4-Ketoproline: An Electrophilic Proline Analog for Bioconjugation

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Installing an electrophilic amino-acid residue can engender a peptide or protein with chemoselective reactivity. Such a modification to collagen, which is the most abundant protein in animals, could facilitate the development of new biomaterials. Collagen has an abundance of proline-like residues. Here, we report on the incorporation of an electrophilic proline congener, (2S)-4-ketoproline (Kep), into a collagen-mimetic peptide (CMP). An ab initio conformational analysis of Kep revealed its potential to be accommodated within a collagen triple helix. A synthetic CMP containing a Kep residue was indeed able to form a stable triple helix. Moreover, the condensation of its carbonyl group with aminooxy-biotin did not compromise the conformational stability of the triple helix. These data encourage the use of 4-ketoproline as an electrophilic congener of proline. © 2015 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 104: 110–115, 2015.

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Additional Supporting Information may be found in the online version of this article.

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INTRODUCTION

The side chains of several natural amino acids contain nucleophiles. In contrast, the side chains of proteinogenic amino acids lack reactive electrophiles, other than half-cystines. As a result, natural proteins are largely inert to nucleophiles. Hence, the site-specific incorporation of an electrophilic amino acid into a protein can enable chemoselective conjugation with an exogenous nucleophile.[1]

Our research group is interested, in particular, in chemoselective reactions with collagen, which is the most abundant protein in animals and has substantial utility as a biomaterial.[2–4] Collagen is a fibrous protein with a sequence comprised of the repeating triplet Xaa-Yaa-Gly. Because (2*S*)-proline (Pro) and (2*S*,4*R*)-4-hydroxyproline (Hyp) derivatives are the most prevalent residues in the Xaa and Yaa positions of collagen, we had special interest in incorporating a reactive electrophilic center into a proline congener. Toward this end, we set forth to probe the attributes of (2*S*)-4-ketoproline (Kep), which is also known as (2*S*)-4-oxoproline, in a collagen-mimetic peptide (CMP).

Kep has received scant attention in the context of peptides and proteins.[1,5] This amino acid was used in a study of the conformational preferences of collagen prolyl 4-hydroxylase (P4H),[6] is the ultimate product of the hydroxylation of (2*S*,4*S*)-4-fluoroproline by P4H,[7] and has been elaborated into a wide variety of other proline derivatives.[8] The amino acids HHypOH and HKepOH are interconverted by an NAD-dependent enzyme,[9] and Kep is a component of some actinomycin antibiotics.[10,11] Kep has not, however, been incorporated into a protein.

Here, we explore the conformational preferences of Kep using hybrid density functional theory (DFT) calculations and nuclear magnetic resonance (NMR) spectroscopy. These analyses are performed on the well-established AcXaaOMe model system[2,12] with Xaa = Kep. We then show that the collagen triple helix tolerates the incorporation of a Kep residue in each strand. Finally, we find that this electrophilic Pro derivative allows for conjugation to collagen without disrupting its triple-helical structure.

MATERIALS AND METHODS

Computational Methodology

The conformational preferences of AcKepOMe were examined by hybrid DFT calculations as implemented in Gaussian '03.[13–15] Geometry optimizations and frequency calculations at the B3LYP/6-311+G(2d,p) level of theory were performed on the cis and trans geometries. Frequency calculations on the optimized structures yielded no imaginary frequencies, indicating true stationary points on the potential energy surface. The resulting self-consistent field energies were corrected by the zero-point vibrational energy determined in the frequency calculations.

General Experimental Methodology

Commercial chemicals were of reagent grade or better, and were used without further purification. In synthetic procedures, the term "concentrated under reduced pressure" refers to the removal of solvents and other volatile materials using a rotary evaporator at water aspirator pressure (<20 torr) while maintaining the water-bath temperature below 50 °C. Residual solvent was removed from samples using the vacuum (<0.1 torr) achieved by a mechanical belt-drive oil pump.

Semi-preparative high-performance liquid chromatography (HPLC) was performed with a Macherey–Nagel C8 reversed-phase column. Analytical HPLC was performed with an Agilent C8 reversed-phase column. Linear gradients of solvent A (H_2O with 0.1% v/v trifluoroacetic acid (TFA)) and solvent B (CH_3CN with 0.1% v/v TFA) were used for HPLC analysis and purification.

