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Proline 4-Hydroxylase: Stereochemical Course of the Reaction

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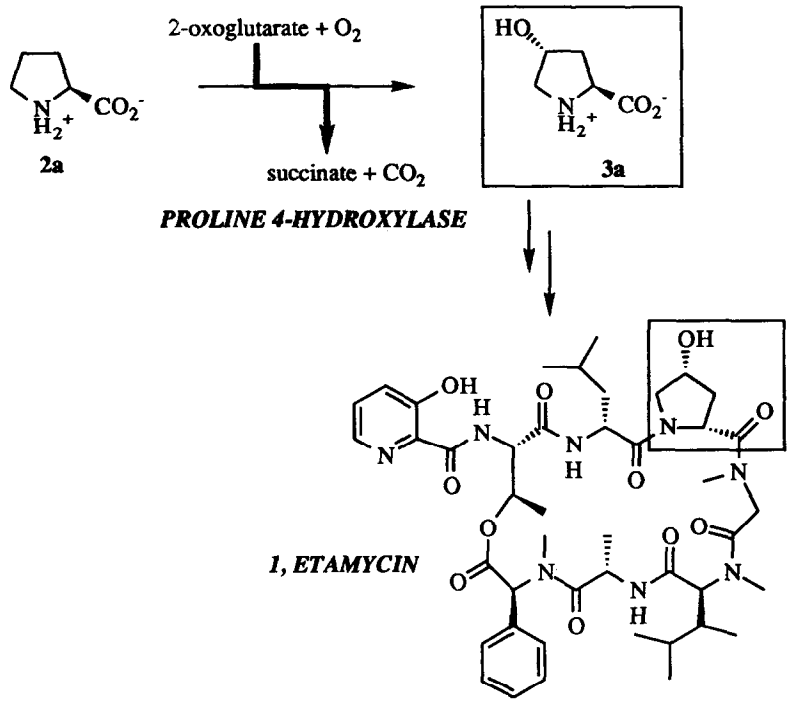
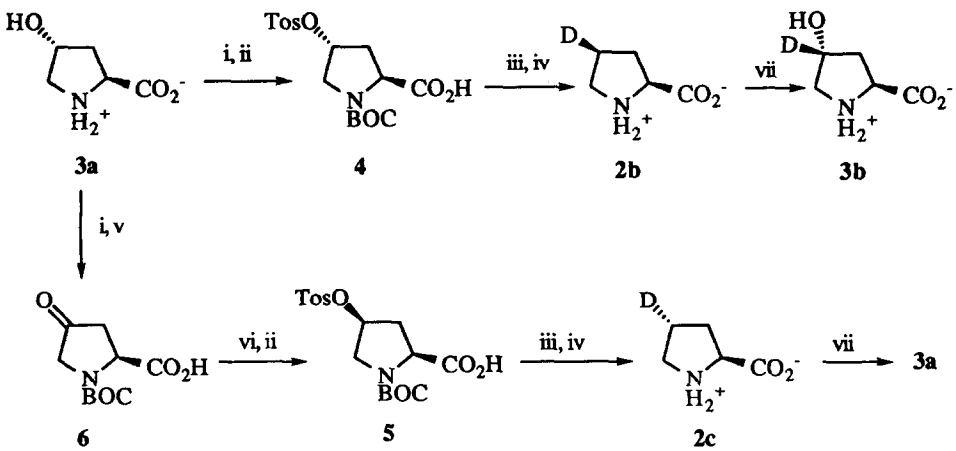
Abstract: The stereochemical course of the hydroxylation of (*S*)-proline by proline 4-hydroxylase from *Streptomyces griseoviridus* P8648 has been investigated using (2*S*, 4*S*)-[4-$^2H_1$]-proline and (2*S*, 4*R*)-[4-$^2H_1$]-proline and found to occur with retention of stereochemistry at C-4 of proline.

