept on wet ice and used the same day. MMP activity was initially evaluated using the Knight SSP and compared with prior data ^[8a, 8b]. In this way, activity towards the substrate was used as an indicator of enzyme integrity, rather than TIMP titration, as performed previously.^[19a]

Enzyme Kinetics

Kinetic parameters were determined with a BioTek Synergy 4 plate reader and Gen5 1.11, Gen5 2.00, and Gen5 2.01 software using $\lambda_{\text{excitation}} = 324$ nm and $\lambda_{\text{emission}} = 393$ nm as described previously.^[8a, 26] A range of peptide concentrations was created by diluting a 100

μ M stock solution of peptide 1:1 twelve times and leaving overnight at 4 °C to allow the proper folding of the peptide triple-helix. 76 μ L of each peptide solution was loaded in 12 different wells in a 384 well plate. The plate was read to obtain initial fluorescence in relative fluorescence units (RFUs). 4 μ L of 20 $\times$ enzyme stock solution was added to each well and incubated at 25 °C. The fluorescence was monitored continuously for 15 min to determine initial reaction rates. The kinetic protocol at 25 °C had 30 sec shaking followed by each well read every 20 sec. Plates were stored at ambient temperature for 24 h before a final reading. All measurements were done in triplicate.

To determine rates of hydrolysis (Corr V_i ; μ M/sec), RFU was plotted over time (sec), slopes were divided by fluorescence change after 24 h, and then multiplied by the substrate concentration (μ M) {Corr V_i = (Slope/Change in RFU) $\times$ [peptide]}. Kinetic parameters were evaluated from Lineweaver-Burke, Hanes-Woolf, and Eadie-Hofstee linear plots as well as non-linear Michaelis-Menton regression using GraphPad Prism 5 and 6. When kinetics parameters from the above four modes correlated well, the experiment was considered a success. However, when linear analysis displayed significant exponential character, enzyme concentration was reduced and the experiment repeated until linearity was obtained.

Inhibition Studies

A series of inhibitor concentrations were prepared diluting a 50 μ M stock solution of inhibitor 1:5 eleven times and leaving overnight at 4 °C to allow the proper folding of the peptide triple-helix. 38 μ L of each inhibitor solution and 4 μ L of 20 $\times$ enzyme stock solution were mixed and loaded in 11 different wells in a 384 well plate. A mixture of 38 μ L of TSB*Zn buffer and 4 μ L of enzyme was placed in the twelfth well to obtain uninhibited enzyme activity. Based on prior studies demonstrating that phosphinate inhibitors of Zn^{2+} metalloproteinases are slow binding,^[27] the plate was incubated for 1 h at ambient temperature. Residual enzyme activity was monitored by adding equal volume of fTHP-15 solution in TSB*Zn buffer to produce a final concentration below K_M . Initial velocity rates were determined from the first 15 min of hydrolysis (when product release is linear with time) and RFU was plotted over time (sec). Plates were stored at ambient temperature for 24 h before a final reading. Enzyme activity was determined as {Corr V_i = (Slope/Change in RFU) $\times$ [enzyme]}. Percentage of activity was calculated as {(inhibited activity/uninhibited activity) $\times$ 100}. K_i values were calculated by using GraphPad Prism.

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Figure 1.

Heterotrimeric, triple-helical peptide inhibitors. The sequences are derived from the MMP cleavage sites in types I-III collagen. In the heterotrimeric constructs, the inhibitory chain (containing the phosphinate moiety) may be placed distinctly in one of three strands, originating from the (top) Lys47 ε-azido group, (middle) Lys47 α-amino group, or (bottom) Lys48 ε-azido group. Blue spheres indicate the sites of click ligation. O = Hyp.

Figure 2.
CD spectra of THCP6, THCP8, THCP9, and THCP12. Conditions are given in Experimental Section.

Figure 3. Thermal transitions as monitored by CD spectroscopy for THCP6, THCP8, THCP9, and THCP12. Conditions are given in Experimental Section.

Scheme 1.

Schematic of the click-based assembly of THCP6 and THCP7. Assembly was achieved with DSP3 or DSP4 and SSP1 (see Experimental Section), where $n = 5$ (DSP3) or 6 (DSP4).

Scheme 2.

Schematic of the click-based assembly of THCP8. Assembly was achieved with DSP4 and SSP2 (see Experimental Section).

Scheme 3.

Schematic of the click-based assembly of THCP9. Assembly was achieved with DSP5 and SSP2 (see Experimental Section).

Scheme 4.

Schematic of the click-based assembly of THCP12. Assembly was achieved with SSP10 and SSP11 (see Experimental Section).

Table 1

Inhibition of MMPs.

Enzyme	Inhibitor	$K_i^{(app)}$ (nM)
MMP-1	THCP8	NI
MMP-3	“	$18,400 \pm 11,600$
MMP-13	“	136 ± 8.7
MT1-MMP	“	382 ± 111.1
MMP-1	THCP9	NI
MMP-3	“	$12,700 \pm 4,700$
MMP-13	“	106 ± 71.3
MT1-MMP	“	210 ± 42.4
MMP-1	THCP12	NI
MMP-3	“	NI
MMP-13	“	180 ± 62.4
MT1-MMP	“	320 ± 56.0
MMP-1	$\alpha 1(I-III)Gly\psi\{PO_2H-CH_2\}Leu$ THPI	7.8 ± 1.03
MT1-MMP	“	120 ± 22.3

NI = No inhibition.