Photophysical Studies of Luminarosine—A New, Highly Fluorescent Ribonucleoside with Pteridine-like Betaine as the Aglycone

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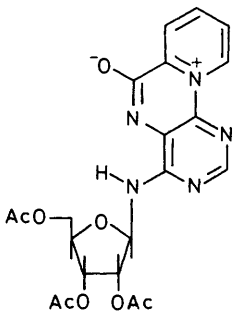
The solvent and pH dependence of the absorption and fluorescence spectra, fluorescence quantum yields and lifetimes, and fluorescence quenching behaviour of 4-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosylamino)pyrido[2,1-*h*]pteridin-11-ium-6-olate, its α-anomer, the parent ribonucleoside 4-(β-D-ribofuranosylamino)-pyrido[2,1-*h*]-pteridin-11-ium-5-olate (luminarosine) and the aglycone 4-aminopyrido[2,1-*h*]-pteridin-11-ium-5-olate (luminarine) are reported. Spectroscopic properties of these compounds in solvents of different polarities are characterized by evidence of the occurrence of a highly dipolar, charge-transfer excited state within the heterocyclic betaine system. The pH dependence of the absorption spectra of luminarosine and luminarine infer the occurrence of ground state prototropic equilibria in solution involving protonation of the negatively charged oxygen atom followed by tautomerism to a lactam. The excited state prototropic equilibria, as shown by fluorescence properties, seem to be more complex and suggest the possible occurrence of proton-transfer and/or phototautomerization reactions.

Fluorescent derivatives of nucleic acid bases are of particular interest in view of their possible applications in various studies of nucleic acids, especially their conformation and dynamics,[1] non-isotopic detection,[2] and automated sequencing.[3] Very recently, we reported the photochemical transformation of water soluble, blue-emitting, *N*-[2-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosyl)purin-6-yl]pyridinium chloride[4] into another nucleoside that emits intense, green fluorescence, namely 4-(2,3,5-tri-*O*-acetyl-β-D-ribofuranosylamino)pyrido[2,1-*h*]-pteridin-11-ium-5-olate, (**1**), termed 2,3,5-tri-*O*-acetyl-β-luminarosine.[5,6] The α-anomer, (**2**), as well as the parent ribonucleoside, (**3**) (luminarosine), and the aglycone, (**4**) (luminarine), have also been obtained.[5] The interesting emission properties of luminarosine prompted us to undertake detailed photophysical studies of the solvent and pH dependence of the absorption and fluorescence spectra, fluorescence quantum yields, lifetimes, and fluorescence quenching of all the compounds cited above. The results of these studies are presented and discussed in this paper. This work is part of an ongoing project investigating the dynamics and interactions of oligonucleotides using, as a probe, luminarosine spliced into an oligonucleotide chain in a sequence-specific manner.



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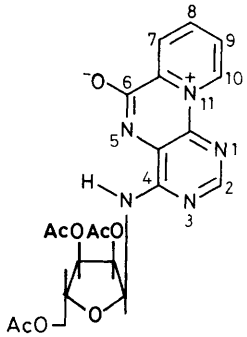

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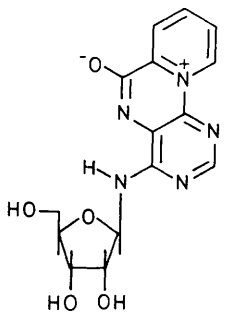

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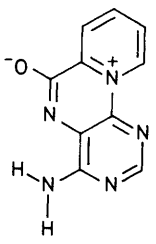
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Experimental

Materials and Methods.—Spectrograde ethanol (95%) and chloroform (Merck) and h.p.l.c. grade methanol and acetonitrile (BDH Omnisolv) were used as received. 1,4-Dioxane (Merck) was purified as described.[7] Water was purified using a Millipore Super-Q system. T.l.c. was performed on Merck precoated silica plates in the following solvent systems: *A* chloroform–methanol 15:1 (v/v) and *B* chloroform–methanol 8:2 (v/v). H.p.l.c. analyses were made using a reversed-phase (CN) column eluted isocratically with a mixture of water and acetonitrile (5:3, v/v) containing 0.05% formic acid.

