5-(2-Chloroethyl)-3-hydroxy-2-methyl-4-pyridinecarboxaldehyde (α^5-Pyridoxal Methyl Chloride) and Its Reaction with N^α-Acetyl-L-lysine to Form a New Cyclic Imino Acid Derivative of Homopyridoxal†

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ABSTRACT: 5-(2-Chloroethyl)-3-hydroxy-2-methyl-4-pyridinecarboxaldehyde (α^5-pyridoxal methyl chloride) has been synthesized and found to react with N^α-acetyl-L-lysine at 25° and pH 8.0. The bright yellow, ninhydrin-positive product Y has been isolated after removal of the acetyl group. Chemical ionization mass spectroscopy and nuclear magnetic resonance (nmr) spectroscopy of derivatives of Y have been used to show that compound Y is 6-(5-amino-5-carboxypentyl)-7,8-dihydro-3-methyl-2,6-naphthyridin-4-ol, a cyclic imino acid derivative of homopyridoxal. The synthesis of compound Y occurs in two steps: the formation of an imine between the carbonyl group of 5-(2-chloroethyl)-3-hydroxy-2-methyl-4-pyridinecarboxaldehyde and the ε-amino group of lysine is followed by an intramolecular alkylation of the imino nitrogen by the chloroethyl side chain to form a stable six-membered ring. Since the product of this reaction is a cyclic imino acid (lysyl) derivative of homopyridoxal, it serves as a useful model of pyridoxal-P enzymes in which the cofactor is usually bound in an imine link with the ε-amino group of a lysyl residue. The cyclic structure of this new derivative Y results in a restriction of the conformation of the C=N bond and of the number of tautomeric forms. Studies of the absorption and fluorescence properties of the different ionic forms of Y have contributed to our understanding of the spectra of pyridoxal imines and have been interpreted in relationship to spectra of pyridoxal-P enzymes.

Studies in our laboratory have been aimed at understanding structure–function relationships of enzymes which contain bound pyridoxal-P. This cofactor serves as an optical probe of the active site. A pyridoxal-P analog, 5-(2-chloroethyl)-3-hydroxy-2-methyl-4-pyridinecarboxaldehyde (α^5-pyridoxal methyl chloride), was synthesized with the design that it would alkylate a group at the active site of a pyridoxal-P enzyme (Miles, 1972).

Model studies of the reaction of α^5-pyridoxal methyl chloride with an amino acid derivative, N^α-acetyl-L-lysine, show that a new intramolecular alkylation reaction occurs. The product is a very stable cyclic imino acid derivative of homopyridoxal. This new compound serves as a useful model in studying spectral properties of imino acid derivatives of pyridoxal and of pyridoxal-P enzymes. Pyridoxal-P has been previously shown to be bound in an imine linkage with the ε-amino group of a lysyl residue in most pyridoxal-P enzymes. The cyclic ring structure of this new *imino acid* derivative of homopyridoxal is the same as that of a cyclic *imine* derivative of homopyridoxal which was prepared by Fisher and Metzler (1969) by a quite different synthetic route.

Methods and Materials

Absorbance spectra were recorded with a Cary 11 spectrophotometer. Fluorescence spectra (uncorrected) were recorded with an Aminco-Bowman spectrofluorimeter on 0.01–0.02 mM solutions of pyridoxal derivatives. The pH dependence of absorbance and fluorescence spectra was determined by diluting aliquots of a stock solution into the buffers recommended by Johnson and Metzler (1970). The pH values of the buffers were determined with a Radiometer pH meter. pK values were evaluated and theoretical titration curves were calculated using the procedures described by Johnson and Metzler (1970). Mass spectra were observed by direct insertion probe using an LKB 9000 mass spectrometer at 70 eV, 60-mA ionizing current with source temperature 290°. Chemical ionization mass spectra were measured on an MS-902 mass spectrometer with isobutane as the reactant gas, using a direct insertion probe with source temperature 260 or 300° (Fales *et al.*, 1969). Nuclear magnetic resonance (nmr) spectra were measured with a Varian HA-100 instrument.[1] Amino acid analyses were performed on the short column of a Beckman-Spinco Model No. 120C amino acid analyzer using the standard accelerated system (120 ml/hr) (Spackman, 1967).[2] The Gibbs reaction was carried out on 0.01–0.05 mM solutions of pyridoxal derivatives according to Hochberg *et al.* (1944) using *N*,2,6-trichloro-*p*-benzoquinoneimine (Eastman). The absorbance at 645 nm was observed for 5 min after the reagents were mixed and the maximum absorbance was recorded. Elemental analyses were performed by J. F. Alicino, New Hope, Pa. Electrophoresis of pyridoxyl derivatives was carried out on Whatman No. 3MM paper in pyridine–acetate buffer at pH 3.6 for 1 hr at 3200 V according to the standard method for peptide maps (Bennett, 1967). Preparative thin-layer chromatography of pyridoxyl derivatives was carried out on silica

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[1] These spectra were generously determined and interpreted by Dr. Robert J. Highet, Laboratory of Chemistry, National Heart and Lung Institute.

[2] These analyses were kindly performed by Mr. George Poy, Arthritis and Rheumatism Branch, National Institute of Arthritis, Metabolism, and Digestive Diseases.

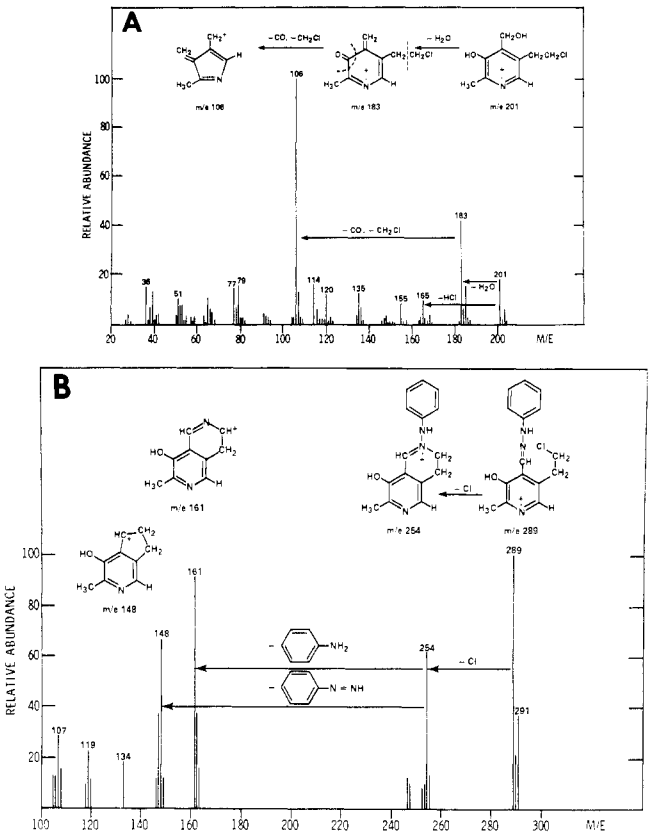


FIGURE 1: Electron impact mass spectra of α^5-pyridoxine methyl chloride above *m/e* 20 (A) and of the phenylhydrazone of α^5-pyridoxal methyl chloride above *m/e* 100 (B).

