Pyridoxal Derivatives as Probes for Water Concentration in Non-aqueous Solvents

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In order to provide a basis for the design of spectofluorimetric indicators for water activity or concentration in organic solvents a series of *O*- and *N*-alkylated pyridoxal derivatives has been prepared. The compounds were examined for their ability to exhibit a shift in fluorescence emission wavelength on complexation with water. The shift in fluorescence on complexation with water previously observed (Yunxiang and Xin, *Talanta*, 1984, **31**, 556) was confirmed for pyridoxal itself but alkylation of the oxygen functionalities blocked the fluorescence shift. In contrast, a strong enhancement in emission intensity but no shift in emission wavelength was observed with the *N*-methylpyridoxal. From these studies, it appears that modification at the nitrogen or, presumably, at C-2 or C-6 are the only available sites for the development of useful compounds from the pyridoxal prototype.

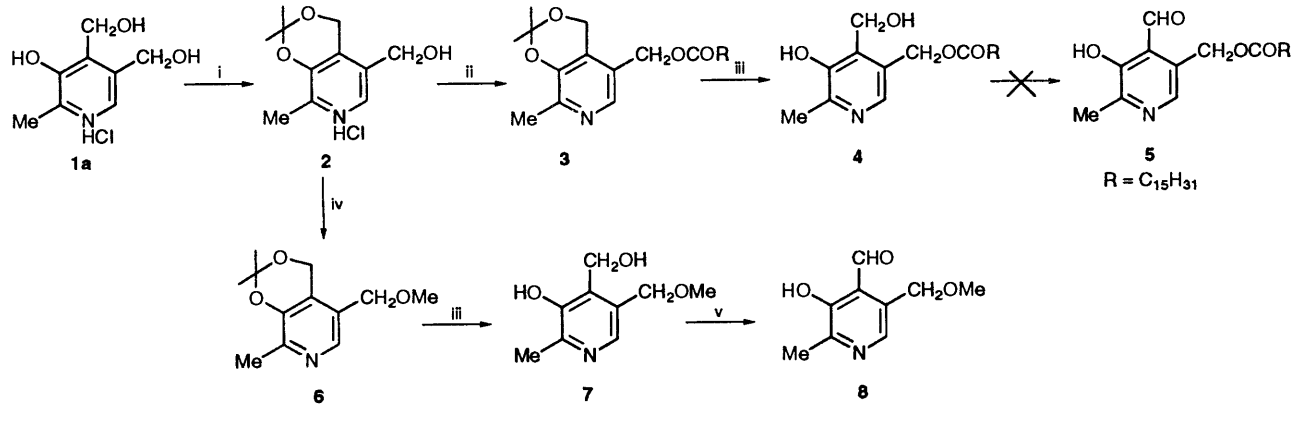
The control of enzyme-catalysed reactions in media of low water content is difficult because of problems associated in taking measurements in the inaccessible small volume of aqueous phase present in reaction mixtures. We have approached this problem for the determination of pH by the design, synthesis, and evaluation of a series of hydrophobic dyes that respond in a well defined way to changes in pH in the reaction mixture.[1] A second parameter that requires to be measured is the water concentration or activity. Water can be measured directly in the reacting system using appropriate sensors[2] and Karl–Fischer[3] titration has been widely used for stock solutions. So far, sensors provide the only method for a direct measurement of water in a typical reaction mixture. It would be valuable, therefore, to have a direct reading indicator for water concentration or activity in parallel with the indicators that have been developed for pH. A particular problem that must be resolved in water analysis in non-aqueous media is to distinguish water from other hydroxylic compounds that may be present in high concentrations, especially alcohols. This paper describes the properties of pyridoxal derivatives that define, in part, the structural features of molecules that might be developed successfully into such indicators.

A few years ago, Yunxiang and Xin[4] reported that spectrofluorimetric measurements of water concentrations in organic solvents was possible by measuring the fluorescence of the exciplex of pyridoxal with water; pyridoxal itself emits at a wavelength of 330 nm when excited at 295 nm whereas the water complex emits at 380 nm. Whilst these wavelengths are too low to provide a generally useful spectrofluorimetric probe for water and the solubility of pyridoxal is too limited, we hoped that by identifying the structural features required for such a response to water, it should be possible to design a molecule that would maintain the sensitivity to water but emit at longer wavelength. Accordingly, we have synthesised and characterised a series of pyridoxal derivatives and evaluated their properties as potential water probes.

