Biosynthesis of 4′-*O*-Methylpyridoxine (Ginkgotoxin) from Primary Precursors†

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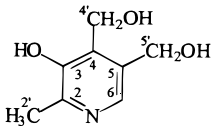
Cell suspension cultures of *Ginkgo biloba* and *Albizia tanganyicensis* were investigated for the presence of 4′-*O*-methylpyridoxine (ginkgotoxin, **2**), the 4′-*O*-methyl derivative of vitamin B_6 (pyridoxine, **1**). The cultures produced the toxin even in the absence of vitamin B_6 (a common additive to plant cell culture media). This indicates that the pyridoxine ring system of ginkgotoxin is synthesized de novo by the cultured cells. A feeding experiment with D-[U-$^{13}C_6$]glucose revealed that the mode of incorporation of label into the pyridoxine moiety of **2** matched that observed for **1** in *Escherichia coli*. Thus, the data obtained in this investigation provide independent proof supporting the current hypothesis on vitamin B_6 biosynthesis. The 4′-*O*-methyl group of ginkgotoxin (**2**) was labeled from L-[methyl-$^{13}C_1$]methionine. This indicates that ginkgotoxin is likely to be derived by 4′-*O*-methylation of pyridoxine (**1**). The *G. biloba* cell suspension culture may be a suitable system to get further insight into vitamin B_6 and/or ginkgotoxin biosynthesis.

4′-*O*-Methylpyridoxine (**2**) is one of several vitamin B_6 derivatives with an antivitamin character, which cause symptoms of B_6 deficiency in mammals. Vitamin B_6-antimetabolites are potential inhibitors of all enzymes dependent on pyridoxal-5′-phosphate or pyridoxamine-5′-phosphate as cofactor.[1] Of the known 4′- and 5′-substituted analogues of vitamin B_6 (pyridoxine, **1**), 4′-*O*-methylpyridoxine (ginkgotoxin, **2**) is the most potent neurotoxin.[2]

After ingestion, this toxic principle causes convulsions as the cardinal symptom in humans and may even lead to death. It was first isolated several years ago from *Ginkgo biloba* L. (maidenhair tree; Ginkgoaceae) seeds[3,4] and later from the leaves of this species as well.[4] Besides ginkgotoxin, the seeds *of Albizia tanganyicensis* Bak. f. (Leguminosae) contain the 5′-acetylated derivative 5′-*O*-acetyl-4′-*O*-methylpyridoxine (**3**).[5]



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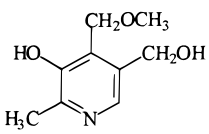

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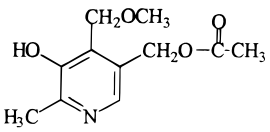

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Although ginkgotoxin (**2**) may be suitable for the study of the biosynthesis of vitamin B_6 and its derivatives in higher plants, no reports dealing with the biosynthesis of this compound have been published so far. In contrast, in bacteria the biosynthesis of the B_6 vitamins pyridoxine (**1**), pyridoxal, and pyridoxamine and their 5′-phosphorylated derivatives has been investigated for several years.[6,7] One of the key experiments performed to elucidate the origin of the vitamin B_6 carbon skeleton in *Escherichia coli* showed that it was assembled from one two-carbon unit and two three-carbon units, which are derived from glucose. The C_2 unit is incorporated into carbon atoms C-2′ and C-2 of pyridoxine (**1**), whereas the two C_3 units are incorporated into carbon atoms C-3, C-4, C-4′ and C-5′, C-5, C-6, respectively. This indicates that the only two carbon−carbon bonds newly formed during the biosynthesis of **1** are those between C-2/C-3 and C-4/C-5.[8] Of these, the bond between carbon atoms 2 and 3 is likely to be generated before the pyridine ring is formed because a ^{13}C-labeled sample of 1-deoxy-D-xylulose, a sugar derived from a C_2 and C_3 unit,[6] was shown to be incorporated intact into carbon atoms C-2′, C-2, C-3, C-4, C-4′ of pyridoxine (**1**).[9] Thus, this five-carbon sugar or its phosphorylated derivative is assumed to be a precursor of pyridoxine. Experiments aimed at elucidating the origin of the remaining C_3 unit, comprising carbon atoms C-5′, C-5, and C-6, indicated that this part of the pyridoxine molecule (**1**) is derived from 4-(phosphohydroxy)-L-threonine.[10−12]

