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Tricine as a convenient scaffold for the synthesis of C-terminally branched collagen-model peptides

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Abstract

A novel and convenient method for the synthesis of C-terminally branched collagen-model peptides has been achieved using tricine (*N*-[tris(hydroxymethyl)methyl]glycine) as a branching scaffold and 1,2-diaminoethane or 1,4-diaminobutane as a linker. The peptide sequence was incorporated directly onto the linker and scaffold during solid-phase synthesis without additional manipulations. The resulting branched triple-helical peptides exhibited comparable thermal stabilities to the parent, unbranched sequence, and served as substrates for matrix metalloproteinase-1 (MMP-1). The tricine-based branch reported herein represents the simplest synthetic scaffold for the convenient synthesis of covalently linked homomeric collagen-model triple-helical peptides.

Graphical abstract

Keywords

Triple-helical peptide; collagen; matrix metalloproteinase; peptide synthesis

Collagen is the most abundant structural protein in all animals.¹ In humans it is the most prevalent component of the extracellular matrix (ECM).¹ The defining feature of collagen is

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an elegant structural motif in which three parallel polypeptide strands in a left-handed polyproline II-type helical conformation coil about each other with a one-residue staggered to form a right-handed triple-helix.^{1, 2}

Synthetic triple-helical peptides (THPs) that mimic the structure of native collagens have been used to investigate collagen-protein interactions as well as collagen structure and stability.³ Our laboratory has been particularly interested in utilizing triple-helical peptides as model substrates for studying the mechanism of collagenolysis.^{4–8}

Numerous scaffolds (templates) have been used to create stable THPs.⁹ The main categories of template strategies involve (a) a scaffold to which the peptides are covalently attached or (b) the use of metal ions for non-covalent association. The covalent templates include Lys-Lys or Glu-Glu branching,^{10–21} double disulfide knots,^{22–29} *cis,cis*-1,3,5-trimethyl-cyclohexane-1,3,5-tricarboxylic acid (Kemp triacid),^{30–38} tris(carboxyethoxymethyl)aminomethane (TRIS),^{39–41} tris(2-aminoethyl)amine (TREN),^{42, 43} cyclotrimerarylene (CTV),⁴⁴ and a macrocyclic scaffold.⁴⁵ Alternatively, metal ions such as Ca²⁺, Ru²⁺, Ni²⁺, Fe³⁺, Cd²⁺, Hg²⁺, Cu²⁺, Zn²⁺, or Ln³⁺ have been used in conjugation with amino acids, hydroxamic acid, catechol, or pyridyl or bipyridyl functionalities to create or stabilize multistrand linkages.^{40, 41, 46–52}

An *N*- or *C*-terminal template is expected to align and entropically stabilize the THP and thus enhance triple-helical thermal stability.¹⁰ Templated triple-helices have been utilized to enhance the binding of triple-helices to surfaces and for developing triple-helices as affinity chromatography ligands.^{53, 54} Such utilization of template triple-helices can allow for the creation of novel biomaterials and the isolation of receptors that bind to collagen. Herein, we present a convenient strategy for the synthesis of *C*-terminally branched, homotrimeric THPs compatible with 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase chemistry.

While constructing peptoid-containing THPs using solid-phase methodology,⁵⁵ we considered incorporating tricine, *N*-[tris(hydroxymethyl)methyl]glycine, as a building block using a simple method that is compatible with existing Fmoc chemistry. Tricine, commonly used as a buffering agent,⁵⁶ is scarcely soluble in organic solvents. The addition of protecting groups that would make tricine suitable for Fmoc solid-phase peptide synthesis would require several synthetic manipulations. This potential inconvenience was bypassed by the direct, on-resin synthesis of a tricine residue using the submonomeric method typically applied for solid-phase peptoid synthesis (Scheme 1).^{57, 58}

The synthesis of the THP started with the coupling of a *C*-terminal Tyr residue to serve as a chromophore for eventual peptide concentration determination. Subsequently, after coupling of bromoacetic acid using *N,N*-diisopropylcarbodiimide, the bromine was substituted with TRIS to afford a tricine molecule (Scheme 1).

During the course of our investigations, we found that the resin-bound terminal hydroxyl groups could be easily converted into chlorides using triphenylphosphine and trichloroacetamide *via* previously reported in-solution conditions.⁵⁹ This method was found to be mild and suitable for solid-phase synthesis on acid-labile resins. The resin-bound

tricine was treated with triphenylphosphine and trichloroacetamide in CH_2Cl_2 in order to convert the terminal hydroxyl groups to chlorides (Scheme 1).