Table 1 Absorption and emission maxima for pyridoxal derivatives

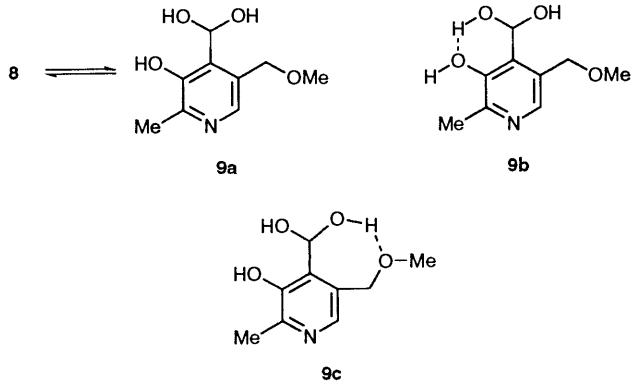
Compound	λ_{ex}/nm	λ_{em}/nm	λ_{em}–H_2O complex/nm
1b	295	330	380
1c	295	330	360
8	295	330	—
10	295	330	330
12	300	330	330
13	300	420	420
Pyridine-4-carbaldehyde	281	—	—

Synthesis of Pyridoxal Derivatives.—The molecules studied together with their absorption and emission maxima are shown in Table 1. Several of these compounds are new and others have been fully characterised for the first time. Scheme 1 shows the



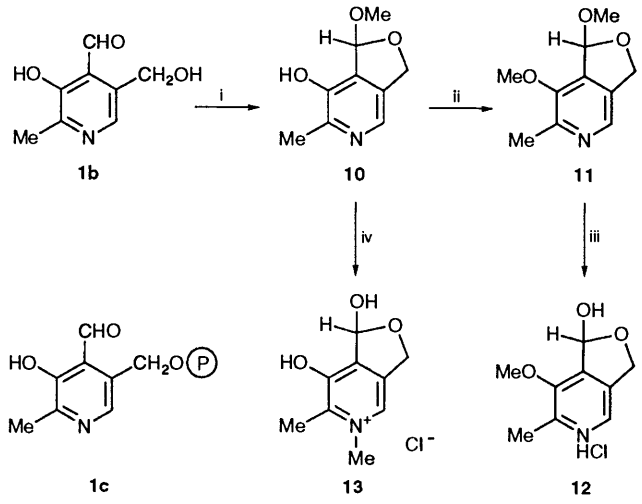
Scheme 1 *Reagents:* i, acetone; ii, $C_{15}H_{31}COCl$, pyridine; iii, EtOH–HCl; iv, diazomethane; v, MnO_2

reaction sequences used. We initially sought to prepare a hydrophobic analogue of pyridoxal and this was approached by protection of the C-3 phenol and C-4 hydroxymethyl groups of pyridoxine **1a** with acetone to give the hydrochloride **2**.[5] The acetal was acetylated with palmitoyl (hexadecanoyl) chloride in pyridine solution[6] to form the ester **3** from which the protecting group was removed with ethanolic hydrogen chloride[7] affording the alcohol **4**, the immediate precursor of the required hydrophobic derivative. Several attempts were made to oxidise this compound to the corresponding aldehyde **5** using manganese dioxide and Pfitzner–Moffatt conditions but none was successful, either starting material or decomposition products being obtained. The problems with this reaction could be associated with the lability of an alkyl ester and, therefore, an alternative route was sought using ether derivatives of the C-5 hydroxymethyl group. Although only the methyl ether was investigated, this position appears to be one of the most important in the fluorescence behaviour (see below) and the synthesis of hydrophobic derivatives at this position is, therefore, irrelevant to the problem at hand. Treatment of the acetal-protected pyridoxine **2** with diazomethane furnished the methyl ether **6** from which the protecting group was readily removed to give the diol **7**. Oxidation of compound **7** with manganese dioxide[8] led to a low yield of a product which was not the expected simple aldehyde **8**. The NMR spectrum showed that the compound **9a** was the major product by the almost complete absence of the expected aldehyde proton. An alternative route to the aldehyde **8** involves the direct methylation of the pyridoxal **1b** but attempts at this reaction were unsuccessful. A possible structure for the product of oxidation of the diol **7** is the hydrate **9a**. This structure can account for the ^{1}H NMR spectrum observed in terms of two hydrogen bonded structures represented by **9b** and **9c**.