NMR spectra were recorded at ambient temperature in $CDCl_3$ with a Bruker DMX-400 Avance spectrometer (^{1}H, 400 MHz; ^{13}C, 100.6 MHz). Mass spectrometry was performed with a Micromass LCT (electrospray ionization, ESI) or an Applied Biosystems Voyager DE-Pro (matrix-assisted laser desorption/ionization, MALDI) mass spectrometer.

N-Acetyl-(2*S*)-4-ketoproline Methyl Ester (2)

Jones oxidation of AcHypOMe (1) provided access to AcKepOMe (2), as shown in Scheme 1. AcHypOMe (1.45 g, 6.5 mmol) was dissolved in acetone (100 mL), and the resulting solution was cooled to 0 °C. Jones reagent (4.6 mL) was added, and the reaction mixture was stirred for 1 h at 0 °C. Isopropanol (10 mL) was added to quench the reaction. The reaction mixture was filtered and concentrated under reduced pressure. The resulting solid was dissolved in water (30 mL) and extracted with EtOAc (2 × 20 mL). The organic layer was dried over anhydrous $MgSO_4$(s) and concentrated under reduced pressure. The product was isolated by flash chromatography over silica gel (0:10→1:19 v/v MeOH/CH_2Cl_2) affording AcKepOMe (**2**) (0.60 g, 50%) as a yellow oil. ^{1}H NMR ($CDCl_3$, 400 MHz): δ Rotamer A 2.99 (s) and Rotamer B 3.00 (s; total = 3H), Rotamer A 3.61 (d, J = 18.6 Hz, 0.2H) and Rotamer B 3.50 (dd, J = 3.1 and 19.1 Hz, 0.8H), Rotamer A 3.91 (dd, J = 10.2 and 18.6 Hz, 0.2H) and Rotamer B 3.81 (dd, J = 10.6 and 19.1 Hz, 0.8H), Rotamer A 4.67 (s, 0.6H) and Rotamer B 4.63 (s, 2.4H), Rotamers A and B 4.64–5.07 (m, 2H), Rotamer A 5.73 (dd, J = 1.3 and 10.2 Hz, 0.2H) and Rotamer B 5.89 (dd, J = 3.1 and 10.6 Hz, 0.8H). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 22.5, 22.6, 40.5, 41.8, 52.0, 52.9, 53.3, 53.6, 55.2, 57.4, 170.1, 170.2, 171.0, 171.8, 207.0, 207.1. ESI–MS: m/z $[M + H]^+$ calcd for $C_8H_{12}NO_4$ 186.0761, found 186.0755.

Measurement of $K_{trans/cis}$ for AcKepOMe (2)

AcKepOMe (**2**) (~5 mg) was dissolved in D_2O or $CDCl_3$ (~0.8 mL). ^{1}H NMR spectra were acquired and processed with the program NUTS from Acorn NMR (Livermore, CA). Values of $K_{trans/cis}$ were determined from the relative areas of *trans* and *cis* peaks.

N-Fluorenylmethyloxycarbonyl-(2*S*)-4-ketoproline

FmocKepOH was synthesized as a white solid by Jones oxidation of FmocHypOH using conditions that enabled access to BocKepOH.[16] ^{1}H NMR ($CDCl_3$, 400 MHz): δ 2.61 (d, J = 19.3 Hz, 0.4H) and 2.80 (d, J = 19.3 Hz, 0.6H), 2.95 (dd, J = 10.5 and 19.0 Hz, 1H), 3.76–4.02 (m, 2H), 4.17–4.31 (m, 1H), 4.44–4.68 (m, 2.3H), and 4.82–4.92 (m, 0.7H), 7.09 (bs, 1H), 7.28–7.47 (m, 4H), 7.55 (m, 2H), 7.77 (m, 2H). ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 40.2, 41.1, 47.1, 52.4, 52.6, 55.7, 56.0, 68.0, 68.5, 120.3, 124.8, 124.9, 125.0, 127.3, 128.1, 141.5, 141.5, 143.4, 143.5, 155.7, 175.5, 207.1. ESI–MS: m/z $[M + Na]^+$ calcd for $C_{20}H_{17}NO_5Na$ 374.1, found 373.9.