It has been reported that a chloroplast preparation obtained from either spinach or maize converts glyceraldehyde-3-phosphate, pyruvate, and glycine in the presence of MgATP into vitamin B_6. When pyruvate was omitted from the incubation mixture, pyridoxine formation ceased, but could be restored by addition of 1-deoxy-D-xylulose.[13] This incubation mixture was, therefore, the first system in which enzymatic synthesis of vitamin B_6 was claimed, although a rigorous proof of the structure of the reaction product was not provided. Very recently however, Laber et al. reported the synthesis of pyridoxine-5′-phosphate in the presence of 4-(phosphohydroxy)-L-threonine, 1-deoxy-D-xylulose-5-phosphate, and the *E. coli* enzymes PdxA and PdxJ.[14] In an attempt to establish another system suitable for the investigation of vitamin B_6 biosynthesis, we have raised cell suspension cultures of *G. biloba* and *A. tanganyicensis*. We expected that the ginkgotoxin (**2**) found in

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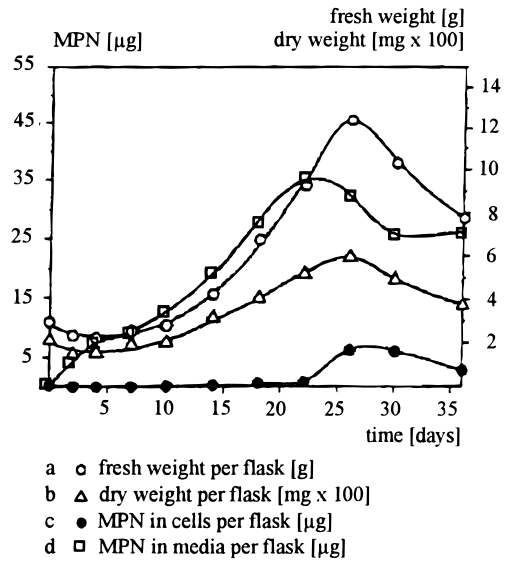

Figure 1. Growth curves and ginkgotoxin production of a *Ginkgo biloba* cell suspension culture in 50 mL MSwoP medium. Points represent single determinations that were repeated twice with essentially the same result. MPN = Ginkgotoxin (**2**).

both plant species[5,15] would be generated by *O*-methylation of the 4′- hydroxymethyl group of pyridoxine (**1**). Moreover, it needed to be established that the heterocyclic ring system of the toxin is assembled from primary precursors in the same way as observed for pyridoxine (**1**) in *E. coli*.

This was indeed found when ^{13}C-labeled samples of D-glucose and methionine were administered to the *G. biloba* cell culture. Although any label from D-[U-$^{13}C_6$]-glucose would be highly diluted by the degradation products of sucrose (30 g/L) present in the cell culture medium, incorporation of glucose-derived fragments into the toxin was observed. Analysis of the ^{13}C NMR spectra of ginkgotoxin (**2**) isolated from this experiment suggests that the labeling patterns of the pyridoxine moiety in ginkgotoxin (**2**) and of pyridoxine (**1**) in *E. coli* are identical.

Results and Discussion

Analysis of the *A. tanganyicensis* and *G. biloba* cell suspension cultures showed that both produced ginkgotoxin (**2**). Cultured cells from both plant species excreted the toxin into the culture medium, and only a minor amount was extractable from the cell material. The *G. biloba* culture was the more productive of the two, with a yield of more than 500 μg/L of medium, whereas *A. tanganyicensis* produced only about 30 μg/L (maximum) of medium. The cells were grown in modified Murashige and Skoog's medium[16] without pyridoxine (MSwoP). Growth of the cells was also possible on 4X medium.[17] However, when pyridoxine was omitted from this medium, the amount of ginkgotoxin produced by the *G. biloba* culture dropped from 760 μg/L to 400 μg/L of medium.

