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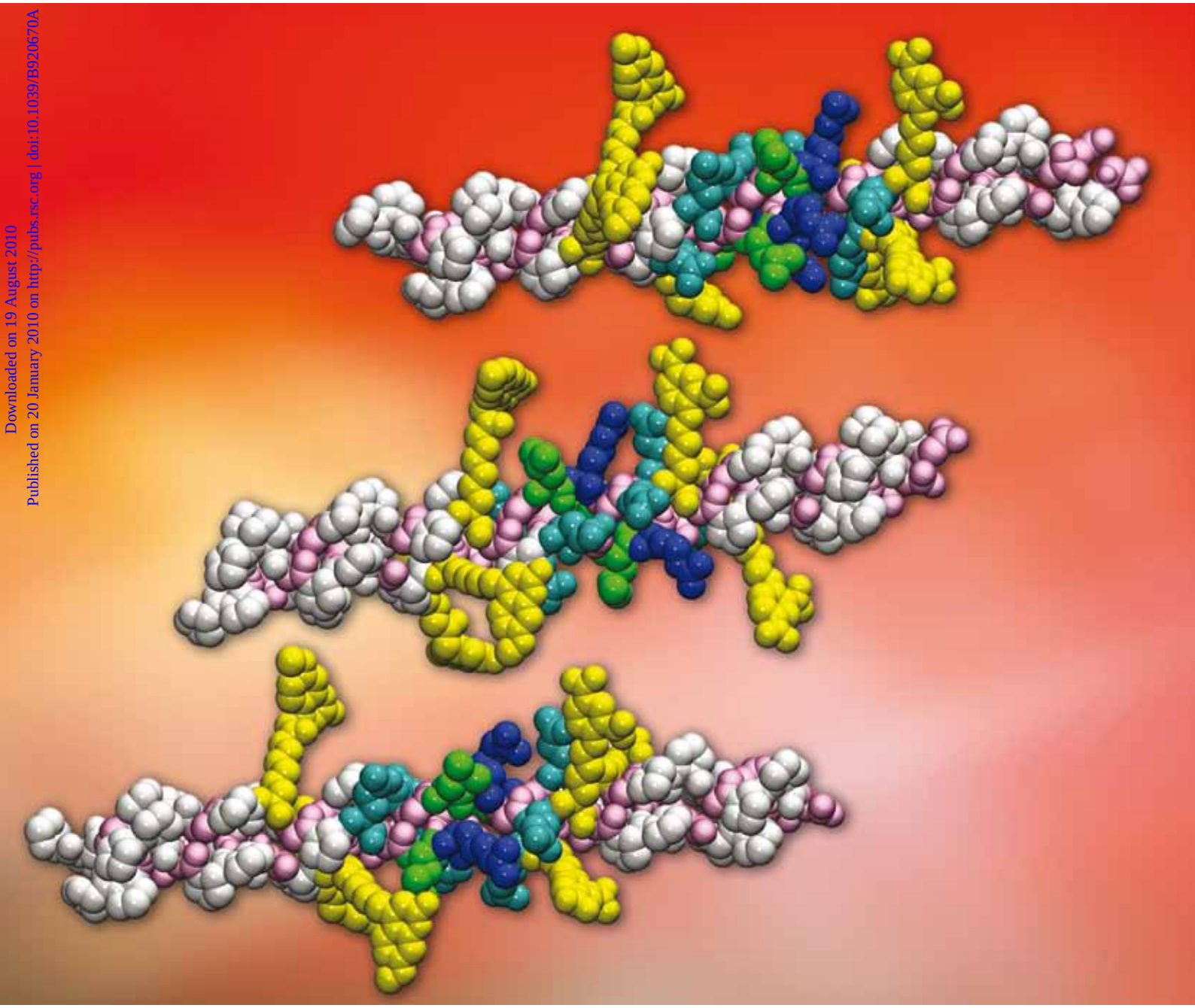

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Synthesis and biological applications of collagen-model triple-helical peptides

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Triple-helical peptides (THPs) have been utilized as collagen models since the 1960s. The original focus for THP-based research was to unravel the structural determinants of collagen. In the last two decades, virtually all aspects of collagen structural biochemistry have been explored with THP models. More specifically, secondary amino acid analogs have been incorporated into THPs to more fully understand the forces that stabilize triple-helical structure. Heterotrimeric THPs have been utilized to better appreciate the contributions of chain sequence diversity on collagen function. The role of collagen as a cell signaling protein has been dissected using THPs that represent ligands for specific receptors. The mechanisms of collagenolysis have been investigated using THP substrates and inhibitors. Finally, THPs have been developed for biomaterial applications. These aspects of THP-based research are overviewed herein.

1 Introduction

The collagen family is a diverse group of proteins made up of at least 28 members.[1-5] Collagens are composed of three α chains of primarily repeating Gly-Xxx-Yyy triplets, which induce each α chain to adopt a left-handed polyPro II helix. Three left handed chains then intertwine to form a right-handed superhelix. The collagen triple-helix (Fig. 1) is important for the integrity and workings of multiple connective tissues, including skin, bone, cartilage, tendon and dentin. Most collagens assist in anchoring cells to the extracellular matrix and some function in cellular regulation.

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Collagens have been classified according to their α chains.[4] Homotrimeric collagens (*i.e.*, types II and III) have three α chains of identical sequence. Heterotrimeric collagens have either two α chains of identical sequence (designated α1) and one α chain of differing sequence (designated α2), such as type I, or three α chains of differing sequence (designated α1, α2, and α3), such as type VI.[6] Collagens are further classified into subfamilies, based on their quaternary structure. These subfamilies include fibrillar, fibril associated with interrupted triple-helices (FACIT), short chain, basement membrane, multiplexins, and membrane associated with interrupted triple-helices (MACIT).[6] The most common collagens (types I, II, III, V, and XI) have fibrillar structures.

The triple-helical motif is also found in a variety of non-collagenous proteins, such as macrophage scavenger receptors types I and II and bacteria-binding receptor MARCO, complement component C1q, pulmonary surfactant apoproteins A and D, acetylcholinesterase, bovine conglutinin, collectin-43, ficolins, aggretin, ectodysplasm, and mannose binding protein.[1,7,8]

To fully investigate the structural and biological roles of collagen and collagen-like proteins, triple-helical peptides (THPs) or "mini-collagens" incorporating collagen-model sequences and three-dimensional structure have been constructed. The synthesis and application of triple-helical, collagen-model peptides was comprehensively reviewed in 1995-1996.[7,9] Subsequent reviews have focused on collagen biochemistry captured *via* THP constructs.[5,10-13] Thus, this perspective will primarily emphasize synthetic research conducted in the last decade and recent biological studies. Many of the details pertaining to THP construction and application are included herein. The topics covered include methodologies for assembling THPs (associated triple-helical peptides, templated triple-helical peptides, and triple-helical peptides of higher order structure), structural studies of THPs, biological studies of THPs (adhesion receptor binding, protease substrates and inhibitors, and lipoprotein-, glycosaminoglycan-, nucleic acid-, and other protein-collagen interactions), and miscellaneous applications of THPs.

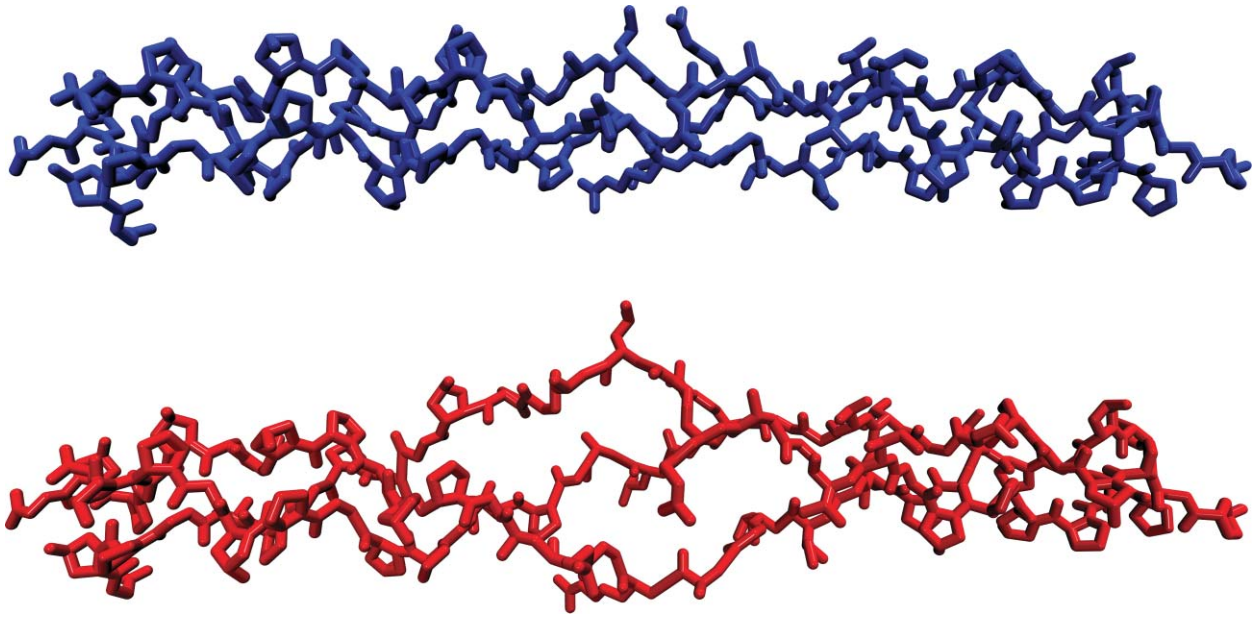


Fig. 1 "Ball and stick" computer generated models of (top) a continuous collagen triple-helix (peptide T3-785, 3[(Pro-Hyp-Gly)$_3$-Ile-Thr-Gly-Ala-Arg-Gly-Leu-Ala-Gly-(Pro-Hyp-Gly)$_4$][325]) and (bottom) an unwound (heat denatured) version of the same sequence.

2 Associated triple-helical peptides

It has long been noted that peptides containing repeating sequences of Gly-Pro-Pro or Gly-Pro-4*R*-Hyp [where 4*R*-Hyp is (2*S*,4*R*)-4-hydroxyproline] were capable of self-association in aqueous solution to form stable collagen-like triple-helical structures.[9] The assembly of such sequences is now almost entirely based on stepwise solid-phase methodology using *tert*-butyloxycarbonyl (Boc) or 9-fluorenylmethoxycarbonyl (Fmoc) chemistries.[14-20] To obtain THPs of reasonable stability, the self-association approach typically "sandwiches" a collagen-model sequence between repeats of Gly-Pro-Hyp (Fig. 2). Self-associated triple-helical peptides have often been used to study the structural aspects of collagen, where the peptide contains either a repeating tripeptide sequence or a "host-guest" sequence such as (Pro-Hyp-Gly)$_n$-Xxx-Yyy-Gly-(Pro-Hyp-Gly)$_n$.[21-23]

Although the majority of self-associated THPs have been homotrimers, a few examples of self-associated heterotrimers have been reported. In most cases, the heterotrimer was not isolated in pure form, but rather studied structurally as a mixture. Highly stable heterotrimers have been assembled from 1 : 1 : 1 of (Asp-Hyp-Gly)$_{10}$, (Pro-Lys-Gly)$_{10}$, and (Pro-Hyp-Gly)$_{10}$ (T_m = 65.0 °C) or (Asp-Hyp-Gly)$_{10}$, (Pro-Arg-Gly)$_{10}$, and (Pro-Hyp-Gly)$_{10}$ (T_m = 54.0 °C).[24,25] The stability of the former heterotrimer was comparable to (Pro-Hyp-Gly)$_{10}$ homotrimer (T_m = 67.5 °C).[25] NMR structural analysis revealed that the Asp and Lys side-chains formed a network of ionic hydrogen bonds, most likely accounting for the heterotrimer stability.[26] A stable heterotrimer (T_m = 28 °C) was also obtained from a 1 : 2 mixture of (Pro-Pro-Gly)$_7$ and (4*S*-Flp-4*R*-Flp-Gly)$_7$ [where 4*S*-Flp is (2*S*,4*S*)-4-fluoroproline and 4*R*-Flp is (2*S*,4*R*)-4-fluoroproline].[27] A mixture of (Pro-Hyp-Gly)$_{10}$ and (Pro-Pro-Gly)$_{10}$ (either 1 : 2 or 2 : 1) formed stable heterotrimers, with T_m dependent upon the scanning speed.[28,29] A heterotrimer composed of a 2 : 1 mixture of (Gly-Pro-Hyp)$_3$-Gly-Met-Hyp-Gly-Val-Gly-Glu-Lys-Gly-Glu-Hyp-Gly-Lys-Hyp-(Gly-Pro-Hyp)$_2$-Gly-Tyr and

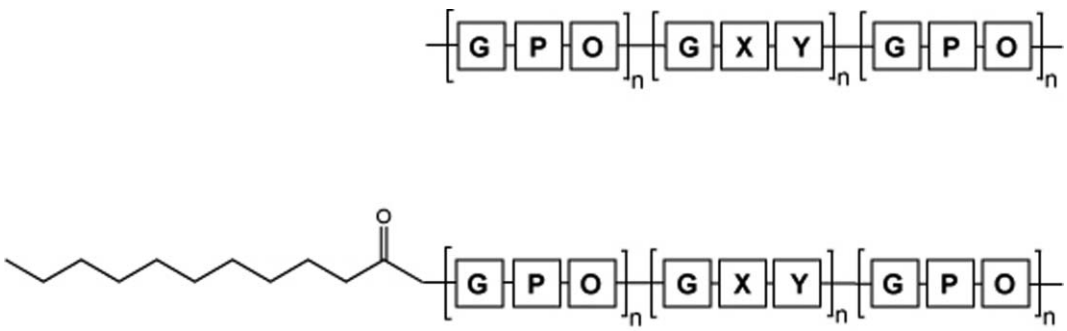


Fig. 2 Modular structures of (top) sandwiched associated THP and (bottom) sandwiched associated triple-helical peptide-amphiphile. The associated THP features repeats of Gly-Pro-Hyp [(GPO)$_n$] on both the *N*- and *C*-termini to induce or stabilize triple-helical structure, and a diverse collagen-like sequence [(GXY)$_n$] in the middle for structural and/or biological studies. The peptide-amphiphile additionally possesses a pseudo-lipid attached to the *N*-terminus to further enhance triple-helical stability *via* hydrophobic interactions.