Peptide Synthesis

Ac-(ProProGly)$_{10}$-NH_2 (**[Pro]CMP**) was synthesized as described previously.[17] Ac-(ProProGly)$_4$-KepProGly-(ProProGly)$_5$-NH_2 (**[Kep]CMP**) was synthesized on a solid phase by condensation of FmocProProGlyOH,[18] FmocKepOH, FmocProOH, and FmocGlyOH as appropriate with an Applied Biosystems Synergy 432A Peptide Synthesizer. The first ProProGly unit was loaded onto Fmoc-amide resin using standard coupling conditions. Additional FmocProProGlyOH units for the "host" triplets were coupled using standard coupling conditions, as were individual FmocProOH, FmocKepOH, and FmocGlyOH amino acids for the "guest" triplet. FmocKepOH was either double- or quadruple-coupled, and unreacted sites on the nascent peptide were capped subsequently by acetylation. The FmocProProGly trimer immediately following the Kep residue was double-coupled. The final three FmocProProGly triplets were coupled under standard coupling conditions, and unreacted sites on the peptide were capped by acetylation before cleavage. During the peptide synthesis, Fmoc-deprotection was achieved by treatment with piperidine (20% v/v) in dimethylformamide (DMF). The Fmoc-tripeptides (3–4 equiv) were converted to active esters by treatment with *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate, *N*,*N*-diisopropylethylamine, and 1-hydroxybenzotriazole. Couplings were allowed to proceed for 45–60 min. N-Terminal acetylations were achieved by

using acetic acid (3 equiv) and standard coupling reagents. Peptides were cleaved from the resin with 38:1:1 TFA/H_2O/TIPS (3 mL), precipitated from *tert*-butyl methyl ether at 0 °C, and isolated by centrifugation. Semi-preparative HPLC was used to purify the peptides at 60 °C after preheating solutions to 70 °C to disassemble any oligomers. **[Kep]CMP** was condensed with aminooxy-biotin by heating their solution in methanol for 30 min to form **[biotin–Kep]CMP** (Scheme 2). **[Kep]CMP** and **[biotin–Kep]CMP** were purified by semi-preparative HPLC with a flow rate of 4.0 or 5.2 mL/min and a gradient of 10–75% B over 45 min. Purified peptides were >90% pure as judged by analytical HPLC and MALDI–TOF mass spectrometry (*m*/*z*): $[M + Na]^+$ calcd 2607.3, found 2608.3 for **[Kep]CMP**; $[M + Na]^+$ calcd 2991.5, found 2992.2 for **[biotin–Kep]CMP**. We note that the oxime linkage in **[biotin–Kep]CMP** is labile to hydrolysis,[19] and minor degradation products were observed by mass spectrometry.

Conformational Analyses by CD Spectroscopy

CD spectra were recorded with an Aviv 202SF circular dichroism spectrometer. For the analyses, peptides were incubated at 4 °C for ≥48 h in either 50 mM HOAc, pH 3, or 10 mM PBS, pH 7.4. CD spectra were recorded in 1-nm increments with a 5-s averaging time, 1-nm bandpass, and 0.1-cm pathlength. Solutions were heated in 3 °C increments with a ≥5-min equilibration at each step. The ellipticity near 225 nm was monitored with a 5-s averaging time, 1-nm bandpass, and 0.1-cm pathlength. Values of T_m, which is the temperature at the midpoint of the thermal transition between the folded and unfolded states, were determined to ±1 °C by fitting the data to a two-state model.[20]

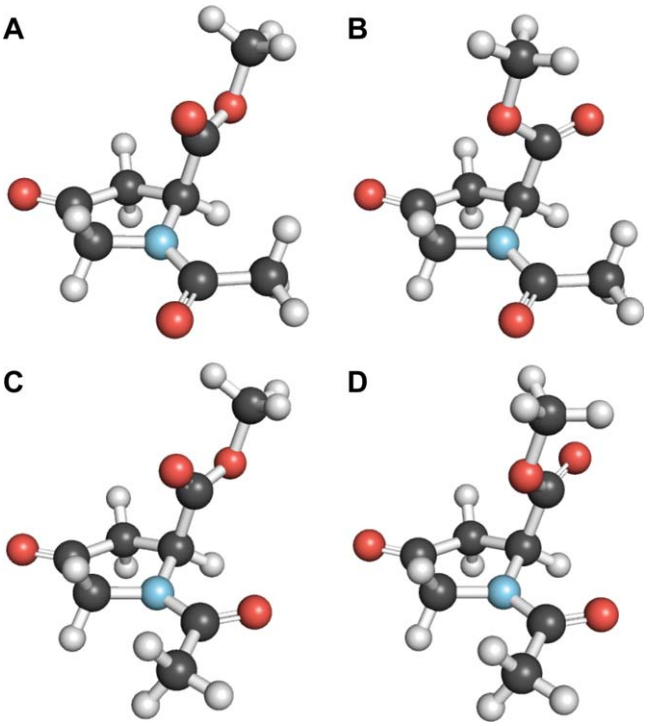


FIGURE 1 Calculated minimal energy conformations of AcKepOMe: (A) *cis*-distal (B) *cis*-proximal (C) *trans*-distal (D) trans-proximal. Calculations were performed at the B3LYP/6-311+G(2d,p) level of theory.

