SYNTHESIS OF [4'-^{13}C] PYRIDOXOL

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SUMMARY

5-Cyano-3-hydroxy-2-methylpyridine-1-oxide, after O-methylation with dimethylsulfate, was reacted regiospecifically with sodium [^{13}C] cyanide to give [4'-^{13}C] 4,5-dicyano-3-hydroxy-2-methylpyridine. The latter compound was successively transformed to [4'-^{13}C] pyridoxol and [4'-^{13}C] pyridoxal phosphate.

Key words: [4'-^{13}C] pyridoxol, [4'-^{13}C] pyridoxal phosphate.

INTRODUCTION

As part of a general program involving the study of enzymatic reactions by nuclear magnetic resonance techniques using ^{13}C enriched substrates, inhibitors and coenzymes, the formyl group of pyridoxal phosphate (1) was chosen as the enriched center in order to monitor, for example, the formation of a Schiff base between this aldehyde and the ε-amino group of lysine present in the apoenzyme (1, 2). According to literature procedures (3-6), pyridoxal phosphate (1) is obtained by selective oxidation of pyridoxol (2), followed by phosphorylation. We describe a short and direct method in which the labeled atom was introduced after the construction of the aromatic skeleton, thus reducing the synthesis of ^{13}C pyridoxol (2) to three simple chemical steps (Scheme 1).

RESULTS AND DISCUSSION

The preparation of [4'-^{13}C] pyridoxol (2) described below was based on a synthetic strategy involving a "C_7N" moiety and a "C_1 labeled" unit. Using

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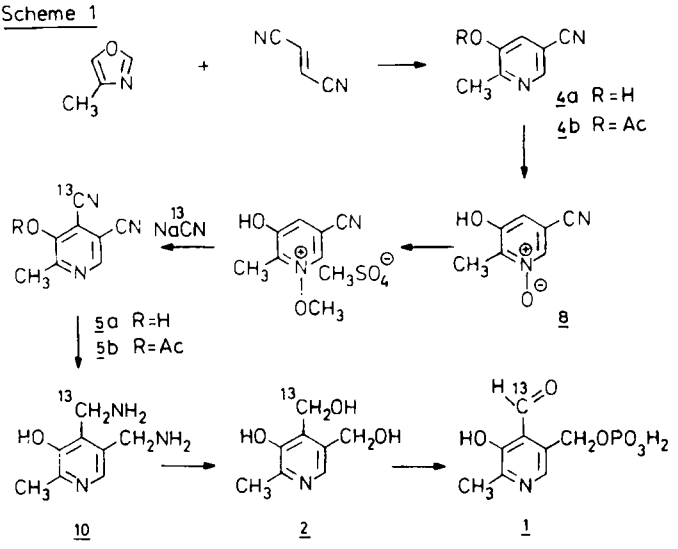
Scheme 1



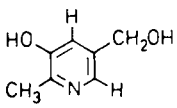
sodium [^{13}C] cyanide as the "C_1 labeled" building block, this synthetic approach requires that substituents attached to the "C_7N" moiety correspond as closely as possible to those of the target molecule (2). To this end, an attempt to cyanolate 3-hydroxy-5-hydroxymethyl-2-methylpyridine (3) failed. Accordingly, 5-cyano-3-hydroxy-2-methylpyridine (4a) was selected as a second candidate for this purpose. During a study concerned with different cycloaddition reactions, Yoshikawa et al. (7) reported the formation of compound 4a. However, this chemical structure was deduced only by transforming it into the dicyano derivative 5a (8), identified (mixed melting point) with an authentic sample (9). Since the proposed intermediates are crucial to a labeled synthesis, rigorous evidence for their structures was required. Indeed, should structure 6 be the product of the cycloaddition, the above mentioned reactions should also give the same dicyano derivative 5a as the product, but applied to a labeling process, the undesired isomer 7 would be obtained. Yoshikawa's work was repeated and the structure of compound 4a was confirmed by examination of its ^1H NMR spectrum (Table 1) in which each aromatic proton (δ 7.29 and 8.22) exhibits a coupling constant of 1.8 Hz, a value characteristic for two meta protons.