Pro-Hyp-Gly-Asp-Hyp-(Gly-Pro-Hyp)$_2$-Gly-Ile-Ser-Leu-Lys-Gly-Glu-Glu-Gly-Pro-Hyp-Gly-Pro-Ala-Gly-Pro-Hyp-Gly-Tyr-Hyp-Gly was found to have a T_m value of 14.5 °C.[30] Additional heterotrimers have been created *via* 1 : 2 mixtures of diverse sequences.[24]

Gly-Pro-Hyp repeats may not enhance triple-helical sequences with sufficient thermal stability for biological studies. Self-associated peptides have been further stabilized by the addition of lipophilic molecules at the *N*-terminus of the peptide. These "peptide-amphiphiles" (PAs) have incorporated an amino acid sequence with the propensity to form a triple-helix as the polar head group and a dialkyl or monoalkyl hydrocarbon chain as the non-polar tail (Fig. 2).[14,15,31,32] Desirable peptide head group T_m values can be achieved for *in vivo* use, as triple-helical PAs have been constructed with T_m ranging from 30 to 70 °C.[14,15,18,33-41]

The stability of associated THPs has also been modulated by pH or photolysis. To create a triple-helix that was pH dependent, Hyp was modified by addition of a carboxylate by O-alkylation.[42] Interestingly, the incorporation of 1 or 3 Hyp(CO_2) residues within a Pro-Hyp-Gly template did not result in a pH-sensitive triple-helix. However, acetyl-[Pro-Hyp(CO_2)-Gly]$_7$-OH formed a triple-helix with a T_m = 17 °C at pH 2.7 but had no triple-helical structure at pH 7.2.[42] pH-dependent triple-helical stability was also observed for (Pro-4*R*-Amp-Gly)$_6$ sequences, where 4*R*-Amp is (2*S*,4*R*)-4-aminoproline.[43,44] Modification of the sequence (Gly-Pro-Hyp)$_3$-Gly-Cys-Hyp-Gly-Pro-Hyp-Gly-Pro-Cys-(Gly-Pro-Hyp)$_5$-Gly-Gly-NH_2 with an azobenzene bridge between the Cys residues resulted in a THP whose stability decreased up irradiation at $\lambda = 330$ nm at 27 °C.[45] Unfortunately, the poor solubility of this peptide prohibited quantitative analysis of its stability.[45]

In all of the above cases, fairly standard solid-phase synthetic conditions have been utilized for THP assembly. Notable modifications include extended coupling times for Hyp and Pro derivatives and extended Fmoc deprotection times and/or the use of diaza(1,3)bicyclo[5.4.0]undecane (DBU) for deprotection [2% DBU plus 2% piperidine (to scavenge dibenzofulvene)].[46-48] Although the use of Hyp without side-chain protection has been described,[16,47,49] the commercial availability of Boc-Hyp(Bzl) and Fmoc-Hyp(*t*Bu) and subsequent lack of potential interchain hydrogen-bonding and side-chain esterification during synthesis has lead to the predominant use of these derivatives.[17,20,49,50] The synthesis and incorporation of 4*R*-Hyp analogs and derivatives, such as 3-Hyp, 4*S*-Hyp [(2*S*,4*S*)-Hyp; alloHyp; hyp], 4-*cis*-Hyp [(2*R*,4*R*)-Hyp], 4*R*-Flp, 4*S*-Flp (flp), 3*S*-Flp, 4,4-difluoroproline (Dfp), 4-oxoproline, 4*R*-Clp [(2*S*,4*R*)-4-chloroproline], 4*S*-Clp (clp), and 4*R*-Amp has been described.[27,37,43,51-55] These secondary amino acids have been utilized primarily for studies on triple-helix stability (see below), although in one case Flp was used to examine the effects of triple-helix stability on melanoma cell binding.[37] Finally, preparation of side-chain protected or glycosylated Fmoc-Hyl derivatives has been described,[56-61] and Hyl(Gal) has been incorporated into a THP.[62]

3 Templated triple-helical peptides

Numerous approaches have been described by which THPs are nucleated and/or stabilized by covalent attachment to a template. In its simplest form, covalent attachment of three strands stabilizes a triple-helix.[11,12,63] To study the nucleation step for the folding of collagen, a protocol was developed in the 1980s for the liquid phase synthesis by which three peptide strands were covalently linked *via* a *C*-terminal branch.[64,65] The *C*-terminal branch was expected to align and entropically stabilize the *C*-terminus of the THP and thus enhance triple-helical thermal stability, and to provide a model of the disulfide-linked *C*-terminus of type III collagen. Branching was achieved by selective deprotection of Lys Nα- and ε-amino groups. Our laboratory evaluated systematically several general solid-phase methods for synthesizing covalently branched THPs that incorporate native collagen sequences. Branching of three peptide strands from one initial chain required three different protecting group strategies (Fig. 3): *N*α-amino protection (**A**), Lys *N*ε-amino side-chain protection (**B**), which must be stable to the *N*α-amino group removal conditions, and *C*α-carboxyl protection (**linker**), which must be stable to both the *N*α- and *N*ε-amino protecting group removal conditions. Four different synthetic schemes were employed, with the only common protecting group strategy being Fmoc for **A**.[46,47,49] The combinations were: (a) **B** the Boc group, **C** the 2,6-dichlorobenzyl (Dcb) group, and the 4-hydroxymethylphenylacetic acid (PAM) **linker**; (b) **B** the Boc group, **C** the allyl group, and allyl-based 4-trityloxy-*Z*-but-2-enyloxyacetic acid **linker**; (c) **B** the allyloxycarbonyl (Aloc), **C** the *t*Bu group, and the 4-hydroxymethylphenoxy (HMP) **linker**; and (d) **B** the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl (Dde) group, **C** the *t*Bu group, and 2-methoxy-4-alkoxybenzyl alcohol (SASRIN) **linker**. Tyr was incorporated prior to branching to provide a convenient chromophore for eventual concentration determination. In the case of (a), branching was achieved by synthesizing Fmoc-[Lys(Boc)]$_2$-Tyr(Dcb)-Gly-PAM resin and deprotecting the *N*α- and *N*ε-amino groups with TFA. The peptide-resin was cleaved with TFMSA. In the case of (b), branching was achieved by synthesizing Fmoc-[Lys(Boc)]$_2$-Tyr(Al)-Gly-allyl resin and deprotecting the *N*α- and *N*ε-amino groups with TFA. The THP was side-chain deprotected with TFA while still resin-bound, and cleaved with $(Ph_3P)_4Pd$. In the case of (c), both Aloc groups of [Lys(Aloc)]$_2$-Tyr(*t*Bu)-Gly-HMP resin were removed with $(Ph_3P)_4Pd$. In the case of (d), both Dde groups of [Lys(Dde)]$_2$-Tyr(*t*Bu)-SASRIN resin were removed with hydrazine. For all THPs, Ahx was incorporated onto all three amino termini to provide a flexible spacer.

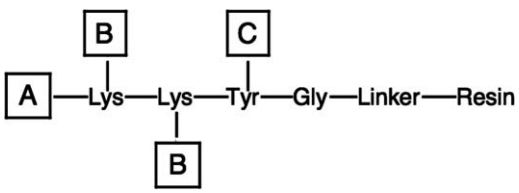


Fig. 3 Template for *C*-terminal branching strategy.

The four strategies indicated that *C*-terminal templates can be incorporated into THPs by a variety of approaches. Due to the mild conditions, we currently favor the method used to assemble the Fmoc-[Lys(Dde)]$_2$-Tyr(*t*Bu)-SASRIN branching template. We have subsequently improved yields by switching from the Dde group to the ivDde group, which has greater stability to the conditions of Fmoc chemistry.[66] A total of 28 different branched THPs have been reported by our laboratory.[34-36,46-48,50,62,67-70] The

solid-phase branching strategy has also been used by other groups, based on Fmoc/Lys(Dde).[71-74]

Numerous other templated approaches have been described, including the introduction of a di-Lys or di-Glu template at the *C*- or *N*-terminal regions of the three peptide chains,[75-77] a double disulfide "knot" at the *C*-terminal region of the three peptide strands,[78,79] or a *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid (Kemp triacid; KTA),[80-83] cyclotriveratrylene,[84] tris(2-aminoethyl)amine (TREN),[85] macrocyclic,[86] or Boc-β-Ala-tris(carboxyethoxymethyl)aminomethane [Boc-β-Ala-TRIS-$(OH)_3$][87] template linked to the *N*-terminus of three peptide chains (Fig. 4).

Tanaka *et al.*[88] utilized chemoselective ligation to create a triple-helical di-Lys branched peptide in aqueous solution. Collagen-like sequences were synthesized with an *N*-terminal Cys residue, while the branch structure had three bromoacetic acid molecules

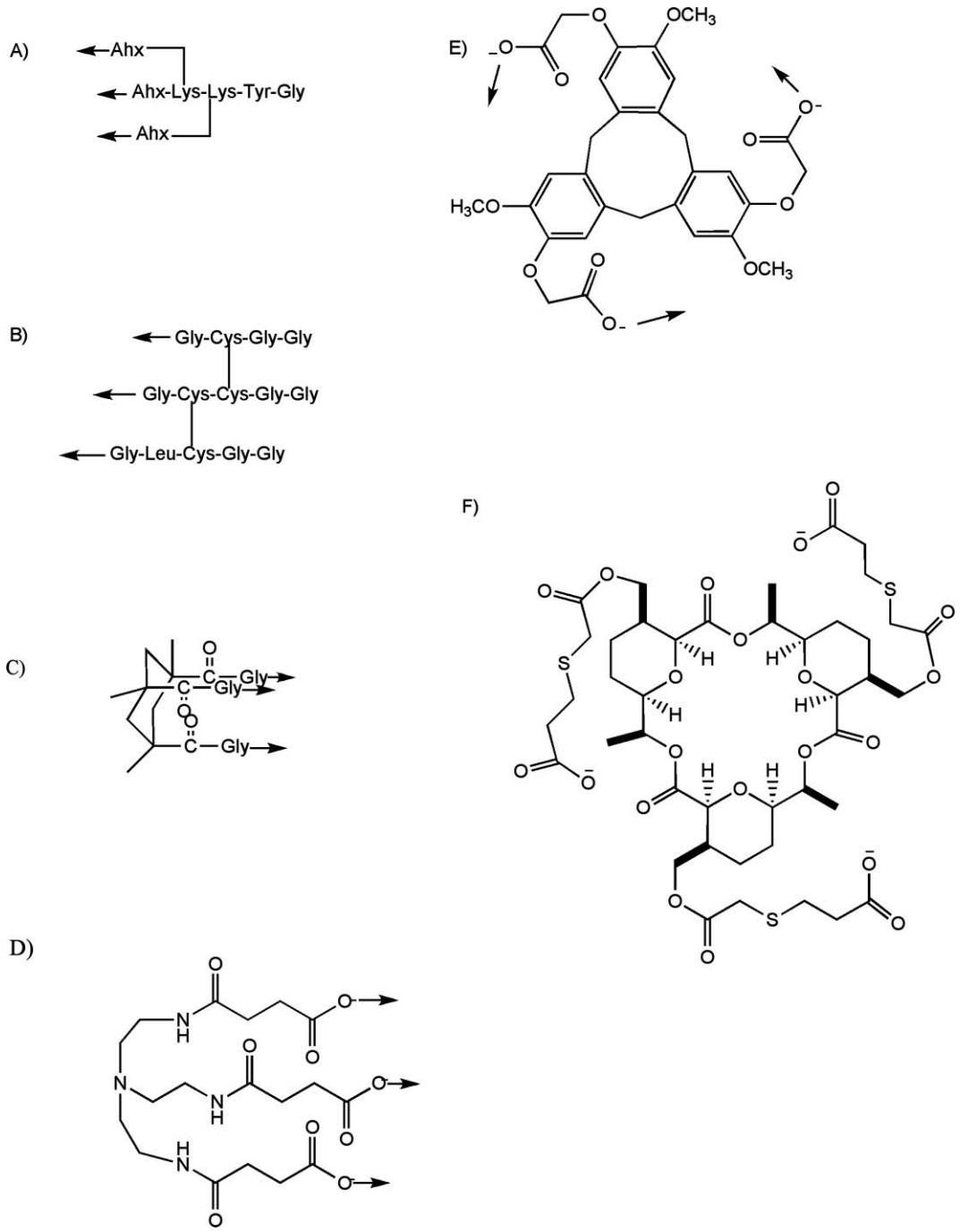


Fig. 4 Templates used for the chemical synthesis of triple-helical peptides: (A) di-Lys branch after coupling to 6-aminohexanoic acid (Ahx); (B) disulfide bridge (cystine-knot); (C) *cis*,*cis*-1,3,5-trimethylcyclohexane-1,3,5-tricarboxylic acid (KTA) after coupling to Gly; (D) tris(2-aminoethyl)amine (TREN) after coupling to succinic acid; (E) cyclotriveratrylene (CTV) after coupling to α-bromoethanoic acid; and (F) macrocyclic. The arrows indicate the direction of collagen-like sequence incorporation.

coupled to the spacer.[88] The individual chains were then ligated to the branch in $NaHCO_3$/Gdn·HCl solution, forming thioether bonds.

