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Species Responsible for the Fluorescence of 3,N^4-Ethenocytidine

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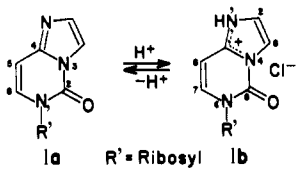
Abstract: The fluorescence properties of 3,N^4-ethenocytidine (ε-cytidine), substituted derivatives, and closely related two-ring heterocycles have been examined. The chloroacetaldehyde-modified cytidine is fluorescent only in its protonated form (**1b**). The fluorescence emission maximum is 340 nm and the pK_a* is 4.0, very close to the value for the ground state. N1-alkylation of ε-cytidine at the same position as protonation (**2** and **3**) makes reversion to the nonfluorescent type of structure **1a** impossible, upon changing the pH, and accordingly the fluorescence emission characteristics are preserved over a wide range of pH. The presence of the n → π* transition of the carbonyl group in ε-cytidine is considered to be responsible for the lack of fluorescence in neutral solution. Even ε-cytidine hydrochloride has a low fluorescence quantum yield ($\Phi < 0.01$) and a short fluorescence lifetime ($\tau = 30 \pm 5$ ps). Ring substitution produces a red shift of the π → π* transition, due to inductive or mesomeric effects, and a clear improvement in the fluorescence emission characteristics; e.g., 2-acetylamino-5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidine (**9**), in its protonated form, shows $\Phi = 0.85$ and $\tau = 4$ ns. Imidazo[1,2-*a*]pyridine, a close model for ε-cytidine lacking the carbonyl group and accordingly the n → π* transition, has a high fluorescence quantum yield and long lifetime in either neutral solution or organic solvents. Both N1-protonated (**10**) and N1-alkylated (**11**) imidazo[1,2-*a*]pyridines have emission maxima similar to that observed for ε-cytidine hydrochloride but higher quantum yields.

It has been shown that the 3,N^4-ethenocytosine (ε-cytosine) moiety can replace *adenine* in nucleotides for enzymatic phosphorylation[1,2] and photophosphorylation[3a] and in dinucleotide derivatives as substrates for certain nucleases.[3b] In addition, the structural analogue of NAD^+, nicotinamide 3,N^4-ethenocytosine dinucleotide (ε-NCD^+),[4] had activity comparable with the natural coenzymes in several enzyme systems including representative dehydrogenases.[5] If the chloroacetaldehyde-modified cytidine (ε-Cyd)[6-8] and related nucleotides[1,2,5] are to be investigated as fluorescent as well as biologically active probes, this requires an understanding of the excited state of ε-Cyd (**1a**) or its conjugate acid (**1b**) because changes in quantum efficiencies, lifetimes, and spectra



1a R' = Ribosyl **1b**

reflect environmental conditions surrounding the fluorophore. To contribute to such an understanding, the present work examines the effect of pH in aqueous solution and of solvent polarity on the fluorescence properties of 3,N^4-ethenocytidine, related derivatives, and model compounds. This study follows

on the examination of the fluorescence properties of 1,N^6-ethenoadenosine and closely related compounds.[9]

Experimental Section

3,N^4-Ethenocytidine hydrochloride (1b), prepared as previously described,[7] was highly purified for use in the fluorescence studies presented herein and for the x-ray study described in the accompanying earlier article:[10] NMR (D_2O) δ 7.14 (br d, 1, J_{78} = 8.0 Hz, 8-H), 7.84 (d, 1, J_{23} = 2.5 Hz, 2-H), 8.12 (dd, 1, J_{23} = 2.5 Hz, J_{38} = 0.6 Hz, 3-H), 8.30 (d, 1, J_{78} = 8.0 Hz, 7-H).

Dioxane used as a solvent was heated at reflux over sodium and distilled. All buffer solutions were prepared from commercial reagents of high purity in glass-distilled water.

5,6-Dihydro-1-methyl-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidinium Chloride (3,N^4-Etheno-N^4-methylcytidinium Chloride) (2). The pH of a solution of 100 mg of 3,N^4-ethenocytidine hydrochloride (**1b**)[7] in 4–5 ml of water was adjusted to 9.9–10.0 with 1 M aqueous sodium carbonate. The solution was evaporated. The residue was dried by two successive azeotropic distillations with ethanol and finally heated under reflux with 40 ml of absolute ethanol protected from moisture. Methyl iodide (5 ml) was added to the boiling mixture. After 11 h, the reaction mixture was filtered, concentrated to about 15 ml, and cooled, and the colorless crystals of the methiodide were collected. Two additional crops of crude product were obtained by concentration of the filtrate to give a total yield of 94 mg (69%). After one recrystallization from aqueous ethanol, pure product (68 mg, 50%) was obtained as colorless needles, mp 200 °C. 3,N^4-Etheno-N^4-methylcytidinium chloride (**2**) was prepared by application of the iodide to a column of Dowex 1-X2 (chloride form) and elution with water. The chloride was collected in the first few fractions from the column. Combination, evaporation, and recrystallization from aqueous ethanol and ether yielded the hygroscopic product: mp 196–198 °C after swelling at 171–172 °C; NMR (D_2O) δ 4.00 (s, 3, CH_3), 7.14 (br d, 1, J_{78} = 8.0 Hz, 8-H), 7.82 (d, 1, J_{23} = 2.5 Hz, 2-H), 8.12 (dd, 1, J_{23} = 2.5 Hz, J_{38} = 0.6 Hz, 3-H), 8.34 (d, 1, J_{78} = 8.0 Hz, 7-H); λ_{max} (pH 7.0 and 0.1 M HCl) 307 nm (sh) (ϵ 7700), 292.5 (13 200), 288 (13 000), 250 (sh) (4100); λ_{max} (0.1 M NaOH) 275 nm (ϵ 9900); mass spectrum (10 eV) *m/e* 149 (B^+).

Anal. Calcd for $C_{12}H_{16}ClN_3O_5$: C, 45.36; H, 5.08; N, 13.23. Found: C, 45.12; H, 5.06; N, 12.95.