RESULTS AND DISCUSSION

We began our studies by analyzing the conformational properties of the Kep derivative AcKepOMe (**2**) in the gas phase with DFT calculations. Typical 4-substituted Pro derivatives display either the C^γ-endo or the C^γ-exo ring pucker.[21,22] In these conformations, C^γ of the pyrrolidine ring is located on either side of the plane formed by the rest of the ring constituents. Such conformations are not readily accessible to AcKepOMe, due to the presence of an sp^2 hybridized carbon atom at C^γ along with an amide nitrogen that likewise has sp^2 hybridization. Hence, we expected the pyrrolidine ring conformations adopted by AcKepOMe to be significantly more planar than those of typical C^γ-substituted Pro derivatives. Geometry optimizations validated this hypothesis, and showed that only C^α is oriented out of the plane of the ring, and only slightly so (Figure 1). Four energy minima were located for AcKepOMe, differing in their φ, ψ, and ω dihedral angles, where φ, ψ, and ω refer to C'_{i-1}–N_i–C^α_i–C'_i, N_i–C^α_i–C'_{i+1}–O_{i+1}, and C^α_{i-1}–C'_{i-1}–N_i–C^α_i, respectively.

Our *ab initio* calculations indicated that the trans conformation of AcKepOMe is favored over the cis conformation by 1.5 kcal/mol. Previously, we showed that the trans conformation in AcProOMe is stabilized by the delocalization of electron density from the amide oxygen into the antibonding orbital of the ester carbonyl group.[22–24] Natural Bond Orbital[25–27] analyses indicate that this $n \rightarrow \pi^*$ interaction stabilizes the trans conformation of AcKepOMe by 0.22 kcal/mol. This stabilization energy is lower than that in AcProOMe (0.40 kcal/mol),[22] presumably due to a less favorable distance and angle between the donor oxygen and the acceptor carbonyl group in the trans conformation of AcKepOMe. In addition, we found that the main-chain torsion angles adopted by trans AcKepOMe are $\varphi = -72°$ and $\psi = 156°$. These values are close to the φ and ψ angles found in the Xaa position of the collagen triple helix ($\varphi = -75°$ and $\psi = 164°$),[28] suggesting that Kep could be acceptable there.

Next, we synthesized AcKepOMe (Scheme 1). NMR spectroscopy revealed that the *trans* geometry of AcKepOMe is significantly different in energy than the cis geometry, in both $CDCl_3$ and D_2O. The values of the equilibrium constant for peptide bond isomerization determined in $CDCl_3$ and D_2O were similar: $K_{trans/cis} = 4.3$ and 3.6, respectively. These values are likewise similar to those obtained previously for AcProOMe in $CDCl_3$ and D_2O.[22]

Taking into account our conformational analyses, we proceeded to incorporate Kep into the Xaa position of a CMP. We synthesized **[Kep]CMP** via solid-phase peptide synthesis, along