Assembly of THPs using a Glu-Glu *N*-terminal branch required two peptide types, one containing a Glu α-thioester at its *N*-terminus and one without the Glu.[75] Crosslinking was achieved by silver ion activation of the Glu thioester group, followed by nucleophilic attack of the *N*-terminal amino group from the other peptide. The process was repeated using the Glu α-thioester peptide to create a trimeric structure. Minimally protected peptides (restricted to amino group protection) allowed for high solubility and reactivity during crosslinking. The more simplified *N*-terminal di-Glu template Gly-Phe-Gly-Glu-Glu-Gly was assembled by solid-phase Fmoc methodology, isolated as Fmoc-Gly-Phe-Gly-Glu-Glu-Gly, and acylated to three peptide strands simultaneously on the solid-phase using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt).[77]

The synthesis of an *N*- and *C*-terminal cross-linked THP has been described.[76] Boc-Ser(*t*Bu)-OSu was reacted with Fmoc-Lys-OH to obtain Fmoc-Lys[Boc-Ser(*t*Bu)]-OH. Collagenous linear peptides of the sequence Cys-Gly-(Gly-Pro-Hyp)$_n$-Lys(Ser)-NH_2 (n = 3-10) (**1**) were then synthesized using Fmoc chemistry.[76] The trivalent linker, tris-bromoacetylated Lys-Lys dimer **2**, was prepared on MBHA resin. The tris-aminooxyacetylated branch-peptide **3** was synthesized by a similar method using Boc-aminooxyacetic acid instead of bromoacetic acid. Peptide **1** and the bromoacetyl peptide **2** were reacted to yield (Gly-Pro-Hyp)$_n$[N] (**4**).[76] Sodium periodate (3 equiv per Ser residue) was added to a solution of peptide **4** to convert the Ser residues into aldehydes, producing peptide **5**. Peptide **5** and peptide **3** were dissolved in sodium acetate buffer, and after 3 h, the product (Gly-Pro-Hyp)$_n$[NC] (**6**) was obtained.[76]

Extensive studies have been performed by Moroder and colleagues for creating *C*-terminally branched heterotrimeric THPs using disulfide bonding (cystine knots).[3,17,78,89-91] In one representative example, the following peptide chains were constructed by solid-phase methodology: α1, which contained either 3 or 5 Gly-Pro-Hyp repeats on the *N*-terminus of Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Cys(Acm)-Gly-Gly-OH, where Acm is acetamidomethyl; α2, which contained 3 or 5 Gly-Pro-Hyp repeats on the *N*-terminus of Gly-Pro-Gln-Gly-Leu-Leu-Gly-Ala-Hyp-Gly-Ile-Leu-Gly-Cys(Acm)-Cys(S*t*Bu)-Gly-Gly-OH; and α1′, which contained either 3 or 5 Gly-Pro-Hyp repeats on the *N*-terminus of Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg-Gly-Val-Val-Gly-Leu-Cys(S*t*Bu)-Gly-Gly-OH. All three peptides were synthesized using Fmoc chemistry. Couplings were performed with HBTU/HOBt except for Cys residues, which were coupled as pentafluorophenyl esters to minimize racemization. The three chains were assembled into the heterotrimer by stepwise regioselective crosslinking. Peptide α1 was treated with 3-nitro-2-pyridine-sulfenyl chloride (Npys-Cl) to convert Cys(Acm) to Cys(Npys). Peptide α2 was treated with P(C_4H_9)$_3$ to remove the S*t*Bu group. Peptides α1 and α2 were reacted at pH 4.5 to form a dimer. The dimer was treated with Npys-Cl to convert the α2 chain Cys(Acm) to Cys(Npys). The α1′ chain was treated with P(C_4H_9)$_3$ to remove the S*t*Bu group. Peptide α1′ and the dimer were reacted at pH 4.5 to form a trimer. The heterotrimer containing 3 Gly-Pro-Hyp repeats was obtained in 72% yield over all crosslinking reactions.[78,89] A similar strategy was utilized to construct heterotrimeric THPs possessing *N*-terminal cystine knots.[92]

A more simplified, oxidative approach has been utilized for the assembly of cystine knot homotrimeric THPs.[93,94] Oxidation with aldrithiol has been utilized for the assembly of heterotrimeric THPs possessing cystine knots, with isolation of the desired heterotrimer achieved by size-exclusion chromatography in combination with RP-HPLC, MS, and NMR spectroscopic analyses.[95]

Two synthetic routes have been used to prepare KTA template-assembled collagen-based structures. The first method utilized solid-phase methodology exclusively. Boc-(Gly-Pro-Hyp)$_n$-MBHA resin was prepared and the Boc group removed. KTA-(Gly-OH)$_3$ was coupled using DIPCDI and HOBt for 3 d.[96] Structures containing 3 or 6 Gly-Pro-Nleu repeats (where Nleu is *N*-isobutylglycine) or (Gly-Nleu-Pro)$_n$ were also synthesized using this approach.[82,83] The KTA-(Gly-OH)$_3$ coupling was allowed to proceed for 1-2 d.

The second method to connect the KTA template and peptide-peptoid chains involved peptide bond formation in solution. Peptide-peptoid chains were assembled by solid-phase methods and cleaved from the resin as *N*-terminal free amine and *C*-terminal amidated forms. The KTA-(Gly-OH)$_3$ template and free-amine peptide-peptoid chains were then coupled in solution using EDC and HOBt.[82]

The *N*-terminal TREN template was prepared by coupling tris(2-aminoethyl)amine to monobenzylated succinate, and removing the benzyl ester groups by hydrogenation.[85] TREN-(suc-OH)$_3$ is then acylated to three peptide strands simultaneously on the solid-phase.[85] The macrocyclic template was coupled to the *N*-termini of collagen-model peptides in solution using PyBOP and DIEA.[86] In similar fashion, the cyclotriveratrylene template was coupled in solution using BOP.[84] Boc-β-Ala-TRIS-(OH)$_3$ was coupled to peptides in solution using 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one (DEPBT).[87]

The effect of templates is often to enhance the thermal stability of collagen-like sequences. For example, the THP acetyl-Gly-Gly-(Pro-Hyp-Gly)$_5$-NH_2 has a T_m value of 9.2 °C, while templated versions of the same sequence, using either (+)CTV or KTA, had T_m values of 58 and 62 °C, respectively.[84] Similarly, (Gly-Pro-Hyp)$_6$ has a T_m = 25.4 °C, (Pro-Hyp-Gly)$_6$ with a *C*-terminal branch has a T_m = 39.4 °C, (Gly-Pro-Hyp)$_6$ with an *N*-terminal branch has a T_m = 56.2 °C, and (Gly-Pro-Hyp)$_6$ with both an *N*- and *C*-terminal branch has a T_m = 69.7 °C.[73,76] An *N*-terminal macrocyclic template induced triple-helical structure into the sequence Gly-(Pro-Pro-Gly)$_7$-NH_2 with a T_m = 39.9 °C, whereas the non-templated sequence was not triple-helical.[86] Triple-helices containing peptoid residues, such as *N*-isobutylglycine (Nleu), have been stabilized by templates. Acetyl-(Gly-Nleu-Pro)$_6$-NH_2 has a T_m = 26 °C, while KTA-[Gly-(Gly-Nleu-Pro)$_6$-NH_2]$_3$, TREN-[suc-(Gly-Nleu-Pro)$_6$-NH_2]$_3$, and Boc-β-Ala-TRIS-[(Gly-Nleu-Pro)$_6$-OCH_3]$_3$ had T_m values of 36, 46, and 33 °C, respectively.[83,85,87] The thermal stability of the α2β1 integrin binding sequence (Gly-Pro-Hyp)$_3$-Gly-Phe-Hyp-Gly-Glu-Arg-(Gly-Pro-Hyp)$_3$ was enhanced by an *N*-terminal Gly-Phe-Gly-Glu-Glu-Gly template, resulting in an increase in T_m from 25 to 44 °C.[77] Acetyl-(Pro-Hyp-Gly)$_5$-Pro-Cys(S*t*Bu)-Cys(S*t*Bu)-Gly-Gly-Gly-NH_2 has a T_m = 20.3 °C, while the

double disulfide linked [acetyl-(Pro-Hyp-Gly)$_5$-Pro-Cys-Cys-Gly-Gly-Gly-NH$_2$]$_3$ has a T_m = 68.1 °C.[93] Formation of the cystine knot in (Gly-Pro-Pro)$_3$-Gly-Pro-Arg-Gly-Glu-Lys-Gly-Glu-Arg-Gly-Pro-Arg-(Gly-Pro-Pro)$_3$-Gly-Pro-Cys-Cys-Gly increases T_m from 35 to 43 °C.[94]

"Templated" triple-helices have also been constructed using metals. The addition of dopamine on the *N*-terminus of (Gly-Nleu-Pro)$_6$, followed by incubation with Fe^{3+}, resulted in a non-triple-helical peptide becoming triple-helical with a T_m value of 28 °C.[97] Addition of 2,3-dihydroxybenzoic acid on the *C*-terminus of Boc-β-Ala-TRIS-[(Gly-Nleu-Pro)$_6$]$_3$, followed by incubation with Fe^{3+}, resulted in a stabilized triple-helical structure (22 °C increase in T_m).[97] In similar fashion, addition of hydroxamic acid on the *C*-terminus of Boc-β-Ala-TRIS-[(Gly-Pro-Nleu)$_6$]$_3$, followed by incubation with Fe^{3+}, resulted in a stabilized triple-helical structure (7 °C increase in T_m).[98]

4 Triple-helical peptides of higher order structure

Collagen triple-helices are well known to interact and form higher order structures, such as fibrils. THPs have been found to form higher order structures based the presence of secondary amino acids and an appropriate distribution of hydrophobic and elżectrostatic residues.[99] Consequently, a number of approaches have been described by which triple-helical peptides are directed to form higher order structures. Longer THPs have been constructed using THP self-assembly driven by hydrophobic interactions at the *N*- and *C*-termini, for either pentafluoroPhe-(Gly-Pro-Hyp)$_{10}$-Phe or Phe-(Gly-Pro-Hyp)$_3$-Gly-Gln-Hyp-Gly-Leu-Hyp-Gly-Leu-Hyp-(Gly-Pro-Hyp)$_4$-Gly-Tyr.[100-102] The Phe-pentafluoroPhe stacking interaction resulted in THP aggregates of 260 nm in diameter which were fibril-like in nature.[100] Micron-length fibers of ~70 nm in diameter have been produced following thermal annealing of (Pro-Arg-Gly)$_4$-(Pro-Hyp-Gly)$_4$-(Glu-Hyp-Gly)$_4$.[103] In similar fashion, electrostatic interactions of the cystine knot THP (Gly-Pro-Pro)$_3$-Gly-Pro-Arg-Gly-Glu-Lys-Gly-Glu-Arg-Gly-Pro-Arg-(Gly-Pro-Pro)$_3$-Gly-Pro-Cys-Cys-Gly resulted in the formation of nanorods and microfibrils of 6 μm in length and 130 nm in width.[94] Metal coordination has been utilized to form microflorettes from THPs.[104] The (Pro-Hyp-Gly)$_9$ was modified by the addition of His$_2$ at the *C*-terminus and nitriloacetic acid (NTA) at the *N*-terminus. Introduction of Zn^{2+}, Cu^{2+}, Ni^{2+}, or Co^{2+} resulted in the formation of higher order structures; in the case of Zn^{2+}, microflorettes were observed with diameters >5 μm.[104]

To study platelet aggregation, THPs have been crosslinked *via* *N*- and/or *C*-terminal Cys or Lys residues to obtain higher order structures.[19,71,74,105-114] Cys crosslinking has been achieved *via* 3-(2-pyridyldithio)-propionic acid *N*-hydroxysuccinimide ester,[19,74,106,108-111,113,114] while Lys crosslinking has utilized glutaraldehyde or disuccinimidyl glutarate.[19,106,108]

Polymerization of THPs *via* native chemical ligation has created nanofibers of 10-20 nm.[115] In similar fashion, disulfide-linked trimeric peptides possessing self-complementary sequences have been designed and produced fibrils of >400 nm in length or supramolecules of up to 14 μm in diameter.[116,117] Self-complementary sequences have also been utilized to produce collagen-like gels from THPs.[118] The most straightforward construction of higher order structures was achieved by direct polycondensation of (Pro-Hyp-Gly)$_n$ with 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide and HOBt.[119] Final molecular weights of over 10 kDa were obtained for polycondensation of (Pro-Hyp-Gly)$_5$ and (Pro-Hyp-Gly)$_{10}$ in DMSO for 48 h at 20 °C, and similar results were obtained in phosphate buffer (pH = 7.4) using 50 mg ml^{-1} Pro-Hyp-Gly.[119] The poly(Pro-Hyp-Gly)$_{10}$ aggregates formed nanofiber-like structures of 10 nm in diameter.[119]

Radial growth of THPs has been achieved by metal-triggered assembly. Hyp was replaced with a bipyridyl-modified Lys (Byp) to create (Pro-Hyp-Gly)$_4$-Pro-Byp-Gly-(Pro-Hyp-Gly)$_4$, and association of THPs promoted by addition of Fe(II).[120] Fibers were obtained of 3-5 μm in length and ~10 nm in width. Combining the internal incorporation of Byp with His$_2$ at the *C*-terminus and nitriloacetic acid at the *N*-terminus [NTA-(Pro-Hyp-Gly)$_4$-Pro-Byp-Gly-(Pro-Hyp-Gly)$_4$-His-His-NH$_2$] produced a THP capable of forming a metal-triggered three-dimensional collagen-like network.[121]

A host-guest approach was used to examine supramolecular assemblies formed by THPs.[122] The host peptide was (Glu)$_5$-(Gly-Xxx-Hyp-Gly-Pro-Hyp)$_6$-(Glu)$_5$, and the guest residues were Ala, Pro, Ser, and Val. The authors have found that no supramolecular order occurs when Xxx = Ser, a similar, banded spherulite order was seen for Xxx = Ala or Pro, and a nematic order was observed for Xxx = Val. In addition, the (Glu)$_5$ ends were required for assembly formation. The supramolecular structures corresponded to those observed for type I collagen *in vitro*.

THP dendrimers have been constructed from *N*-(benzyloxycarbonyl)-tris(carboxyethoxymethyl)aminomethane (*Z*-TRIS-[OH]$_3$). *Z*-TRIS[OH]$_3$ was coupled to the *N*-terminus of THPs in solution, the Z group removed, and the dendrimer formed by addition of trimesoyl chloride.[123] THP dendrimers have also been assembled using a poly(amidoamine) dendrimer, with subsequent crosslinking by tissue transglutaminase.[124]

5 Structural stability studies

The use of THPs for X-ray crystallographic analysis of triple-helix conformation has been reviewed recently,[125] and thus will not be covered here. Of particular note is the continued evaluation of the collagen triple-helix as being either of 7/2-helical symmetry, 10/3-helical symmetry, or a combination of both.[126-128] As proposed based on structural analysis,[129] interstrand hydrogen bonds formed between the GlyN-H and XxxC=O are critical for triple-helix stability, as replacement of a central amide bond of (Pro-Pro-Gly)$_{10}$ with either an ester or (*E*)-alkene, or replacement of the central Pro-Pro with a Pro-*trans*-Pro isostere, substantially destabilizes the THP.[130-132]

One of the most intensive areas of THP research is the role of hydrogen bonding *versus* inductive effects in triple-helix stabilization.[5] The non-native amino acid 4*R*-Flp has been shown to induce hyperstability in the triple-helix of (Pro-4*R*-Flp-Gly)$_{10}$ compared to (Pro-4*R*-Hyp-Gly)$_{10}$.[133,134] Alternatively, 4*S*-Flp destabilizes the triple-helix of (Pro-4*S*-Flp-Gly)$_7$ compared to (Pro-4*R*-Hyp-Gly)$_7$.[135] More specifically, substitution of all of the 4*R*-Hyp residues in (Pro-4*R*-Hyp-Gly)$_{10}$ or (Pro-4*R*-Hyp-Gly)$_7$ by 4*R*-Flp increased T_m by 22 and 9 °C, respectively.[133-135] Conversely, an analogous substitution in (Pro-4*R*-Hyp-Gly)$_7$ by 4*S*-Flp dramatically decreased T_m by greater than 26 °C.[135] The relative effects of 4*R*-Hyp, 4*R*-Flp, and 4*S*-Flp coincide with

their propensity for forming *trans*-peptide bonds compared to *cis*-peptide bonds.[135,136] A similar correlation has been found using additional Pro and Hyp analogs.[137,138] This "inductive effect," based on the electronegativity and stereochemistry of the 4-substituent in the pyrrolidine ring system, is an important contributor to triple-helix stability.[133,134,136,139] $K_{trans/cis}$, the pucker of the pyrrolidine ring (*exo versus endo*), and the phi and psi torsion angles are interdependent parameters.[137,140] Consideration of these parameters provides a more complete understanding of the forces that contribute to the stability of the triple-helix.[125,141]

Thermodynamic analyses of Hyp- and Flp-containing THPs indicated that the enhanced stability of $(Pro\text{-}Hyp\text{-}Gly)_{10}$ compared to $(Pro\text{-}Pro\text{-}Gly)_{10}$ is enthalpically driven, while the enhanced stability of $(Pro\text{-}4R\text{-}Flp\text{-}Gly)_{10}$ compared to $(Pro\text{-}Pro\text{-}Gly)_{10}$ is entropically driven.[142,143] Based on these results, it was proposed that stabilization of triple-helices by Flp and Hyp are mechanistically distinct, with the former driven by hydrophobic effects and the latter by hydrogen bonding. However, studies with Dfp [which has a similar hydrophobicity as 4*R*-Flp but a $K_{trans/cis} = 3.6$, similar to Pro (4.6) but much lower than 4*R*-Flp (6.7)] demonstrated similar T_m values for THPs containing Dfp or Pro in the Yyy position, which were 6 °C lower than that observed for the 4*R*-Flp-containing THP.[55] Thus, hydrophobicity is not the dominant driving force in the stability of Flp-containing THPs.

