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Pyrrolopyridine Derivatives from Pyridoxal 5′-Sulfate†

Robert D. Scott, Hiroshi Ueno, and David E. Metzler*

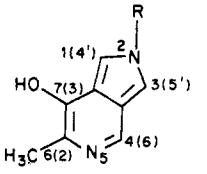
ABSTRACT: A fluorescent derivative of lysine-258 isolated from the active site of aspartate aminotransferase modified by treatment of the apoenzyme with pyridoxal 5′-sulfate has been characterized as a substituted 2*H*-pyrrolo[3,4-*c*]pyridine. Similar pyrrolopyridines are produced in up to 20% yield by reaction of pyridoxal sulfate with simple alkylamines or with amino acids including lysine. The latter forms two products, one of which is identical with that isolated from the enzyme. The pyrrolopyridine derived from ethylamine has been characterized by proton and ^{13}C NMR, ultraviolet–visible, and mass spectroscopy and by its chemical reactions.

Pyridoxal 5′-sulfate reacts with the apoenzyme form of cytosolic aspartate aminotransferase to generate a fluorescent chromophore with an unusual absorption spectrum (Yang et al., 1974). A similar chromophore is generated with cysteine and other β-substituted amines and also by reactions of a 5-carboxyethenyl analogue of pyridoxal phosphate (Miura & Metzler, 1976).

Peptic hydrolysis of aspartate aminotransferase modified with pyridoxal sulfate led to the isolation of fluorescent peptides that were shown to contain a derivative of the active site Lys-258 (Schmidt et al., 1980, 1982). After further degradation of the peptides by acid hydrolysis a derivative of lysine that retained the characteristic absorption spectrum and fluorescence of the chromophore observed for the intact modified protein was isolated.

We have discovered that simple alkylamines also react with pyridoxal 5′-sulfate although with less distinct changes in the spectrum than are observed with cysteine. From such reactions we have obtained compounds with spectral properties closely resembling those of the product formed from apo aspartate aminotransferase and pyridoxal sulfate. Lysine itself reacts under the same conditions and forms two fluorescent compounds that can be separated by thin-layer chromatography. One of these is identical with the compound isolated by degradation of the modified aspartate aminotransferase.

The products obtained from alkylamines have been studied by NMR and mass spectroscopy. The chromophore present in these compounds and in the modified lysine is shown to be a substituted 2*H*-pyrrolo[3,4-*c*]pyridine ring.



1, R = $-CH_2CH_3$
2, R = $-CH_2CH_2CH_3$
3, R = $-(CH_2)_4CHCOO^-$ with NH_3^+

The numbering is that of the pyrrolopyridine (Patterson et al., 1960) with the more familiar numbering of pyridoxine given in parentheses. Preliminary reports have been published (Scott et al., 1981, 1982).

Experimental Procedures

Pyridoxal 5′-sulfate and the 5′-*trans*-carboxyethenyl analogue of pyridoxal phosphate were prepared in this laboratory according to published procedures (Yang et al., 1974; Miura & Metzler, 1976). Deuterium oxide "100%" was purchased

† From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011. *Received May 18, 1982.* This work was supported by Grant AM-01549 from the National Institutes of Health.

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from Merck Sharp & Dohme Canada Ltd., Montreal, Canada. All other chemicals were obtained from commercial sources and were of reagent grade or better.

Many of the nuclear magnetic resonance (NMR) spectra were taken on a Bruker WM300 spectrometer by using 5-mm sample tubes. Chemical shifts were recorded in parts per million (ppm) relative to an external standard of tetramethylsilane [Me_4Si (TMS in the figures)] in $CDCl_3$ in a capillary tube. So that an undesirably high HDO peak could be avoided, 100% D_2O was used as the solvent, which also provided the internal deuterium lock signal. The value of pD was estimated by adding 0.41 to the reading on a Radiometer Model PHM64 pH meter. The pD was adjusted by addition of DCl or NaOD. A 30° flip angle (2.1-μs pulse) and a 0.1-s delay time were used for most spectra that were measured with water saturation.

The proton NMR spectra of **1** (Figure 5) were run on a JEOL FX90Q spectrometer at 89.55 MHz by using 2- or 5-mm tubes. One of the spectra was measured on about 19 μmol of compound in 0.5 mL of H_2O at pH 1. The conditions employed were similar to those used in the inverse recovery method: a 180°–τ–90° pulse sequence with a 90° flip angle (52 μs) and τ = 0.68 s. Data from 100 scans were accumulated on an 8K computer memory. Proton decoupled carbon-13 NMR spectra were also run on the JEOL spectrometer at 22.5 MHz by using either 2- or 5-mm tubes. The internal standard was dioxane, whose chemical shift was assumed to be 66.5 ppm.

Samples of pyrrolopyridines were prepared for NMR by concentrating to 0.1 mL under reduced pressure, adding either D_2O or 0.005 M DCl to bring the volume to 5 mL, and again reducing the volume to 0.1 mL. This cycle was repeated 4 or 5 times. Lyophilization was avoided because it seemed to promote decomposition. A 60° flip angle (15-μs pulse) and a 0.2-s delay time were used.

