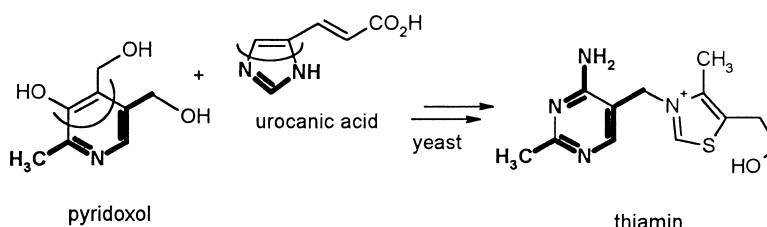Article

Biosynthesis of Vitamin B in Yeast. Derivation of the Pyrimidine Unit from Pyridoxine and Histidine. Intermediacy of Urocanic Acid

Johannes Zeidler, Brian G. Sayer, and Ian D. Spenser

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 4 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

Biosynthesis of Vitamin B₁ in Yeast. Derivation of the Pyrimidine Unit from Pyridoxine and Histidine. Intermediacy of Urocanic Acid

Johannes Zeidler, Brian G. Sayer, and Ian D. Spenser*

Contribution from the Department of Chemistry, McMaster University, Hamilton, Ontario, Canada L8S 4M1

Received April 29, 2003; E-mail: spenser@mcmaster.ca

Abstract: Incorporation studies with ^{13}C-, ^{15}N-, and ^{2}H-labeled substrates, followed by NMR analysis, show that the pyrimidine unit of thiamin (Vitamin B₁) originates from a C_5N fragment, derived from C-2′,2,N,C-6,5,5′ of pyridoxol (Vitamin B₆) and an N−C−N fragment derived from L-histidine. Urocanic acid serves as an intermediate on the route of the N−C−N fragment of histidine into the thiamin pyrimidine.

Introduction

It is now well documented[1] that, in bacteria on one hand and in yeasts on the other, each of the two subunits of thiamin (Vitamin B₁) (**2**), the pyrimidine and the thiazole, is generated by entirely different routes. In *Escherichia coli*, the primary precursors of the thiazole unit have been identified as tyrosine, 1-deoxy-D-xylulose, and a sulfur source, whereas, in yeasts, glycine, a pentulose, and an S-source serve as the substrates. The pyrimidine unit in bacteria is generated from 5-aminoimidazolyl ribotide by an intramolecular rearrangement. Much less is known about the biosynthesis of the pyrimidine unit in yeast.
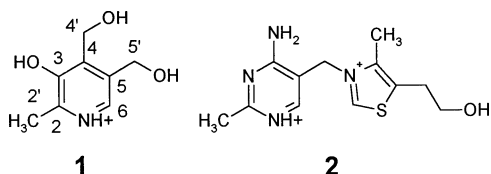
Our original investigations on the origin of thiamin in yeast were carried out by incubations with ^{14}C-labeled substrates, followed by chemical degradation of the radioactive products to locate the sites of labeling.[2,3]

Our recent investigations on the biosynthesis of thiamin[4] and of pyridoxamine[5,6] in yeast started with a study of the mode of incorporation of ^{13}C-labeled glucose, pentoses, and glycerol, determined by high field NMR. It became evident that the incorporation patterns of these compounds within the pyrimidine unit of thiamin corresponded to those observed within pyridoxamine. This led us to the notion that pyridoxine (**1**) might be involved in the biosynthesis of the thiamin pyrimidine. We soon discovered that this was not an original idea (see below).

We here present data that add to the evidence that in yeast the thiamin pyrimidine unit is derived from two fragments, one of which arises by degradation of pyridoxine, the other by degradation of histidine. Whereas the source and the eventual fate of the two fragments are clear, attempts to identify intermediates, representing degradation products of pyridoxine and of histidine that correspond to the fragment units, were unsuccessful. Urocanic acid (**7**), a histidine metabolite in which the imidazole ring of histidine remains intact, serves as an intermediate. A chemically rational process, leading to the generation of the thiamin pyrimidine from pyridoxine (**1**) and histidine (**6**), is proposed.

The existence of a possible metabolic relationship between thiamin and pyridoxine (Vitamin B₆) in yeasts had been noted more than half a century ago,[7] and interaction of the two vitamins in *Saccharomyces sps.*[8] and in *Neurospora sps.*[9] has been repeatedly studied since then. Several possible rationalizations for this apparent relationship have been advanced, among them the notion "that one of the vitamins might serve as the precursor of an intermediate from which the other may be synthesised",[8a] but no conclusive interpretation has been offered.

The first indirect evidence in support of the notion that pyridoxine served as a precursor of the pyrimidine unit of thiamin in *S. cerevisiae* emerged as recently as 1993: It was found on the basis of mass spectrometric analysis that the presence of pyridoxol in the incubation medium suppressed the

(1) Reviews: (a) White, R. L.; Spenser, I. D. In *Escherichia coli and Salmonella*; Neidhardt, F. C., Ed.; ASM Press: Washington, DC, 1996; 2nd Edition, Vol. 1, pp 680−686. (b) Spenser, I. D.; White, R. L. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 1032−1046.
(2) (a) White, R. L.; Spenser, I. D. *Biochem. J.* **1979**, *179*, 315−325. (b) *ibid., idem. J. Am. Chem. Soc.* **1982**, *104*, 4934−4943.
(3) Grue-Sørensen, G.; White, R. L.; Spenser, I. D. *J. Am. Chem. Soc.* **1986**, *108*, 146−158.
(4) Himmeldirk, K.; Sayer, B. G.; Spenser, I. D. *J. Am. Chem. Soc.* **1998**, *120*, 3581−3589.
(5) Gupta, R. N.; Hemscheidt, T.; Sayer, B. G.; Spenser, I. D. *J. Am. Chem. Soc.* **2001**, *123*, 11353−11359.
(6) Zeidler, J.; Gupta, R. N.; Sayer, B. G.; Spenser, I. D. *J. Org. Chem.* **2003**, *68*, 3486−3493.

(7) Schultz, A. S.; Atkin, L.; Frey, C. N. *J. Bacteriol.* **1940**, *40*, 339−346.
(8) (a) Moses, W.; Joslyn, M. A. *J. Bacteriol.* **1953**, *66*, 197−203. (b) *ibid.; idem.* **204−209**. (c) Kishi, T. *Vitamins (Japan)* **1969**, *40*, 385−391. (d) Minami, J.; Kishi, T.; Kondo, M. *J. Gen. Microbiol.* **1982**, *128*, 2909−2917.
(9) (a) Stokes, J. L.; Foster, J. W.; Woodward, C. R., Jr. *Arch. Biochem.* **1943**, *2*, 235−245. (b) Harris, D. L. *Arch. Biochem. Biophys.* **1952**, *40*, 294−304. (c) *ibid.; idem.* **1956**, *60*, 35−43.

Scheme 1. Correspondence of the [13]C Incorporation Patterns of D-Glucose, D-Ribose, and Glycerol within Vitamins B_6 (**1**) and B_1 (**2**)
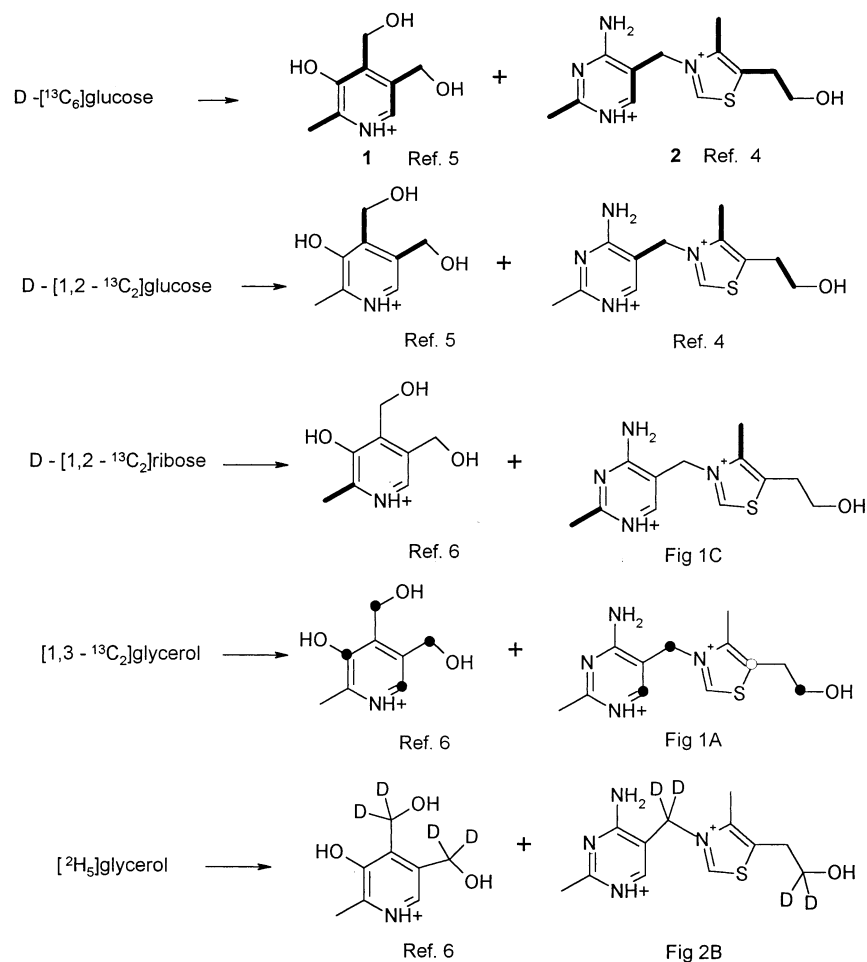
entry of ^{15}N from [15]NH_4Cl into N-1 of the pyrimidine ring of thiamin.[10] Evidence for the derivation of the C_5N moiety, C-2′,2,N-1,C-6,5,5′, of the pyrimidine ring of thiamin from the corresponding C_5N unit of pyridoxine followed, with the finding, based on mass spectrometric analysis of the isolated thiamin samples, that incubation of the yeast with [^{15}N]pyridoxine[11] and with [2′-13C]-,[11] [6-13C]-,[12] and [5′,5′-2H₂]pyridoxine[12] yielded [1-15N]-, [2′-13C]-, [6-13C]-, and [5′,5′-2H₂]pyrimidine-labeled thiamin, respectively.