Terminal chloride moieties undergo substitution with diamine linkers to afford amino groups ready for further synthesis. In general, the linkers that support branched collagen-like peptides play a crucial role in alignment of the three strands.^{30, 61} For this purpose, 1,2-diaminoethane (DAE) and 1,4-diaminobutane (DAB) were tested as suitable linkers between the tricine branch and the collagen-model peptide sequence. DAE or DAB was incorporated onto the resin-bound tricine, displacing the chlorides (Scheme 1). The resin substitution level was then quantified using a ninhydrin test or, following installation of an Fmoc protecting group, by spectrophotometric analysis of the fulvene-piperidine adduct.⁶⁰

The $\alpha 1(\text{I})772\text{-}786$ collagen sequence, commonly used as matrix metalloproteinase (MMP) model substrate,^{62, 63} was then incorporated onto the tricine scaffold (Scheme 1 and Fig. 1). With the exception of the manual incorporation of tricine, the synthetic route proceeded using standard Fmoc chemistry on a microwave peptide synthesizer (Liberty Blue, CEM Corporation) according to previously published protocols.⁵⁵

The obtained THPs were compared to the unbranched $\alpha 1(\text{I})772\text{-}786$ THP and the Lys-Lys branched $\alpha 1(\text{I})772\text{-}786$ THP (Fig. 1) reported previously.⁵³ The triple-helical conformations of DAE $\alpha 1(\text{I})772\text{-}786$ THP, DAB $\alpha 1(\text{I})772\text{-}786$ THP, and unbranched $\alpha 1(\text{I})772\text{-}786$ THP were evaluated by circular dichroism (CD) spectroscopy. At room temperature, all peptides exhibited typical features of a collagen-like conformation,² such as a large negative molar ellipticity ($[\Theta]$) at $\lambda = 200$ nm and a positive $[\Theta]$ at $\lambda = 225$ nm (data not shown). The thermal stabilities of the peptides were evaluated by measuring $[\Theta]$ at $\lambda = 225$ nm as a function of temperature (Fig. 2). When the temperature was increased from 5 to 70 °C, DAE $\alpha 1(\text{I})772\text{-}786$ THP and DAB $\alpha 1(\text{I})772\text{-}786$ THP showed structural transitions (triple-helix $\rightarrow$ unfolded species) with midpoints (T_m values) of 34 and 35 °C, respectively, compared with 38 °C for the unbranched $\alpha 1(\text{I})772\text{-}786$ THP (Fig. 3). While both DAE $\alpha 1(\text{I})772\text{-}786$ THP and DAB $\alpha 1(\text{I})772\text{-}786$ THP formed relatively stable triple-helices, it was surprising that the tricine branch did not enhance the thermal stability of the $\alpha 1(\text{I})772\text{-}786$ sequence compared with the unbranched THP. This could be due to a slight misalignment of the three peptide strands by the branch, or charge repulsion within the branch under the acidic conditions of the CD spectroscopic experiments.

The characterization of branched THPs by mass spectrometry (MS) presents a challenge as this class of peptides is often difficult to ionize.^{10, 21, 63} After incorporation of tricine and treatment of the peptide-resin with trifluoroacetic acid, analysis of the product indicated tricine-Tyr-NH₂ based on the $[\text{M}+\text{H}]^+$ and $[\text{M}+\text{Na}]^+$ molecular ions observed (Fig. S1). However, after incorporation of diamine linkers the corresponding molecular ions were not detected. Surprisingly, MALDI-TOF MS analysis of fully assembled DAE $\alpha 1(\text{I})772\text{-}786$ THP revealed a prominent molecular ion corresponding to the $[\text{M}+3\text{H}]^{3+}$ species (Fig. 4). The $[\text{M}+3\text{H}]^{3+}$ molecular ion is visible in the mass spectrum at very low laser intensities, giving peak resolution of $R \sim 15000$ FWHM (full width at half maximum) (Fig. 4).