Absorption spectra were measured with a Cary 1501 recording spectrophotometer and were analyzed as described previously (Johnson & Metzler, 1970; Metzler et al., 1973). The method of Nagano & Metzler (1967) was used to evaluate pK values, to plot spectrophotometric titration curves, and to calculate the spectra of the individual ionic forms of the compounds studied.

The mass spectra were obtained with Finnegan 4000 and Kratos (AEI) MS902 mass spectrometers.

Thin-layer chromatography (TLC) was carried out with 8 × 8 cm precoated silica gel plates (E. Merck-Brinkman, silica gel 60) by using primarily the system acetic acid–1-butanol–water (1:4:1 v/v).

Reaction of Pyridoxal 5′-Sulfate with Amines. Solutions of pyridoxal 5′-sulfate at 2.6 × 10^{-4} M and an amine at 0.1 M were allowed to react at 22 °C. The pH was adjusted before the solutions were brought to their final volumes with either formic acid or sodium hydroxide, depending upon whether an amine or an amino acid was used. The reactions and subsequent isolation of the modification product were performed with the exclusion of as much light as possible since the products are photosensitive. The product formation was monitored by measuring the absorbance at 400 nm. When this reached a maximum, the solution was concentrated under reduced pressure to $^1/_{100}$ the original volume. The pH was then lowered to 3 with concentrated HCl. Solutions were always kept at low pH to avoid base-catalyzed decomposition.

The concentrated reaction mixture was fractionated on SP-Sephadex. The 1.3 × 40 cm column, which contained about 0.1 g of the ion exchanger/μmol of pyridoxal 5′-sulfate reacted, was eluted with a 0.007–0.5 M gradient of ammonium formate buffer, pH 2.9. The fluorescent product was identified by its characteristic absorption spectrum (Figure 1) and fractionated by using the ratio of absorbance at 384:282 nm. For the reaction with ethylamine it was eluted at a volume between 1.4 and 1.6 L. The separation was similar to that shown in Figure 3 of Schmidt et al. (1982) except that a large peak of unreacted pyridoxal sulfate emerged very early and another major peak with an absorption maximum at about 312 nm emerged just ahead of the fluorescent compound. The fractions containing the desired product were concentrated to about 20 μmol/mL under reduced pressure by using a 60 °C water bath to heat the sample. The concentrated sample was diluted 50% with 0.01 M formic acid, loaded on a Bio-Gel P-2 column, and eluted with 0.01 M formic acid. A 2.8 × 90 cm column was used to separate 50–70 μmol of the fluorescent product while an 0.8 × 90 cm column was used to separate 10 μmol or less. The fluorescent product, which was eluted at a volume between 500 and 600 mL, was concentrated under reduced pressure. The concentration was estimated by using a molar absorptivity of 5.3 × 10^3 M^{-1} cm^{-1} at 382 nm, pH <5: yield of **1**, 20%; yield of **2**, 12%.

Preparation of 7-Acetoxy-2-propyl-6-methyl-2H-pyrrolo[3,4-c]pyridine (O-Acetyl-2). Acetylation of **2** was accomplished by adding 5 mL of a 1:1 (v/v) mixture of acetic anhydride and pyridine to 1.1 μmol of lyophilized **2**. After standing 12 h at room temperature the mixture was dried under a stream of gaseous N_2. The residue was extracted with chloroform, the dissolved material having an absorption maximum at 327 nm. The chloroform was evaporated by a stream of N_2, and the residue was dissolved in 0.01 M formic acid.

Table I: R_f Values from Thin-Layer Chromatograms

compound	R_f[a]
modified lysine (**3**) from tripeptide	0.115
synthetic pyrrolopyridines from	
lysine	0.09, 0.125
N^{α}-acetyllysine	0.31
ethylamine (**1**)	0.38
n-propylamine (**2**)	0.50
glycine	0.31
alanine	0.37
cysteine	0.09

[a] Solvent: acetic acid–1-butanol–water (1:4:1 v/v).

Results

The fluorescent derivative from Lys-258 of apo aspartate aminotransferase treated with pyridoxal 5′-sulfate was isolated as described by Schmidt et al. (1982). The ultraviolet spectrum of this derivative is shown in Figure 1, where it is compared with spectra of the tripeptide isolated by Schmidt et al. and with that of a synthetic pyrrolopyridine. The proton NMR spectrum is shown in Figure 2. We propose the structure **3** for this compound.