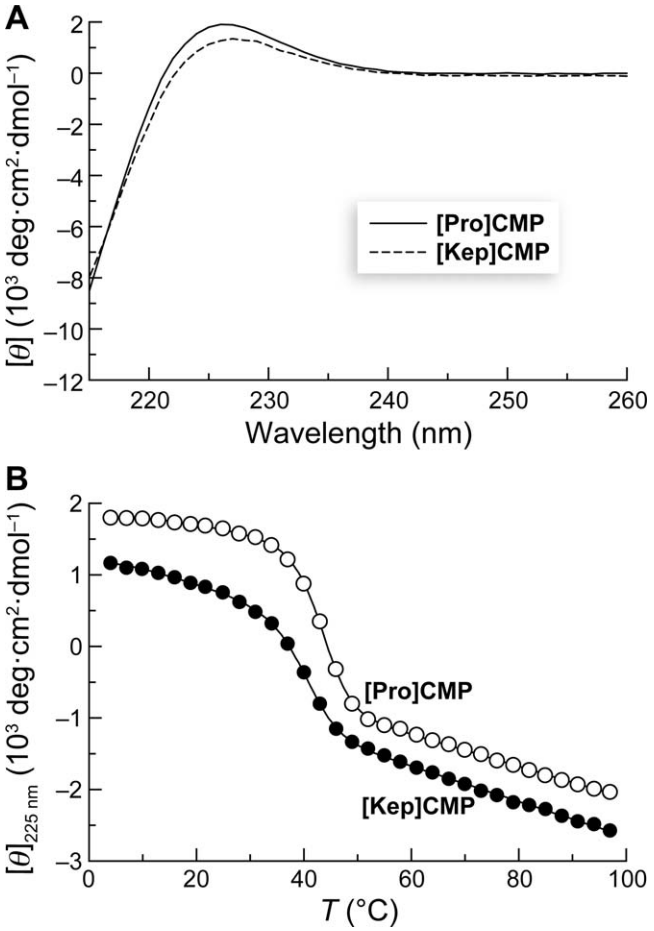


FIGURE 2 Conformational analysis of [**Kep**]**CMP** and [**Pro**]**CMP** by CD spectroscopy. (A) Spectra of peptide solutions (0.2 mM in 50 mM acetic acid) incubated at 4 °C for ≥24 h. (B) Effect of temperature on the molar ellipticity at 225 nm. Data were recorded at 3 °C intervals after equilibration for ≥5 min. [**Kep**]**CMP** and [**Pro**]**CMP** triple helices have T_m values of (40 ± 1)°C and (43 ± 1)°C, respectively.

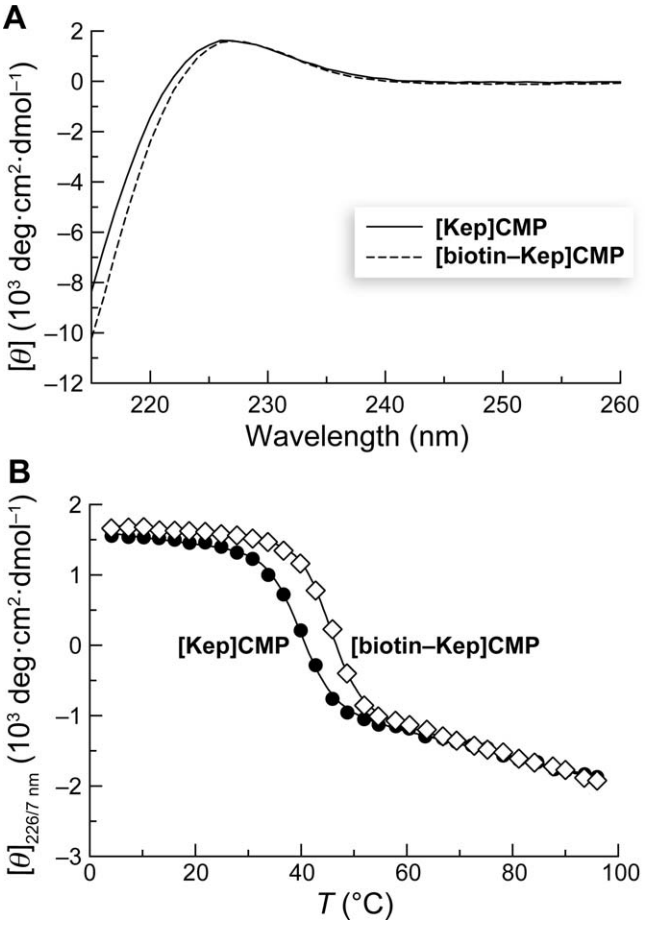


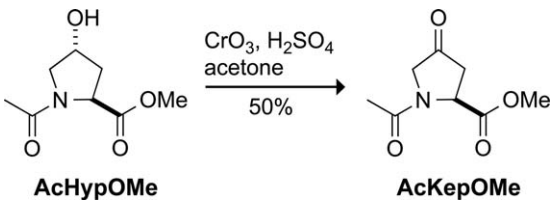
FIGURE 3 Conformational analysis of [**Kep**]**CMP** and [**biotin–Kep**]**CMP** by CD spectroscopy. (A) Spectra of peptide solutions (0.2 mM in 10 mM PBS buffer at pH 7.4) incubated at 4 °C for ≥24 h. (B) Effect of temperature on the molar ellipticity at 226 or 227 nm. Data were recorded at intervals of 3 °C after equilibration for ≥5 min. [**biotin–Kep**]**CMP** and [**Kep**]**CMP** triple helices have T_m values of (45 ± 1)°C and (40 ± 1)°C, respectively.

with [**Pro**]**CMP** as a control peptide. We used circular dichroism (CD) spectroscopy to assess triple-helix formation, as collagen triple helices have a signature CD spectrum with a maximum near 225 nm and display a cooperative transition near 225 nm upon denaturation.