Hydroxylated proline residues are constituents both of certain proteins, such as collagen,[1] and of a number of peptide derived microbial secondary metabolites.[2] In the case of collagen, the biosynthetic route to the 4- and 3-hydroxyproline residues has been extensively studied and shown to occur as post-translational modifications catalysed by prolyl 4-hydroxylase (E.C. 1.14.11.2) and prolyl 3-hydroxylase (E.C. 1.14.11.7) respectively. These enzymes require 2-oxoglutarate and dioxygen as cosubstrates and hence belong to the family of 2-oxo acid and related dioxygenases.[3] In contrast, in the biosynthesis of the antibiotic etamycin **1**, produced by various strains of *Streptomyces griseoviridus*,[4] radiolabelled feeding experiments have revealed that free (*S*)-proline **2a** is initially hydroxylated to give free (2*S*, 4*R*)-hydroxyproline **3a** before incorporation into the macrocycle and epimerisation at the α-centre[5] (Scheme 1). Subsequently, Onishi *et al*[6] reported a crude cell free extract of *S. griseoviridus* P8648 which was capable of converting (*S*)-proline to (2*S*, 4*R*)-hydroxyproline. The crude activity was reported to have similar cofactor/cosubstrate requirements to the mammalian prolyl hydroxylases and thus also belongs to the 2-oxo acid dioxygenase family. Proline 4-hydroxylase has now been purified by us[7] and in this report we describe the strereochemical course of the proline hydroxylation reaction.

The desired substrates **2b** and **2c** have been previously synthesised from (2*S*, 4*R*)-hydroxyproline **3a** by Fujita *et al*,[8] however, this synthesis proceeded via prolinol intermediates, which necessitated undesirable redox manipulations, hence an improved protocol was developed. Thus, (2*S*, 4*R*)-hydroxyproline **3a** was converted to its *N*-BOC, 4-tosyl derivative **4** in two steps[9,10] and deuterium was stereospecifically incorporated by S_N2 displacement of tosylate using lithium triethylborodeuteride in tetrahydrofuran.[11] Trifluoroacetic acid deprotection yielded the 4*S*-diastereomer **2b** [m/z(DCI-NH_3) 119 (0%), 118 (5.5), 117 (MH^+, 100), 116 (3), 115 (5), 114 (1.5), 113 (0)]. For the 4*R*-diastereomer **2c**, *N-tert*-butoxycarbonyl-(2*S*, 4*R*)-hydroxyproline was converted to the 4*S*-tosylate **5** via oxidation to the ketone **6** using Jones reagent in acetone[12] followed by reduction using sodium borohydride in methanol[13] and tosylation.[10] Reactions analogous to those utilised for the other stereoisomer led to the desired product **2c** [m/z(DCI-NH_3) 120 (0%), 119 (1), 118 (5), 117 (MH^+, 100), 116 (3), 115 (4), 114 (1.5), 113 (0)] (Scheme 2). (During the sodium borohydride reduction of ketone **6**, a small amount (*ca.* 15%) of the 4*R*-alcohol was formed, which was removed at the tosylate stage by chromatography.)



Scheme 1



Scheme 2 i, $(^tBuOCO)_2O$, NaOH, $^tBuOH/H_2O$, 18h[9] (Yield=85%); ii, *p*-Toluenesulphonyl chloride, NaOH, H_2O/Et_2O, 24h[10] (**4** Yield=66%; **5** Yield=62%); iii, Lithium triethylborodeuteride, THF, 24h reflux[11] (4*S*-product, Yield=35%, 4*R*-product, Yield=36%); iv, Trifluoroacetic acid, 30 min, (Yield=quant.); v, Jones reagent, acetone, 1h[12] (Yield=73%); vi, Sodium borohydride, MeOH, 0°C, 3.5h[13] (Yield=90%); vii, Incubation with proline 4-hydroxylase - see text. Steps (i) to (vi) unoptimised.

The labelled [4*S*-$^2H_1$] **2b** and [4*R*-$^2H_1$] **2c** (2*S*)-prolines were incubated with partially purified bacterial proline 4-hydroxylase[7] in the presence of the appropriate cofactors.[14] The resultant (2*S*, 4*R*)-hydroxyprolines were derivatised to their *N,O*-bistrifluoroacetyl methyl esters, essentially as described previously,[15] for analysis by gas chromatography/mass spectrometry (BPX5 column, DCI-NH_3) (Table 1). The substrates **2b, 2c** were reisolated from incubations to ensure no loss of label from the (*S*)-prolines had occured during the incubation (Table 2).