Similar fluorescent products form spontaneously at room temperature when pyridoxal 5′-sulfate is mixed with primary amines in large excess. The presence of these compounds was monitored easily by thin-layer chromatography (Table I). The pyrrolopyridines have a brilliant blue-white fluorescence easily seen in solution as well as on TLC plates. The reaction is accompanied by relatively small changes in absorption spectrum; the increase in absorbance at 400 nm can be used to indicate the extent of the reaction. However, at longer times the absorbance decreases again. The rate of reaction increases with increasing pH, but the product is less stable at higher

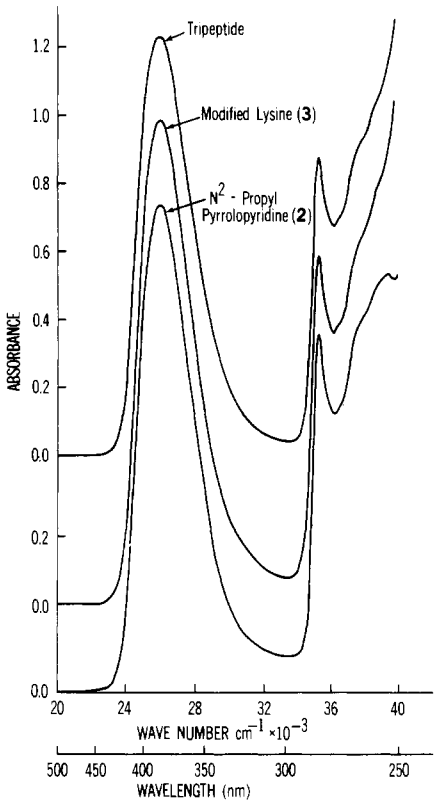


FIGURE 1: Comparison of ultraviolet absorption spectra of the synthetic propylamine derivative **2** with the tripeptide and with the lysine chromophore **3** isolated from aspartate aminotransferase after reaction of the apoenzyme with pyridoxal 5′-sulfate. All spectra were run between pH 2.8 and pH 3.2.

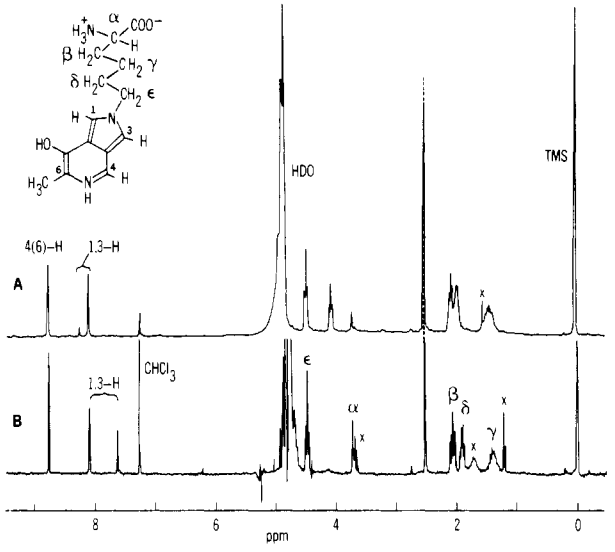


FIGURE 2: The 300-MHz proton NMR spectrum of the isolated lysine chromophore **3**: (A) at pD ~0; (B) at pD 4.7. The resonances of the hydrogens of the α–ε positions are marked. The 4(6)-H and 6′(2′)-CH_3 peaks are readily identifiable in expected positions. The protons giving peaks at 8.1 and 7.6 ppm exchange readily with deuterium of the D_2O at low pH. One proton has been almost completely exchanged for deuterium in (A). Peaks marked X are impurities that were not present in all samples. About 200 nmol of sample was used. The α–γ resonances were assigned according to Bak et al. (1968).

pH. Maximal absorbance increases were obtained in the pH range 9.2–9.5. At pH 9.5 and room temperature the maximum was reached in about 7 h with 0.1 M *n*-propylamine and 2.6 × 10^{-4} M pyridoxal sulfate. Raising the concentration of the pyridoxal sulfate did not lead to any further increase in yield,

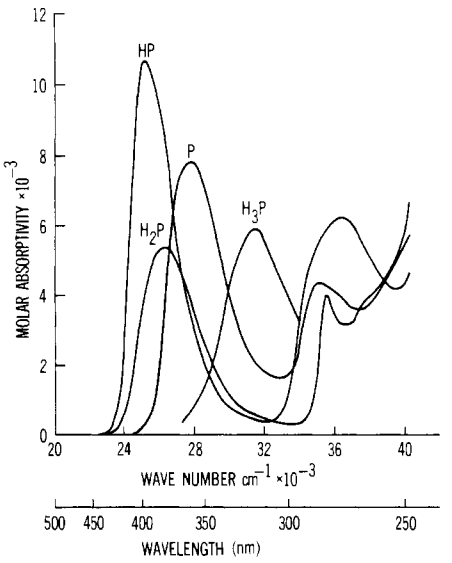


FIGURE 3: Spectra of four ionic forms of the 2-ethylpyrrolopyridine **1**. P, HP, H_2P, and H_3P designate the unprotonated, monoprotonated, diprotonated, and triprotonated forms, respectively. These were evaluated from spectra of 20 solutions ranging from pH −1 to pH 12.2.

but the rate increased with increasing amine concentration up to at least 1 M. A concentration of 0.1 M amine represented a practical compromise between a maximum yield and an undesirably large excess of amine and of its hydrochloride. Methyl-, ethyl-, and *n*-butylamines, glycine, alanine, lysine, N^{α}-acetyllysine, and aspartic acid reacted in the same manner as did propylamine. The reaction with valine was much slower, and little reaction could be detected with ammonia under the same conditions.

