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SUBSTRATE SPECIFICITY OF HUMAN PROLYL-4-HYDROXYLASE

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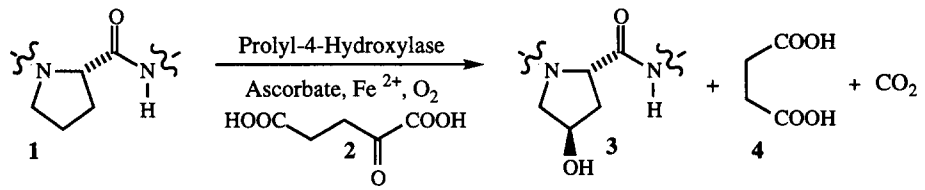
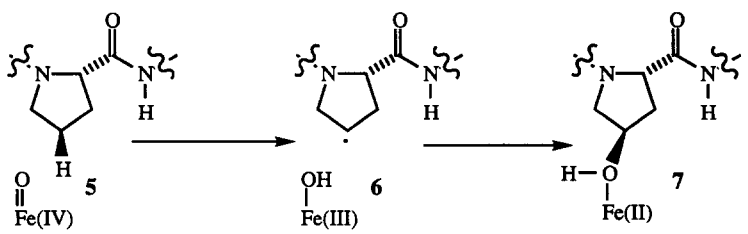
Abstract: Proline analogs (3-F, 3-Cl, 3-Br, 3,3-cyclopropyl, 3,3-methylene, 3-Me, and 4-Me) were synthesized, incorporated into CbzGlyPheXGlyOEt, and tested as substrate analogues/mechanistic probes for the human prolyl-4-hydroxylase catalyzed hydroxylation reaction. With the exception of the 3-fluoroproline containing peptide, none of these peptides were substrates for the enzyme.

Human prolyl-4-hydroxylase catalyzes the hydroxylation of proline residues at the X-Pro-Gly-sequence of procollagen (Scheme 1). This enzyme requires α-ketoglutarate (**2**), Fe(II), oxygen, and ascorbate and catalyzes an essential step in the biosynthesis of collagen, the major protein component of connective tissue.[1] Prolyl-4-hydroxylase is a target for the treatment of diseases involving the uncontrolled proliferation of collagen (fibrotic diseases).[2]



Scheme 1. The reaction catalyzed by human prolyl-4-hydroxylase.

While several examples of α-ketoglutarate dependent dioxygenases have been characterized,[3–6] and simple model systems[7] for the reaction have been described, the mechanism of human prolyl-4-hydroxylase is still poorly understood. A reasonable mechanistic hypothesis involving a hydrogen atom abstraction from the proline by an active site iron (IV) oxo intermediate followed by radical recombination is outlined in Scheme 2.[8] To probe for the intermediacy of the putative C-4 prolyl radical (**6**) proline analogue-containing peptides **16** (methyl cyclopropyl radical rearrangement), **19** (allylic rearrangement), **28** and **29** (β-scission of C-X bond) were synthesized and tested as substrates for the enzyme. In addition, analogs **20**, **22**, and **31** were synthesized to further define the substrate specificity of the enzyme.



Scheme 2. Mechanistic proposal for the prolyl-4-hydroxylase catalyzed reaction.