In the case of the (2*S*, 4*S*)-[4-$^2H_1$]-proline **2b**, the GC-MS analysis indicated retention of the deuterium label (Table 1 entry b) to give labelled product **3b**, whereas, in the case of the (2*S*, 4*R*)-[4-$^2H_1$]-proline **2c**, loss of the deuterium label was observed to give **3a** (Table 1, entry c). In neither case was any loss of label from the proline substrates observed (Table 2, entries d and e). In each case the isotope content of the derivatised reaction products (**3a** or **3b**), as indicated by GC-MS analysis demonstrates that the hydroxylation of (*S*)-proline by proline 4-hydroxylase proceeds with loss of the *pro-R* hydrogen at C-4 with overall retention of stereochemistry.

m/z										355, 356 ~ $[MNH_4]^+$				
% observed	241	242	243	244	245	258	259	260	261	354	355	356	357	358
(a)	20	2	22	7	-	-	2	-	-	-	100	8	-	-
(b)	2	22	12	64	3	-	2	20	2	-	2	100	9	-
(c)	63	22	77	3	-	-	21	1	-	-	100	12	-	-

Table 1 Mass spectra data for *N,O*-bistrifluoroacetyl methyl ester derivatives of (2*S*, 4*R*)-hydroxyprolines from (a) authentic (2*S*, 4*R*)-hydroxyproline **3a**; (b) incubation of (2*S*, 4*S*)-[4-$^2H_1$]-proline **2b** with proline 4-hydroxylase; (c) incubation of (2*S*, 4*R*)-[4-$^2H_1$]-proline **2c** with proline 4-hydroxylase.

m/z	166, 167 ~ $[M\text{-}CO_2CH_3]^+$			226, 227 ~ $[MH]^+$			243, 244 ~ $[MNH_4]^+$				
% observed	166	167	168	226	227	228	242	243	244	245	246
(a)	69	5	-	73	6	-	-	100	8	-	-
(b)	-	33	2	-	64	5	-	-	100	8	-
(c)	3	33	2	2	66	7	-	3	100	8	1
(d)	-	22	-	-	45	1	-	3	100	7	-
(e)	2	31	2	2	63	6	-	1	100	9	-

Table 2 Mass spectra data for *N*-trifluoroacetyl methyl ester derivatives of (*S*)-prolines from (a) authentic (*S*)-proline **2a**; (b) authentic (2*S*, 4*S*)-[4-$^2H_1$]-proline **2b**; (c) authentic (2*S*, 4*R*)-[4-$^2H_1$]-proline **2c**; (d) incubation of (2*S*, 4*S*)-[4-$^2H_1$]-proline **2a** with proline 4-hydroxylase; (e) incubation with (2*S*, 4*R*)-[4-$^2H_1$]-proline **2c** with proline 4-hydroxylase.

The observation that the hydroxylation of (*S*)-proline **2a** by proline 4-hydroxylase proceeds with retention of stereochemistry at C-4 is in accord with previous studies on hydroxylation reactions catalysed by 2-oxo acid dependent dioxygenases, including mammalian prolyl 4-hydroxylase.[8,16-21] In addition, the desaturative cyclisations to form the clavams[22] and the penams,[23] which are catalysed by related dioxygenases, have been shown to proceed with retention of stereochemistry at carbons which are oxidised. The only exceptions to this general trend have been observed in the ring expansion of penicillin N to deacetoxycephalosporin C[24-26]

(catalysed by deacetoxy/deacetylcephalosporin C synthase) and for the second (S containing) ring formation in tripeptide bicyclisation reactions with certain unnatural substrates as catalysed by isopenicillin N synthase.[27] It is noteworthy that the former exception involves a rearrangement via a postulated free radical and both exceptions involve carbon-sulphur rather than carbon-oxygen bond formation.