1-Benzyl-5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidinium Chloride (N^4-Benzyl-3,N^4-ethenocytidinium Chloride) (3). 3,N^4-Ethenocytidine hydrochloride[7] (303 mg, 1 mmol) was converted to the dry base as described above. A solution of the dried residue in 10 ml of dimethylacetamide with 0.48 ml (4 mmol) of benzyl bromide was stirred magnetically for 13 h. The reaction mixture was filtered, 50 ml of ether was added, and the solid was collected, dissolved in water, and treated with Darco. The filtrate was concentrated and passed through a Dowex 1-X2 column (chloride form). The eluted fractions were combined and evaporated, and the residue was recrystallized from aqueous ethanol as colorless needles: mp 150–151 °C; yield 228 mg (57%); NMR (D_2O) δ 5.59 (s, 2, CH_2), 7.20 (br d, 1, J_{78} = 8 Hz, 8-H), 7.46 (s, 5, C_6H_5), 7.87 (d, 1, J_{23} = 2 Hz, 2-H), 8.15 (dd, 1, J_{23} = 2 Hz, J_{38} = 0.6 Hz, 3-H), 8.37 (d, 1, J_{78} = 8 Hz, 7-H); λ_{max} (pH 7.0 and 0.1 M HCl) 313 nm (ϵ 8900), 294 (13 700), 287 (13 000), 253 (sh) (4300); λ_{max} (0.1 M NaOH) 278 nm (ϵ 10 100). Anal. Calcd for $C_{18}H_{20}ClN_3O_5 \cdot H_2O$: C, 52.99; H, 5.30; N, 10.05. Found: C, 52.49; H, 5.38; N, 10.20.

5,6-Dihydro-5-oxo-6-propylimidazo[1,2-*c*]pyrimidine Hydrochloride (3,N^4-Etheno-1-propylcytosine Hydrochloride) (4). This compound was prepared from 1-propylcytosine[11] by the same procedure employed for ϵ-cytidine and was recrystallized from methanol–ethyl acetate: mp 300 °C; yield 77%; NMR [$(CD_3)_2SO$] δ 7.08 (dd, 1, J_{78} = 8.0 Hz, J_{38} = 0.6 Hz, 8-H), 7.97 (d, 1, J_{23} = 2.5 Hz, 2-H), 8.20 (m, 2, 3-H and 7-H); NMR (free base) [$(CD_3)_2SO$] δ 6.74 (dd, 1, J_{78} = 8.0 Hz, J_{38} = 0.6 Hz, 8-H), 7.44 (d, 1, J_{23} = 1.5 Hz, 2-H), 7.56 (d, 1, J_{78} = 8.0 Hz, 7-H), 7.83 (dd, 1, J_{23} = 1.5 Hz, J_{38} = 0.6 Hz, 3-H); λ_{max} (0.1 M HCl) 290 nm (ϵ 12 000), 255 (sh); λ_{max} (pH 7.0) 272 nm (ϵ 11 600); λ_{max} (0.1 M NaOH) 271 nm (ϵ 11 600). Anal. Calcd for $C_9H_{12}ClN_3O_5 \cdot 0.25H_2O$: C, 49.55; H, 5.77; N, 19.26. Found: C, 49.55; H, 5.66; N, 18.98.

6,6-Trimethylenebis(5,6-dihydro-5-oxoimidazo[1,2-*c*]pyrimidine) Dihydrochloride [1,1′-Trimethylenebis(3,N^4-ethenocytosine) Dihydrochloride] (5). This compound was made in a similar manner from chloroacetaldehyde and 1,1′-trimethylenebis(cytosine)[11] and was recrystallized from methanol–ethyl acetate: mp 240 °C dec; yield 85%; NMR (CF_3COOH) δ 7.18 (dd, 1, J_{78} = 8.0 Hz, J_{38} = 0.6 Hz, 8-H), 7.76 (d, 1, J_{23} = 2.5 Hz, 2-H), 8.05 (d, 1, J_{78} = 8.0 Hz, 7-H), 8.12 (dd, 1, J_{23} = 2.5 Hz, J_{38} = 0.6 Hz, 3-H); λ_{max} (0.1 M HCl) 290 nm (ϵ 24 000), 255 (sh); λ_{max} (pH 7.0) 272 nm (ϵ 21 300); λ_{max} (0.1 M NaOH) 272 nm (ϵ 21 500). Anal. Calcd for $C_{15}H_{16}Cl_2N_6O_2 \cdot H_2O$: C, 44.90; H, 4.52; N, 20.94. Found: C, 45.23; H, 4.62; N, 20.80.

5,6-Dihydro-5-oxoimidazo[1,2-*c*]pyrimidine (3,N^4-Ethenocytosine) (6). This compound was first obtained in this laboratory by Cole[12] by treatment of cytosine with aqueous ethanolic chloroacetaldehyde at pH 3.5 for 6 weeks. Filtration at the end of this period gave the product in 25% yield. Recrystallization of the base from ethanol afforded an analytically pure sample: mp 278 °C; NMR [$(CD_3)_2SO$] δ 6.63 (dd, 1, J_{78} = 8.0 Hz, J_{38} = 0.6 Hz, 8-H), 7.28 (d, 1, J_{78} = 8 Hz, 7-H), 7.40 (d, 1, J_{23} = 1.5 Hz, 2-H), 7.80 (dd, 1, J_{23} = 1.5 Hz, J_{38} = 0.6 Hz, 3-H); λ_{max} (EtOH) 288 nm (sh), 276 (sh), 268 (ϵ 11 000); λ_{max} (EtOH, 0.1 M HCl) 301 nm (sh), 286 (ϵ 10 500), 251 (sh), 245 (5000); λ_{max} (EtOH, 0.1 M NaOH) 292 nm (sh), 285 (ϵ 11 800), 282 (sh). Anal. Calcd for $C_6H_5N_3O$: C, 53.33; H, 3.73; N, 31.10. Found: C, 53.24; H, 3.82; N, 31.17.

