Bioorganic & Medicinal Chemistry Letters, Vol.3, No.6, pp. 1189-1192, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00

REDUCTION OF Δ^1-PYRROLINE-2-CARBOXYLIC ACID TO PROLINE BY AN *ESCHERICHIA COLI* PROLINE AUXOTROPH.

Mark L. Lewis, Steve L. Martin, Christine J. Rowe,
John D. Sutherland*, Esther J. Wilson and Martin C. Wright.

The Dyson Perrins Laboratory and The Oxford Centre for Molecular Sciences, South Parks Road, Oxford OX1 3QY, U.K..

(*Received in USA 25 January* 1993)

ABSTRACT.

A new synthesis of Δ^1-pyrroline-2-carboxylic acid **2** and various isotopomers is described; the conversion of **2** to proline by *Escherichia coli* is shown to proceed by a direct reduction.

INTRODUCTION.

In the bacterium *Escherichia coli* proline is normally synthesised from glutamate by the action of three enzymes[1], Fig. 1. The *pro* B gene product catalyses an ATP-dependent kinasing reaction to give γ-glutamyl phosphate which is then reduced by the *pro* A gene product to give γ-glutamyl semialdehyde. Spontaneous dehydrative cyclisation of γ-glutamyl semialdehyde provides Δ^1-pyrroline-5-carboxylic acid **1**[2] which is then reduced by the *pro* C gene product to proline.

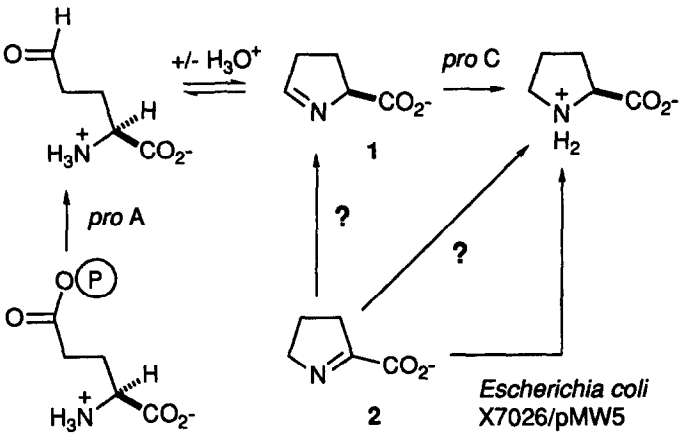

Fig. 1 Proline Biosynthesis

In connection with studies on new, unnatural biosynthetic routes to proline we have recently found that an isomer, Δ^1-pyrroline-2-carboxylic acid **2**, of the natural proline precursor **1**, is capable of sustaining growth of an *Escherichia coli* proline auxotroph, X7026 which has had the *pro* A and *pro* B genes deleted but retains the *pro* C gene[3]. This is in contrast to previously reported results

employing less well characterised auxotrophs and **2** prepared by enzymatic oxidation of proline[4]. We have isolated a plasmid, pMW5, which increases growth on **2**, by selection from an *E. coli* genomic library maintained in strain X7026[5]. The insert in pMW5 appears to be associated with conferring an increase in membrane permeability to **2** rather than with a specific reductase. In order to ascertain whether **2** is converted to proline by direct reduction or *via* initial isomerisation to **1** followed by reduction catalysed by the *pro* C gene product we sought a synthesis of isotopically labelled **2** which had more flexibility than existing routes[4,6].

It has been reported that barium hydroxide catalysed hydrolysis of 3,3-dichloro-2-piperidone **3** yields **1**[7,8] however we have found that the isomeric structure, **2**, is infact produced, Fig. 2.

Reduction of **2** to proline can be achieved by catalytic hydrogenation or, more conveniently, by treatment with sodium borohydride. We have also found that the interconversion of **1** (an unstable compound that can be purchased as the 2,4-dinitrophenylhydrazone and liberated by stirring with acetophenone[9]) and **2** cannot be brought about by treatment with acid or base. Exchange of the C3-protons of **2** for deuterons can be effected by treatment with barium deuteroxide. **3** can be prepared by treatment of 2-piperidone **4** with PCl_5 followed by chlorine[10].