TABLE 1. ^{1}H NMR Spectra of Relevant Synthetic Intermediates

Compound	Solvent	4-H	6-H	2'-Me	Others
2	D_2O*	—	8.18 s ($w_{1/2}$ 1.6)	2.70 s	4'-H:5.02 s 5'-H:4.81 s
^{13}C-2	D_2O*	—	8.18	2.70 s	4'-H:5.02 d (147) 5'-H:4.81 s
3	D_2O*	7.18 bd (1.8)	7.54 bd (1.8)	2.36 s	5'-H:4.51
4a	$CDCl_3$ CD_3OD	7.29 bd (1.8)	8.22 bd (1.8)	2.54 s	—
4b	$CDCl_3$ CD_3OD	7.70 bd (1.8)	8.65 bd (1.8)	2.53 s	3-OAc:2.42
5a	$CDCl_3$ CD_3OD	—	8.38 s ($w_{1/2}$ 1.6)	2.63 s	—
^{13}C-5a	$CDCl_3$ CD_3OD	—	8.38 d (1.6)	2.63 s	—
5b	$CDCl_3$	—	8.83 s ($w_{1/2}$ 1.6)	2.63 s	3-OAc:2.52
8	$CDCl_3$ CD_3OD	7.15 bd (1.8)	8.20 bd (1.8)	2.51 s	—

Chemical shifts are in δ units and refer to TMS, coupling constants (in Hz) are in parentheses (s = singlet, d = doublet, bd = broad doublet, and $w_{1/2}$ = half width).

*Methyl group of ethanol (δ 1.20) used as internal standard.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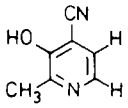
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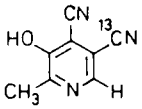
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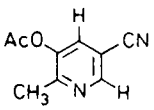
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It is known that in the pyridine series a cyano group cannot normally be introduced under Reissert conditions. However, in a method discovered independently by Okamoto and Tani (10) and Feely and Beavers (11), an alkoxy heterocyclic quaternary salt can be used for this purpose. Since the nucleophilic species has the choice between the ortho or para positions, some regioselectivity must be exercised during the reaction. More precisely, para-cyanolation was favored by performing the reaction at room temperature, in water at basic pH, and by using a bulky counter-anion (12). Using a 2.6 excess of cyanide, 5-cyano-3-hydroxy-2-methylpyridine-1-oxide (8), previously O-alkylated with dimethylsulfate, has been reported to produce the 4,5-dicyano derivative 5a (8). Optimization of the conditions was performed in our laboratory and a highly regiospecific reaction was obtained. Moreover, assuming that the phenolic substituent is neutralized after the N-oxide alkylation and before the cyanolation steps, the excess of cyanide can be decreased to 1.5, a more convenient ratio for labeled syntheses. The high regiospecificity of this reaction was deduced from the ^{1}H NMR spectrum obtained after the cyanolation step. No signal for the ortho-cyanolation product was observed and the aromatic chemical shift of the product confirms the para-cyanolation. Moreover, when an aliquot of compound 5a was acetylated to 5b, the ^{13}C NMR spectrum compared favorably with the values obtained for compound 4b (Table 2). Using literature data (13), the theoretical chemical shifts of compounds 5b and 9 were calculated and compared with the data observed experimentally for the dicyano compound. Clearly, structure 5b (rather than 9) was obtained.

Compound 5a was transformed into pyridoxol (2) following the literature methods (9, 14, 15) which involved hydrogenation of the nitriles and transformation of the resulting amino groups into benzylic alcohols. Droplet countercurrent