The u.v.-visible absorption spectra were measured at room temperature using a Cary 118 spectrophotometer. The fluorescence excitation, emission, and polarization spectra were measured at room temperature and at 77 K using a Spex Fluorolog 222 spectrofluorometer. Spectroscopic measurements at 77 K were carried out in rigid ethanol glasses in a quartz tube of 5 mm path length using a quartz Dewar cold-finger assembly. The emissions from quartz or solvent alone were found to be insignificant. Fluorescence yields were determined relative to

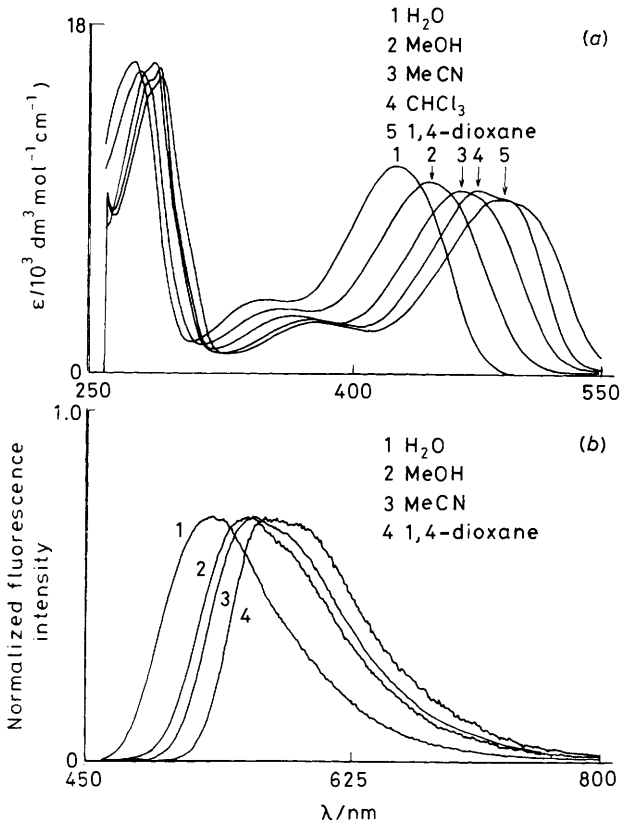


Figure 1. Quantitative absorption (*a*) and normalized fluorescence (*b*) spectra of (**1**) in various solvents.

fluorescein (Φ = 0.92 in 0.1 mol dm^{-3} NaOH).[8] Fluorescence lifetime measurements were made by using picosecond laser excitation and a single photon-counting detection system. Details of the experimental set-up and the analysis of fluorescence decay curves have been reported elsewhere.[9] The reduced χ^2 values were used as a measure of the 'goodness-of-fit', and were within 1.0–1.3 for both single and double exponential decay functions. The errors in the recovered lifetimes are less than 5% for the longer-lived decays and do not exceed 15% for the short-lived components.

Compounds (**1**) and (**3**) have been synthesized previously[5] and were used, after their purity had been checked by means of t.l.c., without further purification.

Preparation of (**2**). 2,3,5-Tri-*O*-acetyl-β-luminarosine (**1**) (300 mg, 0.64 mmol) was dissolved in 80% aqueous acetic acid (20 cm^3) and the solution left for 5 h at 25 °C. T.l.c. (system *A*) and h.p.l.c. analyses revealed that the equilibrium, β ⇌ α, was established after *ca.* 3 h. The solution was diluted with water (100 cm^3) and concentrated under vacuum to remove an excess of acetic acid.

The residue was treated with water (100 cm^3) and extracted with chloroform (3 × 50 cm^3). The chloroform layer was washed with water, dried with Na_2SO_4, and evaporated under reduced pressure to give a mixture of two anomers (280 mg) in a ratio of α:β *ca.* 55:45, as revealed by h.p.l.c. measurements. The mixture of the two anomers was separated by means of preparative t.l.c. to give the desired α-anomer (120 mg, 40% yield), identical in all respects with the authentic sample of (**2**) obtained previously.[5]

Preparation of (**4**). 2,3,5-Tri-*O*-acetyl-β-luminarosine (500 mg, 1.06 mmol) was dissolved in 0.1 mol dm^{-3} aqueous trichloroacetic acid (60 cm^3) and the solution heated to about 90 °C. After 3 h t.l.c. analysis revealed almost quantitative transformation of (**1**) into (**4**) (R_f = 0.22 in system *B*). The reaction mixture was neutralized with $NaHCO_3$ and concentrated under reduced pressure. The residue was treated with methanol (100 cm^3). The white precipitate was filtered and the methanolic filtrate chromatographed (after concentration) on preparative silica plates (Merck, 4 plates) in system *B* to give pure (**4**) (187 mg, 83% yield) identical (t.l.c., ^{1}H and ^{13}C n.m.r.) with the authentic sample of luminarine obtained previously[5] but with improved spectral purity.