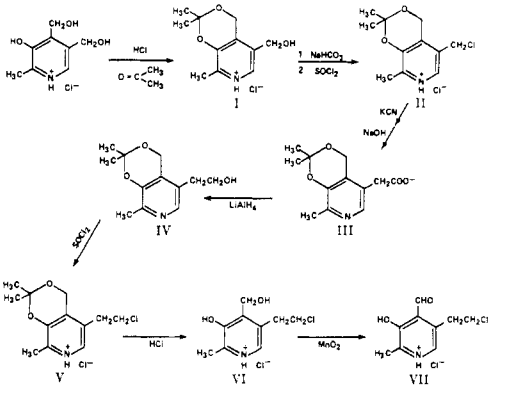
gel G plates (20 × 20 cm, 1 mm thick) prepared by Analtech, Inc., in $CHCl_3$–CH_3OH, (5:1) (solvent I) or in $CHCl_3$–CH_3OH–NH_4OH–H_2O (50:60:15:10) (solvent II). Pyridoxyl derivatives were detected by ultraviolet (uv) fluorescence, ninhydrin, and Gibbs reagent. Compounds were recovered from thin-layer plates by scraping off the silica, packing it into a 0.5 × 5 cm column and eluting with about 1 ml of dimethoxyethane or methanol. Pyridoxyl derivatives were also purified by chromatography on Amberlite CG-50 (200–400 mesh) (Mallinckrodt). The method of Dempsey and Snell (1963) was modified by the use of a linear gradient between water and 1 N acetic acid.

ε-Pyridoxyl-L-lysine was synthesized by a modification of the methods of Dempsey and Christensen (1962) and of Dempsey and Snell (1963). To 76.5 mg of N^{α}-acetyl-L-lysine (Calbiochem) and 60 mg of KOH in 3 ml of methanol was added 107 mg of pyridoxal hydrochloride (Sigma). After 15 min the solution was centrifuged. The supernatant was treated with 20 mg of $NaBH_4$, taken to dryness, dissolved in 1 ml of 5.7 N HCl, and hydrolyzed for 2 hr at 110°. The hydrolysate was fractionated on a 10-ml column of Amberlite CG-50 (see above). The fractions from the main peak which gave positive reactions on paper to ninhydrin and Gibbs reagent were lyophilized.

Results

Synthesis of 5-(2-Chloroethyl)-3-hydroxy-2-methyl-4-pyridinecarboxyaldehyde (α^5-Pyridoxal Methyl Chloride). The synthesis of α^5-pyridoxal methyl chloride (VII) is outlined in Scheme I. Compounds I–IV have been previously reported.

SCHEME I: Synthesis of α^5-Pyridoxal Methyl Chloride.



Pyridoxine derivatives with extensions of the carbon side chain at position 5 and containing hydroxyl and halogen functions at the terminal C-5 were mentioned in an abstract by Korytnyk *et al.* (1964). α^4,3-*O*-Isopropylidenepyridoxine·HCl (I) was prepared from pyridoxine·HCl (Eastman) and converted to the free base by treatment with sodium bicarbonate as described by Korytnyk and Wiedeman (1962) and modified as described by Mühlradt and Snell (1967). This product was converted to α^4,3-*O*-isopropylidene-α^5-pyridoxine chloride·HCl (II) as described by Tomita *et al.* (1966). Extension of the 5 side chain by one carbon to make α^4,3-*O*-isopropylidene-α^5-pyridoxine formic acid (III) was also carried out as described by Tomita *et al.* (1966). This product was converted to α^4,3-*O*-isopropylidene-α^5-pyridoxine methyl alcohol (IV) by reduction with $LiAlH_4$ as described by Korytnyk *et al.* (1967).

α^4,3-O-Isopropylidene-α^5-pyridoxine Methyl Chloride Hydrochloride (V). To 0.5 g (2.24 mmol) of isopropylidenepyridoxine methyl alcohol (IV) in 10 ml of benzene was added 0.175 ml (0.287 g, 2.42 mmol) of thionyl chloride. The mixture was stirred for 20 min at 75°. The precipitated compound was collected by filtration and washed with ether. The yield was 90%, R_F 0.97, in solvent I, mp 161–162°. *Anal.* Calcd for $C_{12}H_{17}NO_2Cl_2$: C, 51.8; H, 6.2; N, 5.0; Cl, 25.5. Found: C, 51.6; H, 6.3; N, 4.9; Cl, 25.7.

α^5-Pyridoxine Methyl Chloride Hydrochloride (VI). Isopropylidenepyridoxine methyl chloride·HCl (V), 0.4 g in 4 ml of 0.2 N HCl, was heated for 25 min in a boiling-water bath. The solution was concentrated to dryness *in vacuo* at 78° over P_2O_5: R_F 0.67, in solvent I, mp 158–160°. *Anal.* Calcd for $C_9H_{13}NO_2Cl_2$: C, 45.5; H, 5.5; N, 5.9; Cl, 29.8. Found: C, 45.8; H, 5.6; N, 5.9; Cl, 28.0. α^5-Pyridoxine methyl chloride was also analyzed by electron impact mass spectroscopy. The spectrum (Figure 1A) showed molecular ion peaks at *m/e* 201 and 203 (^{35}Cl and ^{37}Cl). The first two fragmentation peaks at *m/e* 183 and 185 can be ascribed to the loss of H_2O while the peak at *m/e* 165 corresponds to the loss of HCl. The latter is seen at *m/e* 36 and 38. The major fragmentation peak at *m/e* 106 has been previously observed in mass spectra of pyridoxine and pyridoxamine (DeJongh *et al.*, 1966) and involves the loss from the ($M - H_2O^+$) of CO from the 3 position and benzylic cleavage of the CH_2Cl group from the C-5 side chain.

5-(2-Chloroethyl)-3-hydroxy-2-methyl-4-pyridinecarboxaldehyde (α^5-Pyridoxal Methyl Chloride Hydrochloride) (VIII). The dried α^5-pyridoxine methyl chloride (VI), about 0.2 g, ground to a powder, was stirred with 0.2 g of MnO_2 A [prepared as described by Harfenist *et al.* (1954)] and 4 ml of