With this experience, an alternative route to the synthesis of 3-methyl ethers of pyridoxal (*e.g.* **12**) and its analogues was sought. Pyridoxal was converted directly into the hemiacetal **10** by treatment with dimethyl sulfate in the presence of sodium carbonate.[7] The course of this methylation reaction was unexpected. Presumably, the phenolate acts as a base deprotonating the hemiacetal hydroxy group which is, in turn, methylated (Fig. 1). The acetal was converted into the C-3 methyl ether **11** with diazomethane and finally, into the hemiacetal **12** with hydrochloric acid. *N*-Methylpyridoxal **13** was prepared by alkylation of the protected acetal **10** with methyl iodide followed by ion exchange eluting with dilute hydrochloric acid to cleave the acetal and convert the iodide into the chloride salt **13**.

Fluorescence Properties.—Preliminary experiments were undertaken to establish suitable test conditions for the study of the analogues prepared and related available compounds. The limited solubility of pyridoxal in non-polar organic solvents made it necessary to concentrate upon a moderately polar



Scheme 2 *Reagents:* i, Me_2SO_4, Na_2CO_4; ii, diazomethane; iii, HCl; iv, MeI

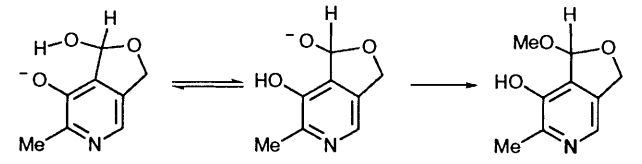


Fig. 1

solvent and tetrahydrofuran (THF) was selected. Although all possible permutations of substitutions of pyridoxal have not been examined, the results establish the necessary functional groups required for a fluorescence based assay. We were able to reproduce the reported results[4] with pyridoxal and one of the derivatives prepared was also found to show an enhancement of fluorescence in the presence of added water. Most significantly, pyridine-4-carbaldehyde and the 5-methyl ether **8** (and the related compounds **9a** and **9b**) did not respond, the former having no fluorescence emission and the latter showing no enhancement of fluorescence in the presence of water. Pyridoxal shows an emission at 330 nm when excited at 295 nm; on complexation with water in organic solvents, the emission is shifted to 380 nm. Blocking the phenolic hydroxy group as in compound **12** gave a molecule that showed enhanced emission at 330 nm but no additional peak at 380 nm. On the other hand, the acetal **10** behaved similarly to pyridoxal, but only on addition of acid. *N*-Methylpyridoxal **13** also showed a strong enhanced fluorescence in the presence of water and the wavelength of emission was on the edge of the visible region. Unlike pyridoxal itself, there was no shift in emission wavelength on complexation. Lipophilic *N*-alkyl pyridoxal derivatives might, therefore, be suitable for measuring water in organic solvents provided that self association into micellar or reverse micellar structures can be avoided by using branched alkyl chains. Conversely, long alkyl chains could provide molecules that would be useful probes of phenomena at the interface between micelles and the bulk solvent.

These results suggest that all of the oxygen functional groups in pyridoxal play a role in producing the enhanced fluorescence at longer wavelength. No enhancement of fluorescence was observed if the 5-hydroxymethyl group was blocked (compounds **8** and **9**); thus, the 5-hydroxymethyl function is essential. No long wavelength peak was observed when the 3-hydroxy group was blocked as in compound **12**; this hydroxy group is thus not essential for detectable complexation but is not optimal. Only the hemiacetal **10**, which presumably yields pyridoxal in the presence of acid and water, and the *N*-methyl derivative **13** were effective. Previous studies[4] had used

pyridoxal hydrochloride as the probe molecule. We prepared the free base and were hence able to show that it was essential that the active molecules (**1b**, **10**) be present in their protonated forms; either the hydrochlorides could be dissolved in THF, or mineral acids could be added to solutions of the free bases. Sulfuric, nitric, and hydrochloric acids were all effective.