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REFERENCES & NOTES

1. For a recent review see Kivirikko, K. I.; Myllylä, R.; Pihlajaniemi, T. Hydroxylation of Proline and Lysine Residues in Collagens and other Animal and Plant Proteins. In *Post-translational Modifications of Proteins*; Harding, J. J.; Crabbe, M. J. C. Eds.; CRC Press: Boca Raton, Florida, 1992; Chapter 1.
2. *Biochemistry of Peptide Antibiotics*; Kleinkauf, H.; von Döhren, H. Eds.; W. de Gruyter: Berlin; New York, 1990.
3. For recent reviews see Jefford, C. W. *Advances in Detailed Reaction Mechanisms*, **1992**, *2*, 149-187 and Prescott, A. G. *J. Exptl. Bot.* **1993**, *44*, 849-861.
4. Haskell, T. H.; Maretzki, A.; Bartz, Q. R. Viridogrisein: Chemical Studies. In *Antibiotics Annual 1954/1955*; Welch, H.; Marti-Ibañez, F. Eds.; Medical Encyclopedia, Inc.: New York, 1955; pp. 784-789.
5. Katz, E.; Kamal, F.; Mason, K. *J. Biol. Chem.* **1979**, *254*, 6684-6690.
6. Onishi, M.; Okumura, Y.; Okamoto, R.; Ishikura, T. *Biochem. Biophys. Res. Comm.* **1984**, *120*, 45-51.
7. Baldwin, J. E.; Lawrence, C. C.; Field, R. A.; Schofield, C. J. Details will be published elsewhere.
8. Fujita, Y.; Gottlieb, A.; Peterkofsky, B.; Udenfriend, S.; Witkop, B. *J. Am. Chem. Soc.* **1964**, *86*, 4709-4716.
9. Keller, O.; Keller, W. E.; van Look, G.; Wersin, G. in *Org. Synth. Coll. Vol. VII*, Freeman, J. P., Editor-in-Chief, John Wiley and Sons, Inc.: New York, 1993; pp. 70-75.
10. Rudolph, M.; unpublished procedure.
11. Krishnamurthy, S.; Brown, H. C. *J. Org. Chem.* **1976**, *41*, 3064-3066.
12. Patchett, A. A.; Witkop, B. *J. Am. Chem. Soc.* **1957**, *79*, 185-192.
13. Andreatta, R. H.; Nair, V.; Robertson, A. V.; Simpson, W. R. J. *Aust. J. Chem.* **1967**, *20*, 1493-1509.
14. Incubations were performed at 26 °C in TES buffer (25 mM, pH 7.5), with shaking (200 r.p.m.) and contained, in a total volume of 0.2 ml, (2*S*, 4*S*)-[4-$^2H_1$]-proline **2b** or (2*S*, 4*R*)-[4-$^2H_1$]-proline **2c** (1 mM), catalase (0.2 mgml^{-1}), iron(II)ammonium sulphate (0.5 mM), 2-oxoglutarate (0.5 mM) and partially purified proline 4-hydroxylase (67 units[a]). After four hours, protein was precipitated with acetone (70%v/v) and the hydroxyproline in the supernatant derivatized as described in the text. ([a] 1 unit = 1 pmol of (2*S*, 4*R*)-hydroxyproline formed per minute from (*S*)-proline).
15. Tredget, E. E.; Falk, N.; Scott, P. G.; Hogg, A. M.; Burke, J. F. *Anal. Biochem.* **1990**, *190*, 259-265.
16. Leete, E.; Lucast, D. H. *Tetrahedron Lett.* **1976**, *38*, 3401-3404.
17. Pascal Jr., R. A.; Han, H. *J. Org. Chem.* **1990**, *55*, 5173-5176.
18. Englard, S.; Blanchard, J. S.; Midelfort, C. F. *Biochemistry* **1985**, *24*, 1110-1116.
19. Baldwin, J. E.; Merritt, K. D.; Schofield, C. J.; Elson, S. W.; Baggaley, K. H. *J. Chem. Soc., Chem. Commun.* **1993**, 1301-1302.
20. Stubbe, J. *J. Biol. Chem.* **1985**, *260*, 9972-9975.
21. Townsend, C. A.; Barrabee, E. B. *J. Chem. Soc., Chem. Commun.* **1984**, 1586-1588.
22. Basak, A.; Salowe, S. P.; Townsend, C. A. *J. Am. Chem. Soc.* **1990**, *112*, 1654-1656.
23. For a recent review see Baldwin, J. E.; Bradley, M. *Chem. Rev.* **1990**, *90* 1079-1088.
24. Townsend, C. A. *J. Nat. Prod.* **1985**, *48*, 708-724.
25. Townsend, C. A.; Theis, A. B.; Neese, A. S.; Barrabee, E. B.; Poland, D. *J. Am. Chem. Soc.* **1985**, *107*, 4760-4767
26. Pang, C.-P.; White, R. L.; Abraham, E. P.; Crout, D. H. G.; Lutstorf, P. J.; Morgan, P. J.; Derome, A. E. *Biochem. J.* **1984**, *222*, 777-788.
27. See Baldwin, J. E.; Schofield, C. J. The Biosynthesis of β-Lactams. In *The Chemistry of β-Lactams*; Page, M. I. Ed.; Blackie Academic and Professional: Glasgow, 1992; Chapter 1 and references therein.

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