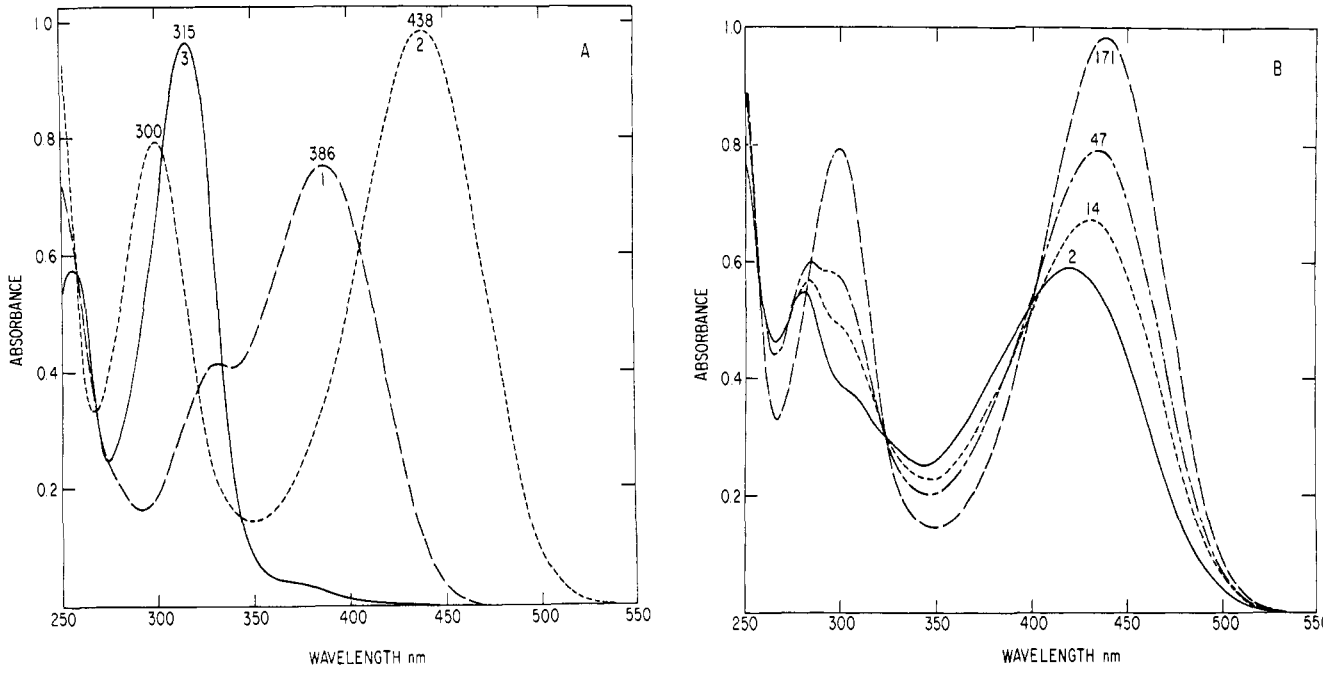


FIGURE 2: Spectra of α^5-pyridoxal methyl chloride and its products of reaction with N^α-acetyl-L-lysine. Reaction conditions are given in the text. All spectra were recorded on 1:100 dilutions in 0.05 M potassium phosphate (pH 7.0). Figure 1A shows spectra of α^5-pyridoxal methyl chloride before reaction (curve 1), 3 hr after addition of N^α-acetyl-L-lysine (curve 2), and after treatment of the reaction mixture in curve 2 with $NaBH_4$ (curve 3). Figure 1B shows spectra recorded at the indicated number of minutes after addition of N^α-acetyl-L-lysine to α^5-pyridoxal methyl chloride.

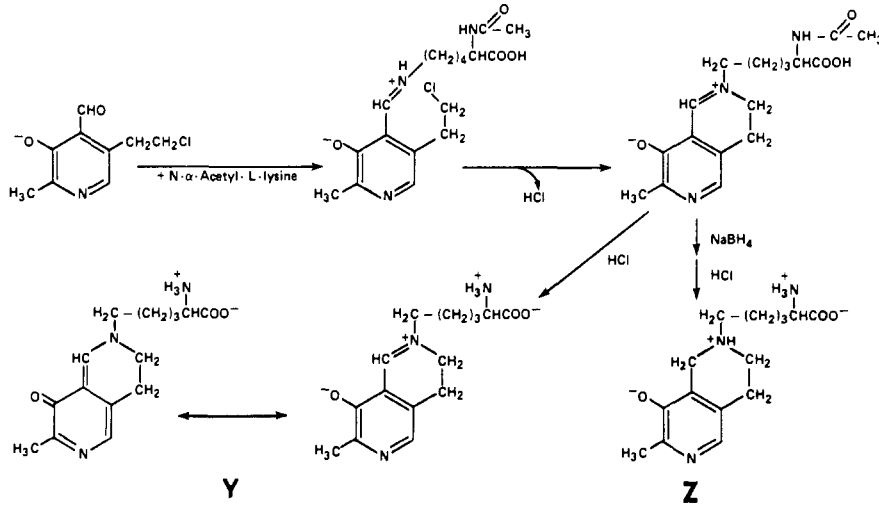
1,2-dimethoxyethane for 24 hr. The suspension was filtered and the precipitate was washed with about 5 ml of dimethoxyethane. The combined filtrates were concentrated to about 0.5 ml, applied to a 20 × 20 cm, 1 mm thick, silica gel thin-layer plate, and developed in solvent I. The yellow band (R_F 0.84) was scraped off and the compound eluted with dimethoxyethane. The solvent was concentrated to dryness and VII was dissolved in 1 ml of either ethanol or dimethoxyethane. The yield of this step was only 10–30%. An aliquot of the concentrated solution was analyzed by electron impact mass spectroscopy. The spectrum (not shown in a figure) was characterized by molecular ion peaks at *m/e* 199 and 201 (^{35}Cl and ^{37}Cl) with relative abundance of 65 and 23, respectively. The main fragmentation peak at *m/e* 163 (relative abundance 100) corresponded to the loss of HCl.

α^5-Pyridoxal methyl chloride (VII) was also characterized by its more stable phenylhydrazone hydrochloride. A solution of about 8 mg of VII in 0.25 ml of ethanol was treated with a solution of 16 mg of phenylhydrazine·HCl in 0.2 ml of 60% ethanol containing 0.2 N HCl. The resulting yellow phenylhydrazone hydrochloride was collected by filtration and washed with cold 0.05 M HCl. The yield was 60%, mp 217–219° dec. The electron impact mass spectrum of the phenylhydrazone was characterized by molecular ion peaks at *m/e* 289 and 291 (^{35}Cl and ^{37}Cl) (Figure 1B). The first fragmentation peak at *m/e* 254 (relative abundance 62) can be ascribed to the loss of Cl^-. HCl is a more common fragmentation product and was found in the spectrum of α^5-pyridoxal methyl chloride described above. The loss of Cl^- is probably due to an intramolecular alkylation reaction of molecular ion with the formation of a stable six-membered ring as shown in Figure 1B. This reaction is analogous to reactions of α^5-pyridoxal methyl chloride with amino acids (see below). Further fragmentation products at *m/e* 161 and 148 can be ascribed to loss of aniline and phenylazine ($C_6H_6N_2$) as shown in Figure 1B. Elemental analysis of the phenylhydrazone hydrochloride for chloride demonstrated that approximately 2 mol of chloride was indeed present. *Anal.* Calcd for $C_{15}H_{17}N_3OCl_2$: Cl, 21.7. Found: 19.2. This indicates that the phenylhydrazone of α^5-pyridoxal methyl chloride still contains the chloroethyl side chain. The cyclization reaction shown in Figure 1B must occur in the mass spectrometer.