The fluorescent products were isolated by chromatography on an SP-Sephadex column at pH 3 followed by filtration on a column of Bio-Gel P-2 (see Experimental Procedures). The absorption spectra all resembled those of the product from propylamine (compound **2**, Figure 1) and from ethylamine (compound **1**, Figure 3). At all pH values these spectra are closely similar to those of the peptides isolated from the pyridoxal sulfate modified aspartate aminotransferase and of the lysine chromophore **3** obtained by degradation of the tripeptide (Figure 1). From the pH dependence of the spectrum p*K* values of ca. −0.8, 6.3, and 11.3 have been estimated for the ethylamine product **1**. The p*K* of 6.3 is almost identical with that of 6.4 found for the tripeptide (Schmidt et al., 1982) and nearly the same as that of 6.1 reported by Yang et al. (1974) for the related chromophore generated from cysteine. The latter also had a high p*K*, estimated as 12.25. The modified enzyme, presumably in denatured form, displayed a high p*K* of about 12.2, but the lack of isosbestic points did not permit estimation of a single p*K* corresponding to that of 6.3 in **1** (Yang et al., 1974). The calculated spectra of the individual ionic forms of **1** are given in Figure 3.

The proton NMR spectrum of the synthetic product **1** obtained from ethylamine is shown in Figure 4, and the ^{13}C NMR spectrum is given in Table II. An NMR titration curve is presented in Figure 5. The low-pH spectrum (Figure 5) shows that the 4(6)-H and 6′(2′)-CH_3 resonances are fairly close to but somewhat shifted from the expected positions for ordinary 3-hydroxypyridines. These are 8.74 and 2.49 ppm for **1** and 8.32 and 2.75 ppm, respectively, for the cation of pyridoxamine (Korytnyk & Ahrens, 1970). The β-methyl and α-methylene resonances of the ethyl group are present as expected at 1.61 ppm (triplet, J = 7.32 Hz) and 4.45 ppm

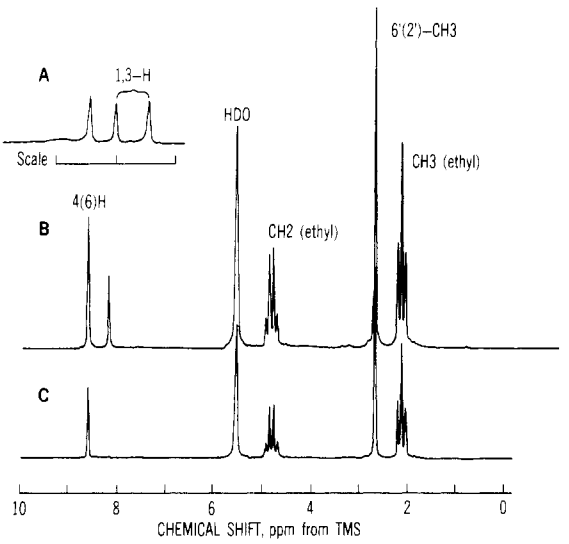


FIGURE 4: The 90-MHz proton NMR spectrum of the 2-ethylpyrrolopyridine **1**: (A) downfield region in H_2O at pH ~0.5; (B) in D_2O at pD ~0.5 immediately after preparation of the sample; (C) in D_2O after 24 h. Spectra were obtained with 19 μmol of compound.

Table II: Carbon-13 Chemical Shifts[a]

chemical shifts (ppm), rel to Me_4Si		
for **1**	for pyridoxine[c]	assignment[b]
11.7		CH_3 of ethyl group
15.7	13.6	6'(2')-CH_3
46.8		CH_2 of ethyl group
110.3	56.2	1(4') or 3(5')
117.3	129.2	4(6)
118.1	136.2	3a(5)[d]
118.2	139.9	7a(4)
120.2	57.4	1(4') or 3(5')
132.3	142.1	6(2)
143.1	152.1	7(3)[d]

[a] The spectrum was obtained at pD ~0.5 on 55 μmol of sample. [b] The numbering is that of the pyrrolopyridine ring with the numbering of the pyridine ring as used for vitamin B_6 derivatives in parentheses. C-1 and C-3 were identified by their loss of intensity when the attached protons exchanged out. [c] Obtained on the Bruker WM-300 spectrometer at pH 2.6. These values are in satisfactory agreement with those published by Mantsch & Smith (1979). [d] Tentative assignments based on comparison with those of pyridoxine.

(quartet), respectively. In addition there are two exchangeable protons at 7.6 and 8.1 ppm in the aromatic region. We identify these with the 1(4') and 3(5') protons of the pyrrolopyridine ring of the proposed structure. The peak at 7.6 ppm exchanges out rapidly at low pH and was stable only when the spectrum was recorded in H_2O (Figure 4A). In D_2O it disappeared at low pH with a half-time of about 1 h at pH 0–1. The 8.1-ppm peak disappeared more slowly, the half-time being about 4.5 h at pH 0–1. At pH 6.5 both protons exchanged out with a half-time of about 7 h.