The effects of single site substitution of naturally occurring amino acids on triple-helix stability have been examined previously using a host-guest approach, where either Pro in the Xxx or Hyp in the Yyy position of a Gly-Pro-Hyp triplet is replaced by the "guest" residue.[21-23] A comprehensive ranking of triple-helix propensity was established based on homotrimeric host-guest THPs.[11,22,23] Of the 20 natural amino acids studied, none provided enhanced stability compared with Gly-Pro-Hyp. The most stable residues in the Yyy position were Hyp > Arg > Met, while for the Xxx position Pro > charged residues > Ala > Gln.[22,23,125] An algorithm for the prediction of THP stability based on sequence was subsequently developed.[144] Surprisingly, 4*R*-Flp in the Yyy position was slightly destabilizing compared with Hyp within a host-guest THP, in contrast to $(Pro\text{-}4R\text{-}Flp\text{-}Gly)_n$ and $(Pro\text{-}Hyp\text{-}Gly)_n$ THPs.[145] It was suggested that different mechanisms are in place when Pro-4*R*-Flp-Gly is inserted within $(Pro\text{-}Hyp\text{-}Gly)_n$ compared with $(Pro\text{-}4R\text{-}Flp\text{-}Gly)_n$.[145]

THP host-guest studies revealed that electrostatic effects can contribute favorably to the stability of THPs.[125,144,146] Favorable electrostatic interactions were observed for Gly-Lys-Asp and Gly-Arg-Asp within the acetyl-$(Gly\text{-}Pro\text{-}Hyp)_3$-Gly-Xxx-Yyy-$(Gly\text{-}Pro\text{-}Hyp)_4$-Gly-Gly-$NH_2$ host.[147] Conversely, guests Gly-Arg-Lys, Gly-Lys-Arg, and Gly-Glu-Asp exhibited charge repulsion.[147] The sequence Gly-Pro-Lys-Gly-[Asp/Glu]-Hyp was found to be as stabilizing as $(Gly\text{-}Pro\text{-}Hyp)_2$, presumably due to interchain ion pairs.[148] Arg in the Yyy position can offer high stability, possibly based on its ability to form a hydrogen bond with C=O in a neighboring strand.[125,149] Heterotrimeric host-guest peptide studies demonstrated that an increased number of Arg residues decreased thermal stability for an acetyl-$(Gly\text{-}Pro\text{-}Hyp)_3$-Gly-Pro-Yyy-$(Gly\text{-}Pro\text{-}Hyp)_4$-Gly-Gly-$NH_2$ sequence.[92] For example, if Yyy = Hyp in all three chains, $T_m = 47.2$ °C, whereas replacement of Hyp by Arg in one chain, two chains, or three chains resulted in $T_m = 44.5$, 40.8, and 37.5 °C, respectively. The role of electrostatic effects for stabilizing the collagen-like domain of *Streptococcus pyogenes* cell surface protein Scl2 was examined using the THP acetyl-$(Gly\text{-}Pro\text{-}Hyp)_3$-Gly-Lys-Asp-Gly-Lys-Asp-Gly-Gln-Asn-Gly-Lys-Asp-Gly-Pro-Leu-$(Gly\text{-}Pro\text{-}Hyp)_4$-Gly-Tyr-$NH_2$.[150] The Gly-Lys-Asp-Gly-Lys-Asp motif was found to contribute greater stability to the triple-helix than what was predicted based on host-guest studies (see above), and stability was pH dependent, indicating that electrostatic stabilization was an important mechanism for the Scl2 triple-helix.[150]

The effects of D-amino acids on triple-helical stability was examined using the guest triplets Gly-Asp-Hyp and Gly-Asp-Ala with either D- or L-Asp.[151] The D-Asp was significantly destabilizing for the triple-helix, but could be accommodated.[151] Triple-helical peptide-amphiphile substrates were also able to accommodate fluorogenic D-amino acids.[152]

The non-native amino acid *trans*-4-amino-L-proline is more stabilizing than Hyp for Phe-$(Pro\text{-}Yyy\text{-}Gly)_6$ triple-helices.[43] Interestingly, β-D-galactose glycosylation of Thr in the Yyy position of $(Gly\text{-}Pro\text{-}Yyy)_{10}$ greatly enhances triple-helical stability compared to Thr.[153-155] In turn, replacement of the central Gly residue of $(Pro\text{-}Hyp\text{-}Gly)_7$ with either D-Ala or D-Ser resulted in no triple-helix formation in an aqueous environment.[156]

A host-guest approach was used in which $(Pro\text{-}Hyp\text{-}Gly)_7$ was the control peptide, to examine the role of tertiary amides for the stability of triple-helical structure.[157] The middle Pro-Hyp-Gly triplet was substituted by *N*-methylalanine (meAla) in the Xxx or Yyy position, Ala in the Xxx or Yyy positions, or Pro in the Yyy position. Overall, meAla was found to be more destabilizing in either the Xxx or Yyy position than Ala, leading the authors to conclude that the presence of a tertiary amide is not sufficient for conformational stabilization of the triple-helix. These conclusions were at odds with prior results where *N*-isobutylglycine (Nleu) did not dramatically destabilize triple-helical structure, and in fact enhanced it compared with Pro. The authors proposed that Nleu may have conformational preferences that are similar to Pro and Hyp, while *N*-methylglycine (Sar) and *N*-methylalanine (meAla) do not. In a related study, acetyl-$(Gly\text{-}Nleu\text{-}Pro)_3$-Gly-Xxx-Pro-$(Gly\text{-}Nleu\text{-}Pro)_3$-$NH_2$ was used as a host, with the guest being a variety of alkyl and aralkyl peptoid residues.[158] Interestingly, virtually all peptoid residues could be incorporated and stable triple-helices maintained.

Mizuno *et al.* have further examined the stability of triple-helices incorporating 4*R*-Hyp in the Xxx position.[159] For many years, it was believed that peptides containing 4*R*-Hyp in the Xxx position of Gly-Xxx-Yyy repeats did not form collagen-like triple-helices. This group previously demonstrated that acetyl-$(Gly\text{-}4R\text{-}Hyp\text{-}Thr)_{10}$-$NH_2$ forms a stable triple-helix.[154] The Yyy position was varied to Ser, Val, Ala, and alloThr to evaluate the specific interactions that stabilize Gly-4*R*-Hyp-Yyy triple-helices. Only the Thr and Val containing peptides formed stable triple-helices in water. Thus, the contributions to triple-helix stability appear to be both the hydroxyl and methyl groups of Thr, as well as their stereochemical configuration. Thermodynamic parameters further indicated that the hydroxyl group of Thr alone does not account for increased triple-helix stability. Molecular modeling showed that the Thr methyl group covers the interchain hydrogen bond between the carbonyl group of Hyp of the adjacent chain and the amino group of the next Gly residue in the same chain. The distance between the Thr hydroxyl group and the Hyp hydroxyl group is longer than found in typical hydrogen bonds.

Host-guest THPs incorporating either Gly-Trp-Hyp or Gly-Pro-Trp have been studied by a variety of fluorescence measurements.[160] Side chains in the Xxx position were found to be more solvent accessible and considerably more mobile than side chains in the Yyy position.[160]

The host-guest approach has also been used to examine triple-helix nucleation.[23,161] The fast folding THPs were those with Hyp in the Yyy position of (Gly-Pro-Hyp)$_3$-Gly-Xxx-Yyy-(Gly-Pro-Hyp)$_4$.[161] Folding kinetics have also been found to be affected by chain register in heterotrimeric sequences.[162]

Multiple hereditary connective tissue diseases have been linked to collagen mutations.[63] These mutations most often disrupt collagen folding, resulting in its defective structure and function.[6,8,163-165] Mutations of type I collagen genes have been identified in both osteogenesis imperfecta (OI) and Ehlers-Danlos syndrome (EDS).[1,6,8,163-167] OI dominant-negative mutations can occur in either gene that encode the α chains of type I collagen and are typically missense mutations that change the Gly codons in the triple-helical motifs. Gly substitutions result in different effects on helix stability, depending on their location and the newly substituted amino acid.

THPs have been designed to house various Gly substitutions, and NMR and CD spectroscopic studies of such peptides permit the folding upstream, downstream and at the mutation site to be examined.[164,168] By using mutant THPs, steps in the normal nucleation and propagation of triple-helical structures have been elucidated.[169-175] NMR, CD, and X-ray crystallographic studies lend insight into the genotype-phenotype relationship of different collagen mutations with severity of associated disease.[129,176-178] For example, host-guest studies utilizing the THP acetyl-(Gly-Pro-Hyp)$_3$-Zzz-Pro-Hyp-(Gly-Pro-Hyp)$_4$-Gly-Gly-NH$_2$ indicated that destabilization by individual residues compared to Zzz = Gly correlated to the severity of OI mutations, based on the α1(I) chain.[178]

(Pro-Hyp-Gly)$_4$-Pro-Hyp-Ala-(Pro-Hyp-Gly)$_5$, which contains a single interruption of the Gly-Pro-Hyp repeat (Ala for Gly mutation), has a T_m = 28 °C compared to T_m = 60 °C for (Pro-Hyp-Gly)$_{10}$.[169,171] A combination of ^{1}H-NMR and X-ray crystallographic experiments has shown (Pro-Hyp-Gly)$_4$-Pro-Hyp-Ala-(Pro-Hyp-Gly)$_5$ to be only 70-75% triple-helical, with the "interrupted" region a distortion of the triple-helix.[129,169,170] Direct interstrand hydrogen bonds were replaced by interstitial water bridges at the site of this interruption, and hence thermal destabilization of this peptide was due solely to entropic factors.[129,169] Flanking sequences have substantial effects on the relative destabilization of Gly mutations.[177,179] Additional studies have been performed with THPs that model Gly to Ala, Ser, or Cys mutations. Triple-helix nucleation was found to occur on either side of such mutations, but that different nucleation mechanisms may be invoked.[180] The ability to nucleate the triple-helix on the *N*-terminal side of a mutation depends on sequence, with Hyp-rich sequences favoring nucleation.[181]

Analysis of host-guest heterotrimeric constructs have revealed that the greatest decrease in triple-helix stability occurs upon the first mutation of a Gly residue in a single chain, with subsequent mutations in the other two chains only slightly decreasing stability.[182] NMR studies of a Gly to Ala or Ser substitution revealed that hydrogen bonding at the mutation site may involve only one chain, and greater conformational flexibility was observed *C*-terminal to the substitution.[183]

The effects of Gly mutations on mineralization were examined using self-assembled monolayers of THPs. Within the sequence Cys-Gly-Lys-Hyp-(Gly-Pro-Hyp)$_2$-Gly-Glu-Hyp-(Gly-Pro-Hyp)$_2$-Gly-Arg-Hyp-**Gly**-Pro-Hyp-Gly-Pro-Hyp-Gly-Asp-Hyp-(Gly-Pro-Hyp)$_5$, replacement of **Gly** by Ala had little effect on mineral thickness, but replacement by Val or Asp resulted in thinner calcium phosphate minerals.[184] The Ala substitution did produce a lower Ca/P ratio (similar to that observed in OI), while Val or Asp substitution produced higher Ca/P ratios.[184]

Other types of interruptions, such as Gly or Hyp deletions, may have different molecular mechanisms then Gly mutations for their destabilizing effects.[171] THPs have been utilized to study natural breaks in the Gly-Xxx-Yyy repeat that occur in collagens, such as type IV. The most well studied is the Gly-Pro-Hyp-Gly-Ala-Ala-Val-Met-Gly-Pro-Hyp-Gly-Pro-Hyp sequence found in the α5(IV) chain (residues 386-399), where 4 residues are found between Gly residues in the break (G4G interruption). It was observed that the Gly-Pro-Hyp regions that flank the break conform to normal triple-helical structure, and hydrophobic interactions replace the Gly packing within the break a preserve a pseudo-triple-helix.[185,186] Replacement of Gly-Ala-Ala at the site of the break by Gly-Pro-Hyp further decreases the stability of the triple-helix.[185] This suggests that secondary amino acids are unfavorable within a break,[185] a trend that was observed with other triple-helical break sequences of G1G interruptions.[187] However, one distinction of G4G *versus* G1G interruptions is that the hydrophobic residue in the former packs near the helix central axis, while the hydrophobic residue in the latter is found on the helix surface.[187] G1G interruptions create a local region of flexibility within the triple-helix, a process which has been suggested to be important for molecular recognition.[188]

The collagenous domain of mannose binding lectin possesses a Gly54Asp mutation at a frequency as high as 30%.[189] Studies with THPs demonstrated that the mutation only slightly decreased the stability of the triple-helix (T_m difference of 2 °C), but the sequence *N*-terminal to the mutation was disordered compared to the parent THP.[189] The mutation occurs at a junction between highly ordered and weakly ordered helical regions, and thus the mutation is less destabilizing than Gly mutations observed in OI.[189]

6 Adhesion receptor binding to collagen

A great variety of cell surface receptors have been recognized for their collagen binding activity. Cell binding is often facilitated by triple-helical conformation.[190] Identification of collagen binding sites has been aided by THP libraries, including one that covers charge clustered sites in the α1(I) collagen chain[48] and the types II and III collagen Toolkits.[191] The best-characterized cell surface adhesion molecules are integrins, which are heterodimeric proteins composed of one α and one β subunit. The collagen binding integrins include α1β1, α2β1, α3β1, α10β1, and α11β1.[192,193] In an effort to better understand the roles of individual integrins, numerous triple-helical binding sites for the collagen binding integrins have been identified (Table 1). The Gly-Phe-Hyp-Gly-Glu-Arg motif, in triple-helical conformation, has been shown to bind to the α2β1 and α11β1 integrins and recombinant α1 and α2 A-domains.[38,77,109,194-198] The co-crystal structure of a THP

Table 1 Sequences of receptor binding sites within types I-IV collagen. O = 4*R*-Hyp