It was found similarly that the presence of histidine in the incubation medium suppressed the entry of ^{15}N from [15]NH_4Cl into the pyrimidine ring of thiamin.[13] More specifically, the label from DL-[1′,3′-15N₂] histidine entered N-3 and the peripheral NH_2 group of the pyrimidine, and radioactivity from L-[2′-14C]-histidine entered an undetermined site, presumed to be C-4, of the pyrimidine unit.[14]

The results of the experiments here reported confirm and extend the conclusions reached by Yamada and her collaborators.[10−14]

(10) Tazuya, K.; Yamada, K.; Kumaoka, H. *Biochem. Mol. Biol. Int.* **1993**, *30*, 893−899.
(11) Tazuya, K.; Azumi, C.; Yamada, K.; Kumaoka, H. *Biochem. Mol. Biol. Int.* **1994**, *33*, 769−774.
(12) Tazuya, K.; Azumi, C.; Yamada, K.; Kumaoka, H. *Biochem. Mol. Biol. Int.* **1995**, *36*, 883−888.
(13) Tazuya, K.; Morisaki, M.; Yamada, K.; Kumaoka, H. *Biochem. Int.* **1988**, *16*, 955−962.
(14) Tazuya, K.; Yamada, K.; Kumaoka, H. *Biochim. Biophys. Acta* **1989**, *990*, 73−79.

Results and Discussion

Comparison of the Incorporation Patterns of Glucose, Pentoses, and Glycerol into Vitamins B_6 and B_1 in Yeast. The distribution of label within the two vitamins, derived from incubations with glucose, ribose and glycerol, are shown in Scheme 1.

The ^{13}C NMR spectra of pyridoxamine[5] and of the thiamin-pyrimidine,[4] derived from D-[13C₆]glucose in *S. cerevisiae*, each showed incorporation of multicarbon units into C-2′,2 and into C-6,5,5′ (Scheme 1). Furthermore, this incorporation pattern was not changed in either vitamin, isolated either from *S. cerevisiae* or from *Candida utilis*,[15] when incubations with D-[13C₆]glucose were carried out in the presence of several compounds whose relevance in the construction of the skeleton of the vitamins was to be examined. These attempted "displacement experiments" gave negative results either with pyridoxamine[6] or with the thiamin pyrimidine: in *S. cerevisiae*, L-aspartic acid (expt 3), DL-hydroxyaspartic acid (expt 4), DL-isoserine (expt 5), D-erythrose (expt 6), or 1-amino-3-hydroxyacetone (expt 7) or, in *Candida utilis*, fumaric acid (expt 1) or L-homoserine (expt 2) did not reduce or change the mode of incorporation of D-[13C₆]-glucose.

(15) *S. cerevisiae* gave a higher yield of thiamin, and *Candida utilis* gave a higher yield of pyridoxamine. Furthermore, whereas *S. cerevisiae* requires glucose for growth, *C. utilis* can be grown on pentoses as a general carbon source.
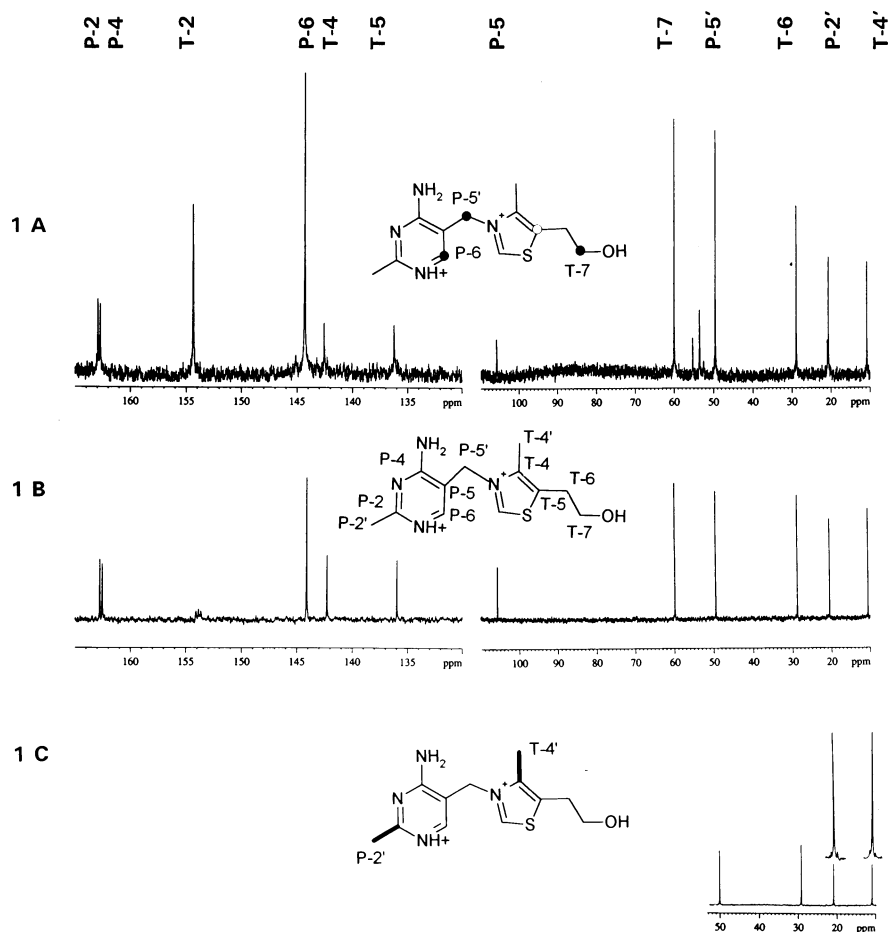
Figure 1. ^{13}C NMR spectra of (A) thiamin derived from [1,3-^{13}C$_2$]glycerol (expt 13), (B) natural abundance ^{13}C thiamin, and (C) thiamin derived from D-[1,2-^{13}C$_2$]ribose (low-frequency region) (expt 12).

The spectra of the vitamin samples derived from D-[1,2-^{13}C$_2$]-glucose showed entry of a C$_2$ unit into C-5,5′ of pyridoxamine[5] and of the thiamin pyrimidine.[4,16]

There was correspondence of the incorporation patterns also in the vitamin samples derived from D-[1,2-^{13}C$_2$]ribose. Incorporation of a C$_2$ fragment into C-2′,2 was observed within pyridoxamine[6] and within the thiamin pyrimidine (expt 12) (Figure 1C).

Incorporation of the label from [1,3-^{13}C$_2$]- and from [^{2}H$_5$]-labeled samples of glycerol also corresponded. In pyridoxamine, ^{13}C enrichment was observed[6] at C-3 and C-4′ and at C-6 and C-5′. The latter two C atoms were enriched in the thiamin pyrimidine (expt 13)(Figure 1A). The deuterium label from [^{2}H$_5$]glycerol was located at C-4′ and at C-5′ in pyridoxamine[6] and, correspondingly, at C-5′ within the thiamin pyrimidine (expt 14)(Figure 2B).
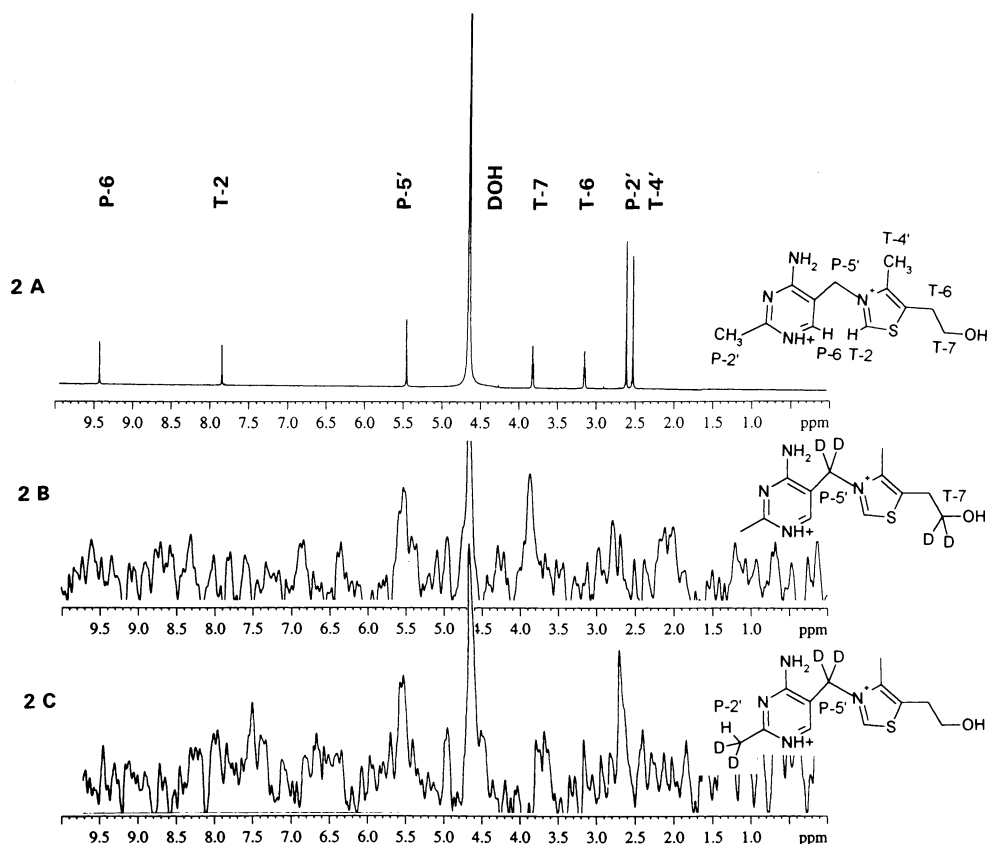
Pyridoxol and 2′-Hydroxypyridoxol as Precursors of the Thiamin Pyrimidine. These observations prompted us to examine whether the incorporation pattern of glucose into thiamin is affected by the presence in the medium of excess unlabeled pyridoxol or 2′-hydroxypyridoxol (Scheme 2). An essential aspect of these experiments was the fact that label from D-[^{13}C$_6$]glucose is incorporated not only into the pyrimidine but also into the thiazole unit of thiamin in characteristic fashion.[4]