The carbon-13 NMR spectrum (Table II) shows the presence of the expected 10 carbon atoms. Two of these, at 110.3 and 120.2 ppm, were not observed in D_2O after the exchangeable protons had been removed. This identifies them as C-1 and C-3. The ethyl carbons are easily assigned. The other aromatic carbons were assigned tentatively by comparison of their positions to those in pyridoxine.

The NMR titration curve of Figure 5 is in good agreement with that obtained from the ultraviolet spectra. The solid lines drawn in Figure 5 are based on "p*K*" values of –0.4, 6.35, and

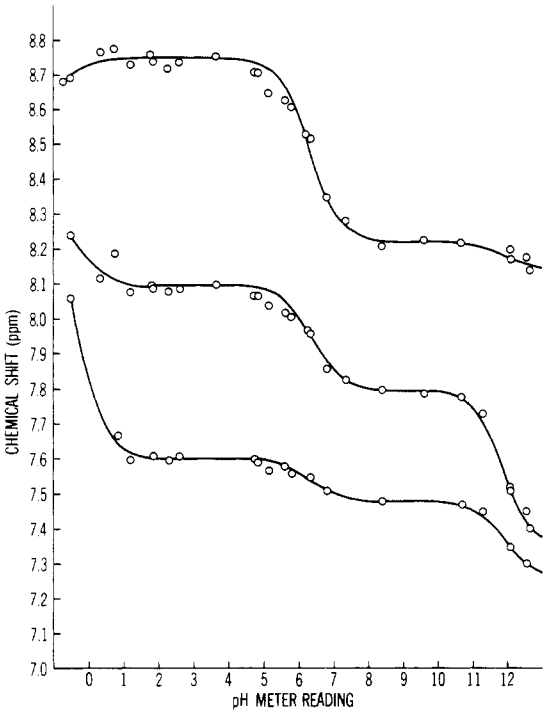


FIGURE 5: NMR titration curve for the 2-ethylpyrrolopyridine **1** in D_2O. Spectra at high pH were recorded before conversion to secondary products had occurred. The pH values are the pH meter readings rather than pD. The solid lines have been fitted to the experimental points by using apparent p*K* values of –0.4, 6.35, and 11.9. The upper curve is assigned to the 4 (6) and the other two to the 1(4') and 3(5') protons. The proton represented by the lowest curve (furthest upfield) exchanges the most rapidly.

11.9. The first two of these values agree well with the spectrophotometric values of –0.8 and 6.3 while there is some discrepancy between the upper values of 11.9 and 11.3 estimated by the two methods. Note, however, that the p*K*s from the NMR titration are based on pH meter readings in D_2O and not on pD.

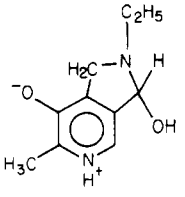
The mass spectrum of **1** contains a strong peak at *m/e* 176, the expected parent ion position. The exact mass was measured as 176.09485 (calculated for $C_{10}H_{12}N_2O$, 176.09497). When a sample of **1** in which the 1 and 3 protons had been completely replaced by deuterium as judged by NMR was subjected to mass spectrometry by using an ordinary capillary sample tube, very little of the expected peak at mass 178 was observed. To clarify this finding we enlisted the aid of Drs. A. Bencsath and F. H. Field at the Rockefeller University Mass Spectrometric Biotechnology Research Resource. They used a ceramic direct insertion tip and desorption chemical ionization (Bencsath & Field, 1981). With this technique the expected ions of $(M + 1)^+ = 179$ for the dideuterated compound were observed clearly. However, the 179 peak dropped rapidly; the 178 peak then rose and again fell to give a 177 peak. Thus, rapid exchange of the 1 and 3 protons occurs in the mass spectrometer.

Besides the parent ion peak at 176 additional peaks at 164 and 147 were present as well as at 192 and 206. These heavier peaks had exact masses of 192.09010 (192.08988 calculated for $C_{10}H_{12}N_2O_2$) and 206.06943 (206.06914 calculated for $C_{10}H_{10}N_2O_3$). Apparently **1** also reacts in the mass spectrometer with oxygen. Two hydrogen atoms must be lost to give the mass 206 peak. The fact that there is almost no 209 peak and mostly the $(M + 1)^+ = 207$ peak in the chemical ionization spectra suggests that it is the 1 and 3 protons that are lost.

Compound **1** is converted by reaction with acetic anhydride to an *O*-acetyl derivative whose mass spectrum has the expected parent ion peak at *m/e* 218, as well as additional peaks at 147, 164, 176 (parent ion of **1**), 192, 206, 234, and 250. The absorption spectrum of the acetylated compound closely resembles that of **1** at low pH, having a peak position of 385 nm and a spike at 284 nm as does **2** itself. However, at pH 8 the absorption maximum is at 345 nm rather than at 400 nm as for **1**. An apparent p*K* of about 7.2 was estimated. The acetylated compound migrated as a single spot of $R_f = 0.2$ and had a green fluorescence in the system acetic acid–1-butanol–water (1:4:1 v/v). Unmodified **1** had an R_f of 0.3 and a blue-white fluorescence.