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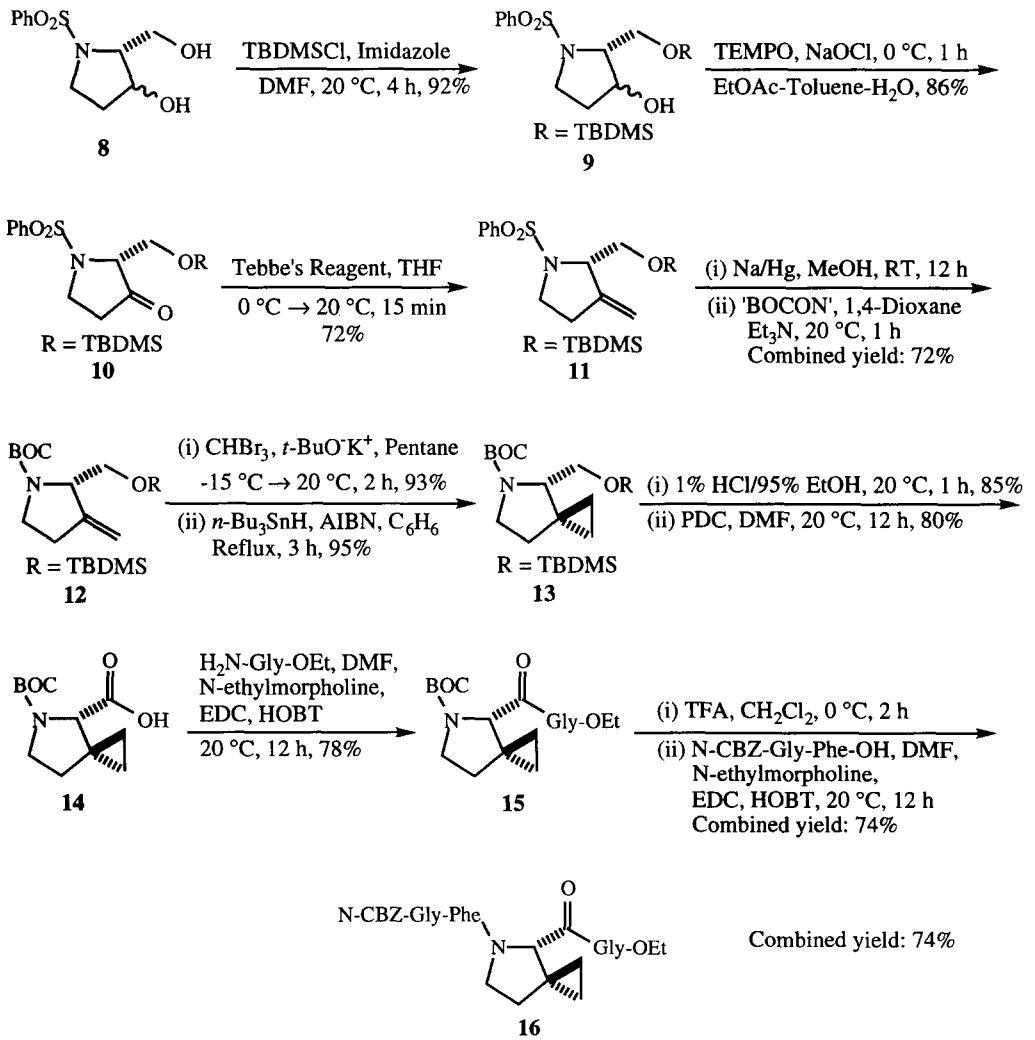
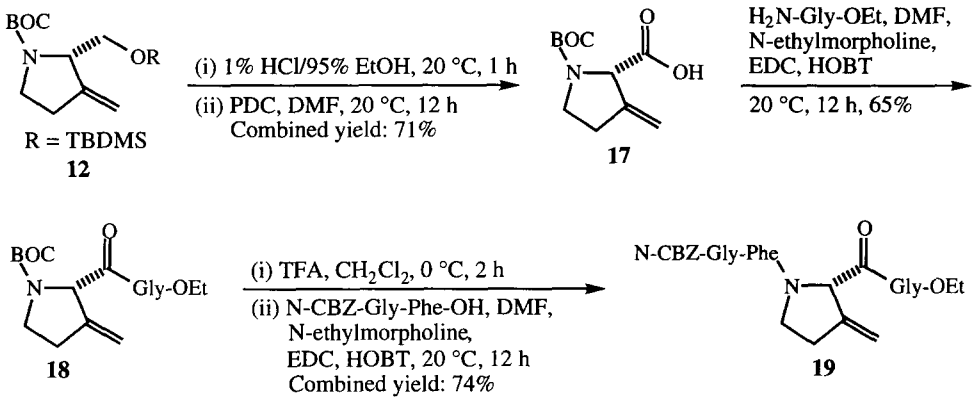
The synthesis of **16** was achieved starting from the diol **8**.[9] The primary alcohol of **8** was selectively protected as a *tert*-butyldimethylsilylether[10] followed by the oxidation of the secondary alcohol using TEMPO-NaOCl[11] to the ketone **10** in a combined yield of 79%. (Scheme 3) The ketone **10** was then reacted with Tebbe's reagent to give the alkene **11** in 72% yield.[12] The sulfonyl group was removed with freshly prepared sodium amalgam[13] and the resulting amine was protected with a *tert*-butyloxycarbonyl group using "BOCON". The combined yield for the two steps was 72%. The *t*-BOC alkene **12** was cyclopropanated using dibromocarbene generating conditions and dehalogenated using *n*-butyltin hydride and AIBN to give **13** in a combined yield of 88%.[14] The *tert*-butyldimethylsilyl protecting group was removed using acidic conditions[15] to give the alcohol which was oxidized with PDC-DMF[16] to the acid **14** in 68% yield. The spirocyclopropyl



Scheme 3. Synthesis of the 3,3-cyclopropylproline containing tetrapeptide **16**.