The absorbance spectrum of the phenylhydrazone hydrochloride derivative of α^5-pyridoxal methyl chloride in 0.5 N H_2SO_4 showed λ_{max} 403 nm (ϵ 22,000 M^{-1} cm^{-1}) and λ_{max} 252 nm (ϵ 10,800 M^{-1} cm^{-1}). These extinction coefficients were used to determine the concentration of a stock solution of α^5-pyridoxal methyl chloride. An aliquot of the stock solution was diluted into 0.5 N H_2SO_4 containing phenylhydrazine hydrochloride in order to form the phenylhydrazone of α^5-pyridoxal methyl chloride in solution by the method of Wada and Snell (1961). Other aliquots of the stock solutions were diluted into appropriate buffers for the spectra and spectrophotometric and fluorimetric titrations which are shown below (Figures 5C and 6C). The spectrum of α^5-pyridoxal methyl chloride in 0.1 N HCl showed λ_{max} 295 nm (ϵ 7050 M^{-1} cm^{-1}) and λ_{max} 335 nm (ϵ 2200 M^{-1} cm^{-1}); the spectrum in 0.1 M potassium phosphate buffer (pH 7.8) showed λ_{max} 388 nm (ϵ 5650 M^{-1} cm^{-1}). Solutions at pH 7.8 were unstable. The peak at 388 nm slowly disappeared (about 50% in 3 hr) and was replaced by a new peak at 323 nm. This spectral change is presumably due to hydrolysis of the chloride group to yield α^5-homopyridoxal. Korytnyk and Ahrens (1971) have synthesized α^5-homopyridoxal and have shown that it forms a stable six-membered hemiacetal with $\lambda_{max}^{pH\,7}$ 255 nm (ϵ 4400) and 312 nm (ϵ 7450).

Reaction of α^5-Pyridoxal Methyl Chloride with N^α-Acetyl-L-lysine. α^5-Pyridoxal methyl chloride (50 μmol in methanol) was taken to dryness *in vacuo* and redissolved in 3.7 ml of 0.02

SCHEME II: Preparation of Compounds Y and Z.



M *N*-α-acetyl-L-lysine (pH 8.3). The pH was maintained at pH 8.0 by addition of 1 N NaOH and spectra were recorded from 250 to 550 nm at intervals on 1:100 dilutions in 0.05 M potassium phosphate (pH 7.0) (Figure 2B). These spectra show that most of the α^5-pyridoxal methyl chloride (curve 1, Figure 2A), which has a maximum absorbance at 386 nm, is rapidly converted to a derivative which has a maximum absorbance at 410 and 283 nm (curve at 2 min, Figure 2B). This derivative is probably a simple imino derivative (Schiff base) formed between the carbonyl group of α^5-pyridoxal methyl chloride and the ε-amino group of N^α-acetyl-L-lysine. 5-Deoxypyridoxal and L-leucine rapidly form a Schiff base in aqueous solution with a similar spectrum when a high concentration of leucine is used (Metzler and Nagano, 1968). A second, slow reaction results in a further increase in the visible absorbance and a shift of the wavelengths of maximum absorbance from 410 to 438 nm and from 283 to 300 nm. This second reaction is either an alkylation reaction or a hydrolysis reaction since an amount of NaOH approximately equivalent to the amount of α^5-pyridoxal methyl chloride was added during this time to maintain the pH at 8.0. After 3 hr no further spectral change or pH change occurred. The product (curve 2, Figure 2A) is probably also a Schiff base since it was reduced with $NaBH_4$ (curve 3, Figure 2A). One half of the reaction mixture was treated with 0.1 ml of 0.1 M $NaBH_4$ and the other was untreated.

In order to determine the stoichiometry of the overall reaction, an aliquot of the reaction mixture after $NaBH_4$ treatment which was equivalent to starting concentrations of 65 nmol of α^5-pyridoxal methyl chloride and 100 nmol of N^α-acetyl-L-lysine was subjected to acid hydrolysis and amino acid analysis on the short column (see Methods and Materials). Lysine (43 nmol) was recovered (elution time = 21 min) and 40 nmol of a new ninhydrin-positive product was found (elution time = 68 min). The estimated concentration of this product is based on the assumption that it contains one free amino group and has the same color value as leucine. Since 57 nmol of *N*-α-acetyl-L-lysine disappeared during the synthesis of 40 nmol of product, the overall reaction probably has a one-to-one stoichiometry.

This stoichiometry and the observed spectral changes are consistent with a reaction mechanism outlined in Scheme II. In this two-step reaction, imine formation is followed by an intramolecular alkylation of the imino nitrogen by the methyl chloride side chain. Hydrolysis or $NaBH_4$ reduction followed by hydrolysis would yield compounds Y and Z, respectively, which each contain one free amino group and a stable, cyclic six-membered ring. The systematic name for compound Y is 6-(5-amino-5-carboxypentyl)-7,8-dihydro-3-methyl-2,6-naphthyridin-4-ol. The systematic name for compound Z is 6-(5-amino-5-carboxypentyl)-3-methyl-5,6,7,8-tetrahydro-2,6-naphthyridin-4-ol.

For mass spectral and nuclear magnetic resonance (nmr) studies it was deemed advisable to isolate the *N*-acetyl derivatives of Y and Z which are intermediates in the synthesis (Scheme II). Thus the $NaBH_4$-treated reaction mixture was first acidified to destroy excess $NaBH_4$. Both the $NaBH_4$-treated and untreated reaction mixtures were taken to dryness and purified by preparative thin-layer chromatography in solvents I and II (see Methods and Materials). The products had R_F values of zero in solvent I (which was used to remove some breakdown products of α^5-pyridoxal methyl chloride) and both eluted at $R_F = 0.8$ in solvent II. The eluted *N*-acetyl derivatives of Y and Z (yield about 70%) were taken to dryness and used for mass spectra or nuclear magnetic resonance spectra or dissolved in 1 ml of constant-boiling HCl and hydrolyzed *in vacuo* for 6 hr. The hydrolysates were further purified by chromatography on Amberlite CG-50 (see Methods and Materials) and the combined chromatographic eluents were taken to dryness *in vacuo* and used to further characterize compounds Y and Z.

Chemical Ionization Mass Spectroscopy of N^α-Acetyl Derivatives of Compounds Y and Z. The proposed structures for compounds Y and Z are confirmed by chemical ionization (CI) mass spectrometry using isobutane as reagent gas and by nuclear magnetic resonance studies (Figures 3A–C and 4). The *N*-acetyl derivative of compound Z was characterized by an M + 1 (M + H) ion at *m/e* 336 which was the most abundant peak in the spectrum (Figure 3A). Two minor peaks at *m/e* 318 and 290 can be ascribed to the loss of H_2O and $COOH_2$ from the M + 1 ion as expected from α-amino acids (Milne *et al.*, 1970). The main fragmentation pathway leads to a major peak at *m/e* 165 which is ascribed to cleavage at the carbon–nitrogen bond at C-6 of the *N*-acetyllysine side chain with hydrogen rearrangement to yield the products shown in Figure 3A. The lysyl fragment (mass 171) is neutral and therefore not observed. An ion is also observed at *m/e* 170 which corresponds to an *N*-acetylpyrrolidinecarboxylic