(16) Whereas satellites due to ^{13}C−^{13}C coupling were readily detectable at C-5′ in both spectra, single carbon enrichment at C-2′ which was observed in the pyridoxamine spectrum was not detectable in the thiamin pyrimidine spectrum, presumably as a result of a much lower level of ^{13}C incorporation.

The presence of the label from [^{13}C$_6$]glucose within the thiazole unit thus serves as internal standard that is required to assess whether the presence of pyridoxol or hydroxypyridoxol in the medium leads to a reduction of the level of incorporation of glucose label into the pyrimidine unit. If the presence of these substrates leads to displacement of glucose-derived ^{13}C within the pyrimidine, relative to its level within the thiazole, this serves as evidence for the involvement of these substrates in the biosynthesis of the pyrimidine unit. Without the presence of an internal standard, any conclusions drawn from such a displacement experiment lie on much weaker foundations.

The NMR spectra of the samples of thiamin isolated from incubations of *S. cerevisiae* with [^{13}C$_6$]glucose in the presence of added pyridoxol (expt 9) (Figure 3B) or added 2′-hydroxypyridoxol (Experiment 10) (Figure 3C) is compared with the spectrum of [^{13}C$_6$]glucose-derived thiamin (Figure 3A) (=Figure 6 of ref 4). It is evident that the satellites at the signals due to the labeled carbon atoms of the pyrimidine unit are greatly reduced in intensity relative to those of the labeled thiazole carbons. Thus, both pyridoxol and hydroxypyridoxol displace the label from [^{13}C$_6$]glucose within the pyrimidine unit.

These results led to the inference that pyridoxol as well as 2′-hydroxypyridoxol serve as intermediates in the biosynthesis of the thiamin pyrimidine. Experiments with labeled samples of these two putative precursors substantiated this inference: Figure 3D shows the ^{13}C NMR spectrum of a thiamin sample isolated from an incubation of *S. cerevisiae* with a sample of

Figure 2. (A) Proton NMR spectrum of thiamin. (B) ²H NMR spectrum of thiamin derived from [²H₅]glycerol (expt 14). (C) ²H NMR spectrum of thiamin derived from [2′,2′,5′,5′-²H₄]-2′-hydroxypyridoxol HCl.[17]

Scheme 2. Derivation of the Pyrimidine Unit of Thiamin from Pyridoxine
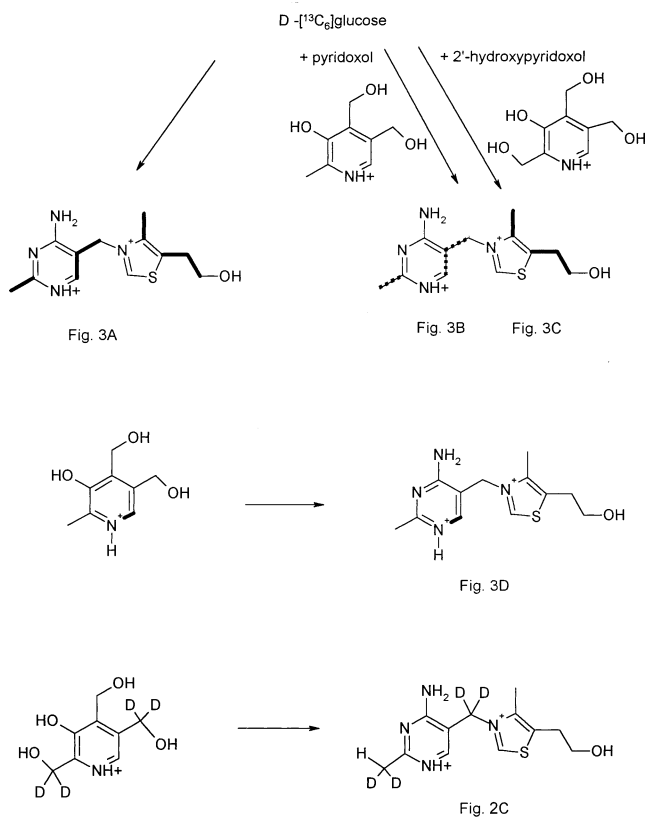
[¹⁵N,6-¹³C]pyridoxol hydrochloride (expt 18). The signal due to C-6 of the thiamin pyrimidine shows a doublet of satellites with a coupling constant of appropriate value ($^1J_{13C,15N}$ = 12.1 Hz) for ¹³C¹⁵N coupling. Thus, the N,C-6 unit of the precursor entered the product without being separated. Similarly, deuterium from [2′,2′,5′,5′-²H₄]-2′-hydroxypyridoxol entered the pyrimidine unit of thiamin with retention of deuterium at the corresponding sites (Figure 2C).[17] The two results provide further evidence that 2′-hydroxypyridoxol and pyridoxol[11,12] serve as intermediates in the biosynthesis of the thiamin pyrimidine in yeast.

Thus, the C₅N unit, C-2′,2,N-1,C-6,5,5′ of the pyrimidine unit of thiamin is not merely of common origin with the corresponding unit of pyridoxol, but is in fact derived from it.

A rational next step was to consider possible degradation reactions of pyridoxine that might yield an intermediate that could serve as a vehicle for the transfer of the C₅N fragment, C-2′,2,N-1,C-6,5,5′, of pyridoxol (**1**) into the pyrimidine unit. We considered *N*-acetyl-1-amino-3-hydroxypropan-2-one (**3**), originating by oxidative degradation of pyridoxol (Scheme 3), as the most likely of such intermediates.

However, since the label from [1,2-¹³C₂]acetate did not enter the pyrimidine unit, either in *C. utilis* (expt 11) or in *S. cerevisiae* (expt 15), since, furthermore, the label from [2,3-¹³C₂]pyruvate, a potential acetyl donor, did not serve as a precursor (expt 16), and since, finally, the label from [2,3-¹³C₂]-4-hydroxy-L-threonine, the progenitor of 1-amino-3-hydroxy-