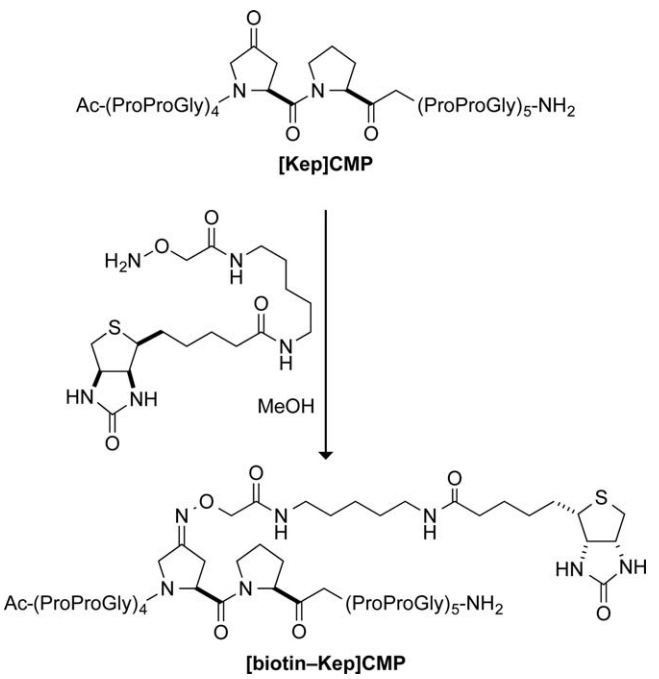
In acetic acid, both [**Kep**]**CMP** and [**Pro**]**CMP** exhibit the signature CD spectrum of a collagen triple helix (Figure 2A). Both also display cooperative transitions at 225 nm upon heating (Figure 2B). These data indicate that [**Kep**]**CMP** and [**Pro**]**CMP** adopt a triple helix at low temperature. Moreover, the thermal denaturation data indicate that triple helices composed of three [**Kep**]**CMP** strands and [**Pro**]**CMP** strands have comparable thermostability.

After finding that a collagen triple helix can accommodate Kep residues, we probed the use of Kep for bioconjugation. Analysis by CD spectroscopy revealed that [**biotin–Kep**]**CMP**, like [**Kep**]**CMP**, exhibits the signature CD spectrum of a triple helix (Figure 3A). The thermostabilty of a [**Kep**]**CMP** triple helix is nearly identical in acetic acid and PBS (Figures 2B and 3B). Most importantly, [**biotin–Kep**]**CMP** displays a cooperative transition upon heating (Figure 3B), and has uncompromised thermostability.

Thus, Kep joins (2*S*,4*R*)- and (2*S*,4*S*)-4-azidoproline[29–33] as a functionalizable amino-acid residue tolerated within a



SCHEME 1 Synthetic route to AcKepOMe.



SCHEME 2

collagen triple helix. Known bioconjugation reactions to Kep and a 4-azidoproline are orthogonal, allowing appendage of two distinct functional groups to collagen. The incorporation of Kep into a triple helix provides an especially facile means to conjugate exogenous alkoxyamines and hydrazines. The carbonyl group in Kep is constrained by its pyrrolidine ring, conveying rigidity beyond that of carbonyl groups in other nonproteinogenic α-amino acids (e.g., formylglycine). This electrophilic Pro derivative could also be used to tether different strands of a collagen triple helix, which is known to enhance thermostability.[34] Finally, the unique stretching vibration of its carbonyl group could provide a subtle probe for monitoring collagen folding with infrared spectroscopy.

CONCLUSION

We have revealed the conformational attributes of a (2*S*)-4-ketoproline residue. These attributes inspired us to perform host–guest studies, which demonstrated that Kep is well-tolerated in the Xaa position of a collagen triple helix. Finally, we found that Kep has utility as an electrophilic residue for conjugation with an alkoxyamine.

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