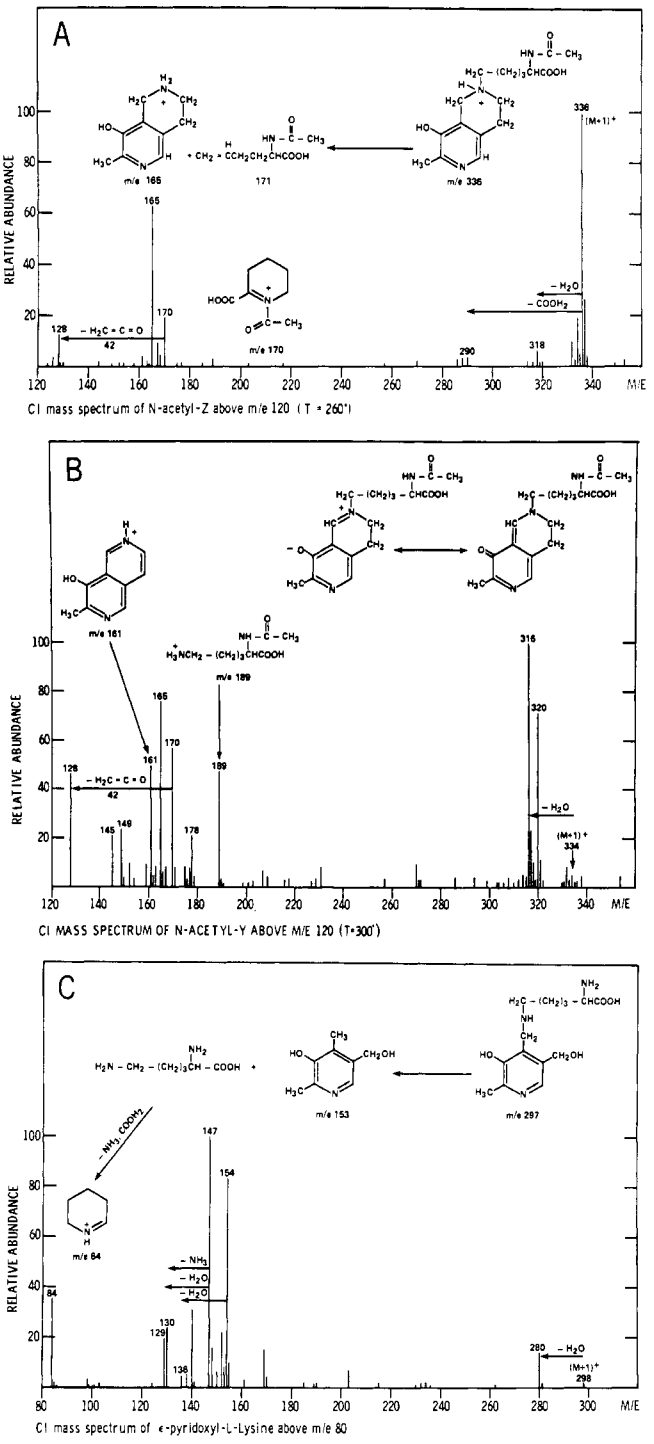


FIGURE 3: Chemical ionization mass spectra of *N*-acetyl-Z above *m/e* 120; $T = 260°$ (A), *N*-acetyl-Y above *m/e* 120; $T = 300°$ (B) and ε-pyridoxyl-L-lysine above *m/e* 80; $T = 200°$ (C).

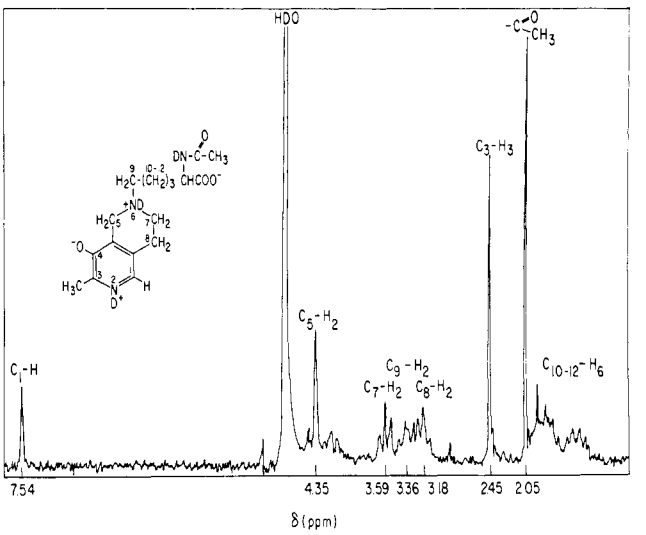


FIGURE 4: 100-MHz nuclear magnetic resonance spectrum of *N*-acetyl-Z in D_2O at pD 6.9.

acid since it loses ketene and CO_2 but its source is uncertain; it may be a pyrolysis product of *N*-acetyl-Z arising in the source.

The *N*-acetyl derivative of compound Y is expected to be much less volatile than Z because of contributions from dipolar resonance structures. No spectra were observed until 300° and at this temperature it is clear that several thermal reactions are occurring. Thus the ion at *m/e* 316 (Figure 3B) represents dehydration of *N*-acetyl-Y and is perhaps the best evidence for the existence of a molecular species of mass 333 since the M + 1 ion at *m/e* 334 is very weak. On the other hand, the presence of ions at *m/e* 165, 170, and 128 as shown by Z itself suggests that some intermolecular reduction has occurred in the source to give a species similar to Z. The intense ion at *m/e* 320 probably results from dehydration of a still further reduced species whose M + 1 ion may be observed at *m/e* 338, the highest mass ion in the spectrum. The source of the hydrogens necessary for these reductions is of course uncertain, but it may be noted that the ion at *m/e* 161 corresponds to M + 1 of fully aromatic 3-methyl-2,6-naphthyridin-4-ol, formation of which would supply an equivalent of hydrogen. Still other evidence for decomposition is provided by the ion at *m/e* 189, protonated lysine.

Similar fragmentation pathways have been observed in the model compound ε-pyridoxyl-L-lysine (Figure 3C) where the main products of fragmentation are lysine + 1 (*m/e* 147) and 4-methylpyridoxine + 1 (*m/e* 154). Loss of H_2O and NH_3 from the lysine + 1 ion and of H_2O from the 4-methylpyridoxine + 1 ion were observed. The $(M + 1)^+$ ion and $(M + 1 - H_2O)^+$ ion at *m/e* 298 and 280 were minor but easily discernible peaks. The pyrrolinium ion, *m/e* 84, has been previously observed as a fragmentation product of lysine (Milne *et al.*, 1970).