The pyrrolopyridines are reasonably stable in acid but at high pH decompose rapidly. The characteristic absorption at 360 nm disappears in an approximately first-order process. At pH 7.5 the half-time is about 19 h whereas at pH 12 it is only 22 min. Preliminary examination of the pH profile suggests that the neutral ionic species decomposes spontaneously at a slow rate and that at higher pH the same form is attacked by OH^-, the rate becoming relatively constant above the p*K* of 11.3. The absorption spectrum after the base-induced decomposition has occurred is broad (Schmidt et al., 1982) and is significantly different at pH 12 than at pH 10.

From a sample of **1** allowed to stand at pH 12.2 for 15 h and chromatographed on Bio-Gel P-2 in 0.01 M formic acid, two or three major compounds and five to six minor products were separated (see Experimental Procedures). The relative amounts of the products vary with pH. The electronic and ^1H NMR spectra of the first of these compounds (**4**) shows that it contains an intact 3-hydroxypyridine ring and probably arose by attack of OH^- on the pyrrole ring of **2**. The NMR resonances at pH 3 in ppm are as follows: 4(6)-H, 8.32; 6(2)-CH_3, 2.66; $-CH_2$ of the ethyl group, quartet, centered at 3.69, $J = 7.3$; CH_3 of the ethyl group, triplet, 1.30. In addition a two-proton singlet at 4.4 ppm, probably representing the 1-CH_2 group, and a weak singlet at 1.7 ppm, possibly a partially exchanged 3-H, were found. The positions of the ethyl group resonances are distinctly shifted upfield from their positions in **1** by 0.73 (CH_2) and 0.27 ppm (CH_3). The absorption maximum was at 289, 317, and 312 nm at low, neutral, and high pH, respectively, with p*K* values of about 4.0 and 7.5. The probable structure is



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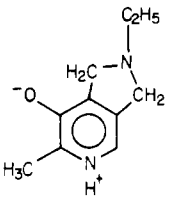
Both the electronic and NMR spectra of a second compound, **5**, produced by basic degradation of **1** show that the 3-hydroxypyridine ring has been destroyed. The absorption maximum at 290 nm at pH 6 is shifted to 286 nm at pH 12. The ring CH_3 resonance is still present in the NMR spectrum at pH 4 but has been shifted upfield to 1.64 ppm. However, the resonances of the ethyl group are close to the same positions as in **1**: CH_2, quartet, 4.15 ppm, $J = 7.3$ Hz in the decomposition product, and 4.45 ppm, $J = 7.32$ Hz in **1**; CH_3, triplet, 1.48 ppm, in the decomposition product, and 1.61 ppm, in **1**. Two single-proton doublets at 7.50 and 7.72 ppm are coupled with $J = 1.98$ Hz and presumably represent the 1 and 3 protons. The positions suggest that the pyrrole ring remains intact in this product and that the attack by hydroxide ion has occurred in the pyridine ring.

Reaction of 2×10^{-4} M **1** and 3×10^{-4} M periodic acid at pH 3 destroyed the 382-nm band with formation of a new band at 312 nm. The reaction was half complete in 15 min. After 14 h the sample was concentrated to a small volume and chromatographed on a column of Bio-Gel P-2 with 0.01 M formic acid. Three major products were separated. The first appears from the absorption spectra to be a 3-hydroxypyridine while the other two have broad absorption bands at 294 and 308 nm, respectively.

As with the products formed from cysteine and from apo aspartate aminotransferase (Yang et al., 1974) compound **1** is resistant to reduction by sodium borohydride. However, when solid borohydride was added gradually at pH 8.4 with frequent adjustment of the pH with formic acid, the absorption at 397 nm decreased slowly over a period of hours and finally disappeared. Passage of the resulting solution through a column of Bio-Gel P-2 separated two to three major bands. The first of these (**6**) had an absorption spectrum typical of a substituted 3-hydroxypyridine with absorption maxima at 286, 315, and 294 nm, respectively, for low, neutral, and high pH. Apparent p*K* values were 3.2 and 9.0. These peak positions are similar to those of the hemiacetal form of isopyridoxal: 283, 311, and 295 nm at low, neutral, and high pH. The NMR spectrum in neutral solution had the expected 4(6)-H resonance at 7.6 ppm and the 6(2)-CH_3 resonance at 2.45 ppm. The ethyl group resonances were as follows: CH_3, 1.3 ppm, $J = 7.3$ Hz; CH_2, 3.2 ppm. The latter is shifted from the position in **1** (4.15 ppm) even more than in the base decomposition product **4**.