(17) Zeidler, J.; Ullah, N.; Gupta, R. N.; Pauloski, R. M.; Sayer, B. G.; Spenser, I. D. *J. Am. Chem. Soc.* **2002**, *124*, 4542−4543.
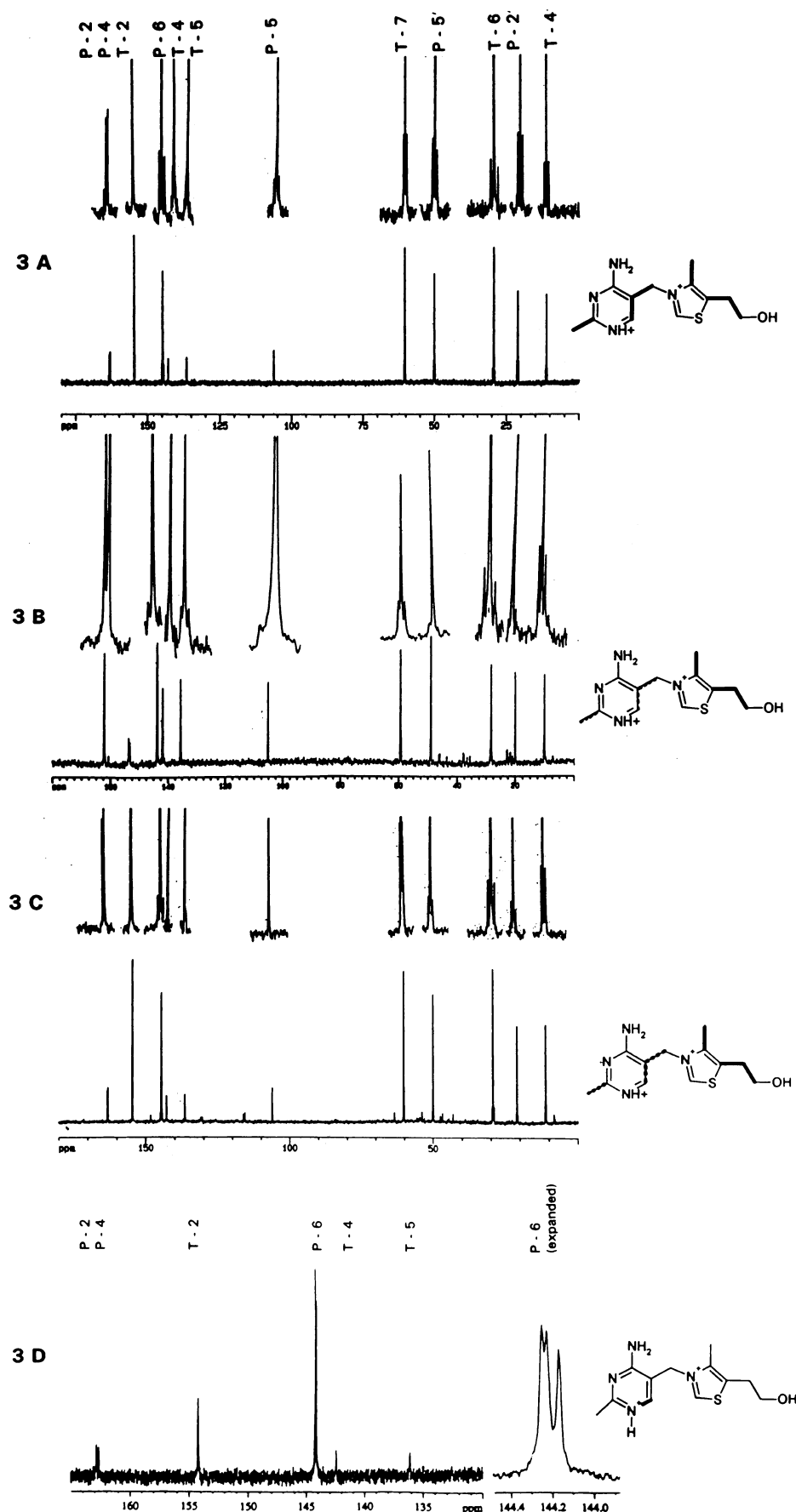
Figure 3. ^{13}C NMR spectra of (A) thiamin derived from D-[[13]C$_6$]glucose,[4] (B) thiamin derived from D-[[13]C$_6$]glucose in the presence of unlabeled pyridoxol (expt 9), (C) thiamin derived from D-[[13]C$_6$]glucose in the presence of unlabeled 2′-hydroxypyridoxol (expt 10), and (D) thiamin derived from [^{15}N,6-^{13}C]-pyridoxol (high-frequency region)(expt 18).

Scheme 3. Hypothetical Oxidative Fragmentation of Pyridoxol (**1**), Followed by the Union of Its C₅N Fragment, Derived from C-2′,2,N,C-6,5,5′, with Formamide (**4**) to Yield a Thiamin Pyrimidine Derivative (**5**)[a]
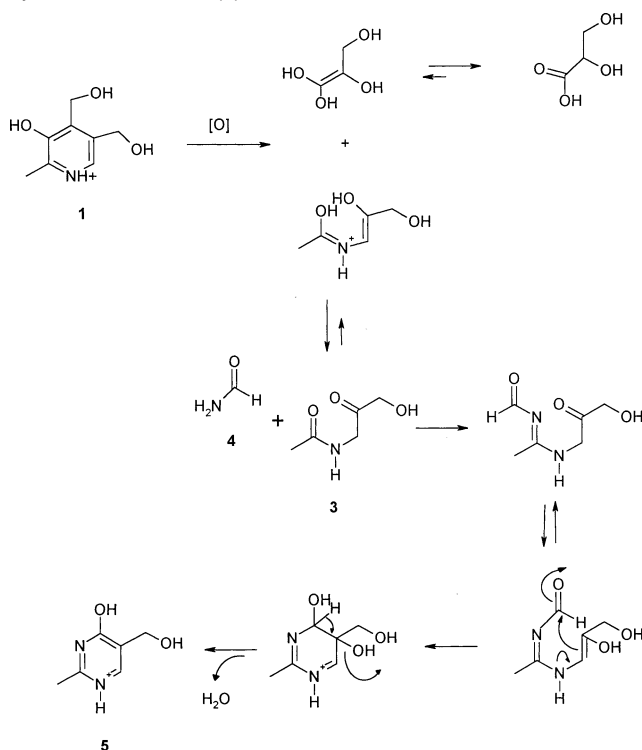
[a] This scheme, which served as our initial working model for the pyridoxol-pyrimidine conversion, was shown to be untenable by subsequent experimental results (see below).

acetone (= 1-amino-3-hydroxypropan-2-one),[18] was not incorporated (expt 17) and 1-amino-3-hydroxyacetone did not displace incorporation of label from [¹³C₆]glucose into C-6,5,5′ of the pyrimidine (expt 7), N-acetyl-1-amino-3-hydroxypropan-2-one could not have been synthesized by the yeast *de novo* by acetylation of hydroxyaminoacetone. If present, the compound could have arisen solely as a pyridoxol degradation product. However, incubation of *S. cerevisiae* with [¹³C₆]glucose in the presence of a sample of N-acetyl-1-amino-3-hydroxypropan-2-one (**3**) (a compound which, surprisingly had not so far been prepared) did not lead to displacement of the [¹³C₆]glucose label from C-2′,2 or from C-6,5,5′ of the thiamin pyrimidine (expt 8).

The inference is inescapable that a discrete C₅N intermediate derived from pyridoxol does not participate in the formation of the thiamin pyrimidine.

Origin of the N-3,C-4,NH₂ fragment of the Thiamin Pyrimidine. It has been known for many years that in yeasts C-4 of the pyrimidine nucleus of thiamin is derived from formate.[19,20] Incubation of *S. cerevisiae* with [¹³C]formate (Scheme 4) in the presence of excess [¹⁵N]ammonium sulfate

gave a thiamin sample whose ¹³C NMR spectrum showed ¹⁵N=¹³C−¹⁵N coupling at the signal due to C-4 of the pyrimidine unit (Figure 2 in ref 4). This experiment was repeated (expt 19) with identical results (Figure 4A, $^1J_{13C,15N}$ = ca. 20, 2.5 Hz, C-4/C-2 = 2.0/1). When a similar incubation was carried out in the presence of excess unlabeled L-histidine (expt 20), the ¹³C spectrum (Figure 4B) showed neither ¹³C enrichment at C-4 (C-4/C-2 = 0.8/1) nor ¹³C,¹⁵N coupling at C-4. Thus, the presence of histidine had prevented entry of administered labeled formate and labeled ammonia into the N=C−NH₂ site of the thiamin pyrimidine. Clearly, histidine serves as an intermediate on the route of formate and ammonia into the pyrimidine unit. This conclusion was confirmed by an experiment with L-[2′-¹³C,1′,3′-¹⁵N₂]histidine (expt 21)(Scheme 5). The ¹³C NMR spectrum of the thiamin from this experiment (Figure 5A) showed ¹³C,¹⁵N coupling at C-4 ($^1J_{13C,15N}$ = 21.6, 2.2 Hz) and C-4 enrichment, relative to C-2 (C-4/C-2 = 2.7/1).

It is important to note here that genetic evidence is available to show that the route to histidine in yeast from adenosine triphosphate plus phosphoribosyl pyrophosphate corresponds to that established in other microorganisms.[21] Thus, C-2 of the histidine nucleus is ultimately derived from formate (via formyltetrahydrofolate), N-1 is derived from the amino group of aspartic acid, and N-3 is derived from the amide group of glutamine. Furthermore, catabolism of histidine (**6**) by the histidine ammonia lyase/urocanase sequence[22] ultimately leads to glutamic acid and formamide[23] and hence to formate plus ammonia.

The question then arises whether the experiments, which show that unlabeled histidine spares the incorporation of labeled formate plus labeled ammonia (expt 20) and that labeled histidine delivers an N=C−NH₂ fragment into the thiamin pyrimidine (expt 21), simply indicate that histidine, when present, serves as a more effective source of formate plus ammonia or whether they do indeed show that histidine per se serves as a precursor.

Two further experiments showed that the latter view is correct (Scheme 6). Administration of [¹³C,¹⁵N]formamide (expt 23) yielded a pyrimidine sample whose spectrum (Figure 6A) showed strong ¹³C enrichment at C-4 (C-4/C-2 = 2.3/1) but no ¹³C,¹⁵N satellites, showing that the ¹⁵N−¹³C unit of the substrate had not been incorporated intact but that it was [¹³C]formate, formed by hydrolysis of the [¹⁵N,¹³C]formamide that had entered the pyrimidine and that the [¹⁵N]ammonium ion so derived had been diluted by the excess unlabeled ammonium ion present in the medium. The significance of this finding will be referred to below.

Furthermore, when L-[2′-¹³C,1′,3′-¹⁵N₂]histidine was administered in the presence of excess formamide (expt 22), the mode of incorporation of L-[2′-¹³C,1′,3′-¹⁵N₂]histidine was unaffected: ¹³C enrichment of C-4 (C-4/C-2 = 3.4/1) as well as ¹³C,¹⁵N coupling at C-4 ($^1J_{13C,15N}$ = 22.4, 3.4 Hz) that had been observed in the experiment in which L-[2′-¹³C,1′,3′-¹⁵N₂]histidine alone was administered was maintained (Figure 5B).