Receptor	Binding site location	Sequence
α1β1, α2β1, α11β1	α1(I)502-507	GFOGER
α1β1, α2β1	α1(III)64-69	GROGER
α1β1, α2β1	α1(IV)382-393	GAOGFOGERGEK
α1β1, α2β1	α1(I)127-135	GLOGERGRO
α1β1	α1(IV)437-448	GPPGDQGPPGIP
	α2(IV)454-465	GAKGRAGFPGLP
α2β1	α1(I)433-438	GADGEA
α2β1	α1(III)115-120	GLOGEN
α2β1	α1(III)522-528	GGPOGPR
α3β1	α1(IV)531-543	GEFYFDLRLKGDK
α2β1, α11β1	Scl1 collagen-like region	GLPGER
GpVI, LAIR-1	α1(III)517-543	GAOGLRGGAGPOGPEGGKGAAGPOGPO
CD44	α1(IV)1263-1277 [IV-H1]	GVKGDKGNPGWPGAP
DDR2	α1(III)397-408	GPRGQOGVMGFO

For type IV collagen, sequence numbers are based on the human α1(IV) and α2(IV) genes.[324]

incorporating Gly-Phe-Hyp-Gly-Glu-Arg and the α2 A-domain revealed that the Glu residue directly coordinated the α2 A-domain metal ion (Mg^{2+}), the Arg residue formed a salt bridge with an Asp residue in the α2 A-domain, and Phe was involved in hydrophobic interactions with the receptor.[195] Changes in receptor conformation upon ligand binding appear initially induced by changes in metal ion coordination.[195,199] Binding also results in changes in the triple-helix main-chain conformation and bending of the triple-helix.[200] The Gly-Phe-Hyp-Gly-Glu-Arg motif is found within type I collagen at α1(I)502-507 and type IV collagen at α1(IV)385-390. A THP model of α1(I)502-516 binds to platelets, a model of α1(I)496-507 binds to endothelial cells, and models of α1(IV)382-393 bind to HT-1080 and melanoma cells.[38,109,201,202]

Other collagen binding motifs have been identified for the α2β1 integrin. Gly-Leu-Hyp-Gly-Glu-Arg from type I collagen has been identified as a ligand for the α1β1 integrin, α1 and α2 A-domains, and α2 I-domain,[38,196] while the Gly-Arg-Hyp-Gly-Glu-Arg motif from type III collagen binds the α1 and α2 I-domains (Table 1).[203,204] Gly-Leu-Pro-Gly-Glu-Arg from the collagen-like region of Scl1 has been shown to bind the α2β1 and α11β1 integrins (Table 1).[205] Gly-Gly-Pro-Hyp-Gly-Pro-Arg from α1(III)522-528 and Gly-Leu-Hyp-Gly-Glu-Asn from α1(III)115-120 binds the α2β1 integrin (Table 1).[71,72,204] Based on the model of type I collagen fibers, the Gly-Phe-Hyp-Gly-Glu-Arg and Gly-Leu-Hyp-Gly-Glu-Arg sequences are accessible for integrin binding.[206]

The α1β1 integrin, either isolated from human placenta or recombinant, simultaneously binds Asp441 from two α1(IV) chains and Arg458 from the α2(IV) chain.[207-209] This type IV collagen region is believed to be a higher affinity α1β1 integrin binding site than the Gly-Phe-Hyp-Gly-Glu-Arg motif found in type IV collagen,[194] although the K_D value for integrin binding to the heterotrimeric THP was 20 μM while binding to homotrimeric Gly-Phe-Hyp-Gly-Glu-Arg occurred with K_D = 1.45 μM.[209-211] Proline hydroxylation is required for α1β1 integrin binding.[212]

The platelet α2β1 integrin binds to the Asp-Gly-Glu-Ala motif in some THP constructs[213] but not others.[109] The melanoma cell α3β1 integrin binds to α1(IV)531-543.[67,69,214,215] Binding of the α3β1 integrin to collagen-derived substrate is not dependent upon triple-helical conformation,[67,214,215] unlike the α1β1 and α2β1 integrins.[38]

Another collagen-binding receptor is the proteoglycan CD44. The overexpression of CD44 is found on a variety of tumor cells,[216] and elevated CD44 expression by 4 to 6-fold is associated with tumor growth and metastasis.[217] CD44 in the chondroitin sulfate proteoglycan (CSPG) modified form is among the receptors uniquely overexpressed in metastatic melanoma.[216] Interestingly, CD44 has recently been revealed as a cancer stem cell marker for at least 6 different tumor types.[218-220] A theory is emerging that CD44 positive cells within a tumor display true stem cell properties such that one cell can give rise to an entire tumor.[218] Additionally, CD44 interaction with hyaluronan induces ankyrin binding to MDR1 (*P*-glycoprotein), resulting in the efflux of chemotherapeutic agents and chemoresistance in tumor cells.[221]

The type IV collagen α1(IV)1263-1277 sequence (Table 1; designated [IV-H1]) promotes melanoma cell adhesion, spreading, and signaling.[33,37,46,62,222-224] Affinity chromatography studies with single-stranded and triple-helical [IV-H1] peptides resulted in the isolation of melanoma cell CD44 receptors, in the chondroitin sulfate proteoglycan (CSPG) form.[62,225,226] Loss of triple-helical structure dramatically reduces melanoma cell adhesion, spreading, and signaling modulated by this ligand.[33,37,46] Melanoma cell responses to the triple-helical [IV-H1] sequence have been compared for the galactosylated *versus* non-galactosylated ligands.[62] Galactosylation was found to strongly modulate adhesion and spreading, both of which were dramatically decreased due to the presence of a single sugar. This study was the first demonstration of the prophylactic effects of ligand glycosylation on tumor cell interaction with the basement membrane, while related reports have shown that (a) tumor cell surface sialic acid reduced binding to type IV collagen[227] and (b) decreased laminin binding glycans results in increased prostate and breast carcinoma motility.[228]

There is a family of receptor tyrosine kinases (RTKs) that have extracellular domain motifs homologous to the dictyostelium discoideum protein discoidin-I.[229] The RTKs with discoidin-I homology have been named discoidin domain receptors (DDRs).[230] Two distinct DDRs have been characterized. DDR1 is expressed in tumor cells themselves, while DDR2 has been detected in the stromal cells surrounding the tumor.[229] Numerous breast carcinoma cell lines, as well as other carcinomas, have been shown to highly express DDR1.[229,231,232]

DDR1 and DDR2 are activated by fibrillar collagens, *i.e.* Tyr phosphorylation of DDR is substantially increased.[230,232,233] Triple-helical conformation is required for collagen to serve as a ligand for DDR1 and DDR2.[230,233,234] The specificity of DDR interaction with collagen is also dependent upon the ligand carbohydrate content. Deglycosylation of collagen by treatment with sodium *m*-periodate resulted in significant reduction of collagen-induced stimulation of DDR.[230] Thus, collagen requires both an intact triple-helical conformation and glycosylation for optimum induction of DDR.

The type III collagen Toolkit was utilized to identify the Gly-Pro-Arg-Gly-Gln-Hyp-Gly-Val-Met-Gly-Phe-Hyp binding site for DDR2 (Table 1).[191,235] Autophosphorylation of DDR2 was induced by this THP.[235] Interestingly, this site bound to DDR2 and induced activation even though it does not contain a glycosylated Hyl residue, and thus appears at conflict with prior studies (see above).

THPs containing Gly-Pro-Hyp repeats alone have been shown to bind platelet glycoprotein VI (GpVI), with two repeats of Gly-Pro-Hyp representing the smallest binding motif.[74,110,111,113] Proline hydroxylation is required for GpVI binding.[212] However, it has been noted that simple Gly-Pro-Hyp repeating sequences are not found in native collagens, and thus GpVI interaction with collagens is more complex than THP models initially revealed.[206] The GpVI binding sequence Gly-Ala-Hyp-Gly-Leu-Arg-Gly-Gly-Ala-Gly-Pro-Hyp-Gly-Pro-Glu-Gly-Gly-Lys-Gly-Ala-Ala-Gly-Pro-Hyp-Gly-Pro-Hyp from type III collagen was recently identified using the Toolkit (Table 1).[114]

Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) binds types I and III collagen with high affinity (K_D = 14.2 and 16.2 nM, respectively).[236] THP models showed LAIR-1 binding to $(Gly\text{-}Pro\text{-}Hyp)_{10}$ but not $(Gly\text{-}Pro\text{-}Pro)_{10}$.[236] Use of the types II and III collagen Toolkits resulted in several binding sites identified for LAIR-1 and LAIR-2 (Table 1).[237] Although a common motif was not observed, the presence of 2-4 Gly-Pro-Hyp repeats within binding sites was found.[237]

Staphylococcus aureus possesses a microbial surface component recognizing adhesive matrix molecule (MSCRAMM), referred to as CNA, that binds to collagen. CNA binds to several THPs, and a crystal structure was obtained for the complex of $CNA_{31\text{-}344}$ and $(Gly\text{-}Pro\text{-}Hyp)_4$-Gly-Pro-Arg-Gly-Arg-Thr-$(Gly\text{-}Pro\text{-}Hyp)_4$.[238] The interactions between the CNA and the THP occurred in the Gly-Pro-Hyp regions, primarily due to hydrophobic interactions with Pro residues and hydrogen bonding with Hyp residues.[238] In similar fashion, the *Yersinia enterocolitica* adhesion A (YadA), a collagen binding trimeric autotransporter, binds to $(Pro\text{-}Hyp\text{-}Gly)_{10}$ with K_D = 0.17-0.28 μM.[239] Specific sequence specificity for YadA binding was not observed.

The binding affinities of receptors for THPs have been determined in several studies. The α1β1 integrin binds to α1(I)127-138 and α1(I)496-507 THPs with K_D = 7.0 and 1.7 μM, respectively.[38] The α2 I-domain binds to α1(I)127-138 and α1(I)496-507 THPs with K_D = 0.40 and 1.1 μM, respectively.[38] The Gly-Arg-Hyp-Gly-Glu-Arg motif is bound by the α1 and α2 I-domains with K_D = 23 and 283 nM, respectively.[203] As mentioned above, the α1β1 integrin binds to [α2(IV)454-465][α1(IV)437-448][α1(IV)437-448] THP with K_D = 20 μM.[209] THP models of α3β1 integrin and CD44/CSPG binding sites have $[cell\ adhesion]_{50}$ values in the range of 0.5-5 μM.[37,46,48,62,67] Two triple-helical peptides derived from type I collagen α1(I) chain sequences were found to inhibit α2 A-domain binding to type I collagen with IC_{50} values of ~5-10 μM.[196] The recombinant α2 A-domain was shown to bind to type I collagen with K_D ~ 6-10 μM.[196] The α1 A-domain has two classes of binding sites within type I collagen, with the higher affinity site exhibiting a K_D = 0.11 μM.[196] Thus, THPs have been shown to have cell binding affinities in the same range as native collagens.

The binding of cell surface receptors to extracellular matrix proteins induces intracellular signaling and ultimately alterations of cellular phenotypes. THPs have been utilized to elucidate collagen-induced cell signaling pathways.[33,38,69,107,110,198,202,206,240-242] Induction of Tyr phosphorylation of focal adhesion kinase (p125FAK) was enhanced and the time of induction was shortened when the α3β1 integrin ligand was in triple-helical conformation.[69] The clustered THP ligand induced more rapid paxillin Tyr phosphorylation than the single-stranded ligand. In addition, paxillin bound directly to pp125FAK. Overall, these studies showed that (a) a model of an isolated sequence from type IV collagen, α1(IV)531-543, can induce α3β1 integrin-mediated signal transduction in melanoma cells and (b) ligand conformation (secondary, tertiary, and/or quaternary structure) can directly influence several α3β1 integrin-mediated signal transduction events.[69] Melanoma cell signal transduction *via* CD44/CSPG has also been studied.[33] A combination of ligand (and hence cell surface receptor) clustering and triple-helical conformation was required for maximum induction of p125FAK phosphorylation by [IV-H1].

THPs were used to examine the expression of matrix metalloproteinases (MMPs) *via* outside-in melanoma signaling.[201,202] Gene expression and protein production of matrix metalloproteinase-1 (MMP-1), MMP-2, MMP-3, MMP-13, and MT1-MMP were modulated with the α2β1-specific THP, whereas the CD44-specific THP yielded significant stimulation of MMP-8 and lower levels of modulation of MMP-1, MMP-2, MMP-13, and MT1-MMP. The profile seen in response to α2β1 integrin engagement is consistent with high invasion potential, in that the MMPs upregulated can participate in the dissolution of basement membrane (type IV) and type I collagen. These results were indicative of specific activation events that tumor cells undergo upon binding to select regions of basement membrane collagen. THP ligands were found to provide a general approach for monitoring the regulation of proteolysis in cellular systems.

The α2β1 integrin THP ligand derived from type I collagen residues α1(I)496-507 was examined for induction of human aortic endothelial cell (HAEC) activation.[198] In addition, a "mini-extracellular matrix" (mini-ECM) composed of a mixture of the α1(I)496-507 ligand and a second, α-helical ligand incorporating the endothelial cell proliferating region of SPARC (**s**ecreted **p**rotein **a**cidic and **r**ich in **c**ysteine) was studied for induction of HAEC activation. Following HAEC adhesion to α1(I)496-507, mRNA expression of E-selectin-1, vascular cellular adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and monocytic chemoattractant protein-1 (MCP-1) was stimulated, while that of endothelin-1 was inhibited. ELISA analysis demonstrated that E-selectin-1 and MCP-1 protein expression was also stimulated, whereas endothelin-1 protein expression diminished. Engagement of the α2β1 integrin *initiated* a HAEC response similar to that of TNFα-induced HAECs, but was not sufficient to induce an inflammatory response. Addition of the $SPARC_{119\text{-}122}$

region had only a slight effect on HAEC activation. Other cell-ECM interactions appear to be required in order to elicit an inflammatory response in HAECs.

The α1(I)496-507 THP was also found to inhibit tube formation, as induced by human umbilical vein endothelial cell (HUVEC) binding to type I collagen.[38] Tube formation, which is a model for angiogenesis, was found to proceed *via* the α2β1 integrin through the p38 MAPK pathway.