Nuclear Magnetic Resonance Spectroscopy of the N^α-Acetyl Derivative of Compound Z.[1] Figure 4 shows the nmr spectrum of N^α-acetyl-Z in D_2O at pD 6.9 at a concentration of about 0.06 M. The assignment of peaks was made by comparison to spectra of other vitamin B_6 derivatives (Korytnyk and Ahrens, 1970), of 7,8-dehydro-3-methyl-2,6-naphthyridin-4-ol, the cyclic imine of Fisher and Metzler (1969) and of 1,2,3,4-tetrahydroisoquinoline in $CDCl_3$. The ratios of the integrals of the peaks are close to the theoretical values for the structure shown. The C_1–H and C_3–H_3 peaks are close in chemical shift to those of the pyridoxal zwitterion model and the C_5–H_2 peak is close to that of the methylene peak of pyridoxamine. The protons on C_7, C_8, and C_9 give three sets of overlapping triplets centered at $\delta \sim 3.59$, 3.36, and 3.18. Although they cannot be easily discriminated, they are characteristic of this type of structure. We found two sets of triplets for 1,2,3,4-tetrahydroisoquinoline at $\delta \sim 2.75$ and 3.1 in $CDCl_3$. Fisher and Metzler (1969) report two sets of triplets at $\delta \sim 3.17$ and 4.03 for the protons on C_8 and C_7 of their cyclic imine.

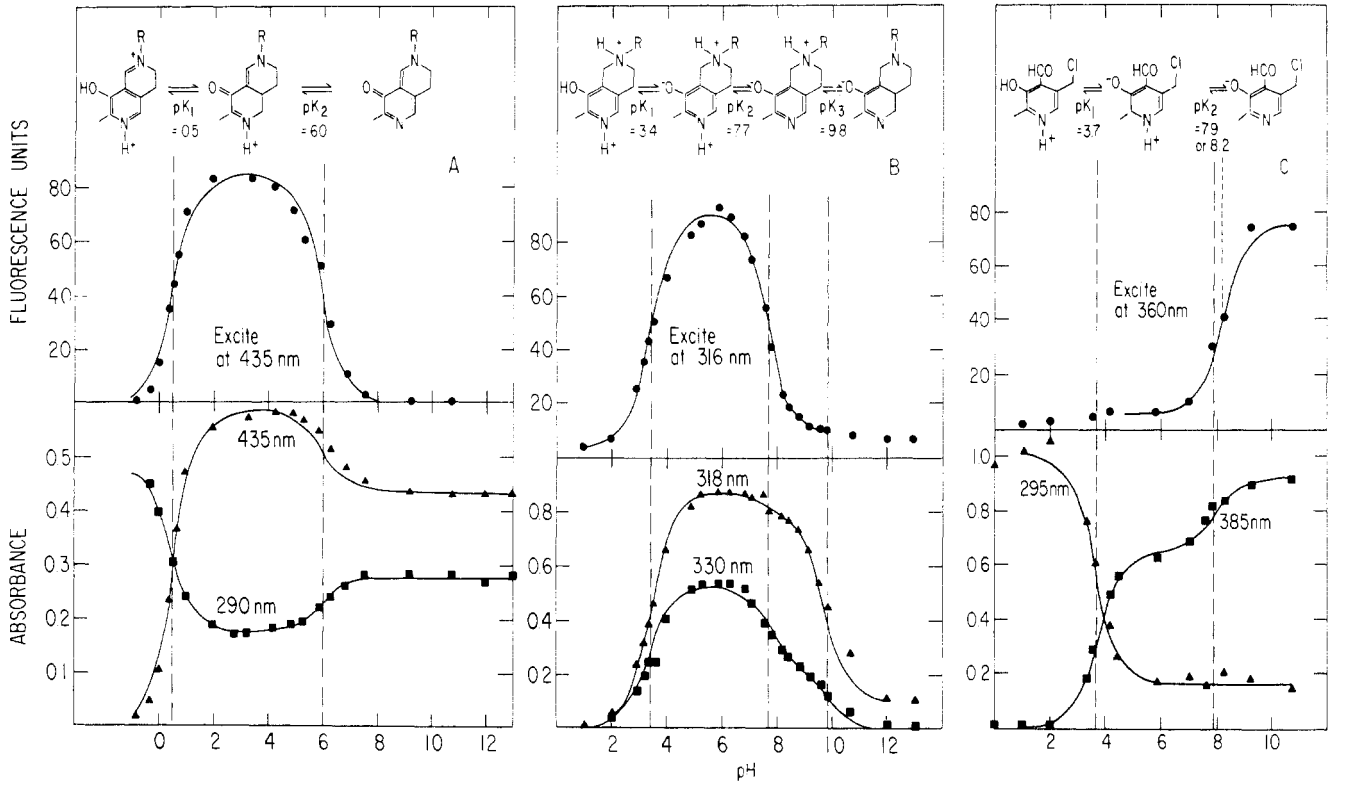


FIGURE 5: Spectrophotometric and fluorimetric titration curves of compound Y (A), compound Z (B), and α^5-pyridoxal methyl chloride (C). Dilutions of stock solutions were made into the following buffers: HCl (pH −0.8–2.0), sodium formate (pH 2.9–3.3), sodium acetate (pH 4.2–5.3), potassium phosphate (pH 5.9–7.8), triethanolamine·HCl (pH 8.2–8.8), sodium bicarbonate (pH 9.2–10.7), and sodium hydroxide (pH 12.0 and 13.0) for absorbance spectra. Absorbance at indicated wavelengths is plotted against pH. The solutions were diluted 4- to 6-fold for fluorescence measurements (○). Excitation was at 435 nm and emission was at 516 nm in part A; excitation was at 316 nm and emission was at 390 nm in part B; excitation was at 360 nm and emission was at 456 in part C. The calculated curves (———) are based on the indicated pK values.

TABLE I: Properties of Synthesized Compounds.[a]

Compound[b]	Amino Acid Anal. Elution Time (min)	Electrophoresis (cm) from Origin	Gibbs Test ϵ_{645} (M^{-1} cm^{-1} × 10^{-4})	Gibbs Test t_{max} (min)	Fluorescence pH	Fluorescence Excitation Max. (nm)	Fluorescence Emission Max. (nm)	Fluorescence Units
X	25	27	2.3	3	5.2	325	400	124
Y	36	26.5	0.4	2	3.5	430	516	331
Z	68	30.5	3.4	0.5	5.2	316	387	103
PM		35	2.2	4	7.0	325	400	91
Arg	41	32						
Lys	21	33.5						

[a] Analyses were carried out as described in Methods and Materials. [b] X = ε-pyridoxyl-L-lysine; Y and Z = synthetic compounds (see Scheme II and text). The concentrations of compounds Y and Z were estimated from their absorbance at 438 and 313 nm, respectively, in 0.1 M potassium phosphate (pH 7.8), using molar extinction coefficients at these wavelengths of 8800 M^{-1} cm^{-1} (Miles, 1972). PM = pyridoxamine.

The upfield part of the spectrum is complicated by the presence of many protons on the lysine moiety.

Other Properties of Compounds Y and Z. Table I compares the electrophoretic mobility, elution on amino acid analysis, reaction with Gibbs reagent, and fluorescence properties of compounds Y and Z with ε-pyridoxyl-L-lysine (compound X), pyridoxamine, and two basic amino acids. Some further details are given in the accompanying paper (Miles, 1972).