(18) (a) Cane, D. E.; Du, S.; Robinson, J. K.; Hsiung, Y.; Spenser, I. D. *J. Am. Chem. Soc.* **1999**, *121*, 7722−7723. (b) Cane, D. E.; Hsiung, Y.; Cornish, J. A.; Robinson, J. K.; Spenser, I. D. *J. Am. Chem. Soc.* **1998**, *120*, 1936−1937.

(19) David, S.; Estramareix, B.; Hirschfeld, H. *Biochim. Biophys. Acta* **1967**, *148*, 11−21.

(20) Uchida, K.; Yamada, K.; Kumaoka, H. *Vitamins (Japan)* **1982**, *56*, 235−240.

(21) (a) Fink, G. R. *Science* **1964**, *146*, 525−527. (b) Daignan-Fortier, B.; Fink, G. R. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6746−6750. (c) Alifano, P.; Fani, R.; Lio, P.; Lazcano, A.; Bazzicalupo, M.; Carlomagno, M. S.; Bruni, C. B. *Microbiol. Rev.* **1996**, *60*, 44−69. (d) Fani, R.; Tamburini, E.; Mori, E.; Lazcano, A.; Lio, P.; Barberio, C.; Casalone, E.; Cavalieri, D.; Perito, B.; Polsinelli, M. *Gene* **1997**, *197*, 9−17.

(22) Rétey, J. *Arch. Biochem. Biophys.* **1994**, *314*, 1−16.

(23) The steps of this pathway (see below) do not appear to have been demonstrated in yeasts.
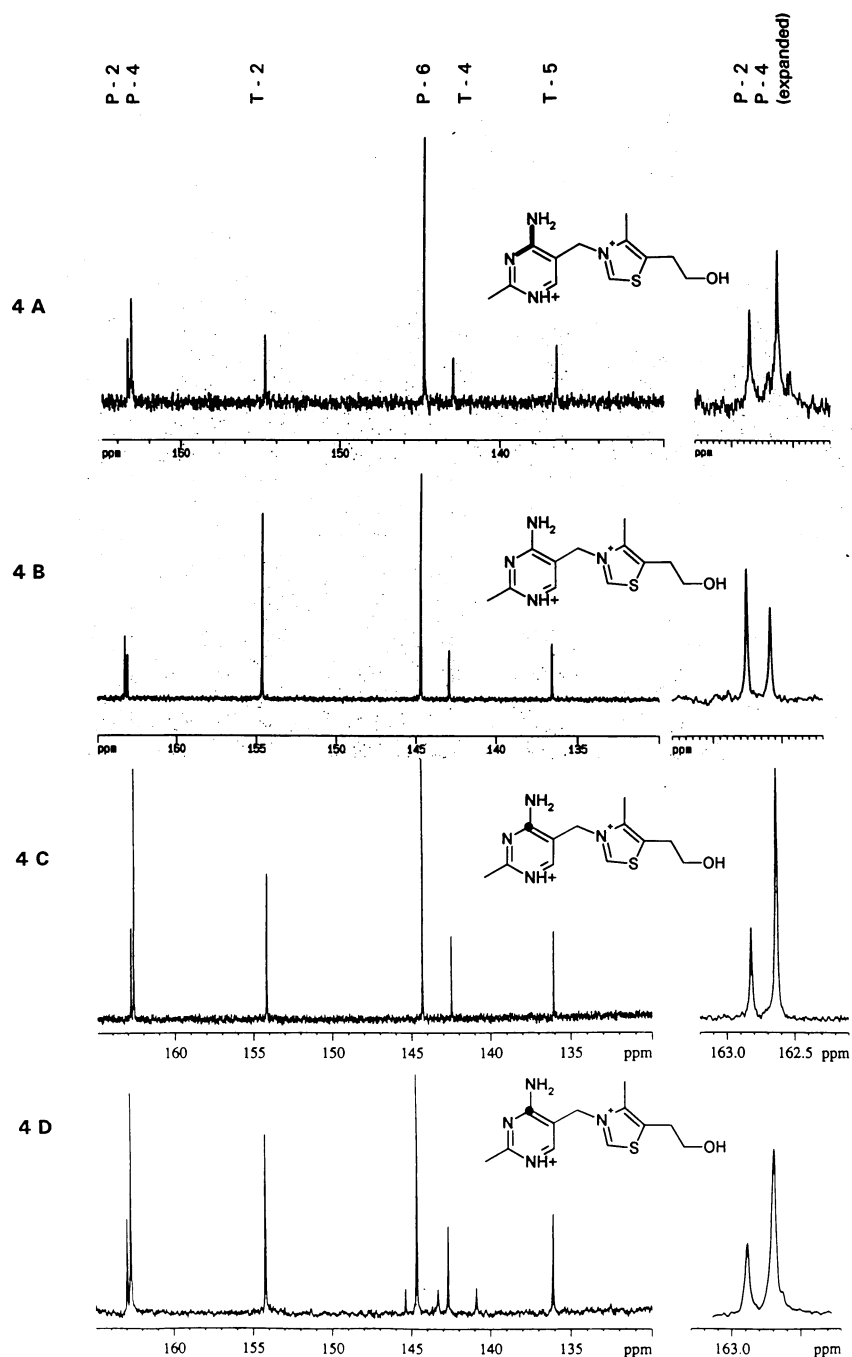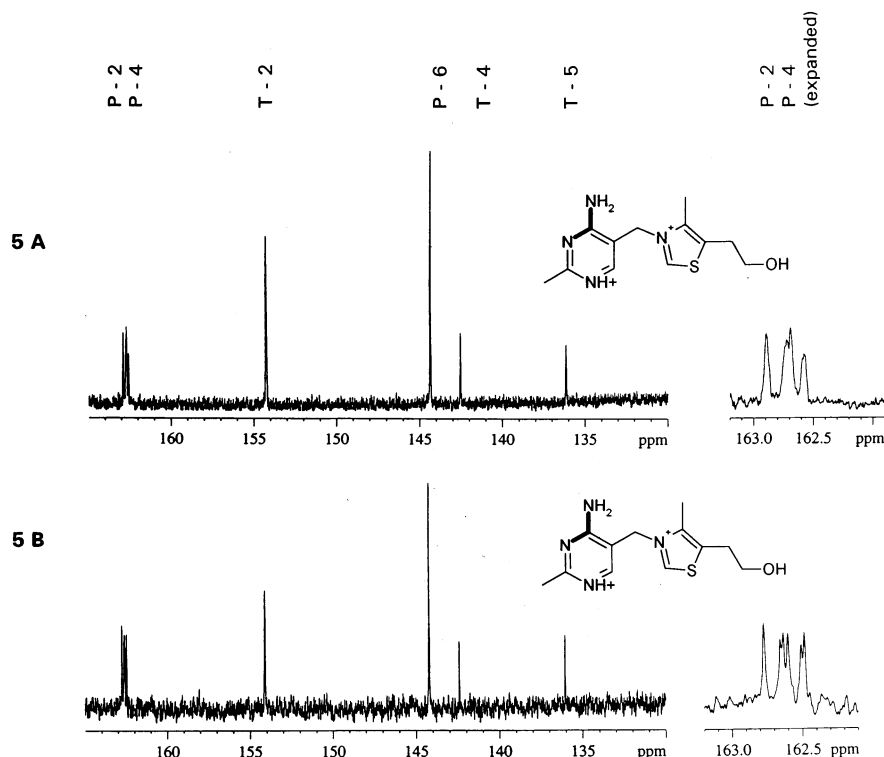
Figure 4. ^{13}C NMR spectra (high-frequency region) of thiamin derived from sodium [^{13}C]formate in the presence of (A) [^{15}N]ammonium chloride (expt 19), (B) [^{15}N]ammonium chloride plus unlabeled L-histidine (expt 20), (C) unlabeled formiminoglutamate (expt 25), and (D) unlabeled imidazoleacetic acid (expt 24).

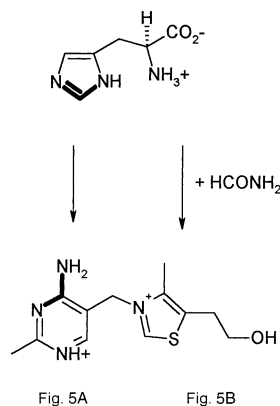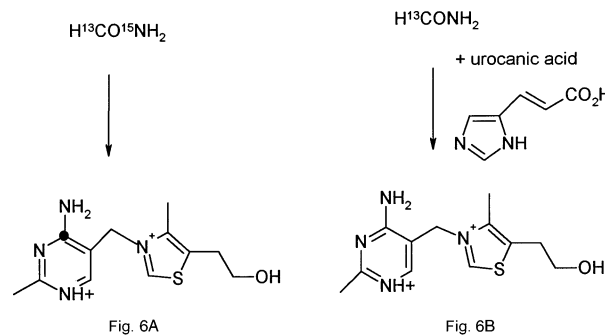**Scheme 4.** Entry of Formate into Thiamin
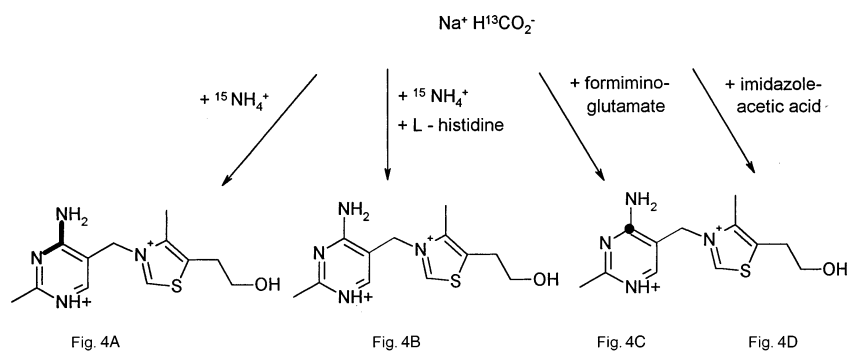
Figure 5. ^{13}C NMR spectra (high-frequency region) of thiamin derived from (A) [2′-^{13}C,1′,3′-^{15}N$_2$]histidine (expt 21) and (B) [2′-^{13}C,1′,3′-^{15}N$_2$]histidine in the presence of unlabeled formamide (expt 22).

