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# 2′-Hydroxypyridoxol, a Biosynthetic Precursor of Vitamins $B_6$ and $B_1$ in Yeast

Johannes Zeidler, Nisar Ullah, Ram Nath Gupta, Richard M. Pauloski, Brian G. Sayer, and Ian D. Spenser*

*Department of Chemistry, McMaster University, Hamilton, Ontario, Canada L8S 4M1*

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It has been established that in *Escherichia coli* and other bacteria the $C_5$-unit C-2′,2,3,4,4′ of pyridoxol (**2**) is derived from 1-deoxy-D-xylulose 5-phosphate (**1**) which, in turn, originates by enzyme-catalyzed condensation, accompanied by decarboxylation, of pyruvic acid (yielding C-2′,2) with D-glyceraldehyde 3-phosphate (yielding C-3,4,4′).[1] In the yeasts *Saccharomyces cerevisiae* and *Candida utilis*, by contrast, the corresponding $C_5$-unit of pyridoxamine (**3**) is derived from an intact pentose or pentulose (**4**), C-1 of which yields C-2′ of pyridoxamine.[2] Thus the oxygen function at C-1 of the $C_5$-sugar, most likely the C-1 hydroxy group of a pentulose, is lost in the course of the biosynthesis of the vitamin. This loss may occur either after formation of the pyridine ring system or before the ring is formed by union of the $C_5$-sugar with the $C_3N$ moiety that generates N-1,C-6,5,5′ of the vitamin.

We now present evidence that in the yeast *S. cerevisiae* ATTC 7752 (= IFO 1234) loss of the C-1 hydroxy group takes place after the pyridine system has been completed: 3-Hydroxy-2,4,5-tri-(hydroxymethyl)pyridine (i.e., 2′-hydroxypyridoxol) (**5**) serves as a precursor of **3**. Furthermore, **5** also lies on the biosynthetic route to the pyrimidine unit (**9**) of thiamin (vitamin $B_1$).

The notion that **5** may be implicated in vitamin $B_6$ biosynthesis was first advanced some 30 years ago: C. J. Argoudelis[3] found that this compound showed vitamin $B_6$ activity in the yeast *S. carlsbergensis*. Somewhat later it was reported[4] that the yeast *Kloeckera apiculata*, which does not synthesize vitamin $B_6$ from simple precursors and, for growth, requires a medium containing pyridoxol, grew on 2′-hydroxypyridoxol in the absence of pyridoxol. When this yeast was incubated with tritium-labeled 2′-hydroxypyridoxol (of undetermined tritium distribution) the $B_6$ vitamers that were isolated from the cell extract contained radioactivity.[4] These results were indicative rather than conclusive, and it is regrettable that at the time this work was not extended "to test the possibility that 3-hydroxy-2,4,5-trihydroxymethylpyridine is a true biological precursor of vitamin B-6"[4] in yeasts.

The results here presented show that this is indeed the case. Deuterium from $^2$H-labeled samples of **5** entered predictable sites of **3** and of the pyrimidine unit (**9**) of thiamin, when cultures of *S. cerevisiae* were incubated with these labeled substrates.

The synthesis of [5′,5′-$^2H_2$]-2′-hydroxypyridoxol ([5′,5′-$^2H_2$]-**5**)[5−7] and [2′,2′,5′,5′-$^2H_4$]-2′-hydroxypyridoxol ([2′,2′,5′,5′-$^2H_4$]-**5**)[8] was carried out by modification of reported methods (see Supporting Information). HPLC traces, the $^1$H, $^2$H, and $^{13}$C spectra,[9] as well as the mass spectra of [5′,5′-$^2H_2$]-**5** and of [2′,2′,5′,5′-$^2H_4$]-**5** showed that the two samples were free of pyridoxol, pyridoxal, or any other contaminants.