Further experiments have been carried out to determine whether any of the commonly encountered components of reaction mixture for enzyme-catalysed reactions in low water media would interfere with the assay. Methanol was found not to interfere with the determination at concentrations up to 20% v/v. This result is consistent with the observations of Yunxiang and Xin who used methanol to introduce pyridoxal into their test solutions of water in very hydrophobic organic solvents. Fatty acids (stearic and myristic) were found not to interfere. Representative alkyl amino acids that might conceivably form Schiff base complexes with pyridoxal (glycine, valine and glycylglycine) also did not interfere. However, aromatic amines such as aniline and phenylhydrazine at up to 40% (v/v) quenched the fluorescence of pyridoxal hydrochloride. With acetic acid at up to 25% (v/v), no separate emission at 380–400 nm was observed but the emission at 330 nm increased. It was not possible to perform experiments in diethyl ether as solvent because of precipitate formation; below water concentrations of 0.45 mol dm^{-3} a yellow precipitate formed and at above 1.5 mol dm^{-3}, phase separation took place. These results suggest that the properties of pyridoxal and its derivatives are particularly suitable for interacting with water to give a spectroscopically detectable complex.

Experimental

Ether refers to diethyl ether; *J* values are given in Hz.

3-Hydroxymethyl-4,5-isopropylidenedioxy-6-methylpyridine Hydrochloride (*Isopropylidenepyridoxine Hydrochloride*) **2**.[5]—Pyridoxine hydrochloride (24.0 g) was suspended in acetone at 0 °C and dry hydrogen chloride gas passed through the suspension for 2 h. After the mixture had been stirred for 2 h, the insoluble product was filtered off and washed with dry ether. On cooling and concentration of the filtrate, the ether washings precipitated a further batch of product giving a total of 27.3 g (Found: C, 53.6; H, 6.6; N, 5.7. $C_{11}H_{16}ClNO_3$ requires C, 53.8; H, 6.6; N, 5.7%); δ_H(90 MHz; CD_3OD) 1.51 [6 H, s, $C(CH_3)_2$], 2.63 (3 H, s, CH_3), 4.94 (2 H, s, CH_2OH), 5.10 (2 H, s, CH_2OC) and 8.05 (1 H, s, 6-H).

3-Hexadecanoyloxymethyl-4,5-isopropylidenedioxy-6-methylpyridine (*Isopropylidenepyridoxine Palmitate*) **3**.[6]—The foregoing ketal hydrochloride **2** (6 g) was dissolved in dilute aqueous sodium hydrogen carbonate (100 cm^3) and the solution extracted exhaustively with chloroform. The combined chloroform extracts were dried (Na_2SO_4) and evaporated to dryness to yield the free base. A portion of the free base (2.52 g) was dissolved in dry pyridine (150 cm^3) to which hexadecanoyl chloride (4.5 cm^3, 18 mmol) was added rapidly. The mixture was stirred at room temp. for 4 days after which time hexane (150 cm^3) was added to it. The organic solution was washed twice with aqueous hydrochloric acid (0.1 mol dm^{-3}) and eight times with aqueous sodium carbonate (1 mol dm^{-3}). The organic layer was dried (Na_2SO_4) and evaporated to give the required *ester* as a clear oil which crystallised from cold methanol (3.5 g), m.p. 48–49.5 °C (Found: C, 72.1; H, 9.9; N, 3.1. $C_{27}H_{46}NO_4$ requires C, 72.3; H, 10.3; N, 3.1%); δ_H(90 MHz; $CDCl_3$) 0.90 (3 H, t, CH_3), 1.2 (26 H, bd, CH_2), 1.49 [6 H, s, $C(CH_3)_2$], 2.36 (3 H, s, CH_3), 2.62 (2 H, t, CH_2CO), 4.74 (2 H, s, CH_2OCO), 4.85 (2 H, s, CH_2OC) and 8.13 (1 H, s, 6-H).