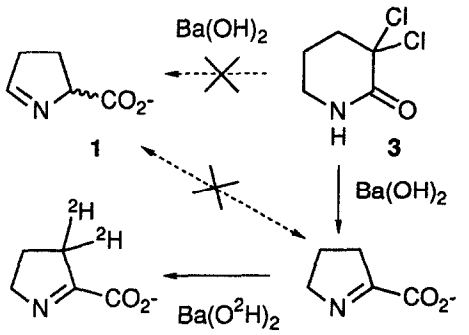

Fig. 2 Dichlorolactam Hydrolysis

We reasoned that as an imino chloride or equivalent is a likely intermediate in this reaction then Beckmann rearrangement of cyclopentanone oxime with PCl_5 followed by chlorination should also produce **3**. This expectation has been realised opening the way to a short synthesis of isotopically labelled variants of **2**, Fig. 3. Deuteration can be introduced at C-5 of **2** by starting from 2,2,5,5-tetradeuterocyclopentanone and at C-3 by hydrolysis of **3** in barium deuteroxide, ^{15}N labelling of **2** can be achieved *via* formation of the oxime of cyclopentanone with ^{15}N-hydroxylamine. The various isotopomers so synthesised have been used in feeding experiments and in the spectroscopic determination of the structures adopted by **2** at various pH values[2].

To differentiate between the two aforementioned routes from **2** to proline, 5,5-dideutero-Δ^1-pyrroline-2-carboxylic acid 5,5-$^2H_2$-**2** was utilised. It was predicted that if there was an isomerase activity then the proline produced would either be unlabelled or monolabelled in the 5-position depending on the stereospecificity of the process. Initial feeding experiments with **2** were hampered by the fact that *E. coli* transformed with plasmid, pMW5, displays an extremely slimy phenotype and does not grow well in liquid medium; accordingly the recombinant organism had to be grown on solid medium containing the unnatural proline precursor. Total soluble protein was isolated from cells grown on 5,5-$^2H_2$-**2** and was hydrolysed by refluxing in 6M HCl. Partial purification of proline was achieved by ion-exchange chromatography on Dowex-50-H^+ resin[11].

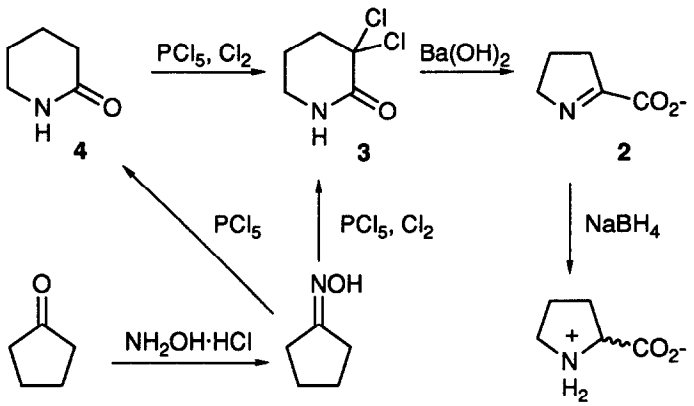

Fig. 3 Synthesis of Δ^1-Pyrroline-2-carboxylic Acid

Examination of the 500 MHz ^{1}H NMR spectrum, Fig.4, revealed that the recovered proline was approximately 60% dideuterated (as adjudged by relative integrations of the H-5 signals at δ 3.4ppm to other signals), the residual material being completely unlabelled.