The crosslinked THPs Gly-Lys-Hyp-(Gly-Pro-Hyp)$_{10}$-Gly-Lys-Hyp-Gly and Gly-Cys-Hyp-(Gly-Pro-Hyp)$_{10}$-Gly-Cys-Hyp-Gly bind to platelet GpVI (see earlier discussion), resulting in activation of the αIIbβ3 integrin and platelet aggregation.[71,106,240] Downstream events linked to GpVI binding include Ca^{2+} mobilization, phosphatidylinositol-3-kinase induced activation of phopholipase C (PLC), activation of protein kinase C, and release of arachidonic acid.[71,106,110,240,242] The (Gly-Pro-Hyp)$_{10}$ motif can induce Tyr phosphorylation of Syk and PLCγ2, but crosslinking or higher order THP structures are required for 5-hydroxytryptamine secretion and platelet activation.[101,107,111] (Gly-Pro-Hyp)$_{10}$ has been shown to induce platelet aggregation when linked to amino-functionalized latex nanoparticles, but acetyl-(Gly-Pro-Hyp)$_7$-Gly does not.[243]

7 Triple-helical substrates

Alterations in activities of one family of proteases, the MMPs (Fig. 5), have been implicated in primary and metastatic tumor growth, angiogenesis, and pathological degradation of extracellular matrix components, such as collagen and laminin.[244-246] The "collagenolytic" MMPs [MMPs that catalyze the hydrolysis of one or more of the interstitial collagens (types I-III) within their triple-helical domain] include the secreted proteases MMP-1, MMP-2, MMP-8, MMP-9, and MMP-13 and the membrane-bound proteases MT1-MMP and MT2-MMP.[247-250] In the cases of MMP-1, MMP-8, MMP-13, MT1-MMP, and MT2-MMP, efficient collagenolytic activity for the isolated enzyme requires both the catalytic (CAT) and hemopexin-like (HPX) domains.[251-256] The linker region between these domains also participates in collagenolysis, either by direct binding of substrate[257] or by allowing for the proper orientation of the catalytic and hemopexin-like domains.[258] The gelatinase members of the MMP family (MMP-2 and MMP-9) possess three fibronectin type II (FN II) inserts within their CAT domains, and these inserts possess similar type I collagen binding sites.[259,260] While collagenolytic MMPs possess common domain organizations, there are subtle differences in their processing of triple-helical substrates.

Our laboratory has previously described a number of fluorescence resonance energy transfer (FRET) THP substrates (fTHPs) based on a consensus types I-III collagen 769-783 region that are either suitable for most collagenolytic MMPs or selective for different collagenolytic MMPs (Table 2).[36,39-41,152,201,202,256,261-263] In addition, we have developed a selective MMP-2/MMP-9/MMP-12 THP substrate, α1(V)436-447 fTHP [(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Pro-Gly~Val-Val-Gly-Glu-Lys(Dnp)-Gly-Glu-Gln-(Gly-Pro-Hyp)$_5$-NH_2].[201] In all cases,

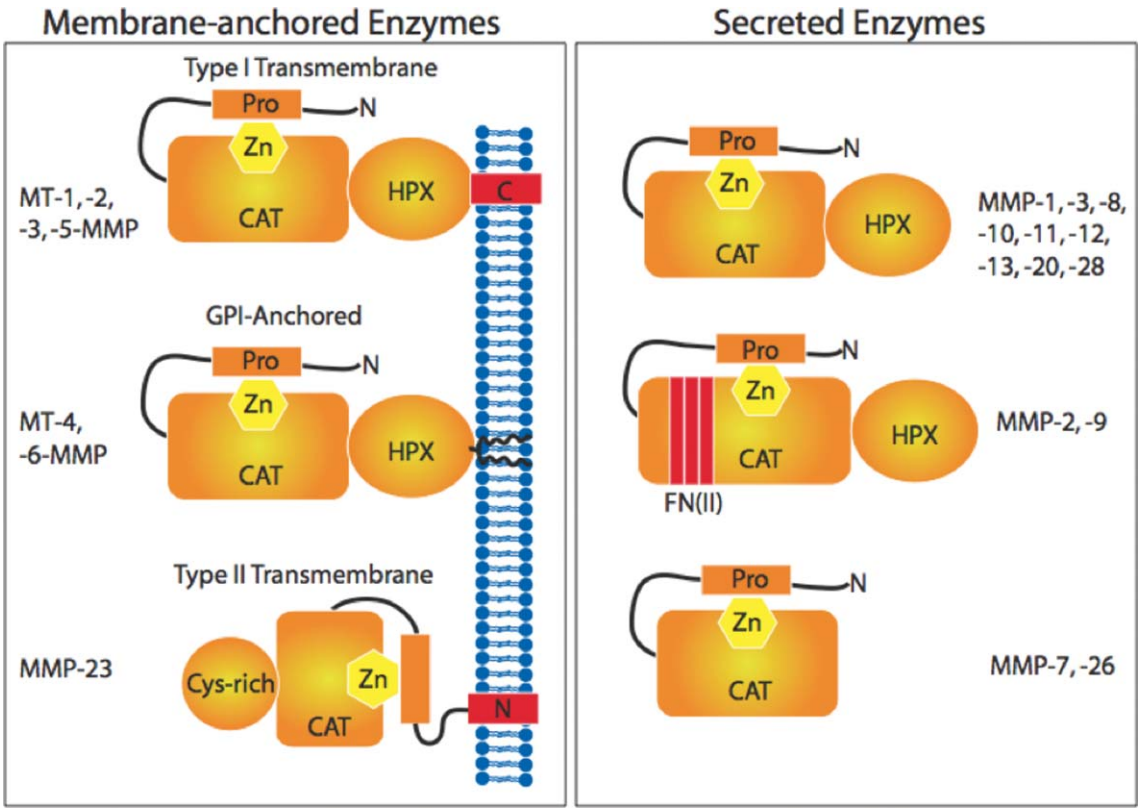


Fig. 5 MMP family members and their structural domains. Reproduced from reference 285 by permission of the American Society for Biochemistry and Molecular Biology.

Table 2 Sequences and stabilities of C_n-(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-P_2-P_1~P_1'-P_2'-P_3'-P_4'-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2 fTHPs

fTHP	P_2-P_1~P_1'-P_2'-P_3'-P_4 Sequence	Peptide T_m/°C	C_6-Peptide T_m/°C	C_{10}-Peptide T_m/°C
fTHP-4	Gln-Gly~Leu-Arg-Gly-Gln	36.5	ND	43.0
fTHP-9	Gln-Gly~**Cys(Mob)**-Arg-Gly-Gln	47.2	ND	60.0
fTHP-10	**Orn**-Gly~Leu-Arg-Gly-Gln	56.6	ND	62.0
fTHP-11	**Orn**-Gly~**Cys(Mob)**-Arg-Gly-Gln	44.2	ND	47.0
fTHP-12	**Leu**-Gly~**Met**-Arg-Gly-Gln	45.0	51.0	ND
fTHP-13	**Val-Asn**~**Phe**-Arg-Gly-Gln	39.0	50.0	ND
fTHP-14	**Val-Asn**~**Phe**-Arg-Gly-**Pro**	ND	46.0	ND

Substitutions relative to fTHP-4 are indicated in bold. ND = not determined, Mob = 4-methoxybenzyl.

FRET is achieved by incorporating (7-methoxycoumarin-4-yl)acetyl (Mca) as the fluorophore in the P_5 position and 2,4-dinitrophenyl (Dnp) as the quencher in the P_5' position (Fig. 6). Due to the differing sequences of the fTHPs, their relative triple-helical thermal stabilities can vary (Table 2). In order to obtain fTHPs that are stable under near-physiological conditions, as well as to examine the effects of substrate thermal stability on MMP activity, we utilized our previously described peptide-amphiphile approach.[14,15,31,32] In addition, the Lys branching protocol has been utilized for the construction of triple-helical MMP substrates in the Fields laboratory,[34-36,264] while the Moroder laboratory has utilized the double disulfide knot to create triple-helical MMP substrates.[3,78,79,89,90]

Through the use of the substrates described in Table 2, we can assign unique triple-helical peptidase behaviors to most of the collagenolytic MMPs.[34-36,39-41,201] MMP-1 has the weakest triple-helical peptidase activity, and has great difficulty in hydrolyzing more thermally stable substrates. MMP-1 does not tolerate long chain, hydrophobic residues interacting with its S_1' subsite nor positively charged residues interacting with the S_2 subsite. MMP-2 possesses many of the characteristics described for MMP-1, but additionally will cleave Gly-Gln bonds. Unlike MMP-1, MMP-2 prefers the combination of Leu in the substrate P_2 subsite and Met in the P_1' subsite compared to Gln and Leu in these respective subsites. MMP-8 is a robust triple-helical peptidase that favors long chain, hydrophobic residues interacting with its S_1' subsite. MMP-9 activity is similar to that of MMP-2, except that cleavage of Gly-Gln bonds is not observed. MMP-13 processes thermally stable sequences efficiently, with little effect of sequence. In contrast to MMP-1, MMP-8, and MT1-MMP, MMP-13

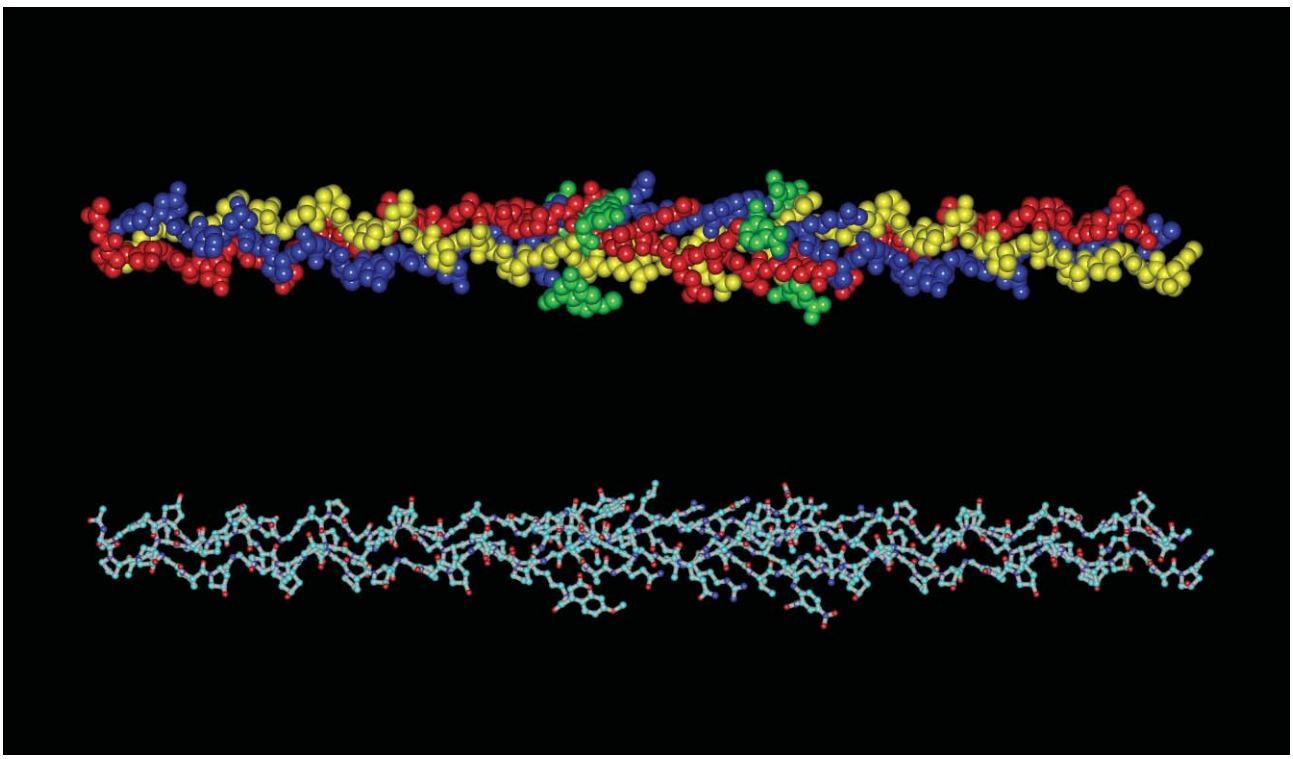


Fig. 6 Space-filling (top) and "ball and stick" (bottom) computer generated models of fTHP-4, 3[(Gly-Pro-Hyp)$_5$-Gly-Pro-Lys(Mca)-Gly-Pro-Gln-Gly~Leu-Arg-Gly-Gln-Lys(Dnp)-Gly-Val-Arg-(Gly-Pro-Hyp)$_5$-NH_2]. For the space-filling model, the individual peptide strands are red, blue, and yellow, while the fluorophore (Mca) and quencher (Dnp) are green. Reproduced from reference 268 by permission of Wiley & Sons.

prefers a positively charged residue interacting with the S_2 subsite, and may favor certain interrupted triple-helical sequences over uninterrupted ones. MT1-MMP is the most robust triple-helical peptidase, and is reasonably sensitive to substrate thermal stability. It disfavors positively charged residues interacting with the S_2 subsite, and is also relatively ineffectual at processing interrupted triple-helical sequences that are cleaved by soluble MMPs. MT2-MMP behaves similarly to MT1-MMP, except that it is less active.

THPs have been utilized recently to identify residues in the HPX domain that participate in collagenolysis.[263] MMP-1 HPX residues Ile290 and Arg291 were found to contribute to recognition of triple-helical structure, and facilitate both the binding and catalysis of the triple-helix.[263] MMP-1 CAT and HPX domains collaborate in collagen catabolism by properly aligning the triple-helix and coupling conformational states to facilitate hydrolysis.[263] The HPX domain is not critical for triple-helical peptidase activity of shorter substrates (such as fTHP-4).[34,36,263] However, as the THP substrate becomes longer and more "collagen-like", the HPX domain becomes more significant for facilitating proteolysis.[79,89,263] The *C*-terminally extended THP used in reference 263 includes Arg-Gly-Glu-Arg residues in subsites P_{14}'-P_{17}'; molecular modeling of fibrillar collagen found this sequence to interact with the MMP-1 HPX domain.[265]

Mammalian collagenases cleave interstitial (types I-III) collagen into 1/4 and 3/4 length fragments. Peptide substrate studies have shown that collagen primary structure is not the basis for discriminatory MMP collagenolytic behavior.[266] For example, MMP-3 cleaves a single-stranded peptide model of the collagen cleavage site at a rate comparable to MMP-1, but MMP-3 does not cleave this sequence in native collagen.[266] Studies with THPs have allowed for the further refinement of a previously described model of the MMP cleavage sites in interstitial collagens.[267,268] This model suggested that all of the information necessary for efficient hydrolysis of collagen is contained in a 24 residue stretch.[267] Cleavage site regions are distinguished by a low content (<10%) of charged residues in addition to being "tightly" triple-helical (high secondary amino acid content) prior to the cleavage site and "loosely" triple-helical (low secondary amino acid content) following the cleavage site (Fig. 7).[267,269,270] One of the implications of this model is that low secondary amino acid content creates a triple-helical region that is distinct, and more flexible, than a high secondary amino acid content region.

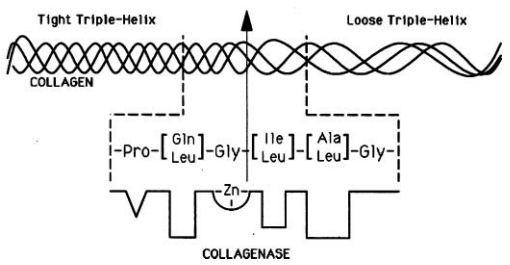


Fig. 7 Model of the collagenase cleavage site in interstitial collagens. The four triplet region that precedes the scissile (Gly~Ile/Leu) bond is rich in secondary amino acids (50% of the Xxx + Yyy residues, Pro always found in subsite P_3), and has a low average side-chain molal volume (<45 ml). The four triplet region that follows the scissile bond is secondary amino acid deficient (a maximum of 2 secondary amino acid residues, not in neighboring triplets). The overall 25 amino acid residue region is hydrophobic, containing a maximum of 2 charged residues. Reproduced from reference 261 by permission of Wiley & Sons.