2,5-Dioxo-6-propyl-2,3,5,6-tetrahydroimidazo[1,2-*c*]pyrimidine (7). This compound was prepared by stirring overnight a suspension of 10.2 g (67 mmol) of 1-propylcytosine[11] and 19.0 g (112 mmol) of chloroacetic anhydride in 340 ml of dry DMF containing 9.1 g of anhydrous K_2CO_3. The solvent was evaporated and the residue was stirred for 1 h in 100 ml of water. The suspension was filtered and dried to give 11.9 g (77%) of N^4-chloroacetyl-1-propylcytosine. Recrystallization from 95% ethanol, with charcoal treatment, gave an analytical sample: mp 170–172 °C; NMR [$(CD_3)SO$, Me_4Si] δ 0.83 (t, 3, CH_3), 1.64 (m, 2, CH_2CH_3), 3.71 (t, 2, $CytCH_2$), 4.70 (s, 2, CH_2Cl), 6.95 (d, 1, J_{56} = 7.0 Hz, 5-H), 8.0 (d, 1, J_{56} = 7.0 Hz, 6-H); mass spectrum (70 eV) *m/e* 229 (M^+). Anal. Calcd for $C_9H_{12}ClN_3O_2$: C, 47.09; H, 5.27; N, 18.30. Found: C, 46.73; H, 5.13; N, 18.16.

A solution of 2.3 g (10 mmol) of N^4-chloroacetyl-1-propylcytosine was heated at reflux in 350 ml of dry acetonitrile under a nitrogen atmosphere for 8 h with magnetic stirring. The solution was concentrated to about half the original volume and stored at −20 °C overnight. The white crystalline solid which separated was collected and dried. Further concentration of liquors and refrigeration gave a second crop. The total yield was 1.5 g (65%) of **7**·HCl: mp 150–152 °C; NMR ($(CD_3)_2SO$) δ 0.87 (t, 3, CH_3), 1.66 (m, 2, CH_2CH_3), 3.88 (t, 2, $CH_2CH_2CH_3$), 4.50 (s, 2, CH_2CO), 6.51 (d, 1, J_{78} = 7.0 Hz, 8-H), 8.45 (d, 1, J_{78} = 7.0 Hz, 7-H), 10.25 (b, 1, NH); mass spectrum (70 eV) *m/e* 193 (M^+ − HCl); λ_{max} (0.1 M HCl) 305 nm (ϵ 16 100), 237 (4800); λ_{max} (pH 7.0) 320 nm (sh), 309 (ϵ 14 700), 218 (4800); λ_{max} (0.1 M NaOH) 300 nm (sh), 288 (sh), 275 (ϵ 7620), 223 (10 700). Anal. Calcd for $C_9H_{11}N_3O_2$ (free base): C, 55.95; H, 5.74; N, 21.75. Found: C, 55.94; H, 5.74; N, 21.57.

2-Chloro-5,6-dihydro-5-oxo-6-propylimidazo[1,2-*c*]pyrimidine (8). A solution of 576 mg (3 mmol) of 2,5-dioxo-6-propyl-2,3,5,6-tetrahydroimidazo[1,2-*c*]pyrimidine (**7**) in 12 ml of phosphorus oxychloride was heated at reflux for 2 h. The solution was concentrated and 0.2 M NaOH was added until the solution was slightly basic. The product which precipitated was collected and dried; yield 598 mg (95%). Recrystallization from water gave an analytical sample: mp 113–115 °C; NMR [$(CD_3)_2SO$] δ 0.88 (t, 3, CH_3), 1.70 (m, 2, CH_2CH_3), 3.90 (t, 2, $CH_2CH_2CH_3$), 6.63 (dd, 1, J_{78} = 8.0 Hz, J_{38} = 0.6 Hz, 8-H), 7.58 (d, 1, J_{78} = 8.0 Hz, 7-H), 7.86 (d, 1, J_{38} = 0.6 Hz, 3-H); λ_{max} (0.1 M HCl) 310 nm (sh), 296 (sh), 286 (ϵ 10 600), 218 (12 900); λ_{max} (pH 7.0), 296 nm (sh), 282 (sh), 276 (ϵ 11 200), 217 (sh); λ_{max} (0.1 M NaOH) 296 nm (sh), 284 (sh), 276 (ϵ 10 700), 214 (12 900). Anal. Calcd for $C_9H_{10}ClN_3O$: C, 51.08; H, 4.76; N, 19.85. Found: C, 51.21; H, 4.82; N, 19.82.

2-Acetylamino-5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo-[1,2-*c*]pyrimidine (9). A solution containing 245 mg (1.0 mmol) of cytidine and 270 mg (1.5 mmol) of *N*-(bromoacetyl)acetamide in dimethylacetamide was heated at 70 °C for 8.5 h. The purple solution was cooled and the solvent evaporated. The residue was applied to a silica gel column packed in benzene–ethanol (8:2). The ribonucleoside was eluted with benzene–ethanol (7:3) in 13% yield: NMR (D_2O) δ 2.20 (s, 3, CH_3), 3.94 (b, 2, 5′-H), 4.30 (b, 2, 2′,3′-H), 6.03 (d, 1, 1′-H), 6.54 (d, 1, J_{78} = 8.0 Hz, 8-H), 7.52 (s, 1, 3-H), 7.62 (d, 1, J_{78} = 8.0 Hz, 7-H); field desorption mass spectrum *m/e* 324 (M^+). The purity of the compound for fluorescence studies was assured by high performance liquid chromatography using an Aminex A-5 cation