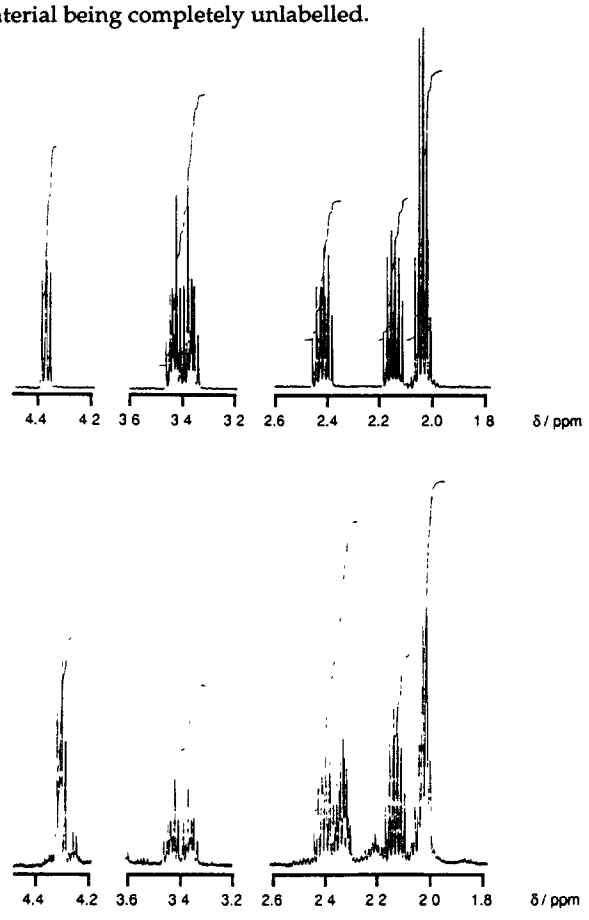

Fig. 4 500MHz ^{1}H NMR of Unlabelled Proline (above) and Biosynthesised Proline (below)

^{2}H NMR spectroscopy confirmed the presence of 5,5-dideuteroproline. Mass spectral analysis was difficult due to the presence of valine, which by serendipity has the same mass as dideuteroproline, in the partially purified sample. These labelling results suggest that the unnatural precursor, **2** is reduced directly to proline in *E. coli* and that isomerisation to **1** followed by reduction catalysed by the *pro* C gene product does not occur *in vivo*. The presumed reversibility of this reduction suggests that the oxidation of proline to **2** may occur in *E. coli* and that **2** should therefore be regarded as a natural product. Studies on two enzymes which are candidates for the *in vivo* reduction are reported in a following paper[12].

ACKNOWLEDGEMENTS.

We thank the SERC for support and Professor J. E. Baldwin for helpful discussions.

REFERENCES & FOOTNOTES.

1 Leisinger, T. in *Escherichia Coli and Salmonella Typhimurium Cellular and Molecular Biology*, Ed. in Chief Neidhart, F. C.; American Society for Microbiology: Washington, D.C., 1987; Vol 1, pp. 345-351.

2 We have shown (3rd paper out of four in this issue) that Δ^1-pyrroline-2-carboxylic acid **2** exists as the cyclic imine carboxylate at physiological pH and hence represent it as such in this paper. It is assumed that Δ^1-pyrroline-5-carboxylic acid **1** also exists as the cyclic imine carboxylate at physiological pH.

3 Kahn, P. L. *J. Bacteriol.* **1968**, *96*, 205.

4 Meister, A. J. *J. Biol. Chem.* **1954**, *206*, 577.

5 1st paper out of four in this issue.

6 Hasse, K.; Wieland, A. *Chem. Ber.* **1960**, *93*, 1686, Häusler, J.; Schmidt, U. *Liebigs Ann. Chem.* **1979**, 1881.

7 Osugi, K. *Yakugaku Zasshi* **1958**, *78*, 1332.

8 Mauger, A. B.; Witkop, B. *Chem Rev.* **1966**, *66*, 47.

9 Mezl, V. A.; Knox, W. E. *Anal. Biochem.*. **1976**, *74*, 430.

10 Wineman, R. J.; Hsu, E.-P. T.; Anagnostopoulos, C. *J. Am. Chem. Soc.* **1958**, *80*, 6233.

11 Hirs, C. H. W.; Moore, S.; Stein, W. H. *J. Am. Chem. Soc.* **1954**, *76*, 6063.

12 4th paper out of four in this issue.