Scheme 5. Entry of Histidine into Thiamin

Thus, the intact N-1′,C-2′,N-3′ fragment of histidine is incorporated into the N-3,C-4,NH$_2$ unit of the thiamin pyrimidine, and histidine serves as an intermediate between formamide (as a source of formate) and the thiamin pyrimidine.

This observation is consistent with the result of an early experiment[24] which demonstrated that in *S. cerevisiae* 4-hydroxy-5-hydroxymethyl-2-methylpyrimidine (**5**), the deamination product of the thiamin pyrimidine, is not an intermediate in the evolution of the latter. Intact incorporation of formamide (**4**) would have been expected to yield the 4-hydroxy derivative (**5**) (Scheme 3).

Union of the Pyridoxine-Derived C$_5$N Fragment with the Histidine-Derived CN$_2$ Fragment. Having drawn the inference that a discrete C$_5$N degradation product of pyridoxol is not involved in the biosynthetic process, a rational approach to this

(24) Baxter, R. L.; Hanley, A. B.; Chan, H. W. S. *J. Chem. Soc., Perkin Trans. 1* **1990**, 2963−2966.
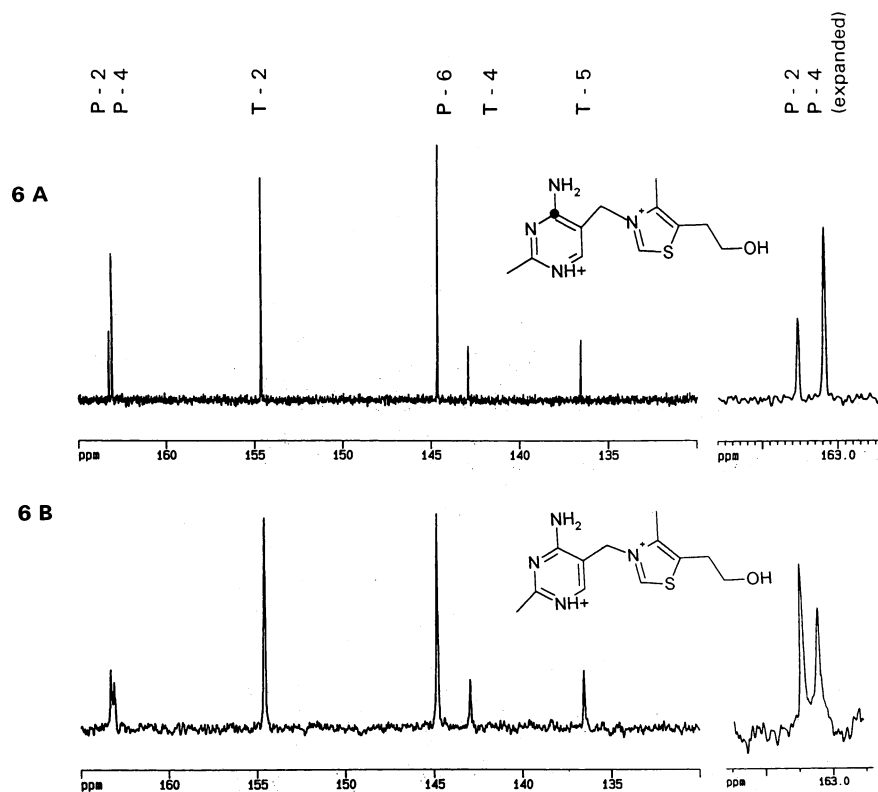
Scheme 6. Entry of Formamide into Thiamin

problem was to consider modes of attack on pyridoxol by histidine metabolites, leading to the transfer of the CN$_2$ unit of histidine into the pyridoxol skeleton, followed by loss of the C$_3$ unit, C-3,4,4′ of pyridoxol.

In evaluating possible intermediates in the reaction, we considered two catabolic reaction sequences of histidine. One of these was its decarboxylation to histamine and the further degradation of the latter to imidazole-4-acetic acid. Involvement of an intermediate of this catabolic sequence was made unlikely when it was found that imidazole-4-acetate did not suppress entry of the label from [^{13}C]formate into C-4 of the pyrimidine (Figure 4D) (expt 24).

The other histidine degradation pathway, involvement of which was considered, was the histidine ammonia lyase/urocanase sequence[22] (Scheme 7), leading to urocanic acid (**7**), imidazolonepropionate (**8**), and formiminoglutamate (**9**), and thence to formamide (**4**) and glutamic acid (**10**), as mentioned earlier.
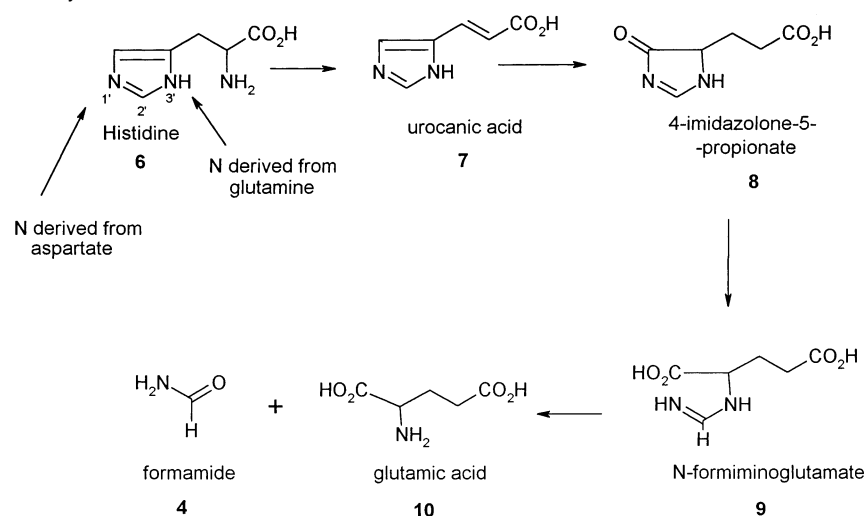
We have already referred to the attempt to show incorporation into the pyrimidine of [^{13}C,^{15}N]formamide (expt 23). The NMR

Figure 6. ^{13}C NMR spectra (high-frequency region) of thiamin derived from (A) $[^{15}N,^{13}C]$formamide (expt 23) and (B) $[^{13}C]$formamide in the presence of unlabeled urocanic acid (expt 26).
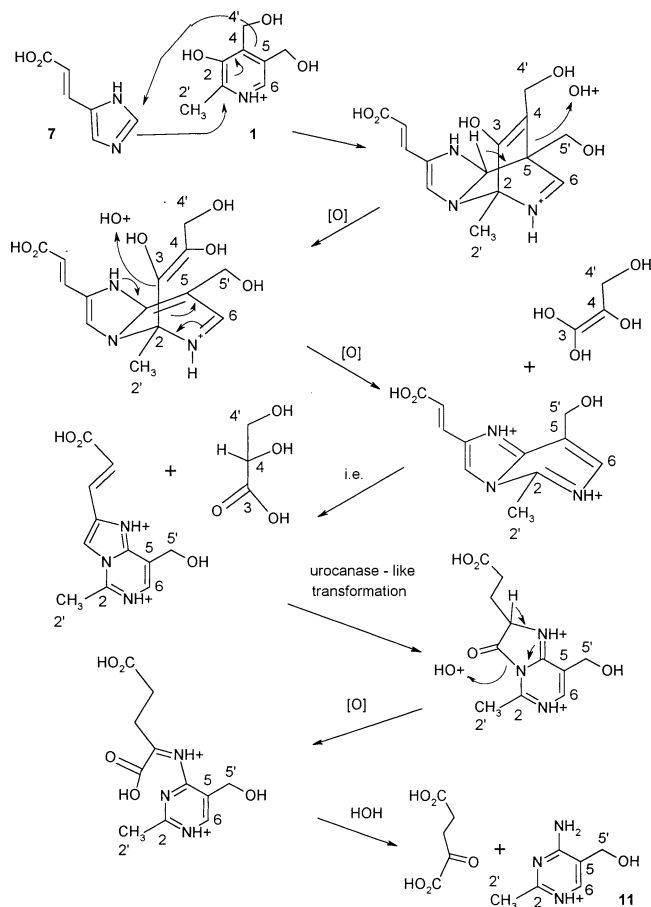
Scheme 7. Urocanase Pathway of Histidine Catabolism

spectrum (Figure 6A) of the latter showed strong ^{13}C enrichment at C-4, relative to C-2 (C-4/C-2 = 2.3/1), but no $^{13}C,^{15}N$ satellites. Thus, formamide was not incorporated as an intact unit, but its ^{13}C was incorporated only after hydrolysis, as formate.