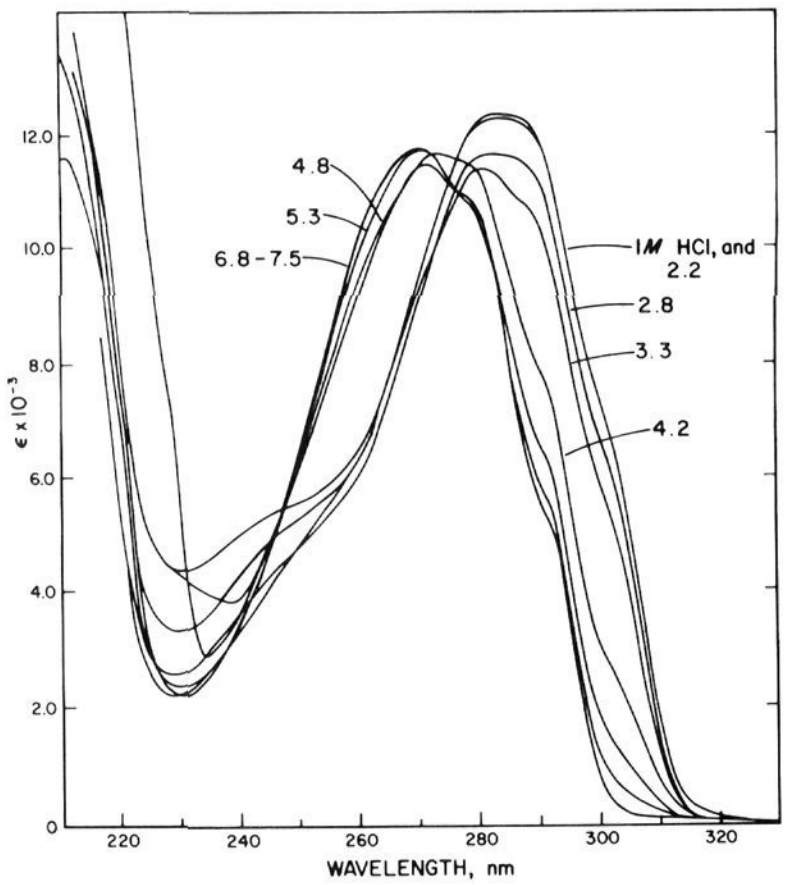


Figure 1. Variation in ultraviolet absorption spectrum of ϵ-Cyd·HCl (**1b**) in water with pH of buffered solution (**1a,b**).

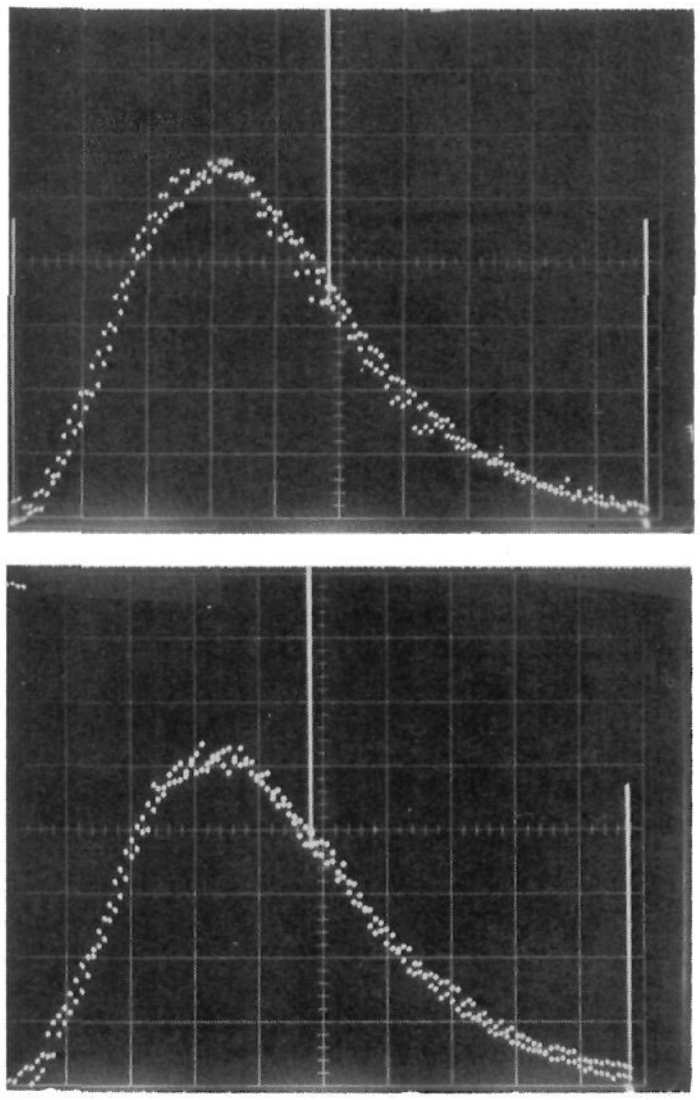


Figure 2. (top) Normalized fluorescence emission spectra (intensity vs. wavelength) of ϵ-Cyd·HCl (**1b**), corrected for background, in 0.5 M HCl and at pH 4.8 at 25 °C (at higher pH's fluorescence emission is too weak to provide accurate measurements). Spectrum was scanned between 305 and 420 nm with excitation at 280 nm. The vertical center marker represents 362 nm. (bottom) Relative fluorescence emission spectra (intensity vs. wavelength) of 3,N^4-etheno-N^4-methylcytidinium chloride (**2**), corrected for background, in 0.5 M HCl and at pH 7.0. Scanning and marker are the same as those of Figure 2 (top).

exchange column equilibrated with 0.4 M ammonium formate, pH 4.7, and elution with the same solvent. Proof of fluorescent purity was available from the identical values of fluorescence lifetimes in 1 M HCl (4.0 ns) determined by phase and modulation measurements using the cross correlation fluorometer described by Spencer and Weber,[13,14] since impure samples may yield differences of 1–3 ns when the two different techniques are used.

Imidazo[1,2-*a*]pyridine hydrochloride (10) was obtained by treatment of 2-aminopyridine with aqueous chloroacetaldehyde, decolorization of the slightly acidic solution with charcoal, evaporation to dryness in vacuo, and recrystallization from ethanol–acetone–ether to give the pure product in 90% yield, identical with that previously reported.[15] Purity was established as for compound **9.** The fluorescence lifetime observed for the free base in cyclohexane (not oxygen purged) was 8.80 ns.