TABLE 2. ^{13}C NMR Spectra of Relevant Synthetic Intermediates

	Compound	C-2	C-3	C-4	C-5	C-6	C-2'	C-4'	C-5'	Others
1	D_2O pH 7.8	151.90	161.79	126.75	135.84	127.35	16.44	197.01	62.84	aldehyde form
1	D_2O pH 7.8	145.47	153.38	137.99	134.50	129.85	15.17	88.57	62.01	hydrate form
2	D_2O	143.50	153.39	141.56	137.50	130.80	15.18	57.92	58.96	
4a	$CDCl_3$ CD_3OD	151.44	152.44	122.39	107.07	141.23	18.17	—	116.07	
4b	$CDCl_3$ CD_3OD	156.54	144.92	132.19	107.73	148.53	19.43	—	115.46	20.29, 167.92
5a	$CDCl_3$ CD_3OD	153.44	156.11	106.94	108.36	142.25	20.01	111.94	113.82	
5b	$CDCl_3$	159.41	146.03	117.78	109.27	149.50	20.13	110.37	112.78	20.13, 167.01
5b	calculated	158.6	147.1	116.5	109.9	150.7	—	—	—	—
9	calculated	154.6	148.5	130.5	112.7	132.8	—	—	—	—

Chemical shifts refer to $CDCl_3$ (δ = 77.0) for organic solutions, and to dioxane (δ = 67.4) for aqueous solutions. Underlined values correspond to the ^{13}C enriched positions.

chromatography (16-18) was used for the final purification of pyridoxol (2).

Labeled pyridoxol (2), key intermediate for the synthesis of pyridoxal phosphate (1), has thus been obtained via three chemical steps. Examination of literature methods reveals that existing ^{13}C or ^{14}C pyridoxol syntheses are longer because the construction of the pyridine ring was included in the labeled synthetic sequences (3, 19, 20).

The conversion of pyridoxol (2) into pyridoxal phosphate (1) was performed following described methods (3-6) in rather low yields. However, the synthetic procedure described in the experimental is as efficient as those reported for enzymatic preparations (21).

The presence of ^{13}C in the synthetic samples was monitored by IR spectroscopy, ^{1}H and ^{13}C NMR measurements and mass spectroscopy.

IR Spectra — Cyano intermediates provide the opportunity to observe the isotopic shifts of the ^{13}CN IR stretching frequency (22). In the case of sodium [^{13}C] cyanide, the starting material, a 40 cm^{-1} shift was observed when compared with unenriched material. While a 30 cm^{-1} shift difference was obtained for the [4'-^{13}C] 4,5-dicyano-3-hydroxy-2-methylpyridine (5a), when compared with natural abundance (5a).

NMR Spectra — Since the NMR data of pyridoxal phosphate (1) and related compounds have been extensively reported (23-25), we restrict our discussion to the ^{13}C-enriched species where the enriched groups are at C-4' (Tables 1 and 2). The ^{1}H coupled ^{13}C NMR spectrum of [4'-^{13}C] (5a) shows the cyano resonance at δ 111.94 as a doublet due to long-range coupling (^{4}J C-4', H-6 = 1.6 Hz). The ^{1}H NMR spectrum showed an identical ^{4}J value for the H-6 resonance at δ 8.38. The ^{1}H coupled ^{13}C NMR spectra of pyridoxol (2) showed a one bond coupling, J C-4', H-4', of 147 Hz. Long-range coupling constants (^{4}J C-4', H-6 = 1 Hz) could only be observed by appropriate exponential multiplication of the FID. In pyridoxal phosphate (1), both the aldehyde group (δ 197.01) and the hydrated form (δ 88.57) gave doublets, ^{1}J C-4', H-4 = 185 Hz.

Mass Spectra — The molecular ion peak in the mass spectra of heteroaromatics is known to be intense (26), exemplified by most of the compounds examined here

TABLE 3. Mass Spectral Data of Cyanoderivatives

Compounds Mol. Weight	4a 134	8 150	5a 159	^{13}C-5a 160
M^+	134 100%	150 25%	159 10%	160 100%
M^+-16 (O)	--	134 50%	--	--
M^+-28 (CO)	106 30%	--	131 100%	132 23%
M^+-29 (C-OH)	105 100%	--	130 80%	131 83%
M^+-16 - 28	--	106 25%	--	--
M^+-16 - 29	--	105 100%	--	--
M^+-55	79 12%	--	104 10%	105 35%
M^+-70	64 25%	--	89 4%	90 12%

(Tables 3 and 4). When the hydrochloride salt of 2 was examined, the $[M + H]^+$ ion is observed. Loss of C-OH (M^+-29) and CO (M^+-28) are characteristic of the phenolic function, while cleavage of the bond beta to the aromatic ring is the general rule for benzylic alcohols (M^+-17, OH). The isotopic enrichments of the labeled compounds, obtained by comparing the intensity of the ^{12}C-M^+ and ^{13}C-M^+ peaks, are approximately 87 and 94% for the dicyano 5a and pyridoxol (2), respectively (cf. 98% ^{13}C in the starting material).