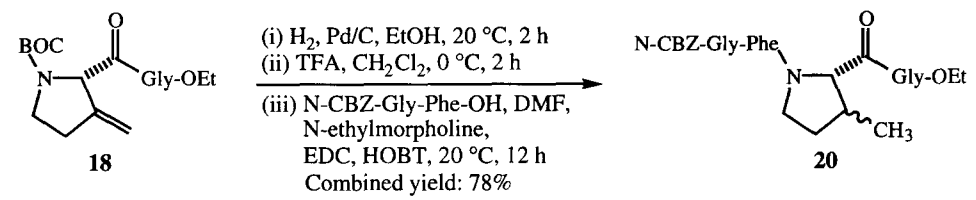
proline analog **14** was then coupled, in 78% yield, with glycine ethyl ester using standard carbodiimide peptide coupling chemistry.[17] The *tert*-butyloxycarbonyl group was removed with trifluoroacetic acid and the resulting amine was coupled with N-CBZ-Gly-L-Phe to give the tetrapeptide **16**. The combined yield for the last two steps was 74%.

To synthesize the 3,3-exomethyleneproline containing peptide **19**, alkene **12** was deprotected using acidic conditions and the resulting alcohol was oxidized to the acid **17** using PDC-DMF in a combined yield of 71% (Scheme 4). The acid was then converted into the tetrapeptide **19** in 48% yield using carbodiimide peptide coupling chemistry.



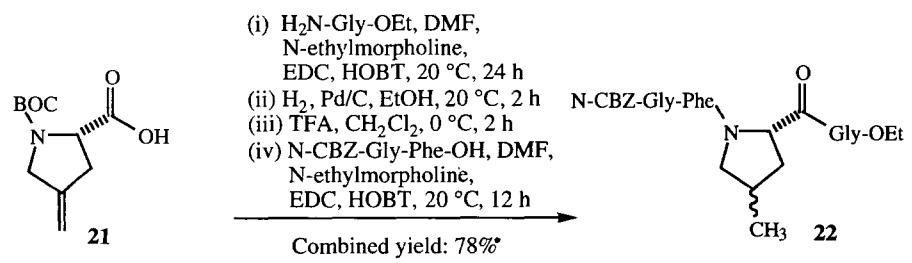
Scheme 4. Synthesis of the 3,3-exomethyleneproline containing tetrapeptide **19**.

The 3-methylproline peptide **20** was synthesized by first reducing the dipeptide **18** with hydrogen and Pd/C,[18] followed by removal of the *t*-BOC protecting group and coupling with N-CBZ-Gly-L-Phe. (Scheme 5) The combined yield for the three steps was 78%.



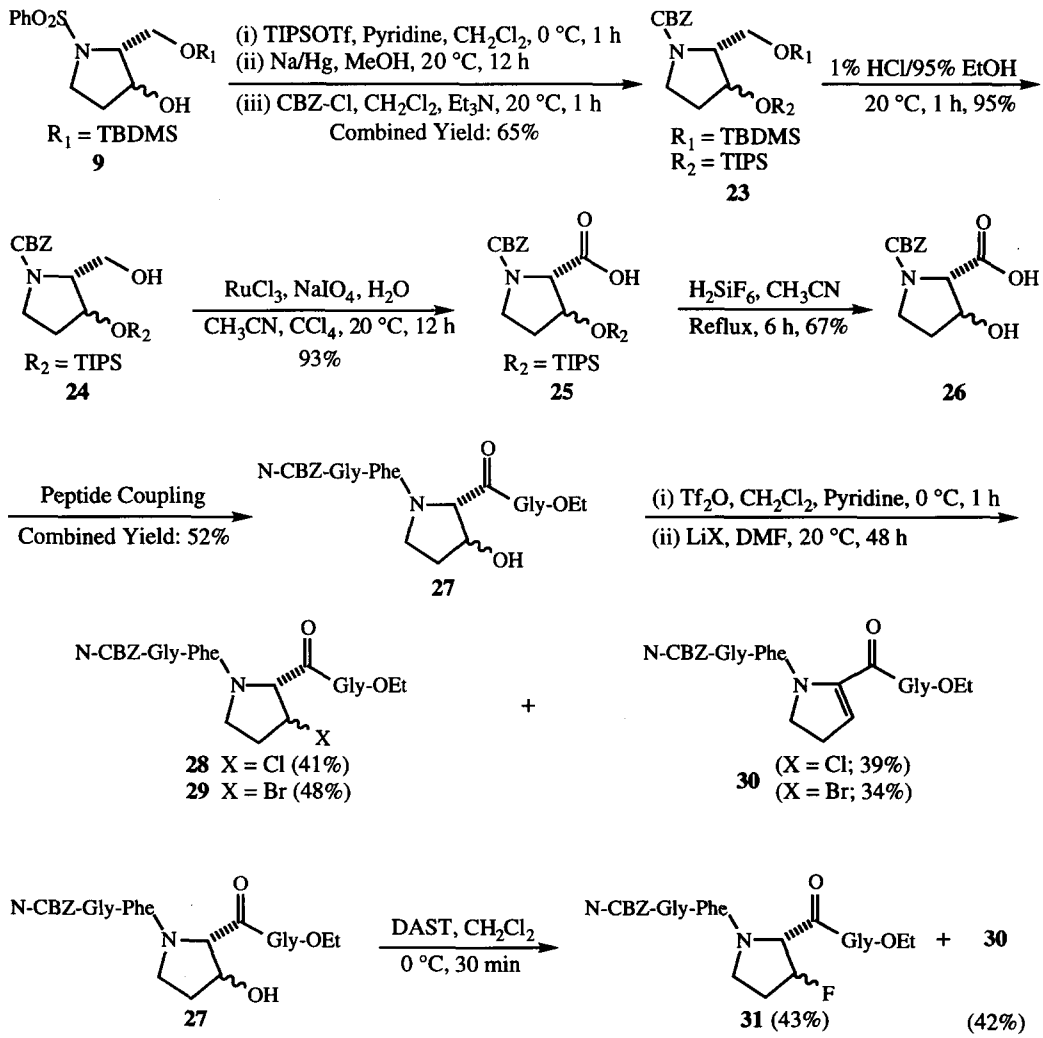
Scheme 5. Synthesis of the 3-methylproline containing tetrapeptide **20**.