1-Methylimidazo[1,2-*a*]pyridinium chloride (11) was made by the method of Paudler and Blewitt.[16]

Methods. Melting points were determined on a Thomas-Hoover capillary melting point apparatus. Proton magnetic resonance spectra were obtained on a Varian HA100 spectrometer. The NMR spectra of the salts were obtained under conditions (approximately 1 M solutions) where the concentration of the nonprotonated species is essentially nil; thus, the comparison of the spectra of an equilibrium-controlled process (hydrohalide salts in D_2O) with a nonequilibrium process (N-methylated compounds in D_2O) is tenable. Mass spectra were obtained by Mr. J. C. Cook and his associates on Varian MAT spectrometers. Molecular ions or base ions were observed in the mass spectra for all compounds. Microanalyses were performed by Mr. J. Nemeth and his staff.

All absorption spectra were taken on a Cary 15 spectrophotometer in aqueous solution (unless otherwise specified) at concentrations of the order of 5×10^{-5} M. For quantitative measurements a specific amount of material was placed inside a volumetric flask and dissolved in the appropriate amount of distilled water. If the spectra were to be determined in water at several pH values, equal aliquots were withdrawn and placed in volumetric flasks. They were diluted to give solutions of known, equal volume. All spectra were determined against the appropriate blank using a matched set of sample and reference cells. All solutions of 3,N^4-ethenocytidine at several pH values used in the absorption and fluorescence studies were prepared by diluting equal aliquots of a solution of the compound in distilled water with a known amount of the appropriate buffer solution (final concentration of buffer = 0.025 M). 3,N^4-Ethenocytidine (**1**) proved to be stable at all pH's indicated in this study.[17] The same procedure was employed with other fluorescent molecules.

Corrected fluorescence emission and fluorescence excitation spectra were acquired on a Spex Fluorolog spectrofluorometer. Technical fluorescence emission spectra were measured on a photon counting scanning spectrofluorometer,[18] interfaced to a Nuclear Data ND 4410 Data Acqusition System so that spectra could be stored in memory and displayed either singly or overlapped for multiple display. Spectra could be normalized, added, subtracted, multiplied, divided, etc., by teletype commands. All fluorescence measurements were made at 20 °C. The fluorescence intensity measured for each solution was normalized for differences in optical density at the exciting wavelength among the samples, and it is therefore a quantitative representation of relative quantum efficiencies. Fluorescence lifetimes were measured on a Model SLM-480 subnanosecond spectrofluorometer.

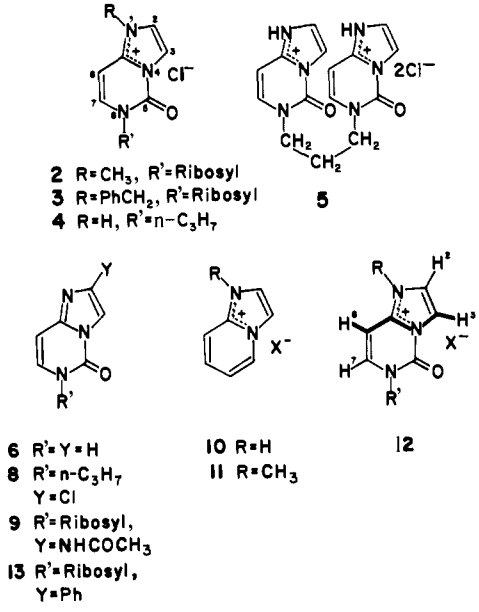
Results and Discussions

In the initial description of the fluorescence properties of 3,N^4-ethenocytidine, we suggested that the protonated form of ϵ-cytidine (**1b**) was responsible for the fluorescence.[7] In the fluorescence results presented here, emission spectra taken at intervals between pH 1.0 and 8.0, upon excitation at 275 nm near the isoabsorptive point (Figure 1), showed that the emission maximum at 340 nm remained constant as the pH was increased from 1.0 to pH 5.2 (Figure 2) while the fluorescence intensity decreased progressively beginning at about pH 3.0 and continuing to pH 5.8, where the fluorescence intensity was about 1% of the intensity at pH 1.0 (Figure 3). The apparent pK_a of the excited state is close to 4.0. This is also the pK_a value of the ground state as determined by titration.[19] The titration curve and the fluorescence emission curve vs. pH are practically superimposable on adjusted scales. The fluorescence

Table I. Fluorescence Data[a]

Compound	pH[b]	Fluorescence emission, nm	Fluorescence excitation,[c] nm
3,N^4-Ethenocytidine hydrochloride or 5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidine hydrochloride (**1b**)	Acid[d]	340	288
	Neutral	None	
3,N^4-Etheno-N^4-methylcytidinium chloride or 5,6-dihydro-1-methyl-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidinium chloride (**2**)	Acid[d]	342	290
	Neutral	342	290
N^4-Benzyl-3,N^4-ethenocytidinium chloride or 1-benzyl-5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidinium chloride (**3**)	Acid[d]	345	295
	Neutral	345	295
3,N^4-Etheno-1-propylcytosine hydrochloride or 5,6-dihydro-5-oxo-6-propylimidazo[1,2-*c*]pyrimidine hydrochloride (**4**)	Acid[d]	340	290
	Neutral	None	
1,1′-Trimethylenebis(3,N^4-ethenocytosine) dihydrochloride or 6,6-trimethylene bis(5,6-dihydro-5-oxoimidazo[1,2-*c*]pyrimidine) dihydrochloride (**5**)	Acid[d]	340	290
	Neutral	None	
3,N^4-Ethenocytosine or 5,6-dihydro-5-oxoimidazo[1,2-*c*]pyrimidine (**6**)	Acid[d]	335	286
	Neutral	None	
2-Chloro-5,6-dihydro-5-oxo-6-propylimidazo[1,2-*c*]pyrimidine (**8**)	Acid	345	294
	Neutral	None	
2-Acetylamino-5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidine (**9**)	Acid	385	305
	Neutral	None	
Imidazo[1,2-*a*]pyridine hydrochloride (**10**)	Acid	339	275
	Neutral[e]	375	295
	Dioxane	375	320, 310
	DMF	375	320
	Dioxane–HCl	335	275
1-Methylimidazo[1,2-*a*]pyridinium chloride (**11**)	Acid[d]	338	290
	Neutral	338	290

[a] In water unless otherwise stated. [b] Acid is 0.05 M HCl (except for **8** and **9**, 1 M HCl; and **10**, 3 M HCl). Neutral is 0.025 M phosphate buffer, pH 7.0. [c] Fluorescence excitation maxima were taken by fixing on the fluorescence emission maximum. [d] Absolute quantum yields are <0.01. [e] Absolute quantum yield is 0.98, τ = 17.2 ns; for the free base in cyclohexane (not oxygen purged), τ = 8.80 ns.