Absorbance and fluorescence properties of compounds Y and Z and of α^5-pyridoxal methyl chloride were determined as a function of pH as shown in Figure 5. Ionization constants were calculated from these data and proposed structures for the different ionized forms are shown. The spectra of the different ionized forms are shown in Figure 6. Table II gives a compilation of the pK values and wavelengths of absorption maxima of the various ionic forms of compounds

Y and Z and the two analogous cyclic compounds **9** and **11** which were synthesized by Fisher and Metzler (1969) in which the R group is H (see structures in Figure 5).

Discussion

The reagent synthesized in this study, α^5-pyridoxal methyl chloride, has not been completely characterized, partly because of its instability in neutral, aqueous solution. However, its structure is supported by mass spectrometry of its precursor, α^5-pyridoxine methyl chloride (Figure 1A), of the free aldehyde, of its phenylhydrazone (Figure 1B), and of its products of reaction with N^α-acetyl-L-lysine (Figures 3A,B). The absorption bands (Figure 6C) and pK values (Figure 5C) for α^5-pyridoxal methyl chloride are similar to those reported by Johnson and Metzler (1970) for 5-deoxypyridoxal. The $pK_1 = 3.7$ for α^5-pyridoxal methyl chloride is about 0.5 pH unit lower than that reported for 5-deoxypyridoxal. Ionization of the pyridinium nitrogen is accompanied by a small change in absorption at 385 nm but by a large increase in fluorescence at 456 nm on excitation at 360 nm.

The structures of the amino acid derivatives Y and Z of α^5-pyridoxal methyl chloride shown in Scheme II are supported by detailed spectral analyses discussed below. The imino acid derivative Y contains the same stable six-membered cyclic imine ring structure as does the imine derivative of homopyridoxal which was previously synthesized by Fisher and Metzler (1969) by a different route. The intramolecular alkylation reaction which results in the formation of this ring structure has not been previously studied. However, the formation of this six-membered cyclic imine is probably especially favored since the six-membered hemiacetal formed from α^5-homopyridoxal is more stable than the five-membered hemiacetal formed from pyridoxal and especially the seven-membered hemiacetal formed from the higher homolog (Korytnyk and Ahrens, 1971).

Although mass spectroscopy has been a powerful tool for structural analysis of many organic compounds including vitamin B_6 derivatives (DeJongh and Korytnyk, 1970), amino acids and their derivatives have frequently given very poor yields of molecular ions in conventional electron impact mass spectrometry. Recently Milne *et al.* (1969) have reported that chemical ionization mass spectroscopy gives high yields of M + 1 ions and simple fragmentation patterns with most of the natural amino acids. Chemical ionization mass spectrometry using isobutane was tried with the model compound ϵ-pyridoxyl-L-lysine (Figure 3C). Although the yield of the M + 1 ion was low, the simple fragmentation was consistent with the known structure. Since preliminary experiments with compounds Y and Z themselves were unpromising, *N*-acetyl derivatives were analyzed. Figure 3A provides excellent confirmation for structure Z, in particular proving that it was a cyclic imine rather than an alternative structure resulting from Schiff base formation and simple solvolysis of the side chain halogen. The mass spectrum of *N*-acetyl-Y (Fig. 3B) was less useful due to the highly polar, involatile nature of *N*-acetyl-Y, but most of the ions could be rationalized on the basis of the proposed structure. The structure of Y therefore rests heavily on the electronic spectra (see below) and on the fact that Y can be converted to Z by $NaBH_4$ reduction.

Further evidence for the proposed structure of compound Z was obtained from the nuclear magnetic resonance spectrum of its *N*-acetyl derivative (Figure 4). Protons characteristic of the pyridoxyl part of the molecule, of the new six-membered ring, and of the *N*-acetyllysyl moiety were identified.

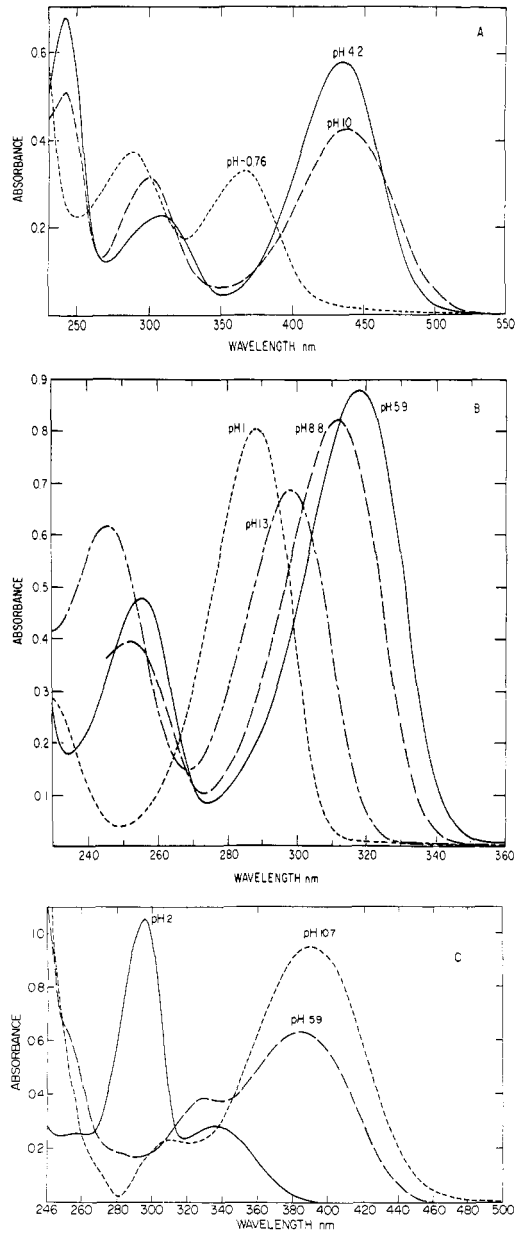


FIGURE 6: Absorbance spectra of different ionic forms of compound Y (A), compound Z (B), and α^5-pyridoxal methyl chloride (C). Spectra were selected from the spectrophotometric titration studies (Figure 5) at the indicated pH values which occur closest to plateau regions in the titration curves.

The most interesting feature of the electronic absorption spectra of compound Y (Figures 5 and 6 and Table II) is the predominance of the intense yellow absorbance band (435–440 nm) over a wide range of pH (1.0–13.0). This absorbance band, which is characteristic of many pyridoxal imines and pyridoxal-P enzymes, has been shown by Heinert and Martell (1962, 1963) to be due to the quinoid structure of the imine of pyridoxal (Scheme III, structure A) rather than to the tautomeric phenolic structure (Scheme III, structure B). Although the quinoid form of a normal pyridoxal imine probably exists in an internally hydrogen-bonded form as shown in Scheme III, Fisher and Metzler (1969) have shown that their cyclic derivative **9** can also absorb at 430 nm even though internal hydrogen bonding is prevented (Scheme IV). Scheme IV shows the structures proposed by Fisher and Metzler (1969) for three of the four ionic forms of **9**. Each of these

TABLE II: Electronic Absorption Properties of the Ionic Forms of Various Pyridoxylimines.[a]