In separate experiments cultures of *S. cerevisiae* ATTC 7752 were grown in the presence of these two labeled substrates, and pyridoxamine and thiamin were isolated. Incubations were carried out as previously reported.[2] The cell digest, after addition of unlabeled pyridoxamine and thiamin pyrophosphate as carriers and after enzymatic hydrolysis, was treated with a weak cation-exchange resin, Amberlite CG50, to isolate bases. The mixture of bases containing thiamin and pyridoxamine was separated on a silica gel column. The separated thiamin chloride HCl was further purified on an alumina column. Pyridoxamine diHCl was isolated from the residual mixture of bases by HPLC, as described earlier.[2]

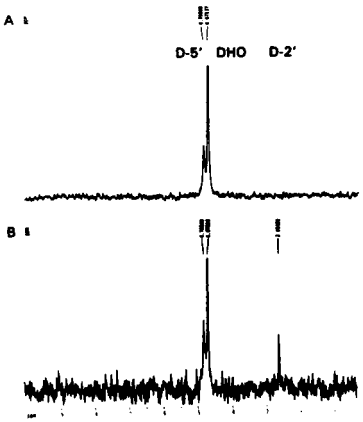
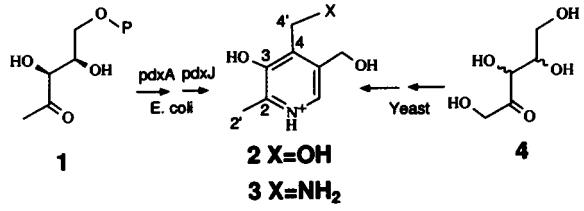



***Figure 1.*** $^2$H NMR (46.07 MHz, $^2$H depleted $H_2O$, 35° C) (A) $^2$H NMR spectrum of pyridoxamine (**3**) diHCl from the incubation with [5′,5′-$^2H_2$]-2′-hydroxypyridoxol HCl. (B) $^2$H NMR spectrum of pyridoxamine (**3**) diHCl from the incubation with [2′,2′,5′,5′-$^2H_4$]-2′-hydroxypyridoxol HCl.

***Scheme 1.*** Origin of the $C_5$ Unit C-2′,2,3,4,4′ of Pyridoxine




The $^2$H NMR spectra of the samples of pyridoxamine diHCl from the two experiments showed the presence of deuterium at the predicted sites. The sample of pyridoxamine diHCl from the experiment with [5′,5′-$^2H_2$]-2′-hydroxypyridoxol (2 × 1 L, 1 g/L labeled substrate, 17 h incubation) contained deuterium at H-5′ (4.80 ppm, with reference to external $D_2O$ at 4.67) (Figure 1A) (cf., $^1$H NMR[10]). Pyridoxamine diHCl from the experiment with [2′,2′,5′,5′-$^2H_4$]-2′-hydroxypyridoxol (2 × 1 L, 1 g/L labeled substrate, 21 h incubation) showed labeling at H-2′ (2.62 ppm) and at H-5′ (4.79 ppm) (Figure 1B). The ratio 5′-$^2$H/2′-$^2$H within the product (0.95/1.00) was identical, within experimental error, with the ratio 5′-$^2$H/2′-$^2$H within the substrate (0.97/1.00). These results provide compelling evidence that **5** was converted directly into **3** and, thus, serves as a precursor of the vitamin.

* Corresponding author. Telephone: (905)525-9140, ext. 23245. Fax: (905) 522-2509. E-mail: spenser@mcmaster.ca.



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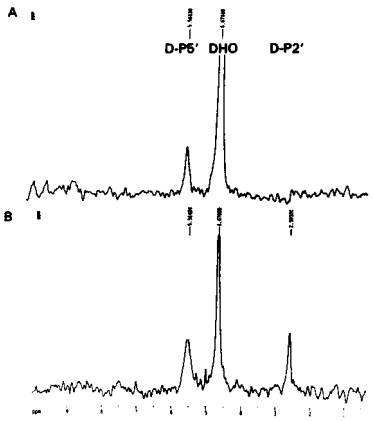
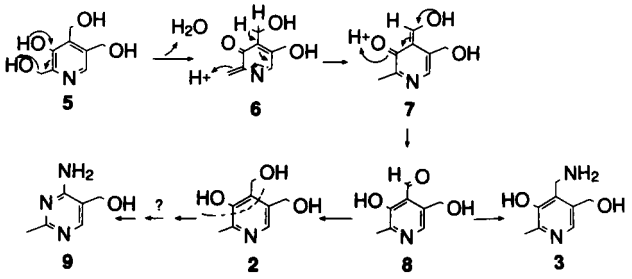