In addition, two two-proton peaks are present at 4.37 and 4.44 ppm. The suggested structure is



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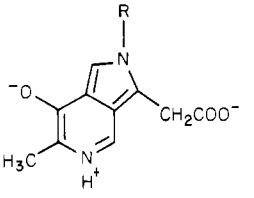
The other products have not been characterized but appear to include one or more of the products of base-catalyzed decomposition.

The 5-*trans*-carboxyethenyl analogue of pyridoxal phosphate reacts with apo aspartate aminotransferase to give a product resembling that obtained with pyridoxal 5′-sulfate (Miura & Metzler, 1976). Fluorescent peptic digestion products resemble those obtained with pyridoxal sulfate (Schmidt et al., 1982). We were also able to show that this compound reacts with various amines to form fluorescent products analogous to those obtained from pyridoxal sulfate. The reactions took place best at pH 9. Thin-layer chromatography gave spots of the R_f values given in Table I. These compounds, which presumably have the structure of **7**, are apparently less stable than the other

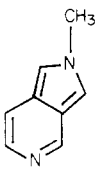


7

pyrrolopyridines, and we have not yet isolated them in pure form.

Discussion

The following evidence supports the proposed pyrrolopyridine structures for the fluorescent compounds produced by the reaction of pyridoxal 5′-sulfate with amines or with apo aspartate aminotransferase. (1) The same unusual and characteristic absorption spectrum is formed in all cases. In the reaction with cysteine the change in spectrum exactly parallels the elimination of inorganic sulfate (Yang et al., 1974). (2) NMR studies show that the intact aromatic ring and the attached ethyl group are present in the products with the expected number of carbon atoms. (3) In addition to the atoms in the ring, there are two additional carbon atoms, each with one attached hydrogen, with aromatic character as judged by NMR peak positions. (4) The proposed structure can be accounted for by a reasonable mechanism of formation, and it is difficult to think of another structure that could be formed and that would also give the observed NMR spectra. (5) The absorption and NMR spectra closely resemble those of 2-methyl-2*H*-pyrrolo[3,4-*c*]pyridine (**8**) reported by Armarego



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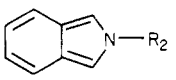
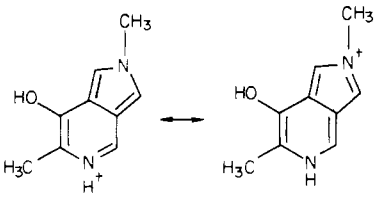


9

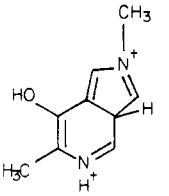
et al. (1972). This includes the presence of the two exchangeable protons at nearly the same positions. The similarity is most striking when our monocationic spectrum ($1 < pH < 6$) is compared with that of the cation of the methylpyrrolopyridine reported by Armarego et al. (1972). Both the chemical shifts in the NMR spectra and the low-energy peak position in the ultraviolet spectrum are in close agreement. (6) The properties of our compounds also resemble those of the related isoindoles (**9**). Again NMR spectra are similar and prominent spikes are present in the ultraviolet spectrum at about 280 nm (Veber & Lwowski, 1964). (7) The observed chemical reactions with nucleophilic reagents are appropriate for the postulated structure. The positions of the ultraviolet absorption bands in the reduction product **6** support the presence of a five-membered ring fused to a 3-hydroxypyridine.

The absorption bands in the ultraviolet spectrum are narrow, suggesting that a single predominate pathway for dissociation does exist. Thus, it should be possible to assign the observed p*K* values of −0.8, 6.3, and 11.3 to definite groups and to establish which are the major tautomers for each ionic form. In view of the very low basicity of pyrroles, the most reasonable structure for the diprotonated form at $2 \leq pH < 5$ is **10**.



10

The proton on the pyridine nitrogen should be held on tightly as a result of the indicated resonance. For the triprotonated form existing in strongly acidic solutions, we suggest structure **11**.



11

The p*K* of 6.3 must be that of the phenolic group. The bathochromic shift of about 1600 cm^{-1} in the lowest energy ultraviolet absorption maximum accompanying dissociation of the phenolic hydroxyl group is less than that for typical 3-hydroxypyridines [about 3300 cm^{-1} (Metzler et al., 1973)]. The p*K* is distinctly higher than that of 3.3–5 observed for 3-hydroxypyridines. Both differences may be attributed to the lowered donation of electrons from the phenolate ion into the ring system as a result of the presence of the pyrrole nitrogen. The shifts of the ultraviolet maximum for dissociation of the dipolar ion of **1** (+2500 cm^{-1}) and of the cation of **8** [+3100 cm^{-1} (Armarego et al., 1972)] are quite similar.

The highest p*K* of 11.3 for **1** must represent the proton on the pyridinium nitrogen. The large increase over the p*K* of 8.67 reported for **8** (Armarego et al., 1972) could reasonably be caused by the presence of the delocalized negative charge of the phenolate ion in **1**. Similar very large effects are seen in simple 3-hydroxypyridines (Metzler et al., 1973).