Results and Discussion

Spectroscopic Properties in Solvents Having Different Polarities.—The absorption and fluorescence spectra of tri-*O*-acetyl-β-luminarosine, (**1**), its α-anomer, (**2**), and ribonucleoside (**3**) have virtually identical shapes and relative intensities. The absorption spectrum of (**1**) is shown in Figure 1(*a*), and important parameters for (**1**) and (**4**) are summarized in Table 1. All four compounds are characterized by two relatively strong transitions, one in the u.v. (λ_{max} 260–280 nm) and the other in the visible (λ_{max} 420–470 nm). These two transitions are separated by a band of lower intensity in the 300–400 nm range. The lowest energy transition undergoes a large blue shift with increasing solvent polarity and is probably due to a strong intramolecular charge-transfer (CT) interaction within the heterocyclic betaine system. In polar, aprotic acetonitrile the maximum is substantially red shifted compared with methanol. This behaviour reflects the greater polarity and concomitant, increasing stabilization of the ground state with increasing polarity and proton donating ability of the solvent. The removal of the sugar moiety from the molecular framework of (**1**), thus leading to (**4**), results in a small red shift in both the absorption and fluorescence bands (see Table 1). This may indicate a greater CT interaction is possible when a primary amine group is present, as in (**4**).

The normalized fluorescence spectra of (**1**) in various solvents are reproduced in Figure 1(*b*). The spectra are characterized by a single, broad emission which shows solvent dependence similar to that observed in the absorption spectra.

The radiative and non-radiative decay constants for (**1**) and (**4**) in the same solvents were calculated from $k_r = \varphi/\tau$ and $k_{nr} = (1 - \varphi)/\tau$. The results, summarized in Table 1, show that (**4**) has consistently larger non-radiative and consistently smaller radiative decay constants than (**1**) in all solvents. There appears to be a general increase in the non-radiative decay rate with decreasing solvent polarity for both (**1**) and (**4**). Chloroform solutions may be slightly anomalous, with respect to both the trend in k_{nr} and the absorption spectra [Figure 1(*a*)]; note the greater resolution in the shoulders of both the u.v. and visible bands, for reasons which are unclear at present.

Low-temperature Spectra.—The low-temperature emission, excitation and excitation polarization spectra of (**1**) and (**4**) were recorded at 77 K in ethanol glasses and were compared with those taken at room temperature (see Figure 2). No phosphorescence could be detected within the sensitivity limits of the instrument. This is taken as a strong indication of the importance of solvent relaxation in stabilizing the excited state in fluid solution, as expected of a polar solvent and a highly dipolar CT excited state. The low-temperature fluorescence spectra show some vibrational fine structure and substantial blue shifts compared with the broad, almost unstructured emissions at room temperature. The lowest energy band in the fluorescence excitation spectrum in each case also shows clear vibrational fine structure and is slightly blue-shifted compared to the room-temperature absorption spectrum. The polarization values in the excitation polarization spectra are large and almost constant across the long-wavelength absorption band.

Table 1. Absorption and fluorescence maxima, quantum yields, and lifetimes of tri-*O*-acetyl-β-luminoarosine (**1**) and luminarine (**4**) in various solvents.

Solvent	Compd.	A_{max}/nm	ε/dm^3 mol^{-1} cm^{-1}	A_{max}/nm	Φ	τ/ns	k_r/10^{-7} s^{-1}	k_{nr}/10^{-7} s^{-1}
H_2O	(1)	425	(10 800)	528	0.62	8.3	7.5	4.6
	(4)	428	(9 000)	533	0.47	7.4	6.3	7.2
CH_3CN	(1)	464	(9 900)	557	0.47	8.4	5.6	6.3
	(4)	468	*a*	564	0.33	7.0	4.7	9.6
CH_3OH	(1)	445	(10 600)	552	0.47	8.0	5.8	6.6
	(4)	449	(8 300)	557	0.33	6.7	4.9	10.0
$CHCl_3$	(1)	473	(9 900)	549	0.53	9.1	5.8	5.2
	(4)	475	*a*	555	0.36	8.6	4.2	7.4
1,4-Dioxane	(1)	486	(9 500)	570	0.39	7.5	5.2	8.1
	(4)	489	*a*	577	0.27	6.2	4.3	11.8

[a] ε could not be determined because of the low solubility of (**4**) in these solvents.