An attempt to displace incorporation of $[^{13}C]$formate by *N*-formiminoglutamate (**9**) (expt 25) gave a sample of thiamin whose NMR spectrum (Figure 4C) showed strong enrichment at C-4, relative to C-2 (C-4/C-2 = 2.8/1). Thus, formimino-glutamate (**9**) does not serve as an intermediate between histidine and the pyrimidine.

A displacement experiment with urocanic acid (**7**) (expt 26) gave an entirely different result: In the presence of this histidine

degradation product, incorporation of $[^{13}C]$formamide into the pyrimidine was entirely repressed (Figure 6B). This suggests that urocanic acid lies on the route. Formation of urocanate by deamination of histidine, catalyzed by histidine ammonia-lyase, appears to be reversible, however,[25] and there is therefore a possibility that histidine is regenerated prior to the release of the N−C−N unit that enters the pyrimidine. An extremely high K_m value for ammonia in the reverse reaction makes it most unlikely, nonetheless, that the reaction is reversible under physiological conditions.[26]

(25) (a) Peterkofsky, A. *J. Biol. Chem.* **1962**, *237*, 787−795. (b) Furuta, T.; Takahashi, H.; Shibasaki, H.; Kasuya, Y. *J. Biol. Chem.* **1992**, *267*, 12600−12605.

Scheme 8. A Proposal for the Mechanism of the Conversion of Pyridoxol (**1**) Plus Urocanic Acid (**7**) into the Pyrimidine Unit (**11**) of Thiamin

Table 1. Incubation of Yeast Cultures with $[^{13}C_6]$Glucose in the Presence of Unlabeled Substrates To Test Possible Diplacement of Glucose-Derived ^{13}C [33]

expt no.	labeled substrate	weight (g/L)	incubation duration (h)	
			A. *Candida utilis*	
1*	D-$[^{13}C_6]$glucose	1	17	no displacement
	D-glucose	4		
	fumaric acid	2		
2*	D-$[^{13}C_6]$glucose	1	17	no displacement
	D-glucose	4		
	L-homoserine	1		
			B. *Saccharomyces cerevisiae*	
3*	D-$[^{13}C_6]$glucose	1	12	no displacement
	D-glucose	4		
	L-aspartic acid	1.5		
4*	D-$[^{13}C_6]$glucose	1	44	no displacement
	D-glucose	4		
	DL-hydroxyaspartic acid	2		
5*	D-$[^{13}C_6]$glucose	1	17	no displacement
	D-glucose	4		
	DL-isoserine	2		
6*	D-$[^{13}C_6]$glucose	1	18	no displacement
	D-glucose	2		
	D-erythrose	1.9		
7	D-$[^{13}C_6]$glucose	1	18	no displacement
	D-glucose	4		
	1-amino-3-hydroxyacetone	1.75		
8	D-$[^{13}C_6]$glucose	1	22	no displacement
	D-glucose	4		
	N-acetyl-1-amino-3-hydroxyacetone	1		
9	D-$[^{13}C_6]$glucose	1	21	displacement Figure 3B
	D-glucose	4		
	pyridoxol HCl	0.3		
10	D-$[^{13}C_6]$glucose	1	17	displacement Figure 3C
	D-glucose	4		
	2′-hydroxypyridoxol HCl	2.1		

We now put forward a hypothetical mechanism for the formation of the thiamin pyrimidine from pyridoxol (**1**) and urocanic acid (**7**), which postulates Diels−Alder-like attack of the latter on pyridoxol, followed by the oxidative decomposition of the urocanate-derived moiety of the adduct, in the manner of the urocanase reaction,[22] eventually leading to the thiamin pyrimidine, glyceric acid, and α-ketoglutarate (Scheme 8).

Experimental Section

²H-, ¹⁵N-, and ¹³C-Labeled Compounds. D-$[^{13}C_6]$Glucose, sodium $[^{13}C]$formate, $[^{13}C,^{15}N]$formamide, sodium $[1,2^{-13}C_2]$acetate, sodium $[2,3^{-13}C_2]$pyruvate, and $[1,3^{-13}C_2]$glycerol were purchased from Cambridge Isotope Laboratories (CIL), Andover, MA; D-$[1,2^{-13}C_2]$ribose and D-$[3,3^{-2}H_2]$glyceraldehyde were purchased from Omicron Biochemicals, Inc., South Bend, IN; and $[^2H_5]$glycerol was purchased from CDN Isotopes, Pointe-Claire, Quebec, Canada. $[2,3^{-13}C_2]$-4-Hydroxy-L-threonine[27] and R- and S-$[1,1^{-2}H_2]$glycerol[28] were synthesized as previously described.

A. $[6^{-13}C,^{15}N]$Pyridoxol Hydrochloride. ¹³C NMR (125.7 MHz, D₂O/DCl) δ: 129.0 ppm (doublet, $^1J_{13C15N} = 12.5$ Hz). The synthesis of this multiply labeled sample of pyridoxol followed a published procedure.[29] The product was obtained from acetaldehyde cyanohydrin in four steps. The cyanohydrin was reacted with $^{15}NH_2^{-13}CHO$ (CIL), to yield $CH_3^{-}CH(CN)^{-15}NH^{-13}CHO$, which was condensed with

fumaronitrile (Aldrich) to yield $[1^{-15}N,6^{-13}C]$-3-amino-4,5-di(aminomethyl)-2-methylpyridine. Reduction with LiAlH₄, followed by treatment with nitrous acid, yielded the desired product.

B. L-$[2'^{-13}C,1',3'^{-15}N_2]$Histidine. ¹H NMR (500 MHz, D₂O) δ: 8.47 (doublet of multiplets, 1H, H-2′; $^1J_{1H,13C} = 220$, $^2J_{1H,15N} = 11$ Hz), 7.23 (m, 1H, H-5′), 4.21 (t, 2H, H-β), 3.25 (m, 1H, H-α). ¹³C NMR (125.7 MHz, D₂O/DCl) δ: 134.3 ppm ($^1J_{13C15N} = 16.8$, $^1J_{13C15N} = 16.8$ Hz). The synthesis of this multiply labeled sample of L-histidine followed a published procedure.[30] The product was obtained from *N*-carbobenzoxy-L-aspartic acid anhydride (Aldrich) in nine steps. One of the ¹⁵N labels was introduced by treating the intermediate, benzyl L-carbobenzoxy-amino-5-chloro-4-oxopentanoate, with potassium $[^{15}N]$phthalimide (CIL). To introduce the second ¹⁵N and the ¹³C atom, the L-2,5-diamino-4-oxopentanoic acid diHCl, obtained by acid hydrolysis of the phthaloyl derivative, was reacted with $KS^{13}C^{15}N$ (prepared from $K^{13}C^{15}N$ (CIL) by treatment with sulfur).

N-Acetyl-3-amino-1-hydroxypropanone (3).[cf.,31] A. DL-N-Acetyl-3-amino-1-*tert*-butyldimethylsilyloxy-2-propanol. DL-N-Acetyl-3-aminopropane-1,2-diol[32] (12 g. 90 mmol) was suspended in CH_2Cl_2. *tert*-Butyldimethylsilyl chloride (TBDMSCl) (15.6 g, 100 mmol) was added with stirring, followed by triethylamine (15.2 mL, 108 mmol) and 4-(dimethylamino)pyridine (DMAP) (431 mg, 3.5 mmol). The mixture

(26) Rétey, J. Personal communication.
(27) Wolf, E.; Spenser, I. D. *J. Org. Chem.* **1995**, *60*, 6937−6940.
(28) Hill, R. E.; Iwanow, A.; Sayer, B. G.; Wysocka, W.; Spenser, I. D. *J. Biol. Chem.* **1987**, *262*, 7463−7471.
(29) Hoshino, J.-i.; Yamamoto, Y.; Hasegawa, T.; Takahashi, S.; Sawada, S. *Biosci. Biotech. Biochem.* **1994**, *58*, 1939−1941.

(30) Furuta, T.; Katayama, M.; Shibasaki, H.; Kasuya, Y. *J. Chem. Soc., Perkin Trans. 1* **1992**, 1643−1648.
(31) Wolf, E.; Kennedy, I. A.; Himmeldirk, K.; Spenser, I. D. *Can. J. Chem.* **1997**, *75*, 942−948.
(32) Crans, D. C.; Whitesides, G. M. *J. Am. Chem. Soc.* **1985**, *107*, 7008−7018.
(33) The labeling pattern within pyridoxamine, isolated from some of these incubations (marked with an asterisk), has been reported in ref 6, where the corresponding experiments are listed, but under experiment numbers that are different from those used in the present tables.