Evidence to support the MMP cleavage site model includes the detection of triple-helical backbone mobilities by one- and two-dimensional NMR experiments using ^{15}N-labeled THPs.[32,173,271] NMR studies of a triple-helical peptide model of the type I collagen cleavage site have shown a reduced triple-helical content in the secondary amino acid poor region.[270,272] Free energy landscape computational analysis indicated the secondary amino acid poor region of the cleavage site can form a structure complementary to the MMP active site.[273] This complementary behavior was not observed for other regions possessing potential cleavage sites.[273]

The lack of charged residues, particularly in the "loosely" triple-helical region, constitutes another mechanism by which the stability of the triple-helix is compromised. Studies with both homotrimeric and heterotrimeric triple-helical peptides have demonstrated enhanced stability based on charged pairs (Asp and Lys).[25,26]

X-Ray crystallographic analysis has indicated that hydration patterns are different in the secondary amino acid poor region of the type III collagen cleavage site compared with secondary amino acid rich regions, in that more ordered water is found in the secondary amino acid poor region.[274] X-Ray crystallographic analysis of a series of host guest peptides revealed that the 1st hydration shell in triple-helical peptides, found at 2.75 Å, is where water molecules directly link to peptide atoms by hydrogen bonds, whereas the 2nd hydration shell, found at 3.55 Å, is where water molecules interact with each other.[275] The introduction of Hyp (as occurs in the "tightly" triple-helical region) causes an increase in the ratio between the number of water molecules in the 1st and 2nd hydration shells.[275] Thus, more peptide-bound water occurs in the "tightly" triple-helical region, and comparatively less in the "loosely" triple-helical region. In addition, NMR hydration experiments revealed that the 1st hydration shell is kinetically labile (upper limits for H_2O residence times in the nanosecond to sub-nanosecond range),[276] and thus hydration-dehydration is probably not rate-limiting during collagenolysis.

Overall, there are numerous structural and hydration differences between secondary amino acid rich and poor regions within triple-helices. The proposed collagen cleavage site model would only be valid if collagenases had extended active or substrate binding sites. Studies using mutant collagens showed that MMP-1, MMP-8, and MMP-13 had active or substrate binding sites that extended at least S_2 through S_8' and conformational restriction of the cleavage site in collagen (by introducing Pro residues in the P′ subsites) eliminated MMP activity.[277-279] THP substrates that are extended by 6 residues in the *N*-terminal or 9 residues in the *C*-terminal direction compared with those given in Table 2 have significantly improved rates of hydrolysis, demonstrating MMP interactions with substrate subsites as far as P_{13} and P_{17}'.[263]

8 THP transition-state analogs

The activity of MMPs can be regulated in numerous ways including gene expression, activation of the zymogen precursors, association with endogenous inhibitors such as the tissue inhibitors of metalloproteinases (TIMPs), or the physical removal of enzyme *via* proteolysis or cellular internalization. One regulatory approach has focused on inhibiting MMPs using THPs. Our

laboratory prefers this approach as it affects only active protease populations and does not disturb the balance of native protease-inhibitor complexes (such as the natural distribution of MMP-TIMP complexes).

Metallo(zinc)-proteases use the nucleophilic attack of a water molecule as one of the steps of amide bond hydrolysis.[280] The tetrahedral intermediate that results from water addition to the amide carbonyl has been the focus of many protease inhibitor designs. Phosphinic peptides/phosphinates [Ψ{PO_2H-CH_2}] have been shown to behave as transition state analog inhibitors of MMPs.[281] Phosphinate THP inhibitors (THPIs; Fig. 8) have several potential advantages over other inhibitor constructs. These analogs incorporate specificity elements for both the S and S′ subsites of the enzyme, while the triple-helical structure interacts with both the active site and secondary substrate binding sites (exosites).[244,282,283]

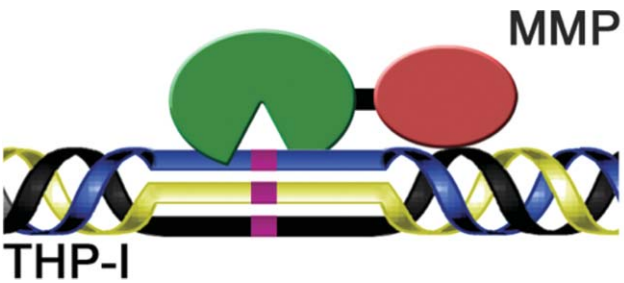


Fig. 8 Model structure of a triple-helical transition state analog MMP inhibitor. The MMP CAT domain is green, the MMP HPX domain is red, and the non-hydrolyzable PO_2H-CH_2 bond is indicated in purple.

In order to create the desired phosphinate transition state analogs, we prepared protected building blocks GlyΨ{PO_2H-CH_2}Val **1a** and GlyΨ{PO_2H-CH_2}Leu **1b** (Scheme 1).[284-286] Briefly, this route consisted of a mild silylation of Fmoc-aminophosphinic acid (**5**)[287] with TMS-Cl and DIEA to give the trivalent phosphinate, which reacted in a Michael-type reaction with an allyl acrylate[288] to afford phosphinic acid **7** as the racemate (Scheme 1). This phosphinate dipeptide analog was protected on phosphorus as the adamantyl ester and the allyl ester was deprotected using $CpRu(CH_3CN)_3PF_6$ catalysis to give the protected phosphinate dipeptide mimic **1a** [(*R*,*S*)-2-isopropyl-3-((1-(*N*-(Fmoc)amino)-methyl)-adamantyl-oxyphosphinyl)propanoic acid] and its Leu analog **1b**.

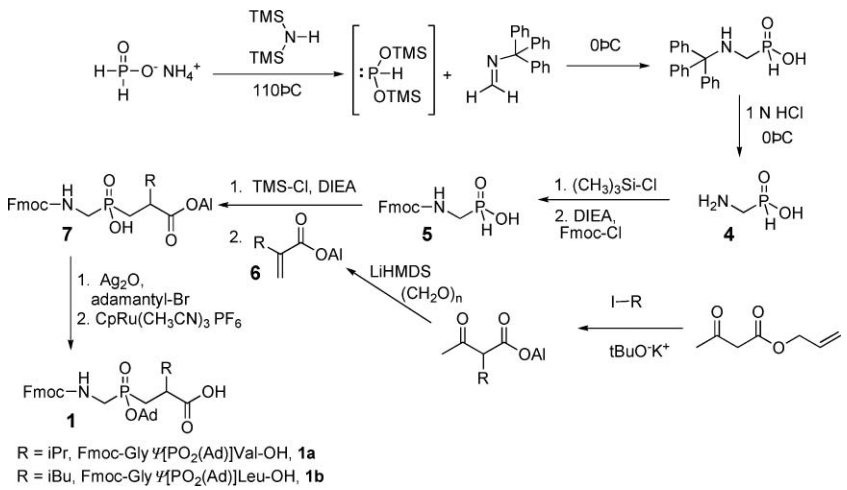
Table 3 Inhibition of MMP-2, MMP-9, and MMP-1

Enzyme	Inhibitor	T/°C	$K_i^{(app)}$/nM
MMP-2	α1(V)GlyΨ{PO_2H-CH_2}Val THP	10	4.14 ± 0.47
″	″	37	19.23 ± 0.6
″	MMP inhibitor III	10	3.17 ± 0.23
″	″	37	0.83 ± 0.03
″	α1(I-III)GlyΨ{PO_2H-CH_2}Leu THP	10	0.18 ± 0.00
″	″	37	0.08 ± 0.01
MMP-9	α1(V)GlyΨ{PO_2H-CH_2}Val THP	10	1.76 ± 0.05
″	″	37	1.29 ± 0.00
″	α1(I-III)GlyΨ{PO_2H-CH_2}Leu THP	10	0.02 ± 0.01
″	″	37	0.09 ± 0.00
MMP-1	α1(I-III)GlyΨ{PO_2H-CH_2}Leu THP	10	7.83 ± 1.03
″	″	37	26.70 ± 5.2
″	MMP inhibitor III	10	2.48 ± 0.35
″	″	37	4.72 ± 0.38

To assemble THPIs, dipeptide mimics **1a** and **1b** were incorporated into peptides by Fmoc solid-phase synthesis. Fmoc-phosphinodipeptide **1a** was utilized to create C_6-(Gly-Pro-Hyp)$_4$-Gly-Pro-Pro-GlyΨ{PO_2H-CH_2}(*R*,*S*)Val-Val-Gly-Glu-Gln-Gly-Glu-Gln-Gly-Pro-Pro-(Gly-Pro-Hyp)$_4$-NH_2 [designated α1(V)GlyΨ{PO_2H-CH_2}Val THPI].[284]

The α1(V)GlyΨ{PO_2H-CH_2}Val THPI (which contains the *S* configuration in the P_1' position, equivalent to an L-amino acid) was initially tested against MMP-2 and MMP-9 (Table 3). Due to the low melting temperature of the potential inhibitor (T_m ~ 25 °C), K_i values were first determined at 10 °C. α1(V)GlyΨ{PO_2H-CH_2}Val THPI was found to be a very effective inhibitor of MMP-2 and MMP-9, with K_i values of 4 and 2 nM, respectively. When inhibition assays were repeated at 37 °C, the K_i value increased for MMP-2 but not for MMP-9 (Table 3). Thus, triple-helical structure modulated inhibition of MMP-2 but not MMP-9.

To determine if an increase in K_i as a function of temperature was a general trend for inhibition of MMP-2, inhibition of MMP-2 by MMP inhibitor III (a hydroxamic acid-Leu-homoPhe dipeptide) was examined. At 10 °C, the K_i value for MMP-2 inhibition was 3 nM (Table 3). Increasing the temperature to 37 °C



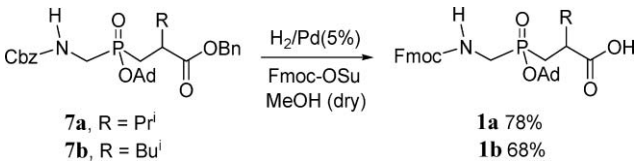
Scheme 1 Synthesis of protected phosphinate amino acids and phosphinodipeptides.

decreased the K_i to 0.8 nM (Table 3). Thus, for a small molecule inhibitor, an increase in temperature slightly increased the affinity towards MMP-2, most likely due to enhanced hydrophobic interactions. This further suggested that the decreased inhibition of MMP-2 by α1(V)GlyΨ{PO_2H-CH_2}Val THPI as a function of increasing temperature is due to unfolding of the inhibitor triple-helical structure.

MMP-1, MMP-3, MMP-8, MMP-13, and MT1-MMP were tested for inhibition by α1(V)GlyΨ{PO_2H-CH_2}Val THPI. No inhibition of MMP-1, MMP-3, or MT1-MMP was observed up to an α1(V)GlyΨ{PO_2H-CH_2}Val THPI concentration of 25 μM. MMP-8 and MMP-13 were inhibited weakly, with K_i values in the range of 50 and 10 μM, respectively. Thus, this study utilized a GlyΨ{PO_2H-CH_2}Val transition state analog to bind selectively at the S_1-S_1' site of MMP-2 and MMP-9. Selective inhibition of these MMPs is desirable, as MMP-2 has been validated as an anticancer drug target, while MMP-9 inhibition may be useful in treating early-stage cancers.[282]

Our second inhibitor design utilized a THP substrate mimicking α1(II)769-783, which is hydrolyzed by MMP-1, MMP-2, MMP-8, MMP-9, MMP-13, and MT1-MMP.[39,40] The P_1-P_1' subsites of the triple-helical peptide, which incorporate Gly-Leu in the substrate, were substituted by a GlyΨ{PO_2H-CH_2}Leu transition state analog. Because the T_m value for α1(V)GlyΨ{PO_2H-CH_2}Val THPI was low (see above),[284] the α1(I-III)GlyΨ{PO_2H-CH_2}Leu THPI incorporated (4*R*)-Flp to enhance triple-helicity.[37,133,134] Thus, the sequence of this inhibitor was C_6-Gly-Pro-Flp-(Gly-Pro-Hyp)$_4$-Gly-Pro-Gln-GlyΨ{PO_2H-CH_2}(*R*,*S*)Leu-Ala-Gly-Gln-Arg-Gly-Ile-Arg-(Gly-Pro-Hyp)$_4$-Gly-Pro-Flp-NH_2, and it exhibited a T_m value of 30 °C.[285] Studies revealed low nM K_i values for inhibition of MMP-1, MMP-2, and MMP-9 (Table 3). Our second transition state analog inhibitor appears to be effective against a broader range of collagenolytic MMPs than the first inhibitor. Interestingly, MMP-1 was sensitive to the triple-helical structure of the inhibitor (K_i increased ~4 times when the inhibitor was thermally unwound), but neither MMP-2 nor MMP-9 was. This contrasts with the sensitivity of MMP-2 to the triple-helical structure of α1(V)GlyΨ{PO_2H-CH_2}Val THPI (Table 3), and indicates that there is *a sequence-dependent sensitivity to triple-helical structure* for some MMPs.

While the eight-step synthesis of the dipeptide analog (3 steps required for fragment **2**) was a fairly succinct route, overall yields starting from fragment **2** were approximately 16% mainly due to the inefficiencies of the last two steps. It appeared that reaction conditions in both these steps are not particularly compatible with the Fmoc group leading to significant deprotection and side-product formation. We reasoned that benzyl ester protection of the *C*-terminus would potentially allow for its removal under the same hydrogenation conditions required for unveiling of the amine.[289] To test our hypothesis, we prepared benzyl ester fragment **7a** (Scheme 2). Catalytic hydrogenation of **7a** with 5% Pd/C under hydrogen in the presence of Fmoc-OSu afforded the desired dipeptide mimic **1a** in good yields (Scheme 2).[289] This one-pot reaction involved the simultaneous removal of the Cbz and benzyl protecting groups and re-protection of *N*-terminus with Fmoc. Importantly, this method does not appear to be sensitive to the steric environment of the ester group. The Gly-Leu dipeptide mimic **7b** was also prepared and underwent efficient bis-deprotection and Fmoc addition to give **1b** (Scheme 2).[289]



Scheme 2 Tandem bis-deprotection and Fmoc attachment leading to protected phosphinodipeptides.