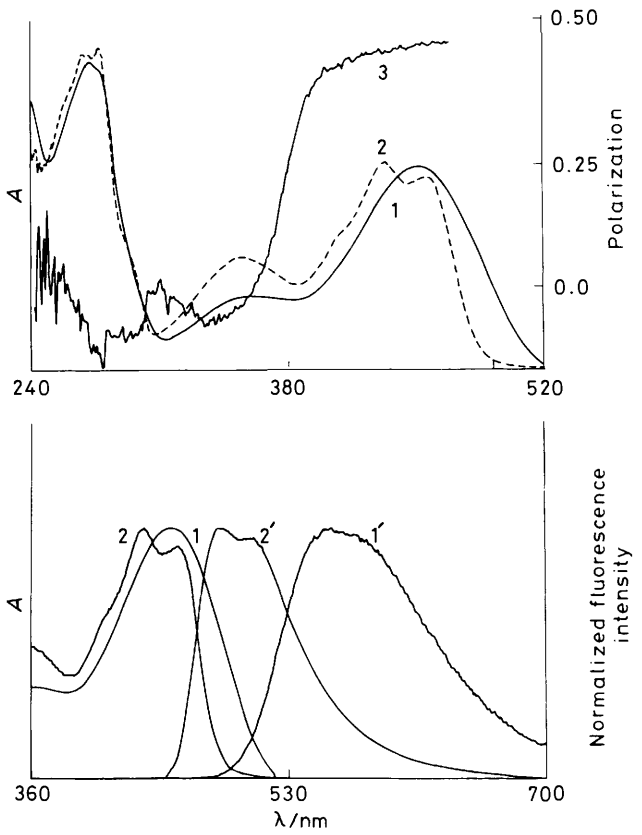


Figure 2. Comparison of room temperature absorption (1) and emission (1′) spectra of (**4**) with low temperature (77 K) fluorescence excitation (2), excitation polarization (3), and emission (2′) spectra.

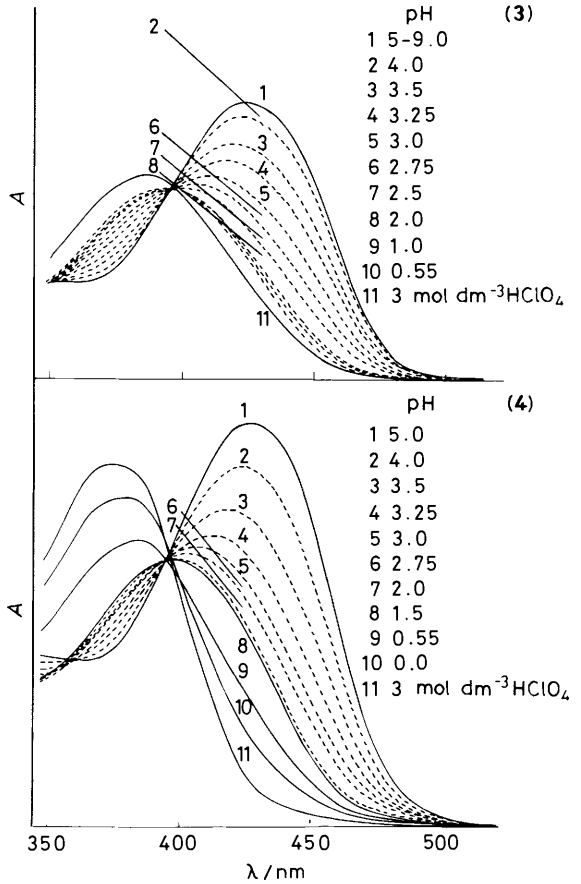
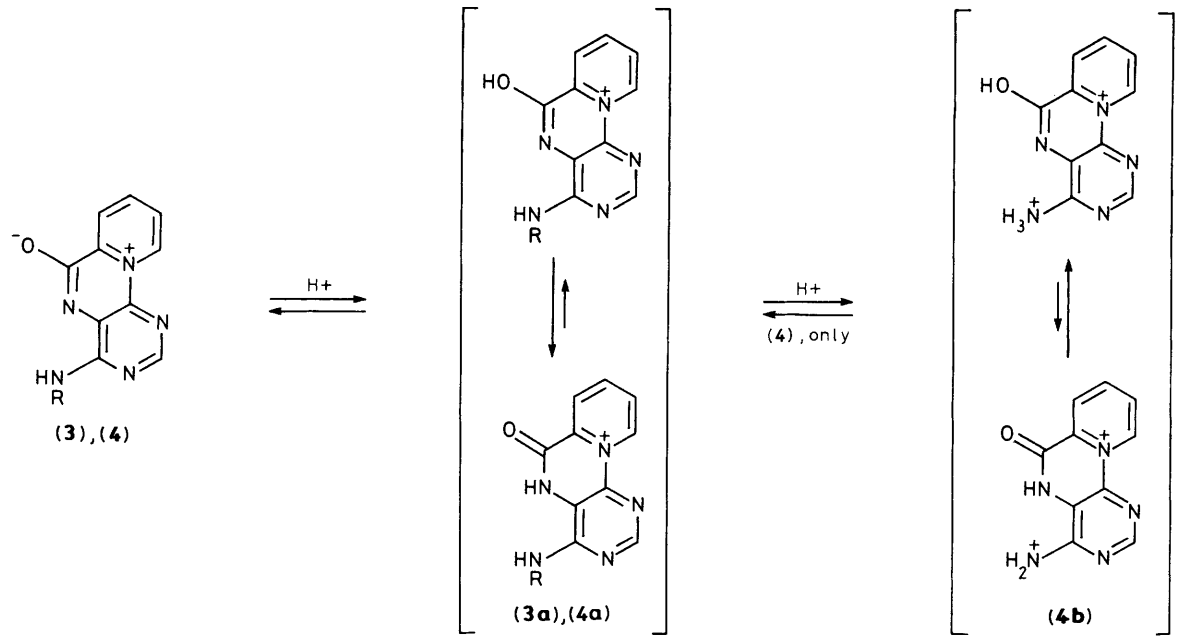