5-Hexadecanoyloxymethyl-3-hydroxy-4-hydroxymethyl-2-methylpyridine **4**.[7]—The above ester **3** (3.0 g) was dissolved in a mixture of dilute aqueous hydrochloric acid (200 cm^3) and ethanol (95%; 500 cm^3) and heated under reflux for 90 min. The solution was cooled and concentrated under reduced pressure. The residue was treated with aqueous sodium hydrogen carbonate until the mixture was alkaline and the product was then extracted with chloroform. Evaporation of the extract gave the required *ester* as a white solid (2.5 g), m.p. 85–87 °C (Found: C, 71.1; H, 10.6; N, 3.2. $C_{24}H_{41}NO_4$ requires C, 70.7; H, 10.1; N, 3.4%); δ_H(90 MHz; $CDCl_3$) 0.85 (3 H, m, CH_3), 1.23 (26 H, bd, CH_2), 2.32 (2 H, t, CH_2CO), 2.44 (3 H, s, CH_3), 4.70 (2 H, s, CH_2OH, 5.30 (2 H, s, CH_2OCO) and 7.90 (1 H, s, 6-H).

3-Hydroxy-4-hydroxymethyl-5-methoxymethyl-2-methylpyridine **7**.—The ketal free base (1 g) prepared from the ketal **2** was dissolved in anhydrous ether (20 cm^3). To this solution, a solution of diazomethane (20 mmol) in ether was added and the mixture stirred at room temperature in the dark overnight. Glacial acetic acid was then added to decompose the excess of diazomethane and the colourless solution evaporated to dryness. The residue was mixed with aqueous sodium carbonate from which the 5-methoxymethyl ether **6** was extracted with ethyl acetate. Evaporation of the solvent afforded an oil which was redissolved in ethanol (95%; 25 cm^3) and treated with dilute aqueous hydrochloric acid (0.01 mol dm^{-3}, 10 cm^3) under reflux for 3 h. The solvent was evaporated under reduced pressure and the oily residue extracted with aqueous sodium carbonate and ethyl acetate. The organic layer was separated, dried, and evaporated to dryness under reduced pressure to give the required *methyl ether* as a white solid (260 mg), m.p. 89–90 °C (methanol–ether); δ_H(90 MHz; $CDCl_3$) 2.45 (3 H, s, CH_3), 3.78 (3 H, s, CH_3O), 4.68 (2 H, s, CH_2OCH_3), 4.78 (2 H, s, CH_2OH), 4.9 (2 H, bd, exch. D_2O, OH) and 7.98 (1 H, s, 6-H).

3-Hydroxy-5-methoxymethyl-2-methylpyridine-4-carbaldehyde **8** *with* **9**.[8]—The methyl ether **7** (100 mg) was dissolved in benzene (100 cm^3) and pyridine (50 cm^3) and active manganese dioxide (0.5 g) was added to the solution which was then heated to reflux with water separation using a Dean–Stark trap. The solution was allowed to cool and aqueous methylamine (1.5 cm^3) added to the mixture which was then heated for 1.5 h with water separation. The mixture was then cooled and a further portion of methylamine solution (1.5 cm^3) added to it. After being heated under reflux for a further 1 h, the mixture was cooled and filtered and the filtrate evaporated to dryness under reduced pressure. The residue was dissolved in dioxane (50 cm^3) containing aqueous hydrochloric acid (0.01 mol dm^{-3}, 10 cm^3) and allowed to stand at room temperature. The solution was made alkaline and extracted with ethyl acetate. Evaporation of the extracts gave the required *aldehyde* as a white solid (5.5 mg), m.p. 165 °C (methanol) (Found: C, 58.7; H, 5.8; N, 7.6. $C_9H_{11}NO_3 \cdot H_2O$ requires C, 59.7; H, 6.1; N, 7.7% Found: M^+, 181.0738. $C_9H_{11}NO_3$ requires *M*, 181.0739); δ_H(250 MHz; $CDCl_3$) aldehyde **8**; 0.10 (s, CHO), 8.70 (s, 6-H), 4.86 (s, CH_2), 3.85 (s, CH_3O), 2.74 (CH_3). Hydrate, major isomer (**9b** or **9c**) δ_H 8.05 (s, 6-H), 6.74 [s, $CH(OH)_2$], 5.25 (5.03, AB q, *J* 12.5), 4.06 (s, CH_3O) and 2.48 (s, CH_3). Hydrate, minor isomer (**9c** or **9b**) δ_H 8.20 (6-H), 6.62 [s, $CH(OH)_2$], 5.40, 5.18 (AB q, *J* 12.2), 3.87 (s, CH_3O) and 2.49 (s, CH_3). Ratio of components (**8**:**9b**:**9c**, 1:6:12).