Growth of the cell culture and the time-dependent formation of ginkgotoxin (**2**) in MSwoP medium is depicted in Figure 1. Because it was intended to feed a D-[U-$^{13}C_6$]-glucose sample to the culture, a parallel experiment was conducted in which an additional amount of 1 g D-glucose was added to the medium. Growth and production of **2** were essentially identical in both media irrespective of the presence of glucose.

The labeled precursors ([methyl-$^{13}C_1$]methionine 150 mg/L medium or D-[U-$^{13}C_6$]glucose 1 g/L medium, respectively) were administered in separate experiments to the culture on day 18 after inoculation, and incubation was

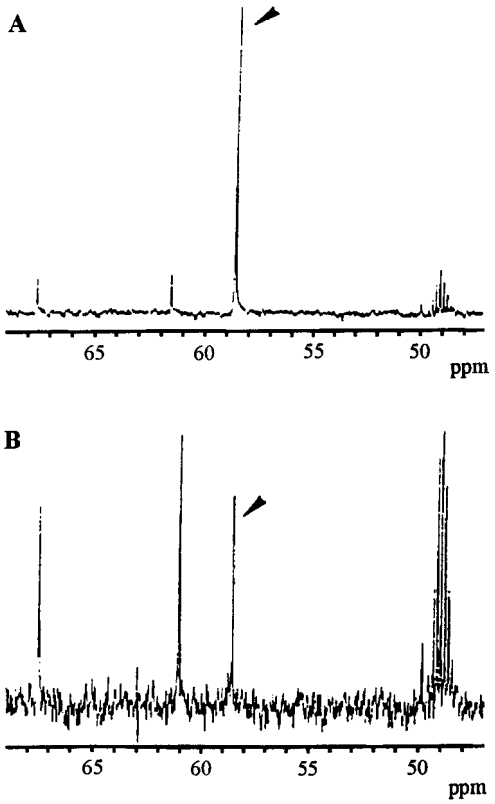

Figure 2. Comparison of a ^{13}C NMR spectrum of 4′-*O*-methylpyridoxine (ginkgotoxin, **2**) isolated from a *Ginkgo biloba* cell suspension culture grown in the presence of L-[methyl-$^{13}C_1$]methionine (A) with that of an unlabeled authentic sample (B). The arrowheads point to the signals of the 4′-*O*-methyl group of **2**.

carried out for 48 h. The ginkgotoxin (**2**) was isolated by a published method,[4] and incorporation of precursors was analyzed by NMR spectroscopy.

Label from the methionine sample was specifically and exclusively incorporated into the 4′-*O*-methyl group of ginkgotoxin (**2**) (Figure 2 A). The signal of this carbon atom appeared as a singlet, which was significantly enhanced when compared to the corresponding signal of an unlabeled authentic sample (Figure 2 B). A specific incorporation of L-[methyl-$^{13}C_1$]methionine of 11% above natural abundance was observed.

Administration of uniformly labeled D-[U-$^{13}C_6$]glucose to the culture also resulted in specific incorporation (0.64% above natural abundance) into ginkgotoxin (**2**). We expected incorporation of multi-carbon fragments from this precursor, and this was indeed observed, inasmuch as satellites adjacent to signals derived from the exocyclic carbon atoms C-2′, C-4′, and C-5′, respectively, were readily apparent (Figure 3). This suggested that the neighboring carbon atoms of the pyridine ring, C-2, C-4, and C-5, respectively, were also labeled. In accord with this prediction, satellites were readily discernible around the signal for C-2 (Figure 4). The observed pattern (coupling constant $^1J_{C-C} = 49$ Hz) suggested that an intact glucose-derived two-carbon fragment had entered C-2′/C-2. In contrast, the signals for C-4 and C-5, respectively, showed no clear satellites, whereas the two neighboring carbon atoms, C-3 and C-6, appeared to be labeled (Figure 4). This might be a consequence of the incorporation of two intact C_3 fragments into the two C_3 units C-6, C-5, C-5′ and C-3, C-4, C-4′. In ^{13}C-labeled isotopomers of such C_3 units, this labeling pattern must result in the formation of a doublet of doublets for the signals of the carbon atoms C-4 and C-5, respectively, in which the central line of such an apparent triplet is