TABLE 4. Mass Spectral Data of Benzylic Alcohols

Compounds Mol. Weight	3 139	2 169 + HCl	^{13}C-2 170 + HCl
$M^+ + H$	--	170 40%	171 80%
M^+	139 100%	--	--
M^+-15 (CH_3)	124 10%	154 100%	155 100%
M^+-17 (OH)	122 15%	152 20%	153 20%
M^+-29 (C-OH)	110 70%	--	--

EXPERIMENTAL

M.p.s are uncorrected. IR spectra were recorded with KBr pellets on a Perkin-Elmer 297 instrument. ^{1}H NMR spectra were determined on a Varian EM-390 instrument. ^{13}C NMR spectra were determined on a Bruker AM500 instrument and mass spectra using a Kratos MS50TA instrument. Droplet countercurrent chromatography was performed on a DCC-300 EYELA instrument, equipped with 625 capillary ϕ1.5 ℓ 400 mm tubes. Analytical TLC was carried out using chromatoplates (100 X 200 X 0.25 mm SiO_2 gel F_{254}). Sodium [^{13}C] cyanide (99 atom % - KOR Isotopes^{TM}, Division of KOR, Incorporated) was used as the labeled starting material.

5-Cyano-3-hydroxy-2-methylpyridine (4a)

4-Methyloxazole (3.08 ml, 37.7 mmol) and fumaronitrile (2.995 g, 38.4 mmol) were reacted as described previously to give 4a (7), 3.536 g, 70% yield, m.p. 246°C (from acetic acid) [lit. m.p. (7) 247°C], IR ν 2240 cm^{-1} CN stretch.

5-Cyano-3-hydroxy-2-methylpyridine-1-oxide (8)

To a solution of 4a (2.999 g, 22.38 mmol) in glacial acetic acid (40 ml) heated to 70°C, 4.5 ml of 30% hydrogen peroxide were added and the solution heated at 100°C for 1 hour. Three successive additions of 30% H_2O_2, 4.5 ml each, followed by reaction at 100°C for 1 hour were performed and after cooling to room temperature, the mixture was reduced to one-half volume by evaporation under vacuum. Overnight crystallization from acetic acid gave 2.43 g of 8, m.p. 275°C [lit. m.p. (8) 278°C]. Yield 72.3%. IR ν 2220 cm^{-1} (CN stretch) and 1220 and 1090 cm^{-1} (aromatic N-O vibrations).

[4'-^{13}C] 4,5-Dicyano-3-hydroxy-2-methylpyridine (5a)

A mixture of 8 (1.001 g, 6.68 mmol) and dimethylsulfate (0.65 ml, 6.68 mmol), prepared and maintained under dry nitrogen, was heated at 95°C for 3 hours then cooled to 4°C. The reaction mixture was dissolved in a minimum volume of cold water containing K_2CO_3 (0.923 g, 6.75 mmol) and this solution was added dropwise to a cooled (4°C) solution of sodium [^{13}C] cyanide (0.502 g, 10.02 mmol) in water (3 ml), kept in ice for two hours and then at room temperature for 24 hours.

After addition of 2N HCl solution until acid pH, the water phase was continuously extracted with ether for 48 hours and the crude extract crystallized from acetone to give the product 5a, m.p. 188°C [lit. m.p. (8) 189°C]. The ^{13}C cyanolation step was performed with an 83% yield (average over 5 labeled syntheses) calculated from the N-oxide 8, or 55% yield if calculated from sodium [^{13}C] cyanide. Attempts to recover unreacted sodium [^{13}C] cyanide were not performed. IR ν 2240 and 2200 cm^{-1} (CN and ^{13}CN stretch) (ν 2245 and 2230 cm^{-1} for unlabeled 5a).

