# An ‘off-on-off’ fluorescent sensor for pH based on ligand–proton and ligand–metal–proton interactions

**Luigi Fabbrizzi,* Francesco Gatti,† Piersandro Pallavicini and Luisa Parodi**

*Dipartimento di Chimica Generale, Università di Pavia, Via. Taramelli 12, I-27100 Pavia, Italy*

A new tripodal tetradentate ligand (**L**) containing two pyridines and two amines as donor groups, and bearing an appended anthracene fragment, has been prepared. In a water–acetonitrile mixture, it displays a characteristic ‘off-on-off’ window behaviour in the $I_f$ *vs.* pH profile. By means of potentiometric titrations, four protonation constants have been determined for **L** and a distribution diagram (% of species *vs.* pH) drawn. Superimposition of the $I_f$ *vs.* pH profile on the distribution diagram suggested that the species responsible for full anthracene emission is $\mathbf{LH_2}^{2+}$, which contains two free pyridines and two protonated amines, whereas the fluorescence quenching is to be associated with the formation of $\mathbf{LH_3}^{3+}$ in the acidic zone and $\mathbf{LH}^{+}$ in basic solutions. Moreover, spectrofluorimetric titrations in the presence of $Zn^{2+}$ (1 : 1 molar ratio) evidenced also in this case an ‘off-on-off’ behaviour in the $I_f$ *vs.* pH profile (with a maximum centred at a lower pH than in the absence of added metal) due to a partial quenching of the fluorescence emission, also in basic conditions. This latter effect has been ascribed to the formation of $[Zn(\mathbf{L})(OH)_n]^{(2-n)+}$ ($n = 1,2$) species.

Systems capable of displaying ‘off-on-off’ fluorescence emission upon variation of pH and thus giving a direct visual indication of pH windows[1] are of interest, in so far as sensing concentration windows of chemical species may have significant implications in the life sciences: biological processes are known to take place only in restricted ranges of species concentration, and, in particular, in restricted pH ranges. With this perspective, some examples of sensors capable of signalling pH windows have been reported, based on single molecules containing one fluorescent fragment, anthracene, and one or more pyridine and amine groups.[1–3] It is well-established that while pyridine is able to quench the fluorescence of anthracene only in its protonated form (pyridinium) through a PET[4] process, amines switch off anthracene emission (again through a PET process) only when not protonated.[5] Thanks to the different p$K_a$ values of $pyH^+$ (5.25 for plain pyridine in 0.1 M $KNO_3$ [6]) and $RR'R''NH^+$ (10.64 for $R = CH_3$; $R' = R'' = H$, in 0.1M KCl [7]), multicomponent molecules of this type display a typical ‘off-on-off’ fluorescence emission profile on going from low to high pH values and *vice versa.*

We considered that, if the fluorescent fragment (anthracene) is appended to a molecular system containing both pyridine and amine groups in such a fashion that metal cations can be bound with a 1 : 1 stoichiometry, a well-defined, more sophisticated ligand–metal–proton system may be obtained. In particular, if $Zn^{2+}$ is employed as the metal, no contributions are expected by the metal itself to the quenching processes (as a matter of fact, for a ligand containing both pyridine and amino groups,[2] it has been demonstrated that coordination to $Zn^{2+}$ revives the quenched anthracene fluorescence in non-aqueous solution). As a first effect, coordination to $Zn^{2+}$ should instead favour deprotonation of the pyridinium group(s), thus lowering the pH at which the ‘off-on’ switching process takes place, and should avoid the PET due to the presence of free amines,[8] in this way possibly deleting the amine-related fluorescence ‘on-off’ switch at high pH values. However, when tri- or tetradentate ligands, in particular, are taken into account, five-coordinate $[(\text{ligand})Zn(H_2O)_n]^{2+}$ ($n = 1,2$) species may exist,[9] in which the coordinated water molecule(s) can release one proton under moderately basic conditions: partial fluorescence quenching may thus be observed in basic solutions after the formation of $[(\text{ligand})Zn(OH)_n]^{(2-n)+}$ ($n = 1, 2$) species, due to the presence of bound $OH^-$ anions.[10] This may reactivate an ‘off-on-off’ behaviour, but quite likely centered on a different pH window with respect to the simple ligand–proton system.

In this work, the new multicomponent ligand **L** has been synthesized, which contains anthracene and a two-pyridine two-amine system. The binding groups are disposed in such a fashion that the tetradentate ligand **L** resembles ligands of the tris(2-aminoethyl)amine (tren) or tris(2-pyridylmethyl)amine (tpa) type, and thus is able to bind transition metals in a 1 : 1 ratio.[9,11] The behaviour of **L** as a base has been examined in 8 : 2 water–acetonitrile mixtures by means of potentiometric titrations, which allowed us to determine the relative stepwise protonation constants. The fluorescence intensity of **L** in the same mixture has been studied as a function of pH and the obtained off-on-off window profile could be explained on the basis of the determined protonation constants. Moreover, $I_f$ *vs.* pH profiles in the same solvent mixture but in the presence of $Zn^{2+}$ (1 : 1 molar ratio) have been measured, finding also in this case an off-on-off profile, which has been related to the formation of OH-containing complex species.