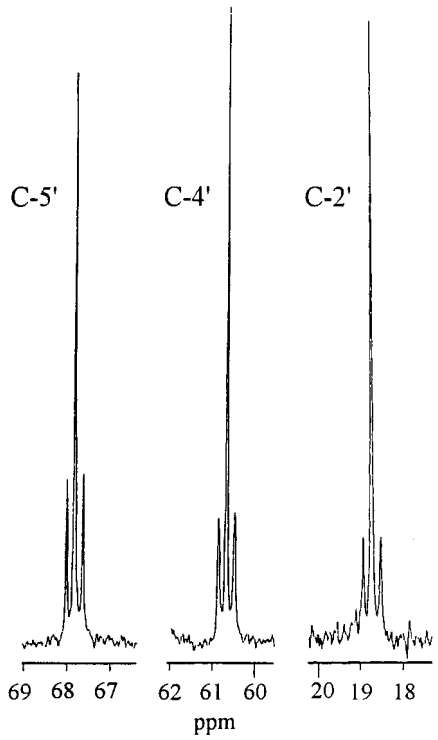

Figure 3. Proton-noise decoupled ^{13}C NMR spectrum of ginkgotoxin (**2**) after administration of D-[U-$^{13}C_6$]glucose to a *Ginkgo biloba* cell suspension culture. Resonances are due to the exocyclic carbon atoms C-2′, C-4′, and C-5′. Spectra were recorded in methanol-d_4.

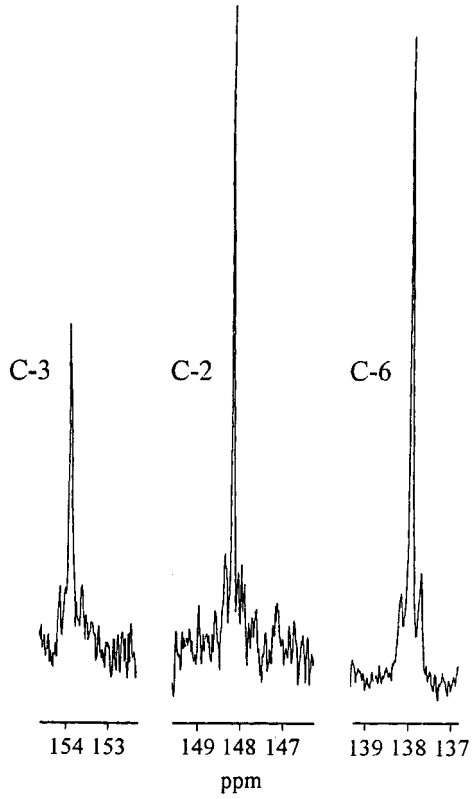

Figure 4. Proton-noise decoupled ^{13}C NMR spectrum of ginkgotoxin (**2**) after administration of D-[U-$^{13}C_6$] glucose to a *Ginkgo biloba* cell suspension culture. Resonances are due to the carbon atoms C-2, C-3, and C-6. Spectra were recorded in methanol-d_4.

coincident with the natural abundance signal for these carbon atoms and, hence, is not readily detectable. Moreover, in such a coupling pattern the outer satellites receive only one-half of the total signal intensity and consequently may not be visible in a sample of limited size (200 μg) owing to a substantially decreased signal-to-noise ratio. A labeling pattern identical to the one suggested by this tentative analysis had previously been observed in pyridoxine (**1**) from a feeding experiment with D-[U-$^{13}C_6$]glucose in a mutant of *E. coli*.[8] A significant diminution in the intensity of satellites around the signals for C-4 and for C-5, when compared to the satellites around the signals for any of the other aromatic carbon atoms, was observed in that work as well. However, this limitation was overcome primarily by a 20-fold larger sample quantity and the resulting improved signal-to-noise ratio of the ^{13}C NMR spectrum.