Table 2. Incubation of Yeast Cultures with Labeled Substrates Other than [^{13}C]Glucose[33]

expt no.	labeled substrate	weight (g/L)	incubation duration (h)	
	A. *Candida utilis*			
11	D-xylose	4	18	no incorporation
(cf expt 15)	Na [1,2-^{13}C$_2$]acetate	1		
12*	D-xylose	1	72	incorporation
	D-ribose	3		Figure 1C
	D-[1,2-^{13}C$_2$]ribose	1		C-4/C-2 = 0.8/1.0
13*	D-xylose	3.5	21	incorporation
	[1,3-^{13}C$_2$]glycerol	1.5		Figure 1A
				C-4/C-2 = 0.9/1.0
14*	D-xylose	3.75	22	incorporation
	[^{2}H$_5$]glycerol	1.25		Figure 2B
	in D depleted water (4.67 ppm)			
	B. *Saccharomyces cerevisiae*			
15	D-glucose	6.25	23	no incorporation
(cf expt 11)	Na [1,2-^{13}C$_2$]acetate	1		
16	D-glucose	6.25	12	no incorporation
	Na [2,3-^{13}C$_2$]pyruvate	1		
17*	D-glucose	4	17	no incorporation
	[2,3-^{13}C$_2$]-4-hydroxy-L-threonine	0.36		
18	D-glucose	5	20	incorporation
	[6-^{13}C,^{15}N]-pyridoxol HCl	0.225		Figure 3D
				($^1J_{13C,15N}$ = 12.1 Hz,
				^{13}C isotope shift 0.018 ppm)
19 (cf ref 4,	D-glucose	5	18	incorporation
expt 2, Figure 2)	Na [^{13}C]formate	1.5		Figure 4A
	^{15}NH$_4$Cl	5		C-4/C-2 = 2.0/1
				($^1J_{13C,15N}$ = ca. 20, 2.5 Hz,
				^{13}C isotope shift 0.015 ppm)
20	D-glucose	5	72	displacement
	Na [^{13}]formate	1.5		Figure 4B
	K$_2$HPO$_4$ (replacing (NH$_4$)$_2$HPO$_4$)	6.7		C-4/C-2 = 0.8/1
	^{15}NH$_4$Cl	5		
	L-histidine	0.35		
21	D-glucose	5	22	incorporation
	L-[2'-^{13}C,1',3'-^{15}N$_2$]histidine HCl	0.20		Figure 5A
				C-4/C-2 = 2.7/1
				($^1J_{13C,15N}$ = 21.6, 2.2 Hz,
				^{13}C isotope shift 0.035 ppm)
22	D-glucose	5	22	incorporation but
	formamide	0.5		no displacement
	L-[2'-^{13}C,1',3'-^{15}N$_2$]histidine HCl	0.28		Figure 5B
				C-4/C-2 = 3.4/1
				($^1J_{13C,15N}$ = 22.4, 3.4 Hz,
				^{13}C isotope shift 0.031 ppm)
23	D-glucose	5	18	incorporation of ^{13}C
	[^{13}C,^{15}N]formamide	0.40		but not of ^{15}N
				Figure 6A
				C-4/C-2 = 2.3/1
24	D-glucose	5	22	incorporation,
	Na [^{13}C]formate	1.5		no displacement
	imidazole-4-acetic acid (Na salt)	1		Figure 4D
				C-4/C-2 = 2.6/1
25	D-glucose	5	44	incorporation,
	Na [^{13}C]formate	1.5		no displacement
	N-formimino-L-glutamate	0.25		Figure 4C
				C-4/C-2 = 2.8/1
26	D-glucose	5	20	displacement
	[^{13}C]formamide	0.5		Figure 6B
	urocanic acid	0.7		C-4/C-2 = 0.8/1

was stirred overnight and was then shaken with water. The aqueous phase was washed with CH$_2$Cl$_2$, and the combined organic phases were washed with water and brine and then dried over MgSO$_4$. The solution was evaporated, and the oily residue was chromatographed on silica gel (CHCl$_3$/MeOH 50/1). The product (6.33 g, 28%) was obtained as a colorless oil that solidified in the refrigerator and melted at room temp. A better yield was obtained on a smaller scale: DL-*N*-Acetyl-3-aminopropane-1,2-diol[32] (1.6 g. 12 mmol), purified by column chromatography (SiO$_2$, CHCl$_3$/MeOH 9/1), was stirred in CH$_2$Cl$_2$ (350 mL) with triethylamine (2 mL, 14 mmol), TBDMSCl (2 g, 13 mmol), and 4-DMAP (60 mg, 0.5 mmol) for 60 h and worked up as

above, yielding 2.37 g (80%) of the product. ^{1}H NMR (200 MHz, CDCl$_3$) δ: 6.37 (br t, 1H), 3.06−3.77 (m, 5H), 1.96 (s, 3H), 0.85 (s, 9H), 0.02 (s, 6H). ^{13}NMR (50.3 MHz, CDCl$_3$) δ: 171.3, 71.0, 65.0, 42.7, 25.9, 18.3, −5.4 ppm.

B. *N*-Acetyl-3-amino-1-*tert*-butyldimethylsilyloxy-2-propanone. A suspension of *N*-chlorosuccinimide (5.27 g, 38.7 mmol) in CH$_2$Cl$_2$ (32 mL) was cooled to 0 °C, dimethyl sulfide (3.93 mL, 53 mmol) was added, and the mixture was cooled further to −20 °C in a cooling bath with MeOH/water (35/65 v/v) and dry ice. A solution of DL-*N*-acetyl-3-amino-1-*tert*-butyldimethylsilyloxy-2-propanol (6.33 g, 25.6 mmol) in CH$_2$Cl$_2$ (9 mL) was added, and the mixture was stirred for

3 h. Triethylamine (5.4 mL, 38.3 mmol) was added, and the cooling bath was then removed. After stirring 1 h, the reaction mixture was diluted with Et_2O (60 mL), washed with HCl (1 M), water, and brine, and dried with $MgSO_4$. Evaporation yielded 6.9 g crude product as a yellow oil, containing some *tert*-butyldimethylsilanol. This sample was used in the next step without further purification. A purified sample (column chromatography on SiO_2, Et_2O) crystallized in the refrigerator and melted at room temp.

^{1}H NMR (200 MHz, $CDCl_3$) δ: 6.62 (br t, 1H), 4.22−4.20 (d, 2H), 1.94 (s, 3H), 0.82 (s, 9H), −0.01 (s, 6H). ^{13}NMR (50.3 MHz, $CDCl_3$) δ: 206.4, 170.4, 68.2, 46.7, 25.6, 22.7, 18.1, −5.7 ppm.

C. *N*-Acetyl-3-amino-1-hydroxy-2-propanone (3). Crude *N*-acetyl-3-amino-1-*tert*-butyldimethylsilyloxy-2-propanone (6.9 g) was stirred in a mixture of acetic acid (240 mL), water (80 mL), and THF (80 mL) at room temp overnight. The solution was concetrated in vacuo, diluted with water, and extracted with $CHCl_3$. The aqueous phase was evaporated, and the yellow oily residue (3.9 g) was chromatographed on silica gel ($CHCl_3$/MeOH 13/1) to yield a colorless oil (2.1 g, 63% based on DL-*N*-acetyl-3-amino-1-*tert*-butyldimethylsilyloxy-2-propanol) that solidified in the refrigerator. Mp 46 °C. ^{1}H NMR (200 MHz, D_2O) δ: 4.27 (s, 2H), 4.00 (s, 2H), 1.91 (s, 3H). ^{13}NMR (50.3 MHz, D_2O) δ: 215.1, 181.5, 72.4, 52.7, 28.3 ppm.

Microorganisms. *Saccharomyces cerevisiae* ATTC 7752 (IFO 1234) and *Candida utilis* ATTC 9256 were cultured in a synthetic minimal medium.[4] The yeast cultures (2 × 1 L) in 2 L conical flasks on a shaking incubator at 32 °C were grown to a cell density of 1.6 OD (629 nm) or higher (12−24 h). The method of workup of the cells has been described previously.[6] Details of the experiments are summarized in Tables 1 and 2.

Isolation of Thiamin. Cells from the yeast culture (2 L) were removed by centrifugation. The cells were suspended in hydrochloric acid (0.1 M), thiamin diphosphate (3 mg) was added as carrier, the mixture was autoclaved, and cell debris was centrifuged off. The pH of the supernatant solution was adjusted to pH 4.7 with acetate buffer solution (thus preventing accumulation of excess base which destroys thiamin), and the mixture was digested with Takadiastase. Column chromatography, first on a weak cation exchanger (Amberlite CG-50) and then on SiO_2, yielded thiamin and pyridoxamine in separate fractions.[6] The fractions containing thiamin (thiochrome test) were combined, the solvent was evaporated at 30 °C, and the residue was dried over NaOH in vacuo. The residue was dissolved in methanol (2 × 250 μL) and applied to an alumina column packed with acetonitrile (1 cm inside diameter, 10 mL neutral Al_2O_3 Brockmann activity 1, 60−325 mesh). The column was eluted with acetonitrile/methanol (3:1 v/v, 50 mL). Thiamin containing fractions were combined and evaporated, the residue was dissolved in methanolic HCl (1%, 200 μL), the solvent was evaporated, and the residue was dried over NaOH. For determination of its NMR spectrum, the dry residue was transferred to a Shigemi tube with 50 + 25 μL of D_2O.

NMR Spectroscopy. Proton decoupled ^{13}C NMR spectra of thiamin were acquired at 125.776 and 150.92 MHz on Bruker spectrometers DRX 500 and AV 600, respectively, using a Shigemi sample tube; pulse width 90° (13.5, 13.8 μs); spectral width 28 985.5 Hz, 36 231 Hz; recycle delay 4.6 s, 4.9 s; digital resolution 0.88 Hz, 0.55 Hz per data point, respectively. Approximately 50 000 transients were required in order to generate the ^{13}C spectra showing satellites.

^{2}H spectra were acquired at 46.07 and 92.12 MHz on Bruker spectrometers AV 300 and AV 600, respectively, using a 30° pulse and a digital resolution of 0.114 and 0.158 Hz per point, respectively. The spectra were obtained at 35 °C and referenced to the residual HDO signal at 4.67 ppm.