Figure 3. The pH dependence of the absorption spectra of (**3**) and (**4**).

This clearly shows that the first absorption band is due to only one electronic transition and also indicates that the absorption and emission transition moments are nearly collinear.

The Acid–Base Dependence of the Absorption and Fluorescence Spectra.—In order to avoid problems associated with base- or acid-catalysed de-*O*-acetylation of (**1**) during spectral measurements at high and low pH, the ribonucleoside (**3**) was used in these experiments. The absorption and fluorescence spectra of (**3**) and (**4**) do not show any changes between pH 5–12 and thus it is concluded that they correspond to the betaine form of (**3**) and (**4**). With decreasing pH the absorption at 425 nm disappears gradually and at pH 1 a new band appears at 394 nm (Figure 3). The clear isosbestic points, at 396 nm in both cases, indicate the occurrence of ground state prototropic equilibria in solution. A further decrease in pH (pH < 1) results in small changes in the absorption of (**3**) but more pronounced changes in (**4**). In 3 mol dm^{-3} $HClO_4$, a new band with a maximum at 374 nm appears in the absorption spectrum of (**4**). The absorption titration curves for (**3**) and (**4**) are shown in Figure 4. In the case of (**3**) one protonation step characterized by a ground state pK_a 3.1 is observed, while in the case of (**4**) two protonation steps occur with pK_a of 3.6 and *ca.* 0.3.

Based on these observations the following Scheme is proposed for the ground state prototropic reactions of (**3**) and (**4**). The first step involves the protonation of the negatively-charged oxygen followed by tautomerization to the more favoured lactam configuration,[10] for both cases. The second protonation step, which only occurs in luminarine, (**4**), involves the primary-amine group. It leads to the dicationic species (**4b**) which, due to



Scheme.