Thus, while the NMR spectra of ginkgotoxin (**2**) derived from D-[U-$^{13}C_6$]glucose were suggestive of an identical labeling pattern of pyridoxine in *G. biloba* and in *E. coli*, conclusive proof was dependent on the establishment of the multiplicity of the satellites in the signals due to C-4 and C-5. Numerous attempts to accumulate evidence for a doublet of doublet coupling pattern in the signals due to C-4 and C-5, respectively, employing various standard NMR techniques such as TOCSY, INADEQUATE, and CC-COSY failed in their 1D as well as their 2D versions.

Resolution of the remaining ambiguities was eventually achieved using a gradient-selected version of the modified HMBC sequence first introduced by Seto[18] for the detection of carbon−carbon long-range couplings in terpenoids biosynthesized by the nonmevalonate pathway. In this modification of the classical HMBC sequence, the low-pass filter is removed, and signals for protons ^1J-coupled to ^{13}C are not dephased and removed via phase cycling.

The results of the application of this pulse sequence to the sample of ginkgotoxin (**2**) isolated from the feeding experiment with D-[U-$^{13}C_6$]glucose are shown in Figure 5. Panel A shows the signal for the protons of the C-2′ methyl group on the horizontal axis and the signals for the carbon atoms C-2 and C-3 on the vertical axis. As expected, HMBC correlations are readily visible from the signal for the protons at C-2′ to both of these carbon atoms via ^2J and ^3J couplings, respectively. Significantly, two additional correlations were observed for C-2 as evidenced by two less intense signals, displaced to higher and lower field, respectively, on the proton chemical-shift axis from the main correlation. The separation of these two satellite signals is 127 Hz, identical to the ^1J coupling constant of the C-2′ protons to the C-2′ carbon atom. The observation of correlations of the ^{13}C NMR signal for C-2 to the ^{13}C satellites of the H-2′ protons suggests that a significant fraction of the molecules carries two contiguous ^{13}C atoms at C-2/C-2′. The probability for the presence of such an isotopomer at natural abundance is 1.21×10^{-4}. This value is too low to allow for the observation of such a species on a sub-milligram sample as in the present case. This analysis suggests again that an intact C_2 unit derived from D-[U-$^{13}C_6$]glucose has been incorporated into C-2′/C-2. However, because C-3 is labeled as well, as indicated by the presence of satellites in the ^{13}C NMR signal for this carbon atom (Figure 4), it might be argued that a C_3 fragment of glucose has been incorporated into C-2′, C-2, and C-3 instead. That it is indeed a C_2 unit and not a C_3 unit incorporated into this portion of ginkgotoxin (**2**) was indicated by the absence of correlations of the ^{13}C NMR signal for C-3 at 160 ppm to the ^{13}C satellites of the ^1H NMR signal for H-2′ (Figure 5A). If C-3 were part of a glucose-derived multi-carbon fragment also labeling C-2/C-2′, such correlations would be expected. Their absence