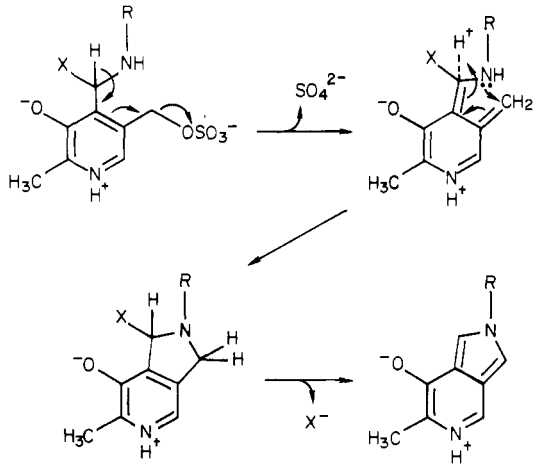
The chemical shifts of the C-1, C-3, and C-4 protons in the NMR spectrum of **1** are strongly influenced by the three successive dissociation steps (Figure 5). The C-4 proton undergoes the smallest changes for the very low pH dissociation from N-2 and the largest for dissociation of the phenolic group. The C-1 and C-3 protons are affected by all three dissociations.

The pyrrolopyridines studied here are all decomposed at high pH, destroyed by periodate, and reduced slowly by sodium borohydride. In each case a variety of products are obtained. While most of the products as yet have not been characterized adequately, it appears that they form two groups. In one the pyrrole ring has been altered and in the other the pyridine ring. Of the former group the base modification product **4** is proposed to be a simple adduct at C-3 that can be visualized as arising from the right-hand resonance form of structure **10**. The reduction product **6** presumably arises by attack of a hydride ion from borohydride on the same carbon. The other class of products may arise in part by nucleophilic attack on C-4 and other positions in the pyridine ring. The left-hand resonance form of **10** provides the necessary reactivity. Lyophilization seems to cause some decomposition of the pyrrolopyridines, and we suspect oxidation by O_2.

Derivatives of 2*H*-pyrrolo[3,4-*c*]pyridine have been prepared previously by reduction of pyridine-3,4-dicarboxylic acid to the bis(hydroxymethyl) compound, conversion to the bis(chloromethyl) derivative, condensation with a primary amine, and dehydrogenation (Armarego et al., 1972). They have also been prepared in about 50% yield by condensation of 5-bromo-3-picolyl chloride with an *N*-alkylaminoacetonitrile followed by ring closure with potassium amide in liquid ammonia (Ahmed et al., 1979).

Our method provides a practical synthetic route to a family of new pyrrolopyridines. Pyridoxal 5′-sulfate is readily made from pyridoxine hydrochloride in a simple three-step synthesis with about 32% yield (Yang et al., 1974). We have obtained compounds **1** and **2** in yields of about 20% from pyridoxal sulfate. Fluorescent derivatives of a wide variety of amines and amino acids can be made in this way. Containing a compact dipolar ionic fluorophore, these compounds could be

Scheme I



of practical value to biochemists. Since the pyrrolopyridine ring is reactive toward nucleophiles, such derivatives might also be affinity reagents for active sites. However, their reactions would cause the absorption and fluorescence to revert to those of a 3-hydroxypyridine.

A possible mechanism of formation of pyrrolopyridines from pyridoxal 5′-sulfate is given in Scheme I. From an NMR investigation of the reaction of cysteine with pyridoxal sulfate it was observed that the 4′ proton of the cyclic thiazolidine adduct of cysteine with the aldehyde disappeared at the same rate as inorganic sulfate appeared in the solution (Yang et al., 1974). This suggested that in aspartate aminotransferase, and also in the nonenzymatic reactions reported here, a nucleophilic group X, perhaps an amino group or –OH, first adds to a Schiff base of the amine with the aldehyde (Scheme I). In the nonenzymatic reaction the carbinolamine (X = OH) would be an intermediate in the formation of the Schiff base.

Elimination of sulfate, the first step in Scheme I, would yield a quinonoid intermediate as postulated by Yang et al. One of the reactions of this intermediate would be ring closure and elimination of group X as indicated in Scheme I. It is not surprising that the reaction is not quantitative and gives other products that have not yet been characterized. However, it is remarkable that the reaction in the active site of aspartate aminotransferase does occur stoichiometrically. A closely similar mechanism would apply to formation of **7** [see Miura & Metzler (1976)]. In this case tautomerization of the initial adduct would cause saturation of the side chain, at the same time producing an *o*-quinonoid analogous to that arising by the elimination of sulfate as shown in Scheme I.

Does formation of these pyrrolopyridines have any biological significance? No formation of these compounds from pyridoxal 5′-phosphate has been observed, but it is possible in principle. Phosphate is usually a poorer leaving group in elimination reactions than sulfate, but there is no reason that an enzyme may not have been developed to catalyze such a reaction. Thus, the occurrence of these compounds among the fluorescent substances found in organisms is a possibility. There is also a possibility that spontaneous or photochemical elimination of phosphate could lead to formation of pyrrolopyridines from Schiff bases of pyridoxal phosphate. Such reactions could even be part of normal catabolic reactions of pyridoxal phosphate.

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