[4'-^{13}C] 4,5-Dihydroxymethyl-3-hydroxy-2-methylpyridine (pyridoxol (2))

To a solution of [4'-^{13}C] 5a (1.076 g, 5.5 mmol) in absolute ethanol (100 ml), conc. HCl (1 ml) and 10% Pd/charcoal (0.850 g) were added. The mixture was hydrogenated at room temperature until 500 ml of hydrogen were absorbed, the catalyst was removed by filtration and the filtrate was then evaporated under vacuum. The residue was dissolved in 2N HCl (80 ml) and a solution of $NaNO_2$ (2 g, 29 mmol) previously dissolved in cold water (20 ml) was slowly added. After 1 hour at room temperature, the mixture was heated in a water bath at 80°C for 30 minutes (9, 14, 15). After cooling, the mixture was evaporated to dryness. Inorganic salts were precipitated by addition of absolution ethanol and then filtered. The solvent was evaporated under vacuum and the residue dissolved in 5 ml of the aqueous phase described below. This crude reaction mixture was purified by droplet countercurrent chromatography (16-18). Elutions were performed in the ascendant mode using 250 ml of the aqueous phase, obtained from the tertiary system ($CHCl_3$/MeOH/H_2O in the ratio 3/3/2 by volume), and the chloroform phase was used as the stationary phase during this separation. Fractions (2 ml) were collected at the rate of 8 ml/hour and monitored by TLC (Silica gel eluted with the chloroform phase described earlier). The resultant [4'-^{13}C] pyridoxol (2) was crystallized from alcohol-acetone m.p. 204°C [lit. m.p. (9) 205°C], 0.3304 g (1.6 mmol) or 29.2% yield from 5a.

[4'-^{13}C] 3-Hydroxy-2-methyl-5-[(phosphonooxy)methyl]-4-pyridinecarboxaldehyde (pyridoxal phosphate) (1)

[4'-^{13}C] Pyridoxal phosphate (1) was prepared from [4'-^{13}C] pyridoxol (2) following literature procedures (3, 4, 27) modified as follows. In a 25 ml

centrifuge tube $KMnO_4$ (0.4939 g, 3.12 mmol) was dissolved in H_2O (15 ml). Na_2SO_3 was added until decoloration of the solution occurred. After centrifugation (5,000 rpm), the supernatant was discarded and the residue washed three times with H_2O (15 ml). After suspension of the residue in water (10 ml), [4'-^{13}C] pyridoxol (2) (0.6425 g, 3.12 mmol) and conc. H_2SO_4 (0.5 ml) were added and the mixture was left at room temperature for 3 hours. The volume of the reaction mixture was then reduced by half by evaporation under vacuum. Phenetidine (0.5 M, 6.5 ml) (3) and sodium acetate (2 M, 9 ml) were added and the mixture kept at 4°C overnight after which the precipitated Schiff base is filtered and dried under vacuum (0.3071 g - 34% yield).

This Schiff base (0.150 g, 0.52 mmol) is dissolved in pyridine (20 ml), then evaporated to dryness. A solution of cyanoethylphosphate (540 mg) in 100 ml pyridine (20 ml) was added and again evaporated under vacuum. Dry pyridine (20 ml) and N,N'-dicyclohexylcarbodiimide (3 g) were added and the reaction mixture allowed to stand at room temperature for 1.5 hours, after which time ice was added and the solution was evaporated to dryness under vacuum. The solid thus obtained was filtered and washed with water (100 ml). The filtrate was freeze dried and the residue dissolved in NaOH (1 N, 10 ml) and refluxed for 10 minutes. After cooling, the mixture was purified by column chromatography on Amberlite CG50 and the fractions containing compound (1) were further purified, again by column chromatography, on Dowex 50W-X4 which afforded pure [4'-^{13}C] pyridoxal phosphate (1) 0.034 g (26.2% yield from the Schiff base or 8.9% yield from pyridoxol (2)).

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