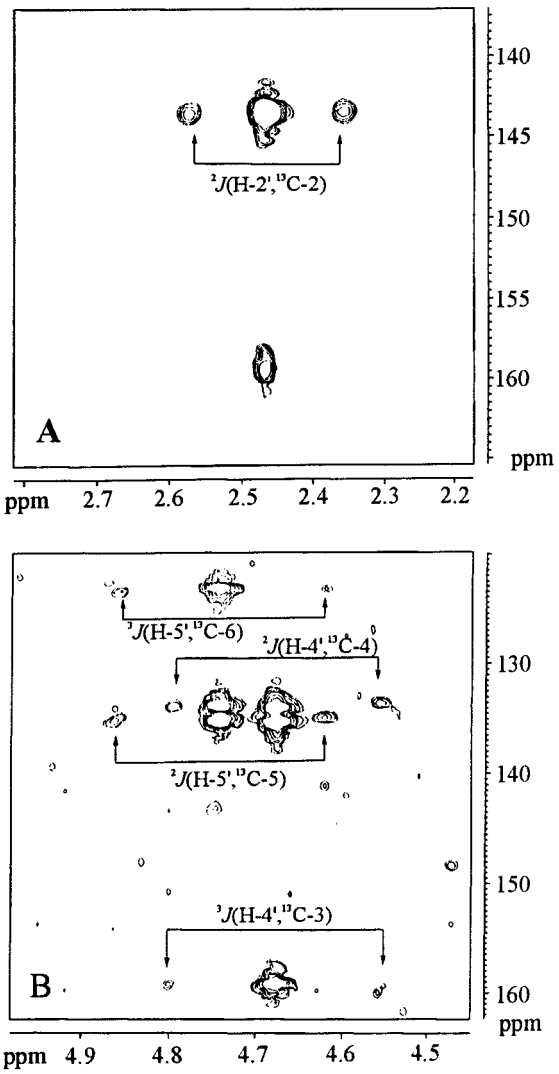

Figure 5. Modified HMBC spectrum of ginkgotoxin after administration of D-[U-$^{13}C_6$]glucose to a *Ginkgo biloba* cell suspension culture. Cross correlations due to the C-2, C-2′ unit (A) as well as the C-3, C-4, C-4′ unit and the C-6, C-5, C-5′ unit (B), respectively. Spectra were recorded in D_2O.

thus proves conclusively that label within C-2/C-2′ is derived from a C_2 fragment of glucose and not from a three-carbon unit. Because C-3 is not coupled to C-2, the satellites around the ^{13}C NMR signal for C-3 must be due to coupling with ^{13}C-enriched carbon atoms at C-4. That the ^{13}C label at C-3 stems from a glucose-derived C_3 fragment incorporated into C-4′, C-4, C-3 was proven conclusively through ^3J correlations of the signals for C-3 at 159 ppm and for C-4 at 133.5 ppm, with the ^{13}C satellites of the signals for the H-4′ protons ($^1J_{C-H}$ = 146 Hz) at 4.65 ppm (Figure 5B). At natural abundance, these correlations would not be visible in a sample of this size owing to the low probability of finding ^{13}C atoms within one molecule at both C-3 and C-4′ or at both C-4 and C-4′. That this analysis is correct can also be shown by inspection of the ^3J correlation of H-4′ to C-5. Thus, protons attached to ^{12}C at C-4′ showed a strong correlation to ^{13}C carbon atoms at C-5 (135 ppm), which proved that this ^3J correlation is observable in a natural abundance sample. However, the correlation of C-5 with H-4′ was not accompanied by the characteristic set of additional, less intense correlations to the ^{13}C satellites of the H-4′ protons. This observation suggests that the carbon−carbon bond between C-4 and C-5 of **2** was newly formed in those molecules derived from labeled glucose.

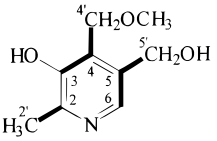

Figure 6. Bond-labeling pattern indicating the origin of ginkgotoxin (**2**) from one C_2 and two C_3 fragments after administration of D-[U-$^{13}C_6$]glucose to a *Ginkgo biloba* cell suspension culture.

Finally, correlations of the signal for C-6 at 126.2 ppm and of the signal for C-5 at 137.0 ppm to the ^{13}C satellites of the signal for the H-5′ protons ($^1J_{C-H}$ = 144 Hz) at 4.73 ppm suggested that an intact C_3 unit derived from glucose entered carbon atoms C-6, C-5, and C-5′. In principle, this labeling pattern should also lead to correlations from C-5′ and from C-5 to the ^{13}C satellites of the ^{1}H NMR signal of H-6. However, owing to the weak intensity and breadth of the ^{1}H NMR signal for this proton, these correlations were not apparent above the noise when a sufficiently low contour level was plotted, although the expected correlations for the natural abundance isotopomers were observable.