Compound[b]	H_3P, λ_{max} (nm)	pK_1	H_2P, λ_{max} (nm)	pK_2	HP, λ_{max} (nm)	pK_3	P, λ_{max} (nm)
9 (ox)	360, 292	1.35	422, 292	5.99	430, 370	8.86	365
Y (ox)	360, 290	0.5	435, 310	6.0	440, 300		
11 (red)	290	3.74	320	8.48	310	10.0	300
Z (red)	289	3.4	318	7.7	310	9.8	299
X (red)	293	2.75	325	7.55			

[a] The four ionic forms of **9** and **11** were designated H_3P, H_2P, HP, and P by Fisher and Metzler (1969); each of these ionic forms bears one fewer proton than the preceding one (see Scheme IV and text). Although compounds X and Y have fewer than four ionic forms, we have designated the fully protonated forms of X, Y, and Z as H_3P, so that the corresponding ionic forms and pK values of the listed compounds can more easily be compared. [b] 7,8-Dihydro-3-methyl-2,6-naphthyridin-4-ol (**9**) and 3-methyl-5,6,7,8-tetrahydro-2,6-naphthyridin-4-ol (**11**) were synthesized by Fisher and Metzler (1969); the data are taken from their paper. Compounds Y and Z have the structures shown in Scheme II; the data for these compounds are taken from Figures 5 and 6. Compound X is ε-pyridoxyl-L-lysine; the data are taken from Forrey *et al.* (1971).

ionic forms bears one fewer proton than the preceding one; they are designated H_3P, H_2P, HP, and P. H_3P, the form on which the phenolic hydroxyl is protonated, is not shown in Scheme IV. The 430-nm absorption band of **9** is intense between pH 2.0 and 5.0, decreases between pH 5.0 and 9.0, and disappears above pH 10.0 (see Table II). The ionic form HP of **9** which predominates at pH 7.0–8.0 appears to be composed of approximately equal amounts of the two tautomeric forms HP-(1) and HP-(2) (Scheme IV) in which either the N-2 or the N-6 is protonated. This proton ionizes with a pK of 8–9 yielding a form P which absorbs at 364 nm.

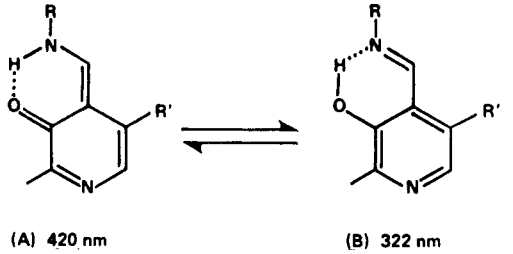
Since compound Y has a substituent on N-6, it is not able to form the tautomeric structure of type HP-(2) (Scheme IV) or the anionic form P. Thus our finding that compound Y has no spectral bands at 360–370 nm supports the structures proposed by Fisher and Metzler (1969) for these species of **9** as well as confirming our structure Y. Compound Y is thus restricted by its structure to exist predominantly in the quinoid tautomeric form shown in Scheme IIIA and Figure 5A over a wide range of pH. This may explain the very weak Gibbs reaction given by compound Y (Table I). The Gibbs reaction is indicative of an unsubstituted position para to a phenolic group (Hochberg *et al.*, 1944) and may thus depend on the presence of the enol form of the phenolic group. The strong Gibbs reaction given by the compound Z indicates that the free phenolic group is indeed present in compound Z.

The protonation of the ring nitrogen (N-2) of compound Y is accompanied by a small change in absorption at 435 nm but by a large increase in fluorescence on excitation at 430 nm (Figure 5A). Arrio-Dupont (1970) has observed that protonation of the ring nitrogen of the imine of pyridoxal-P and valine has a much larger effect on the fluorescence than on the absorption of the imine. Figure 5B shows similar effects of protonation of the ring nitrogen of compound Z on the absorption and fluorescence properties. The absorption bands and pK values for compound Z are similar to those reported by Fisher and Metzler (1969) for **11**. The pK_2 for Z is 0.8 pH unit lower than that for **11** but is closer to that reported by Forrey *et al.* (1970) for ε-pyridoxyllysine.

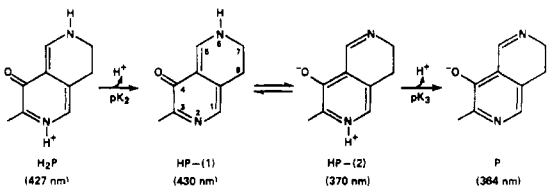
These results have shown that compound Y is a useful model compound for the study of the spectral properties of the imines or Schiff bases of pyridoxal with amino acids or proteins. This compound is stable over a wide range of pH in aqueous solution and does not undergo dissociation as do "normal" Schiff bases of pyridoxal and amino acids; such Schiff bases are usually studied either in organic solvents, in solid films, or in the presence of a large excess of the amino acid. Although these "normal" Schiff bases probably have their C=N bond in a conformation which allows hydrogen bonding with the phenolic hydroxyl as shown in Scheme III, compounds Y and **9** have their C=N bond fixed in the opposite conformation as shown in Scheme IV. A comparison of the spectral properties of Schiff bases in these two different conformations leads to the conclusion that neither internal hydrogen bonding nor a syn orientation of the imine with respect to the phenolic hydroxyl group is necessary for the 430-nm absorption band. The presence of a substituent on the imino nitrogen (N-6) of compound Y further restricts the number of tautomeric and ionic forms in this model compound and forces it to exist predominantly in the quinoid form.

Studies of model imines of pyridoxal have other applications to the understanding of pyridoxal-P enzymes. Although

SCHEME III: Tautomeric Forms of Pyridoxal Amino Acid Schiff Bases (Heinert and Martell, 1962, 1963).



SCHEME IV: Various Ionic and Tautomeric Forms of Compound **9** of Fisher and Metzler (1969).



most pyridoxal-P enzymes have been shown to contain pyridoxal-P in a Schiff base linkage with an ϵ-amino group of lysine, they vary widely in their spectral properties. The most widely studied, glutamic aspartic transaminase, acts as a pH indicator, with its absorption maximum shifting from 420 nm at low pH to 360 nm at high pH upon dissociation of a proton with a pK of 6.3 (Jenkins and Sizer, 1959). Fisher and Metzler (1969) have shown that their cyclic imine acts as a model for this enzyme since it forms the tautomeric form HP-2 (Scheme IV) upon dissociation of a proton with a pK of 6.3. Other enzymes, such as the B protein of tryptophan synthetase, absorb maximally at 415 nm over a wide range of pH. Compound Y serves as a model for these enzymes since it cannot form a tautomeric species absorbing at 360 nm. Another enzyme, aspartate decarboxylase, absorbs maximally at 360 nm over a wide range of pH (Wilson and Kornberg, 1963). Heinert and Martell (1962) studied the spectra of model imines of 3-*O*-methylpyridine-4-carboxaldehyde and found that only the tautomeric form absorbing at 310 nm was formed. Thus studies show that imines of pyridoxal can have markedly different spectra over a wide range of pH depending on what restraints are put on their possible tautomeric forms. The different spectra of these model imines are close to those of some pyridoxal-P enzymes. It seems likely that the environment of the pyridoxal-P imine in the active site of different enzymes can affect which of several possible tautomeric forms will occur.

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