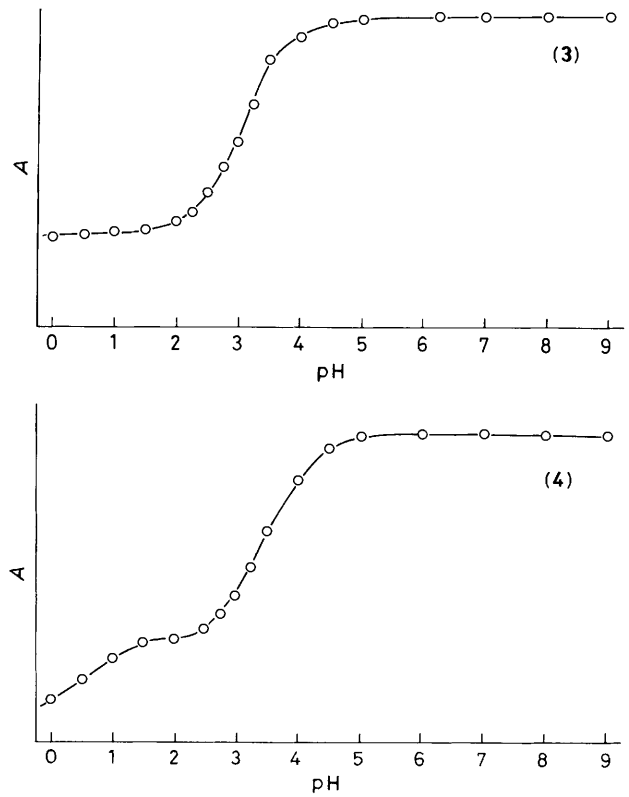


Figure 4. The absorption titration curves for (3) and (4).

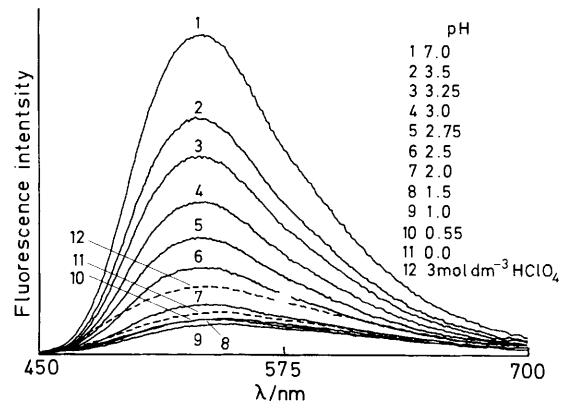


Figure 5. The pH dependence of the fluorescence spectra of (3).

the inductive effect of the ammonium group, presumably exists in the lactam configuration. This is consistent with the observed increase in the intensity of the absorption at 374 nm (3 mol dm^{-3} $HClO_4$) compared to that at 394 nm (pH *ca.* 1) which is attributed to the cationic lactam (**4a**).

The excited state prototropic equilibria seem to be more complex. When excited at the isosbestic point, (**3**) and (**4**) exhibit fluorescence intensities which gradually decrease from pH 5–~1 and then increase at pH < 1 (see Figure 5). Comparison of the normalized fluorescence spectra of (**3**) and (**4**) at pH 7 and 1 and in 3 mol dm^{-3} $HClO_4$ (Figure 6) reveal that the emission broadens and shifts to longer wavelength as the pH decreases from 7 to *ca.* 1, but that additional intensity appears on the short-wavelength side of the spectrum at still higher acidity. A simple explanation is that the spectra at pH 1 contain at least two emissions; a major component with a maximum shifted to the red and a second, minor component which is blue-shifted compared to the emission spectra obtained from pH 5–12. The spectra of the individual components could not be obtained because, even at high acid concentration (see the spectrum in 3 mol dm^{-3} $HClO_4$), the red emission is still dominant.

Fluorescence lifetime measurements show single exponential decays within the pH range 5.0–12.0 with average lifetimes of 7.9 and 7.4 ns for (**3**) and (**4**), respectively. Lowering of the pH (pH < 5.0) results in the appearance of decays which are no longer well-described by monoexponential functions. A better goodness-of-fit is obtained in the pH 5 to 2 range using a bi-exponential model in which a second, short-lived component (see Table 2) appears, and the lifetime of the long-lived component gradually decreases in both cases. At pH ⩽ 2 there is evidence that a third emitting component may be present since at pH 2.0 the pre-exponential factors for the two-

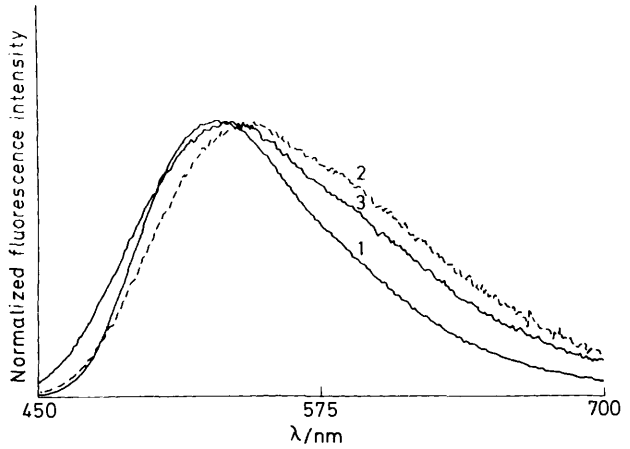


Figure 6. Comparison of the normalized fluorescence spectra of (**4**) at pH 7 (1), pH 1 (2) and in 3 mol dm^{-3} $HClO_4$ (3).