7-Hydroxy-1-methoxy-6-methyl-1H,3H-furo[3,4-c]pyridine (*Pyridoxal Methyl Acetal*) **10**.—Pyridoxal (0.5 g) was dissolved in dry acetone (150 cm^3) and dimethyl sulfate (0.5 cm^3) added to the solution which was then heated under reflux in the presence of sodium carbonate for 2.5 h. After evaporation of the solvent under reduced pressure, the residue was dissolved in water and

the basic solution extracted with several portions of ethyl acetate. The aqueous solution was then evaporated to dryness under reduced pressure and the organic product extracted from the residue with several portions of ethanol. Evaporation, column chromatography of the residue on silica and thick plate chromatography (SiO_2, eluent 7% methanol–ethyl acetate) gave the *methyl acetal* as a white solid (340 mg) (Found: M^+, 181.0739. $C_9H_{11}NO_3$ requires *M*, 181.0739); δ_H(250 MHz; CD_3OD) 2.43 (3 H, s, CH_3), 3.41 (3 H, s, OCH_3), 5.00, 5.16 (AB q, *J* 12.1 CH_2O), 6.19 (1 H, s, CHO) and 7.81 (1 H, s, 6-H).

1-*Hydroxy*-7-*methoxy*-6-*methyl*-1H,3H-*furo*[3,4-c]*pyridine Hydrochloride.* (3-*Methoxypyridoxal Hemiacetal Hydrochloride*) **12**.—To a solution of the acetal **10** (1.0 g) in dry methanol (5 cm^3) a solution of diazomethane in ether (150 cm^3, approx. five-fold excess) was added. The solution was stirred at room temperature in the dark overnight after which the excess of diazomethane was destroyed with dilute acetic acid. The solution was treated with solid sodium carbonate, filtered and evaporated to dryness. Treatment of the residue with warm aqueous hydrochloric acid (1 mol dm^{-3}) on a steam-bath and evaporation to dryness under reduced pressure afforded the *title compound* as a white solid, m.p. 170 °C (decomp.) (water–acetone–ether) (Found: C, 49.5; H, 4.9; Cl, 16.8; N, 6.3. $C_9H_{11}NO_3$·HCl requires C, 49.7; H, 5.5; Cl, 16.3; N, 6.4%); δ_H(90 MHz; D_2O), 3.05 (3 H, s, CH_3), 5.08 (3 H, s, CH_3O), 5.67 (2 H, d, CH_2O), 7.50 (1 H, s, CHO_2) and 8.72 (1 H, s, 6-H).

1,2-*Dihydroxy*-5,6-*dimethyl*-1H,3H-*furo*[3,4-c]*pyridine Chloride* (N-*Methylpyridoxal Chloride*) **13**.—A solution of pyridoxal methyl acetal **10** in acetone was heated under reflux with an excess of methyl iodide for 18 h. The solvent was evaporated and the residue dissolved in water and passed through a cation exchange column, eluting with dilute hydrochloric acid. The eluates were concentrated and freeze-dried to afford the *title compound* as its hemiacetal (Found: C, 45.3; H, 5.9; Cl, 15.5; N, 5.7. $C_8H_{12}ClNO_3$·H_2O requires C, 45.8; H, 5.9; Cl, 15.1; N, 5.9%); δ_H(90 MHz; D_2O) 2.73 (3 H, s, C–CH_3), 4.30 (3 H, s, N–CH_3), 5.31 (2 H, d, *J* 9, CH_2), 6.77 (1 H, d, *J* < 2, O–CH–O) and 8.37 (1 H, s, C–H).

Fluorescence Measurements.—All solvents used were of analytical grade and were dried over activated molecular sieves for 1 week before use. The appropriate quantity of indicator was dissolved in dry methanol (5 cm^3) and diluted to 50 cm^3 with the solvent under study, typically THF. Fresh indicator solutions were prepared each day. Samples of the above indicator solutions (5 cm^3) were transferred into dried vials capped with septa. The required quantity of water was injected into the vial and the solution mixed thoroughly. A portion of this solution was transferred to a dried quartz spectrofluorimeter cell (1 cm^2) and an emission spectrum recorded immediately. The emission spectrum was found to be constant over at least 30 min. Fluorescence spectra were recorded on a Perkin-Elmer LS-5B spectrophotometer equipped with a GP-100 graphics printer. A slit width of 2.5 nm was used for both excitation and emission.

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