A single-stranded peptide model of the α1(I)715-721 collagen sequence has been identified as a ligand for the MMP-2 FN II insert and inhibited MMP-2 gelatinolysis.[290] Our laboratory assembled a triple-helical version of this ligand [α1(I)715-721 THP], and evaluated it for the ability to inhibit MMP-2 and MMP-9 triple-helical peptidase and gelatinase activities.[285] α1(I)715-721 THP inhibited type V collagen-model triple-helical peptidase activity but not interstitial collagen-model triple-helical peptidase activity. To our knowledge, this demonstrated the first use of an exosite binder to selectively inhibit one collagen-based MMP activity but not another.

9 THP models for studying lipoprotein-, glycosaminoglycan-, nucleic acid-, and other protein-collagen interactions

THPs have been used to examine macromolecular interactions in addition to those previously mentioned. Macrophage scavenger receptor (MSR) binds to a variety of polyanionic molecules, including proteins, phospholipids, polysaccharides, and nucleic acids. MSRs contain a transmembrane segment, a coiled-coil α-helical region, and a triple-helical domain. THP models of MSR-1 were used to study binding interactions of acetylated low-density lipoproteins (Ac-LDLs).[88,291] Initially, Ac-LDL was found to bind to a THP model of receptor residues 323-340 [(Gly-Pro-Hyp)$_4$-Gly-Lys-Thr-Gly-Lys-Pro-Gln-Leu-Asn-Gly-Gln-Lys-Gly-Gln-Lys-Gly-Glu-Lys-(Gly-Pro-Hyp)$_3$] (Table 4).[88] Binding did not occur to a single-stranded model of this sequence, nor to a THP in which Lys337 was changed to Ala. The binding affinity (K_D) of Ac-LDL for the MSR THP and mouse peritoneal macrophages was 31 and 3.2 μg ml^{-1}, respectively.[291] Ac-LDL, but not LDL, bound to the MSR THP, and binding was inhibited by dextran sulfate, fucoidan, sulfatides, poly(G), and poly(I), but not poly(C). These binding behaviors were analogous to those exhibited by the native receptor.

MSR has been shown to specifically mediate the uptake of poly(I) and poly(G), but not poly(C), poly(U), or poly(A). A THP model of MSR-1 residues 333-341 [(Pro-Hyp-Gly)$_3$-Pro-Lys-Gly-Gln-Lys-Gly-Glu-Lys-Gly-(Pro-Hyp-Gly)$_4$] was used to study binding interactions of tetraplex nucleic acids.[292] The MSR-1 THP preferably bound tetraplex poly(I) over double-stranded and single-stranded forms (Table 4). The MSR-1 THP interaction with poly(I) was also sequence specific.

THPs have been used to identify and characterize glycosaminoglycan binding sites in collagen and acetylcholinesterase (AChE). A series of THPs was screened to find the high affinity binding site for low MW heparin in type I collagen.[70] The highest affinities were found for α1(I)82-93 THP (K_D = 1.7 μM) and α1(I)925-937 THP (K_D = 1.6 μM) (Table 4), which was a factor of 10 worse than heparin binding to type I collagen (K_D = 165 nM). This result was

Table 4 Sequences of assorted biomolecule binding sites within types I-IV collagen. O = 4*R*-Hyp

Biomolecule	Binding site location	Sequence
Ac-LDL	MSR323-340	GKTGKPGLNGQKGQKGEK
poly(I)	MSR333-341	PKGQKGEKG
heparin	α1(I)82-93	GLPGMKGHRGFS
heparin	α1(I)925-937	GDRGIKGHRGFSG
heparin, heparan sulfate	α1(V)904-918	GKOGPRGQRGPTGPR
heparin, heparan sulfate	AChE146-161	GROGRKGROGVRGPR
heparin, heparan sulfate	AChE251-266	GROGKRGKTGLKGDI
vWF, SPARC	α1(III)399-408	RGQOGVMGFO

For type IV collagen, sequence numbers are based on the human α1(IV) and α2(IV) genes.[324]

not surprising, as type I collagen has multiple heparin recognition sites that may contribute to cooperatively bind heparin. A THP incorporating α1(V)904-918 bound heparin and heparan sulfate, with significant contributions from Lys905, Arg909, and triple-helical structure (Table 4).[293]

The AChE collagen-like tail has two heparin binding sites of differing affinities, one located in the *N*-terminal region of the tail (residues 149-152, Gly-Arg-Lys-Gly-Lys) and one in the *C*-terminal region (residues 254-257, Gly-Lys-Arg-Gly-Lys). THPs were constructed incorporating AChE residues 146-161 [Pro-Hyp-Gly-Arg-Hyp-Gly-Arg-Lys-Gly-Arg-Hyp-Gly-Val-Arg-Gly-Pro-Arg-Gly-(Pro-Hyp-Gly)$_4$] and 251-266 [Pro-Hyp-Gly-Arg-Hyp-Gly-Lys-Arg-Gly-Lys-Thr-Gly-Leu-Lys-Gly-Asp-Ile-Gly-(Pro-Hyp-Gly)$_4$] (Table 4).[294] Heparin, heparan sulfate, and chondroitin sulfate bound to both AChE THPs, resulting in increased T_m values. However, the increase in T_m due to chondroitin sulfate binding was lower, as were the THP $\Delta[\Theta]_{225\ nm}$ values, suggesting a different mode of interaction for chondroitin sulfate compared with heparin and heparan sulfate. Binding of heparin was dependent on triple-helical conformation.[295] A subsequent study utilized THPs starting from the two heparin binding motifs.[296] Triplets were systematically added within a (Gly-Xxx-Yyy)$_8$ template and the effects on thermal stability and heparin binding quantified. Heparin binding was modulated by a combination of sequence and triple-helix stability. The higher heparin binding affinity of the *C*-terminal region (compared with the *N*-terminal region) was achieved when a minimum of 9 residues from that region were incorporated into the THP. Calorimetry indicated a lack of significant enthalpy change as a result of heparin binding, suggesting that binding was largely due to electrostatic interactions.

Heat shock protein 47 (HSP47) is a molecular chaperone that facilitates normal procollagen biosynthesis. HSP47 has been proposed to stabilize correctly folded triple-helical intermediates and/or prevent lateral association of procollagen in the ER. The determinants for binding of HSP47 were evaluated using THPs.[297] HSP47 specificity was found to reside in triple-helical (Pro-Hyp-Gly)$_5$-Xxx-Arg-Gly-(Pro-Hyp-Gly)$_4$ sequences, and the role of Arg in binding was further confirmed by chemical modification of collagen. The binding affinities of HSP47 to type I collagen and Arg-containing THPs were comparable. Subsequent studies demonstrated that triple-helical structure was critical for HSP47 binding, and that only a single Arg residue was needed to promote a specific interaction between a heterotrimeric THP and HSP47.[298] The best binding to HSP47 was observed when Thr or Pro was positioned 3 residues *N*-terminal to Arg and in the same chain.[299] Localization of THP binding to HSP47 was achieved by crosslinking *via* photolysis of *p*-benzoyl-L-Phe (Bpa) residues incorporated within the THP.[298]

A high affinity binding site for von Willebrand factor (vWF) was found using the type III collagen THP Toolkit (Table 4).[300] As the collagen-binding A3 domain of vWF was found to rely on different interactions with collagen than the α2β1 integrin,[301,302] it was expected that the vWF binding motif would differ from the α2β1 integrin binding site found in type III collagen (see Table 1). Although this binding site was the same as identified for DDR2, binding of vWF is dependent upon the Arg, first Hyp, and Val residues, while the Met residue is important for DDR2 binding.[191] The Gly-Val-Met-Gly-Phe-Hyp motif is also bound by SPARC/osteonectin/BM-40 (Table 4).[303] X-Ray crystallographic structural analysis of the SPARC/THP complex revealed that, in contrast to integrin binding, SPARC does not distort the triple-helix, but rather rearranges to form a deep pocket to accommodate the Phe residue from the trailing THP chain.[303]

The THP Gly-Cys-Hyp-(Gly-Pro-Hyp)$_{10}$-Gly-Cys-Hyp-Gly-NH_2, which binds to platelet GpVI (see earlier discussion), is also bound by keratinocyte growth factor (KGF).[304] The non-hydroxylated form of the THP is bound with less avidity by KGF, while a THP possessing the integrin binding Gly-Phe-Hyp-Gly-Glu-Arg motif is not bound by KGF.[304]

THPs possessing (Pro-Hyp-Gly)$_n$ and (Pro-Pro-Gly)$_n$ motifs have been shown to bind to the collagen-binding domain (CBD) of *Clostridium histolyticum* type I collagenase, while non-triple-helical peptides do not.[305] Gly-(Pro-Hyp-Gly)$_8$ was subsequently utilized to map the residues within the CBD that facilitate binding to triple-helices.[306] The most significant contributions to binding activity were observed for residues Thr957, Tyr970, Leu992, Tyr994, and Tyr996.[306] The importance of side-chain hydroxyl groups, specifically that of Tyr994, for interacting with THPs is reminiscent of a similar effect observed *via* mutation of Tyr189 in MMP-8.[40]

10 Miscellaneous applications of THPs

The use of THPs for biomaterial applications is encouraged by the serum protease stability of (Gly-Pro-Pro)$_{10}$.[307] A PA THP incorporating the α2β1 integrin binding site α1(I)496-507 supported human aortic endothelial cell adhesion and spreading at levels similar to type I collagen.[18] Inclusion of a second peptide-amphiphile, which incorporated residues 119-122 from SPARC, provided a mixed PA surface that promoted comparable adhesion and spreading to type I collagen.[18] As discussed earlier, the

α1(I)496-507 THP induced minimal endothelial cell activation.[18,198] The endothelial cell adhesion, spreading, and activation properties of α1(I)496-507 THP were favorable where promotion of wound healing is desired.

Corneal epithelial cell and fibroblast attachment and growth has been studied for surface-bound peptoid-containing THPs.[308] $(Gly\text{-}Pro\text{-}Nleu)_{10}$-Gly-Pro-$NH_2$, when immobilized to ethylene propylene copolymer film, supported cell adhesion and growth. No effect was seen for $(Gly\text{-}Nleu\text{-}Pro)_{10}$ nor analogous non-triple-helical sequences, suggesting a specific interaction based on peptoid location and triple-helical conformation. Adhesion was inhibited by the linear Lys-Asp-Gly-Glu-Ala peptide, indicative of α2β1 integrin involvement (see Table 1). However, the affinity of adhesion was not evaluated, as concentration dependencies were not performed nor was adhesion compared to native collagen.

Immobilization of $(Gly\text{-}Pro\text{-}Pro)_5$-Gly-Phe-Hyp-Gly-Glu-Arg-$(Gly\text{-}Pro\text{-}Pro)_5$-Gly (CMP1) onto poly(3-hydroxybutyrate-*co*3-hydroxyvalerate) (PHBV) microspheres promoted Hep3B liver cell growth and spreading.[309] In addition, cellular activities such as albumin secretion and P-450 activity were maintained for 14 d, suggesting application for liver tissue engineering.[309] In similar fashion, $(Gly\text{-}Pro\text{-}Hyp)_7$-Tyr was copolymerized with poly(ethylene oxide) diacrylate to create PEG hydrogels; chondrocyte encapsulation resulted in increased glycosaminoglycan and collagen production compared with unmodified PEG hydrogels.[310]

Cys-$(Gly\text{-}Pro\text{-}Hyp)_7$ has been attached to gold nanoparticles and used to visualize type I collagen fibers *via* binding to the gap regions.[311]

The collagen-model peptide acetyl-$(Pro\text{-}Pro\text{-}Gly)_5$ and ethylenediamine-core polyamidoamine dendrimer were combined to create a collagen-mimetic delivery vehicle.[312] Although only 5 repeats, the Pro-Pro-Gly sequence appeared to possess triple-helical character, presumably due to surface clustering.[312] Delivery of the model drug rose bengal was temperature dependent, correlating with the triple-helical content of the THP.[312]

The addition of ferrocene (Fc) to the *N*-terminus of $(Pro\text{-}Hyp\text{-}Gly)_n$-Cys was found to increase the peptide triple-helical stability.[313] In turn, Fc-CO-$(Pro\text{-}Hyp\text{-}Gly)_n$-Cys, when prepared as films on gold surfaces, exhibited a linear but weakly distance dependent electron transfer.[314]

The THP acetyl-$(Pro\text{-}Hyp\text{-}Gly)_5$-Pro-Lys-Gly-$(Pro\text{-}Hyp\text{-}Gly)_5$-Ala-$NH_2$ was used as a model for analysis of glycation end products following exposure to glucose, ribose, and glyoxal.[20] Carboxymethyl-lysine was observed following all treatments, while treatment of the THP with glyoxal resulted in the formation of glyoxal lysine dimer.[20] The THP was a good model for collagen advanced glycation end products.

Discussion and conclusions

Due to the application of THPs, collagen-based research has made significant strides in the past decade. The use of THP models has greatly advanced knowledge of both collagen structure and biological activities. Analyses of THPs possessing non-native amino acids reopened discussion on the forces that modulate triple-helical structure and stability. In addition, the unique contributions toward triple-helical stability within heterotrimeric systems have been unveiled. Development of THP model systems has included the implementation of a variety of templates, which serve to further stabilize triple-helical structure. THP templates may ultimately allow for a *reduction* in the number of Gly-Pro-Hyp or Gly-Pro-Flp repeats required for appropriate stability and a more tractable THP synthesis overall. All of the above studies have provided the foundation for development of THP-based biomaterials.

Identification of collagen binding sites has rapidly progressed with the use of the collagen Toolkits.[191] Similarly, THP substrates have allowed for a better mechanistic understanding of collagenolytic proteases. Analysis of receptor and enzyme binding to triple-helices have indicated that such processes have been achieved by convergent evolutionary mechanisms.[306,315]

MMP inhibitor programs began in earnest in the 1980s, using the destruction of ECM components as a model for inhibitor design.[316] Initial clinical trials with MMP inhibitors were disappointing, with one of the problematic features being a lack of inhibitor selectivity.[244,282,283,317,318] Selectivity appears to be a crucial aspect for future MMP inhibitor design, as several MMPs have been validated as targets in certain cancers (MMP-2, MMP-9, MT1-MMP) while others have been found to be host-beneficial and thus anti-targets (MMP-3, MMP-8).[282,319] The development of THPIs has provided truly selective and potent compounds for modulating MMP activities. These results are most promising, as recent studies indicate that inhibition of MMP collagenolytic activity does not led to musculoskeletal syndrome (MSS),[320] a common side effect of prior, small molecule MMP inhibitors. THP substrates also allow for high-throughput screening and identification of exosite binding metalloproteinase inhibitors.[321-323] The use of exosite binding may well represent an important new step for selective inhibitor development.

While many advances have been made, there are some concerns with THP based research. It has been noted that structural studies with THP models may have been biased due to high secondary amino acid content.[125] Identification of binding sites and receptor-mediated signaling has not fully accounted for ligand glycosylation and the role of heterotrimeric structure. While higher order structures have been obtained from THPs, precise control of these macrostructures is not always readily achieved. Nonetheless, the promise of THP based research is great, and the aforementioned shortcomings represent further areas of exploration.

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