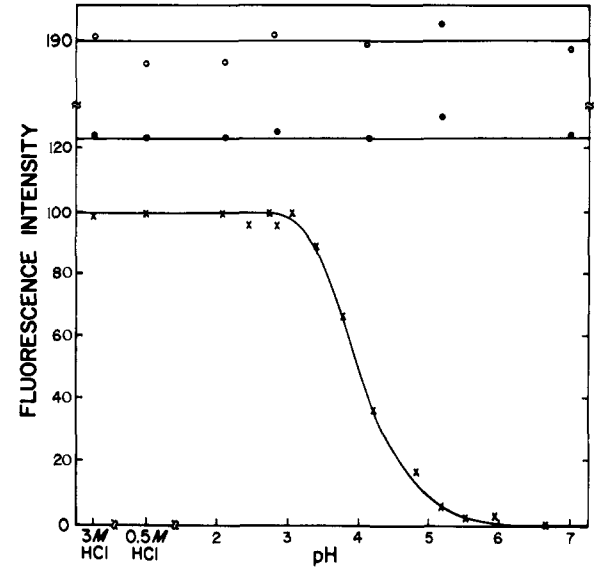


Figure 3. pH dependence of fluorescence emission of ϵ-Cyd·HCl (**1b**) (×), 3,N^4-etheno-N^4-methylcytidinium chloride (**2**) (●), and N^4-benzyl-3,N^4-ethenocytidinium chloride (**3**) (○). The figure shows the relative quantum yields of the compounds.

excitation spectra indicated that the band centered at 288 nm is responsible for the fluorescence (Table I). The blue shift in the absorption spectrum with the loss of fluorescence intensity at 340 nm as 3,N^4-ethenocytidine is deprotonated indicated that only one structure, the protonated form, is the fluorophore.

The fluorescence quantum yield of protonated ϵ-cytidine in aqueous solution is <0.01 and its fluorescence lifetime, as determined by the cross correlation method,[13,14] is 30 ps with a standard deviation of 5 ps.[7b] A similar determination for 3,N^4-etheno-N^4-methylcytidinium chloride (**2**) gives its fluorescence lifetime as 17 ± 3 ps. Fluorescence polarization of ϵ-Cyd·HCl in propylene glycol as a function of temperature confirms the very short lifetime of **1b**. A Perrin plot[20] of $1/P$ vs. T/η, where P is the observed polarization, T the temperature, and η the viscosity of the solvent, shows very little change when the temperature of the system is varied from −50 to 25 °C.

Of the electronic excitations involving the introduction of electrons into antibonding orbitals, in general the lowest energy transition will be from the nonbonding p molecular orbital to the antibonding π^* orbital (n → π^*). However, in complex chromophores like conjugated ketones,[21] the $\pi \rightarrow \pi^*$ transitions tend to pass or hide the n → π^* transitions. It is known that $\pi^* \rightarrow$ n fluorescence is generally not observed because of radiationless deactivation to the triplet level.[20] Since the competitive reactions for the conversion of excited molecules

to the ground state are from the lowest excited state, when the $n \rightarrow \pi^*$ transition is present as the lowest energy band, the molecule is generally nonfluorescent. If changes in pH affect the resonance structure of the molecule,[22,23] such as in the case of ϵ-cytidine, the molecular structure responsible for emission, i.e., for the $\pi^* \rightarrow \pi$ transition, may become the lowest energy band, hiding the $n \rightarrow \pi^*$ transition. The compound may then be appreciably fluorescent.[24] The fluorescence data for 3,N^4-ethenocytidine and related molecules in neutral and acidic media are presented in Table I. In all cases, emission centered at 340 nm is typical in acidic solution.

Results of x-ray crystal structure analysis[10] of ϵ-cytidine hydrochloride have established the site of protonation as N1, as previously proposed (**1b**).[1] Since the cationic form of ϵ-cytidine (**1b**) provides the fluorescence emission centered at 340 nm in aqueous solvents, it was anticipated that N1-alkylation of ϵ-cytidine, i.e., at the same position as protonation, would produce compounds exhibiting fluorescence over a wide range of pH. Reversion to the nonfluorescent structure associated with the base (**1a**) is not possible upon changing the pH of the N1-alkylated derivatives. As expected, the fluorescence emission spectra of 5,6-dihydro-1-methyl-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidinium chloride (**2**) and 1-benzyl-5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidinium chloride (**3**) showed no changes (Figure 3), and the emission maxima remained at 342 and 345 nm, respectively, throughout the pH region 1.0–8.0. The relative yields of fluorescence emission observed in 0.5 M HCl or 3.0 M HCl for **1b, 2,** and **3** were 1.00:1.22:1.90, respectively (Figure 3), while the absolute quantum yields were very low for all three.