The foregoing analysis proves conclusively that the carbon skeleton of the pyridoxine part of ginkgotoxin (**2**) is formed from two glucose-derived C_3 fragments and one C_2 portion. The labeling pattern from D-glucose in ginkgotoxin (**2**) in *G. biloba* was identical to the one observed in pyridoxine (**1**) biosynthesis in *E. coli* (Figure 6). Results from incubations of cell free protein extracts of *G. biloba* cell cultures suggest that in the biogenesis of ginkgotoxin (**2**), *O*-methylation is the final step. Ginkgotoxin (**2**) formation was observed exclusively in incubations in which *S*-adenosylmethionine and either pyridoxine (**1**) or pyridoxine-5′-phosphate were added (data not shown). Interestingly, both pyridoxine (**1**) and pyridoxine-5′-phosphate are substrates for the enzyme activity catalyzing the methylation reaction. This observation suggests that ginkgotoxin may be a dephosphorylation product of 4′-*O*-methylpyridoxine-5′-phosphate. This, in turn, would correspond very well to observations made with *E. coli* that the phosphate group of pyridoxal-5′-phosphate is introduced at an early step in vitamin B_6 biosynthesis.[10,11,14]

Experimental Section

General Experimental Procedures. 1D ^{13}C and 1D ^{1}H NMR spectra were recorded at 20 °C on a 11.75 T spectrometer (GE Omega 500) at 125 MHz (^{13}C). Samples were dissolved in deuteromethanol (99.95 atom % ^{12}C, 99.5 atom % ^{2}H; Sigma, St. Louis, MO) in 3-mm Shigemi tubes (Shigemi Inc., Allison Park, PA). Flip angle: 60°; memory size: 32 k; relaxation delay: 1 s; 108 000 scans.

Modified HMBC spectra were recorded at 20 °C on a 14.10 T spectrometer (Bruker DRX 600) at 600 MHz (^{1}H) and 150 MHz (^{13}C), respectively, using a 2.5-mm dual probehead. The sample was dissolved in deuterium oxide (99.98 atom % ^{2}H; Aldrich, St. Louis, MO).

Establishment and Maintenance of Cell Cultures. Cell suspension cultures were derived from a callus culture of *G. biloba* obtained from "Deutsche Sammlung für Mikroorganismen und Zellkulturen", Braunschweig, Germany. For establishment of cell suspension cultures, 100 mL of MSw-medium[16] without vitamin B_6 (MSwoP) was inoculated with callus material previously crushed under sterile conditions. The medium contained 2,4-dichlorophenoxy acetic acid (2 mg/L) and 6-benzylaminopurine (1 mg/L) as hormones. The cultures were grown in 300-mL Erlenmeyer flasks under permanent illumination (400 lx) at 25 °C and 100 rpm. For maintenance of the cultures, 10−13 g of cells was transferred to fresh medium under sterile conditions in intervals of 10 to 11 days.

Cell cultures of *A. tanganyicensis* were derived from stem and leaf material of plants raised from seeds obtained from Dr. R. Vleggaar, Department of Chemistry, University of Pretoria, Pretoria, South Africa. They were treated as described above for the *G. biloba* cultures.

Cultivation of Cell Suspension Cultures in the Presence of ^{13}C-Labeled Precursors. For each incorporation experiment with ^{13}C-labeled precursors 1 L of MSwoP medium was inoculated with 75 g of cells of a *G. biloba* suspension culture. The cells were grown as described above, but with a shaking frequency of 80 rpm. After 18 days 1.0 g of D-[U-$^{13}C_6$]-glucose or 0.15 g of l-[methyl-$^{13}C_1$]methionine (CIL, Andover, MA) was added under sterile conditions, and the cultivation was continued for 48 h. The cells were harvested by vacuum filtration. The yield of cell mass resulting from 1 L of medium was 160−210 g fresh weight.

Preparative Isolation and Identification of 4′-*O*-Methylpyridoxine (2). After harvest, the cells obtained from a 1-L cell suspension culture were extracted three times with 100 mL of water for 45 min in a boiling water bath. The medium was concentrated to 80−100 mL under reduced pressure in a water bath at 50−60° C. The concentrated medium and cell extracts were combined and adjusted to pH 3. Isolation of 4′-*O*-methylpyridoxine (**2**) was performed as described.[4]

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Note Added in Proof: The enzymic synthesis of pyridoxol phosphate (ref 14) from 1-deoxy-D-xylulose-5-phosphate has also been demonstrated by: Cane, D. E.; Du, S.; Robinson, J. K.; Hsiung, Y.; Spenser, I. D. *J. Am. Chem. Soc.* **1999**, *121*, 7722−7723.

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