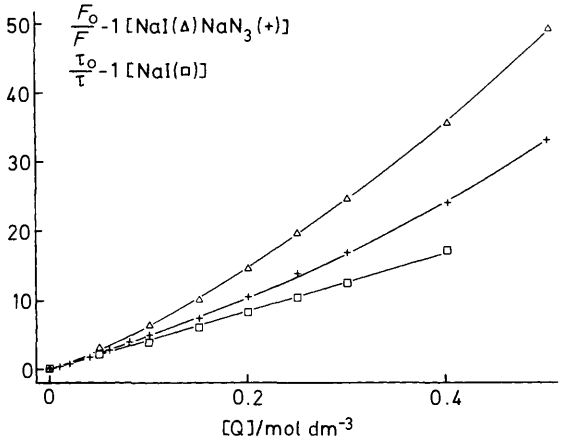


Figure 7. Stern–Volmer plots for the fluorescence quenching of (**4**) by NaI and NaN_3.

Table 2. pH dependence of the fluorescence lifetimes (τ) of luminarosine (**3**) and luminarine (**4**).

	Luminarine (**4**)				Luminarosine (**3**)			
pH	τ_1/ns	$(\alpha_1)^a$	τ_2/ns	$(\alpha_2)^a$	τ_1/ns	$(\alpha_1)^a$	τ_2/ns	$(\alpha_2)^a$
7.0	7.56				7.98			
4.0	7.48	(0.95)	2.08	(0.05)	7.97	(0.90)	0.95	(0.10)
3.5	7.35	(0.58)	2.26	(0.42)	7.91	(0.72)	0.74	(0.28)
3.0	6.90	(0.48)	2.42	(0.52)	7.74	(0.48)	0.76	(0.52)
2.5	6.41	(0.38)	2.64	(0.62)	7.25	(0.27)	0.75	(0.73)

[a] Pre-exponential factors.

component decay rather abruptly reverse their trends with increasing acidity. A third component, if present, would have a lifetime of the order of ≤50 ps, since it could not be resolved temporally from that of the pulse-shape mimic used for deconvolution.[9]

The above observations clearly show the complexity of the excited state prototropic equilibria of these compounds. The observed red shift of the emission coupled with the simultaneous blue shift of the absorption and the appearance of at least two components at low pH suggest the occurrence of excited state proton transfer[11] and/or phototautomerization reactions. Such processes are often encountered in nitrogen-containing heterocycles and have been reported to occur in alloxazine,[12] and

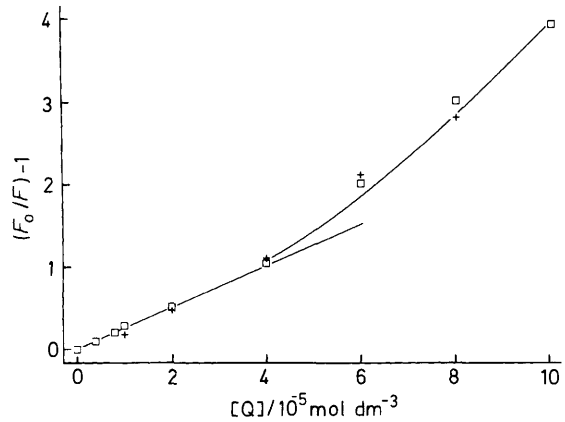


Figure 8. Stern–Volmer plots for the fluorescence quenching of (**3**) (□) and (**4**) (+) by $CuSO_4$.

lumazine.[13] The application of the Förster cycle[14] to estimate the values of excited state pK_a is impossible since fluorescence spectra of the pure components could not be obtained. Indeed, other interpretations of the variations in the fluorescence spectra and lifetimes with pH may be possible. Thus, further investigation is necessary in order to propose any reasonable scheme for the excited state prototropic reactions. It is felt that protecting the lactam system of these compounds by methylation of the lactam oxygen or nitrogen atoms should help solve this problem.

Fluorescence Quenching.—Fluorescence quenching of biopolymers that contain fluorescent moieties is a very useful means of obtaining information regarding the tertiary structure of the biopolymer and the relative accessibility of quenchers to the fluorophore, *i.e.*, location of the fluorophore near the surface or deeper within the biopolymer structure.[15] Therefore, it is important that the quenching behaviour of new fluorophores be examined with a wide variety of quenchers in order to obtain general information concerning the quenching characteristics of the potential fluorescent probe.