The 4-methylproline peptide **22** was synthesized in 78% overall yield from **21** as outlined in Scheme 6.[19]



Scheme 6. Synthesis of 4-methylproline containing tetrapeptide **22**.

For the synthesis of the 3-haloproline containing peptides, alcohol **9** was first protected as the triisopropylsilylether using TIPS-triflate[20] (Scheme 7). The sulfonyl protecting group was then removed with sodium amalgam and the resulting amine was protected with a carbobenzyloxy group to give **23** in a combined yield of 65% for the three steps. The *tert*-butyldimethylsilyl protecting group was then removed using acidic conditions in 95% yield followed by $RuCl_3$-$NaIO_4$ oxidation[21] of the resulting alcohol **24** to give the TIPS protected acid **25** in 93% yield. The triisopropylsilyl group was then removed in 67% yield with fluorosilicic acid to yield 3-hydroxyproline (**26**),[22] which was then converted into the tetrapeptide **27** using standard peptide coupling chemistry. This was then treated with triflic anhydride, followed by displacement of the resulting triflate with LiCl or LiBr in DMF to yield **28** and **29**.[23] The 3-fluoroproline peptide **31** was synthesized in 43% yield by treating **27** with DAST at 0 °C.[24] A side product which was identified as the 2,3-dehydroproline containing peptide **30** was also isolated from each of these reactions.



Scheme 7. Synthesis of 3-chloro, 3-bromo, and 3-fluoroproline containing tetrapeptides (**28**, **29** and **31**).

These peptides[25] were incubated with human prolyl-4-hydroxylase and tested for inhibition of the enzyme and for the formation of new products.[26] The 3-exomethyleneproline peptide **19** was an inhibitor of the enzyme (IC_{50} = 200 μM). The 3-fluoroproline containing peptide **31** was a substrate for the enzyme undergoing hydroxylation at the C-4 position. Peptides **16, 20, 22, 28,** and **29** were not substrates or inhibitors of the enzyme. From these results it is clear that the human prolyl-4-hydroxylase is much less tolerant towards modifications on the proline residue than the bacterial enzyme[27] and that the exploration of the enzyme mechanism using substrate derivatives will require further investigation to identify mechanistically informative substituents that are tolerated by the enzyme.

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25. All peptides were characterized by 400 MHz ^{1}H NMR and FAB-HRMS. Peptides **16** and **19** were purified as a single compound, peptides **20**, **22**, **28**, **29** and **31** were purified as a mixture of the two indicated isomers.

26. The assay procedure involved incubating the peptide with enzyme for 30 min, extracting the reaction mixture with dichloromethane and analyzing by thin-layer chromatography. The enzymatic reaction mixture consisted of peptide (0.85 mM), $FeSO_4$ (0.0025 mM), ascorbic acid (0.1 mM), BSA (0.1 mg), catalase (0.0005 mg), DTT (0.005 mM), α-ketoglutarate (0.025 mM) tris.HCl (50 mM, pH 7.8) prolyl-4-hydroxylase (0.15 mg) in 500 μL volume.

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