Let us consider the basis of the assignment of structure of the alkylated ϵ-cytidines. ϵ-Cytidine (**1a**) was readily converted to its monomethyl or monobenzyl derivative by alkylation with methyl iodide or benzyl bromide, respectively, and conversion to the corresponding chloride. Alkylation could conceivably take place at N1, N4, or O^5 (see **2** and **3** for numbering system.)[16] The assignments of N1 substitution (**2, 3**) were made by comparison of their ultraviolet absorption, fluorescence emission, and nuclear magnetic resonance spectra with those of protonated ϵ-cytidine (**1b**)[10] (Figures 1–3). The alkylated ϵ-cytidine cations exhibited similar spectroscopic behavior. In the NMR spectra, the alkylated compounds showed ring-proton chemical shifts and coupling constants for 2-H, 3-H, 7-H, and 8-H similar to those of ϵ-cytidine hydrochloride (**1b**) and exocyclic proton signals typical for alkyl- or aralkyl-N attachment. Hence, the structure assignments were made as 5,6-dihydro-1-methyl-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidinium chloride (**2**) and 1-benzyl-5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidinium chloride (**3**) (see Experimental Section for NMR chemical shifts and couplings). In addition, these molecules provide examples of cross-ring spin–spin interactions in the NMR or long-range coupling between protons separated by five bonds along an extended zig-zag path (J_{38} = 0.6 Hz). Since ϵ-cytidine hydrochloride is a nearly planar molecule,[10] that is, planar in the imidazole ring and with only 0.037-Å maximum deviation from planarity in the pyrimidine ring, the analogous planarity of the methyl derivative is indicated, because long-range coupling constants are similar for essentially planar configurations but fall off rapidly with departures from coplanarity.[25] If alkylation had occurred at the bridgehead nitrogen, N4, different chemical shifts and different behavior of the long-range coupling between 3-H and 8-H would have been observed. Compounds exhibiting appreciable long-range coupling between protons at the end of a (generally) conjugated chain include benzofuran, indole, indolizine, azaindolizine, benzothiophene, indazole, indene, chromene, quinoline, phenanthrene, and coumarin derivatives.[26–30] The basis for the long-range coupling between 3-H and 8-H is depicted in formula **12.**

The establishment of the structures depended upon the validity of the NMR assignments inter alia. Spin decoupling, using the field sweep method, showed that the smaller splitting (J = 0.6 Hz) in the doublet of doublets at δ 8.12 assigned to the 3-H in **1b** collapsed upon irradiation in the region of the 8-H resonance (δ 7.14). Similarly, irradiation at the center of gravity of the doublet of doublets for 3-H caused collapse of the 8-H signal to a sharp doublet, J = 8.0 Hz. Identical results were obtained in spin decoupling experiments with the methylation product of ϵ-Cyd, namely, 5,6-dihydro-1-methyl-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidinium chloride (**2**). The long-range coupling between 3-H and 8-H confirms these assignments. Long-range coupling between 2-H and 7-H or 8-H was not detected under our experimental conditions.[27,28] The 7-H signal for all the ϵ-cytosine and ϵ-cytidine derivatives invariably moved to lower field in going from unprotonated to protonated species. This behavior is explicable only on the basis of the 7-H assignment and is parallel to the strong downfield shift observed for the 6-H of cytidine under similar conditions.[31] No crossover of the 2-H and 3-H chemical shifts was observed in our series in going from the unprotonated to the protonated species.

Separate replacements of the 2-H and 3-H confirmed the correctness of the NMR assignments throughout the series. In a comparison of the NMR spectra of 2-chloro-5,6-dihydro-5-oxo-6-propylimidazo[1,2-*c*]pyrimidine (**8**) and compound **4** without the chlorine, the long-range coupling remained when the 2-H was replaced. For compound **8,** the 8-H appeared as a doublet of doublets, δ 6.63, J_{38} = 0.6 Hz, J_{78} = 8.0 Hz, coupled with both the 3-H (δ 7.86, J_{38} = 0.6 Hz) and the 7-H (δ 7.58, J_{78} = 8.0 Hz). In a comparison of the NMR spectra of 3-deuterio-5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidine hydrochloride (**1b,** with D at C-3), prepared by the reaction of $ClCD_2CHO$ with cytidine,[32,33] the signal for the 3-H at δ 8.12 in **1b** was lacking in the 3-D compound. The 2-H signal collapsed to a singlet and the 8-H signal collapsed to a doublet, J_{78} = 8.0 Hz, with formal replacement of 3-H by 3-D. Our new NMR results require the interchange of the earlier 2-H and 3-H assignments in this series[6,7a] and indicate that the assignments of Yanai et al.[34a] and of Bartholomew et al.[34b,c] for similar compounds must be reexamined.[34d]

The infrared stretching frequency for the carbonyl group in 3,N^4-ethenocytidine hydrochloride (**1b**), 1730 cm^{-1}, is of interest when it is related to the C–O distance of 1.208 Å observed in the crystal[10] and when both figures are compared with ν_{CO} = 1720 cm^{-1} for cytidine hydrochloride and the corresponding C–O distance of 1.215 Å, which represents an average of 12 N3-protonated cytosine derivatives (including N1-substituted).[10] The experimental values of ν_{CO} = 1652 cm^{-1} and C–O bond length = 1.241 Å for cytidine continue the parallel relation and indicate that the effect of protonation is to shorten the C–O bond and to raise the stretching frequency.

The effect of substitution in the ring system leads to displacement of the $\pi \rightarrow \pi^*$ electronic transition to longer wavelength due to inductive or mesomeric effects. A red shift is observed in the absorption spectra of 2-chloro-5,6-dihydro-5-oxo-6-propylimidazo[1,2-*c*]pyrimidine (**8**), 2-acetylamino-5,6-dihydro-5-oxo-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidine (**9**), and 5,6-dihydro-5-oxo-2-phenyl-6-β-D-ribofuranosylimidazo[1,2-*c*]pyrimidine (**13**).[8] Concomitantly, similar shifts are observed in the fluorescence excitation spectra in acid (Table I). These substitutions result in a modest improvement in the quantum yield in going from **4** to **8** (Φ = 0.02 in 1 M HCl) and in a dramatic improvement in the quantum yield and fluorescent lifetime in going from **1b** to **9** (Φ = 0.85 and τ = 4 ns in 1 M HCl). Similarly, protonated **13** exhibits

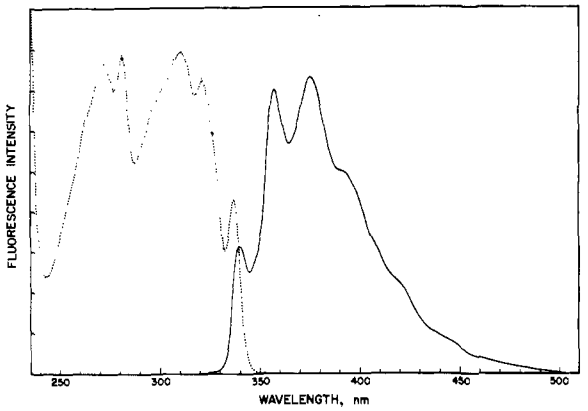


Figure 4. Corrected fluorescence excitation (···) and corrected fluorescence emission (—) spectra for imidazo[1,2-*a*]pyridine (conjugate base of **10**) in cyclohexane. The fine structure is wiped out in protic solvents. See Table I for fluorescence data in other solvents.