In this paper, we present the preliminary results of fluorescence quenching of (**1**)–(**4**) in aqueous solutions by anions such as I^-, Br^-, Cl^-, N_3^-, ClO_4^-, PO_4^{3-}, and NO_3^- as well as by the metal cations, Cu^{2+}, Hg^{2+}, Fe^{2+}, Pb^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Mg^{2+}, Ba^{2+}, Cs^+, and Ag^+.

Among the anions only iodide and azide quench the fluorescence of (**1**)–(**4**). The relevant Stern–Volmer plots are shown in Figure 7. The plot obtained using the lifetime data is linear and gives $k_q\tau = 42.7$ dm^3 mol^{-1} from which a value of $k_q = 5.1 \times 10^9$ dm^3 mol^{-1} s^{-1} is calculated. The plots constructed from the steady-state fluorescence show significant upward curvature indicating that static quenching contributes substantially to the overall quenching process at high quencher concentrations.

Among the metal cations only copper and mercury show significant quenching ability. Fluorescence-quenching data, obtained by intensity measurements alone, give extremely high quenching constants $K_{sv} = 2.6 \times 10^4$ dm^3 mol^{-1} for both mercury and copper cations (*cf.* Figure 8). However, no change occurs in the magnitude of the fluorescence lifetimes over the whole range of concentrations of Cu^{II} and Hg^{II} used. This suggests that there is strong ground state association between (**1**) and (**4**) and these two metal ions. Direct evidence in support of this suggestion comes from an examination of the absorption spectra of solutions of (**1**) and (**4**) containing various amounts of Cu^{II} or Hg^{II}. A gradual decrease of the absorption band at 425 nm, characteristic of luminarosine, with the concomitant

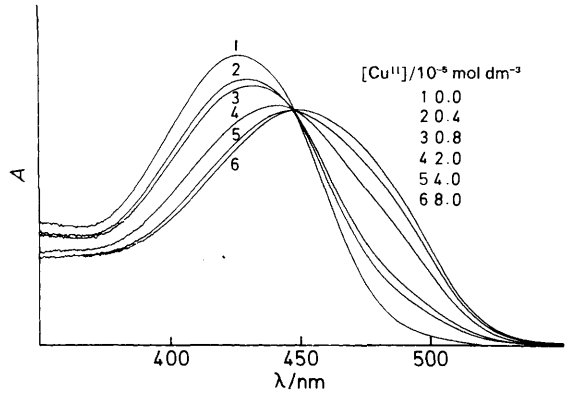


Figure 9. Changes in the absorption spectra of (**4**) upon addition of $CuSO_4$.

appearance of a new band at *ca.* 450 nm, due to the complex (*cf.* Figure 9), occurs with increasing metal concentration. Since the quenching mechanism is purely static, the quenching constants obtained correspond to those for the ground state complexation process.[16] No measurable quenching could be detected in the cases of the other cations. Initial studies of the stability of these complexes as a function of pH showed that the mercury complex is stable over a wide pH range (pH 4–7) while the copper complex undergoes complete dissociation on going from pH 6.7–5.5. The mechanism of formation and the nature of these complexes require further investigation.

Conclusions

The absorption and fluorescence properties of these compounds illustrate their potential in a wide range of applications. The dependence of λ_{em} on solvent polarity suggests that they could serve as fluorescence probes of the polarity of their local environment in solutions. The magnitudes of the fluorescence lifetimes of these compounds fall in a range that is convenient for measurement with a variety of excitation sources. Because of their chemical nature, they can serve as fluorescence probes of nucleic acids and other biologically important molecules. Their large fluorescence quantum yield and good photostability also make them important compounds for use as fluorescent standards. These properties, coupled with the well separated emission and absorption bands, make them potentially useful as lasing systems at wavelength greater than *ca.* 530 nm.

Acknowledgements

We thank the Natural Sciences and Engineering Research Council of Canada for financial support. B. S. thanks the President's NSERC fund of the University of Saskatchewan for providing a postdoctoral fellowship. Partial support from the Polish Ministry of Education (R.P.II.13.2.15) and the Polish Academy of Sciences (CPBR 3.13.4.2.1) is also acknowledged.

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Received 6*th February* 1989; *Paper* 9/00553F