***Figure 2.*** $^2$H NMR (46.07 MHz, $^2$H depleted $H_2O$, 35° C) (A) $^2$H NMR spectrum of thiamin chloride HCl from the incubation with [5′,5′-$^2H_2$]-2′-hydroxypyridoxol HCl. (The structure of the pyrimidine unit of thiamin is shown (**9**)). (B) $^2$H NMR spectrum of thiamin chloride HCl from the incubation with [2′,2′,5′,5′-$^2H_4$]-2′-hydroxypyridoxol HCl.

***Scheme 2.*** Hypothetical Biogenetic Sequence for the Conversion of 2′-Hydroxypyridoxol into Pyridoxol and Thence into the Pyrimidine Unit of Thiamin




A hypothetical mechanism for the conversion of **5** into **2** had been advanced 25 years ago.[4] This involved dehydration of **5**, followed by hydrogenation of **6** at C-2′,2, and then enolization, to yield **2**. Evidence for a reducing enzyme with C-2′,2 specificity in vitamin $B_6$ metabolism does not appear to exist, however. The only known vitamin $B_6$-reducing enzyme in yeasts (pyridoxine 4-dehydrogenase, EC 1.1.1.65) catalyzes the reversible interconversion of **2** and pyridoxal (**8**). Rather than proceeding via reduction at C-2′, a more likely mechanism for the conversion of **5** involves dehydration followed by a series of proton transfers (**5** → **8**, Scheme 2). Such a sequence leads to **8** as the initial $B_6$ vitamer to be formed. While this prediction remains to be tested experimentally, it is relevant that the NADP-specific pyridoxine dehydrogenase (= pyridoxal reductase) that catalyses the interconversion of **2** and **8** in several yeasts[11−14] favors pyridoxol. The kinetic parameters of the enzyme "strongly indicate that the dehydrogenase functions in vivo to reduce pyridoxal to pyridoxine, which is the preferred substrate for pyridoxal (pyridoxine) kinase in yeast".[13]

Deuterium from the two deuterated samples of 2′-hydroxypyridoxol entered predictable sites not only within **3** but also within the pyrimidine unit (**9**) of thiamin (vitamin $B_1$). The $^2$H NMR spectra of the isolated samples of thiamin chloride HCl showed that label from [5′,5′-$^2H_2$]-2′-hydroxypyridoxol was present at H-5′ of the thiamin pyrimidine (**9**) (5.56 ppm)(Figure 2A), while label from [2′,2′,5′,5′-$^2H_4$]-2′-hydroxypyridoxol had entered H-2′ (2.59 ppm) and H-5′ (5.51 ppm)(Figure 2B)(*cf.*, $^1$H NMR[15]). The ratio 5′-$^2$H/2′-$^2$H within the latter product (1.00/0.72) was somewhat higher than that within the [2′,2′,5′,5′] labeled substrate (1.00/0.97), presumably due to deuterium proton exchange at C-2′ in the course of workup.