MS characterization of linear and branched THPs is typically achieved by digestion using trypsin and characterization of the resulting fragments.⁵⁵ The $\alpha 1(I)772-786$ sequence contains an Arg residue at position 780, which represents a potential trypsin cleavage site. DAE $\alpha 1(I)772-786$ THP and DAB $\alpha 1(I)772-786$ THP were digested overnight at room temperature with agarose bead-immobilized trypsin, followed by HPLC analysis (Fig. S2). DAE $\alpha 1(I)772-786$ THP and DAB $\alpha 1(I)772-786$ THP exhibited the same hydrolysis pattern, and peaks were isolated and analyzed by MS (Fig. S3 and S4). Cleavage by trypsin produced the *N*-terminal fragment (Gly-Pro-Hyp)₄-Gly-Pro-Gln-Gly-Ile-Ala-Gly-Gln-Arg (Fig. S2, peaks “a”), as indicated by the molecular ion of $m/z = 1952$ (Fig. S3). Analysis of the remaining fragments (Fig. S2, peaks “b” and “c”) revealed the *C*-terminal branched fragment (peaks “b”) and the uncleaved, parent THP (peaks “c”) (Fig. S4). However, only in the case of DAE $\alpha 1(I)772-786$ THP was the $[M+3H]^{3+}$ molecular ion observed which corresponded to the *C*-terminal branch (Fig. S4, bottom). For DAB $\alpha 1(I)772-786$ THP the *C*-terminal branch molecular ion was not observed. While the obtained analytical data suggested that DAB $\alpha 1(I)772-786$ THP was assembled properly, only DAE $\alpha 1(I)772-786$ THP was pursued further.

To determine if tricine-branched THPs could be used for the development of MMP substrates and inhibitors, DAE $\alpha 1(I)772-786$ THP was incubated with MMP-1 at 25 °C [below the T_m for DAE $\alpha 1(I)772-786$ THP]. MMP-1 is a well known collagenase and its activity has been previously quantified using soluble type I collagen, type I collagen fibrils, and linear and triple-helical collagen-model peptides.^{64–70} HPLC and mass spectrometric analysis revealed that DAE $\alpha 1(I)772-786$ THP behaved similarly to the unbranched $\alpha 1(I)772-786$ substrate (Fig. 5), in that the enzyme catalyzed hydrolysis of both THPs at the Gly-Ile bond. The rate of hydrolysis was slightly slower for DAE $\alpha 1(I)772-786$ THP compared with $\alpha 1(I)772-786$ THP (data not shown). This may be due to the *C*-terminal branch interfering with the collagenolytic mechanism of MMP-mediated hydrolysis.⁶

The $\alpha 1(I)772-786$ THP having a Lys-Lys branch has been described previously.⁵³ It has T_m values of ~43 °C in 0.1% acetic acid/water solution⁵³ and 32 °C in 0.01% (v/v) fluorometric assay buffer.⁶² In comparison to the Lys-Lys branched THP, DAE $\alpha 1(I)772-786$ THP and DAB $\alpha 1(I)772-786$ THP had lower T_m values, most likely due to the smaller number of Gly-Pro-Hyp repeats in DAE $\alpha 1(I)772-786$ THP and DAB $\alpha 1(I)772-786$ THP (four) compared with the Lys-Lys branched THP (six) at the *N*-terminus of the respective THPs. Clustering of Gly-Pro-Hyp repeats greatly stabilizes the triple-helix.^{2, 71}

TREN, which is similar to tricine, has previously been employed as an *N*-terminal branching scaffold for THPs.^{42, 43} However, in this approach, the TREN scaffold, containing succinic acid as a linker, was coupled to the peptidyl-resin over the period of 3 d with a yield of 10–15%.⁴² In similar fashion, coupling of Kemp triacid to peptidyl-resins proceeded over 3 d.^{32, 33} The Fmoc-Gly-Phe-Gly-Glu-Glu-Gly peptide, where the carboxylic acid side-chain of the two Glu residues and the carboxylic acid from the *C*-terminal Gly residue are used to create a trimer branch, was coupled to a peptidyl-resin in 6 h.¹⁹ This approach required separate preparation and isolation of Fmoc-Gly-Phe-Gly-Glu-Glu-Gly.