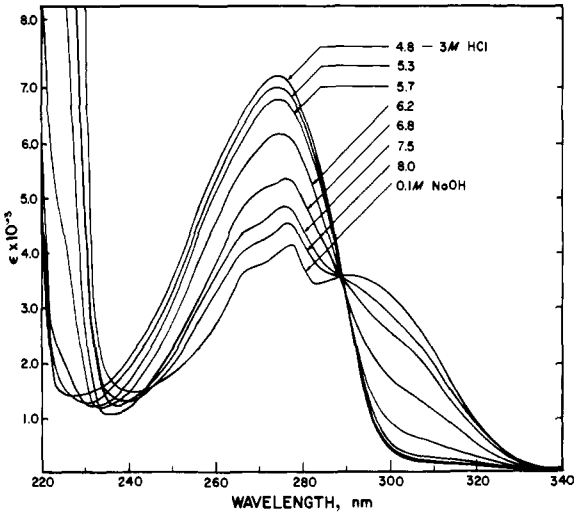


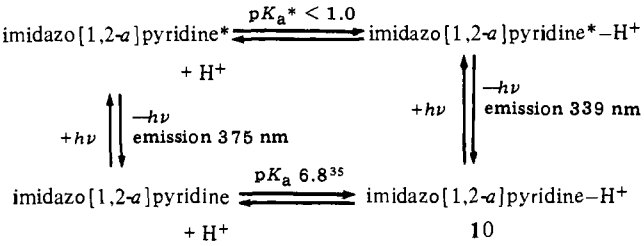
Figure 5. Variation in ultraviolet absorption spectra of imidazo[1,2-*a*] pyridine with pH in aqueous solvents.

greatly improved fluorescence over **1b**.[8] The fluorescence emissions of the protonated forms are also shifted to the red to the extent that compound **9**·HCl has an emission maximum at 385 nm vs. 340 nm for ϵ-Cyd·HCl (**1b**) (Table I). For compound **9**·HCl, the polarization remains constant with wavelength between 340 and 300 nm, and it may be assumed that only one absorption band is present in this region. Below 300 nm the polarization decreases as a result of changes in absorption transitions.

In terms of the fluorescence properties of the protonated form of ϵ-Cyd, the limited solubility of **1b** in nonpolar solvents precluded the possibility of observing the effect of solvents on its fluorescence. We therefore turned to the N1-methylated derivative (**11**) of imidazo[1,2-*a*]pyridine along with the N1-protonated imidazo[1,2-*a*]pyridine (**10**) (Table I) as useful models. Compound **11** showed emission characteristics similar to those observed for N1-protonated or N1-alkylated ϵ-cytidine compounds[35] and gave a higher quantum yield. The lack of a carbonyl group in **10** (and **11**) led us to a better understanding of the fluorescence properties of the ϵ-cytidine cationic forms. In the absence of a carbonyl group, imidazo[1,2-*a*]pyridine is highly fluorescent (Φ = 0.98) in neutral form in aqueous medium or in cyclohexane[36] (Figure 4). For ϵ-cytidine, which contains a carbonyl group, the lowest energy transition is n → π^* and the free base in this case is not fluorescent. Moreover, the carbonyl group is also responsible for the low quantum yield of the protonated form **1b**. The ultraviolet spectra of ϵ-cytidine plotted as a function of pH (Figure 1, see also Figure 3) show that, at pH 3.0 and below, the $\pi \rightarrow \pi^*$ transition has submerged the n → π^* transition and carries major responsibility for the lowest energy ultraviolet radiation absorbed. The qualitative statements concerning the influence of both carbonyl substitution and protonation on the absorption and emission properties of these bicyclic systems will have to suffice until a theoretical treatment is made corresponding to the study of Hug and Tinoco[22,23] that included cytosine and its protonated form.

In contrast to the practically identical values of pK_a and pK_a* observed for ϵ-cytidine, the excited state species of imidazo[1,2-*a*]pyridine has a pK_a* less than 1.0 in aqueous solution, very different from the pK_a value of 6.8 for the ground state (Figure 5)[35] (Scheme I). At pH 2.2, emission at 375 nm from the neutral species was observed. Not until the medium was as acidic as 3 M HCl did the fluorescence emission shift to 339 nm from that of the neutral form at 375 nm. The shorter wavelength marks the region where fluorescence emission is observed from the ϵ-cytosine and ϵ-cytidine cationic forms (e.g., **1b, 2–4**). In an aprotic solvent (dioxane) where dissociation of the cationic species, imidazo[1,2-*a*]pyridine hydrochloride (**10**), would be more difficult, the emission shift to 335 nm was observed when HCl gas was bubbled into dioxane solution.

Scheme I



In conclusion, it is apparent that the 3,N^4-ethenocytidine type of molecule is fluorescent only in cationic form (e.g., **1b, 2–4**), with an emission maximum centered at 340 nm in aqueous solution, close to the emission maximum of indole. Although the fluorescence quantum efficiency changes with pH, the normalized emission spectra are identical and superimposable through the pH range of 1.0–5.2. With a high degree of certainty, only one emitting species is detectable under the matched fluorescence spectra (Figure 2). If no carbonyl group is present and if n → π^* transition is not the lowest energy absorption in the neutral form, fluorescence is restored to the fused six–five ring system, as has been demonstrated with imidazo[1,2-*a*]pyridine. Modification of cytidine and related nucleotides with chloroacetaldehyde yields compounds with interesting enzymatic but weak fluorescence properties. Modification of cytosine-containing compounds with other α-halocarbonyl reagents, since it leads to products that exhibit improved fluorescence properties,[8] may, if the products are active, provide fluorescent probes of biological systems under sufficiently acidic conditions.[19] Practical correlations have resulted from a comparison of the spectroscopic properties with the x-ray crystal structure of ϵ-cytidine hydrochloride.

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