It would thus appear that the $C_5$N-unit C-2′,2,N-1,C-6,5,5′ of 2′-hydroxypyridoxol is incorporated intact into the $C_5$N-unit C-2′,2,N-1,C-6,5,5′ of the pyrimidine unit of thiamin. This observation provides independent confirmation of the results of Kazuko Yamada, and co-workers[16,17] who concluded, on the basis of MS analysis of isotope-enriched samples of thiamin, that in *S. cerevisiae* IFO 1234 label from [2′-$^{13}$C]pyridoxol enters either C-2 or C-2′ of the thiamin pyrimidine,[16] that label from [$^{15}$N]pyridoxol enters N-1,[16] that label from [6-$^{13}$C]pyridoxol enters C-6,[17] and that label from [5′,5′-$^2H_2$]pyridoxol enters H-5′ of the thiamin pyrimidine.[17]

Entry of deuterium from specifically $^2$H-labeled samples of 2′-hydroxypyridoxol into the predicted sites of pyridoxamine and of the pyrimidine unit of thiamin provides the first unequivocal evidence that in yeast 2′-hydroxypyridoxol is an intermediate on the route from a $C_5$-sugar into vitamin $B_6$, and adds to the evidence that pyridoxol serves as a precursor of the thiamin pyrimidine, supplying the $C_5$N unit, C-2′,2,N-1,C-6,5,5′ as an intact unit.

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**Supporting Information Available:** Scheme 3: synthesis of [5′,5′-$^2H_2$]-2′-hydroxypyridoxol HCl. Scheme 4: synthesis of [2′,2′,5′,5′-$^2H_4$]-2′-hydroxypyridoxol HCl (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) NMR spectra: (a) 2′-Hydroxypyridoxol hydrochloride (**5**): $^1$H NMR (200 MHz, $D_2O$) $\delta$ 8.11 (s, 1H)(H-6), 4.90 (s, 2H)(H-2′), 4.86 (s, 2H)(H-4′), 4.68 (s, 2H)(H-5′). $^{13}$C NMR (50.29 MHz, $D_2O$) $\delta$ 151.4, 143.8, 140.8, 137.7, 129.7, 58.2, 57.2, 56.7. (b) [5′,5′-$^2H_2$]-2′-Hydroxypyridoxol hydrochloride: $^1$H NMR (200 MHz, $D_2O$) $\delta$ 8.14 (s, 1H)(H-6), 4.92 (s, 2H)-(H-2′), 4.88 (s, 2H)(H-4′). $^{13}$C NMR (50.29 MHz, $D_2O$) $\delta$ 151.3, 143.8, 140.8, 137.6, 129.7, 58.0 (m, weak signal), 57.3, 56.7. $^2$H NMR (46.07 MHz, $H_2O$, at 35° C) $\delta$ 4.80 (with $D_2O$, $\delta$ 4.67 ppm, as external reference). (c) [2′,2′,5′,5′-$^2H_4$]-2′-Hydroxypyridoxol hydrochloride: $^1$H NMR (200 MHz, $D_2O$) $\delta$ 8.13 (s, 1H)(H-6), 4.91 (s, 2H)(H-4′). $^{13}$C NMR (50.29 MHz, $D_2O$) $\delta$ 151.4, 143.6, 140.8, 137.6, 129.7, 58.0 (m, weak signal), 57.3, 56.1 (quintet, $^1J_{CD}$ = 25.3 Hz). $^2$H NMR (46.07 MHz, $^2$H depleted $H_2O$, at 35° C) $\delta$ 4.92 (D-2′), 4.77 (D-5′), (referenced to external $D_2O$ at 4.67 ppm).

(10) Pyridoxamine dihydrochloride: $^1$H NMR (200 MHz, $D_2O$) $\delta$ 8.21 (s, 1H)-(H-6), 4.83 (s, 2H)(H-5′), 4.40 (s, 2H)(H-4′), 2.68 (s, 3H)(H-2′).

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(15) Thiamin chloride hydrochloride: $^1$H NMR (200 MHz, $D_2O$) $\delta$ 9.69 (s, 1H)(P-6), 8.04 (s, 1H)(T-2), 5.60 (s, 2H)(P-5′), 3.91 (t, 2H)(T-7), 3.22 (t, 2H)(T-6), 2.66 (s, 3H)(P-2′), 2.57 (s, 3H)(T-4′) [P = Hydrogen atoms at the sites of the pyrimidine unit (**9**) of thiamin; T = Hydrogen atoms at the sites of the thiazole unit of thiamin].

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