Other scaffolds were even less convenient to apply, requiring acylation in solution for extended time periods. The Bu₄N salt of CTV triacid was coupled to the *N*-terminus of a protected peptide in solution over 48 h.⁴⁴ In similar fashion, the macrocyclic scaffold was coupled to the *N*-terminus of a peptide in solution for 3 d,⁴⁵ Boc-β-Ala-TRIS was coupled to the *N*-terminus of a peptide in solution for 16 h,^{39, 40} and TREN-succinic acid was coupled to the *N*-terminus of a peptide in solution for 24 h.⁴³

The method presented herein does not require additional protecting groups, unlike the Lys-Lys branching strategy,¹⁰ which relies on two orthogonally protected Fmoc-Lys residues [typically Fmoc-Lys(1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl); ivDde] as well as subsequent deprotection steps (ivDde removal). Also, coupling of the back-to-back Fmoc-Lys(ivDde) residues and Fmoc-6-aminohexanoic acid may require large excesses of reactants and/or repeated coupling steps. The Lys-Lys branching strategy that takes advantage of “click” chemistry typically requires the speciality amino acids 2*S*-amino-6-azidohexanoic acid and (2*S*)-propargylglycinamide.^{20, 21} Overall, the synthesis of *C*-terminally branched THPs using a tricine scaffold is straightforward, efficient, and economical. The tricine-based branch can serve as a synthetic scaffold for developing collagen-model substrates and ligands.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Collagen-model, triple-helical peptides have been assembled on a tricine scaffold.
- Tricine represents the simplest scaffold for triple-helical peptide construction.
- Incorporation of the tricine branch was achieved in one step.
- Tricine-assembled peptides served as matrix metalloproteinase substrates.

Figure 1.

Sequence comparison of (a) $\alpha 1(\text{I})772\text{-}786$ THP, (b) DAE $\alpha 1(\text{I})772\text{-}786$ THP, (c) DAB $\alpha 1(\text{I})772\text{-}786$ THP, (d) Lys-Lys branched $\alpha 1(\text{I})772\text{-}786$ THP. Ahx = 6-aminoheptanoic acid, O = 4-hydroxy-L-proline. The MMP-1 cleavage site is designated by $\downarrow$ arrow.

Figure 2.

Thermal transition curves for $\alpha 1(I)772-786$ THP (black), DAE $\alpha 1(I)772-786$ THP (blue), and DAB $\alpha 1(I)772-786$ THP (red) in 0.5% AcOH at 50 μ M concentration. Molar ellipticities ($[\Theta]$) were recorded at $\lambda = 225$ nm while the temperature was increased from 5 to 70 °C.

Figure 3.
First derivative of the thermal transition curves (Fig. 2) for $\alpha 1(I)772-786$ THP (black), DAE $\alpha 1(I)772-786$ THP (blue), and DAB $\alpha 1(I)772-786$ THP (red).

Figure 4.

MALDI-TOF mass spectrum of purified DAE α 1(I)772-786 THP. The species of $m/z = 3721.0410$ corresponds to $[M+3H]^{3+}$ for the THP (calculated 3720.18). The insert is a magnification of the $m/z = 3721.0410$ peak to reveal the resolution ($R \sim 15000$). The spectrum was recorded on a Bruker Microflex LRF MALDI-TOF spectrometer using α -cyano-4-hydroxy-cinnamic acid as matrix.

Figure 5.

RP-HPLC analysis of DAE $\alpha 1(I)772-786$ THP alone (black) and after 20 h incubation with MMP-1 at 25 °C. Peak “a” corresponds to the *N*-terminal fragment (Gly-Pro-Hyp)₄-Gly-Pro-Gln-Gly, peak “b” the *C*-terminal branched fragment, and peak “c” the intact DAE $\alpha 1(I)772-786$ THP. Conditions used were an Agilent 1260 Infinity analytical HPLC, Phenomenex C18 column (5 μ m, 300 Å, 150 $\times$ 4.6 mm), analytical gradient of 2–98% B in 20 min, flow rate = 1 mL/min, and detection at λ = 214 nm. The solvent system was A = 0.1% TFA in water and B = 0.1% TFA in acetonitrile.

Scheme 1.

Synthetic route for on-resin incorporation of the tricine residue and branching strategy for DAE α 1(I)772-786 THP and DAB α 1(I)772-786 THP syntheses. Conditions were as follows: step 1, (I) Fmoc-Tyr(*t*Bu), HCTU, DIPEA (3 equiv., 60 min) in DMF, (II) 20% piperidine in DMF for 2×5 min; step 2, (I) bromoacetic acid, DIC (5 equiv., 45 min) in DMF, (II) tris(hydroxymethyl)aminomethane (40 equiv.) in DMSO (18 h); step 3, PPh₃ (20 equiv.), Cl₃CCONH₂ (20 equiv.) in DCM (60 min); step 4, diamine (50 equiv.) in DCM (4 h); step 5, peptide synthesis as previously described;⁵⁵ step 6, peptide cleavage and side-chain deprotection as previously described.⁵⁵