## Experimental

### Syntheses

**L** was synthesized as shown in Scheme 1. 2-Pyridinecarboxyaldehyde and 2-(aminomethyl)pyridine were purchased from Aldrich and used as such. *N*-(2-bromoethyl)-9-methyliminoanthracene (**2**) was prepared as described elsewhere.[12]

**Bis(2-pyridinemethyl)amine (1).** 2-Pyridinecarboxyaldehyde ($3.0\ cm^3$, 31.86 mmol) and $3.33\ cm^3$ (31.86 mmol) 2-(aminomethyl)pyridine were dissolved in $200\ cm^3$ of methanol under a nitrogen atmosphere. Stirring at room temperature was continued for 18 h. The solution was treated with an excess of $NaBH_4$ (10 g, added in portions) and heated at reflux for 1 h. The obtained mixture was evaporated to dryness on a rotary evaporator, the residue treated with $30\ cm^3$ $H_2O$

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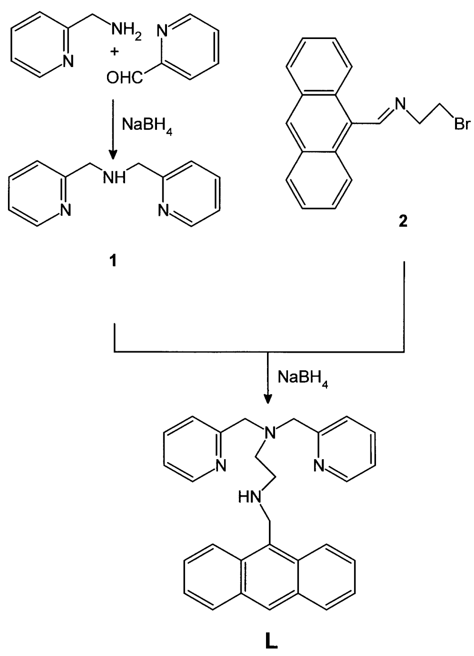
† Present address: University of Manchester, Institute of Science and Technology, PO Box 88, Manchester, M601 QD, UK.



**Scheme 1** Synthesis of ligand **L** (see Experimental for details).

and extracted with 3 × 30 cm$^3$ $CH_2Cl_2$. The combined organic phases were dried on $Na_2SO_4$ and the solvent was removed on a rotary evaporator. The crude product was a yellowish oil, from which the white ammonium salt was obtained pure (as perchlorate) by addition of an excess of concentrated $HClO_4$ to a methanol solution. **1** was then prepared pure from its ammonium salt by dissolving it in 1 M NaOH, extracting with $CH_2Cl_2$, drying the organic phases on $Na_2SO_4$ and removing the solvent under vacuum. Elemental analysis: $C_{12}H_{13}N_3 \cdot 2HClO_4$ required: C 35.95, H 3.74, N 10.49%; found C 35.95, H 3.65, N 10.52%. NMR ($CDCl_3$): δ 8.45 (ddd, 2H, CH in position 6 of the py rings); 7.54 (td, 2H, CH in position 3 of the py rings); 7.26 (ddd, 2H, CH in position 4 of the py rings); 7.06 (ddd, 2H, CH in position 5 of the py rings); 3.89 (s, 4H, py—$CH_2$—NH).

***N*-(2-Anthracene-9-methyl)-*N'*,*N'*-bis(pyridyl-2-methyl)-ethane-1,2-diamine (L).** **1** (336 mg, 1.689 mmol) was dissolved in 40 cm$^3$ toluene and treated with 500 mg (1.60 mmol) of **2** and 200 mg (1.9 mmol) of $Na_2CO_3$. The mixture was stirred at reflux, under a nitrogen atmosphere, for 3 days. After filtration, the solvent was removed with a rotary evaporator and the obtained semisolid dissolved in 60 cm$^3$ of ethanol. The solution was then treated with an excess of $NaBH_4$ (800 mg) in portions, while heated to its reflux temperature. After the addition of $NaBH_4$, the mixture was heated at reflux for a further 2 h, then the solvent removed on a rotary evaporator and the obtained solid treated with 30 cm$^3$ $H_2O$. This mixture was then extracted with 3 × 30 cm$^3$ $CH_2Cl_2$ and the organic phases dried over anhydrous $Na_2SO_4$. The pure product, **L**, was obtained after filtration and removal of the solvent on a rotary evaporator. All attempts to prepare an ammonium salt of **L** were unsuccessful. MS (ESI): 433 (**LH**$^+$). NMR ($CDCl_3$): δ 8.5 (d, 3H, CH of the py and anthr rings); 8.25–8.4 (m, 4H, CH of the anthr rings); 7.9–8.0 (m, 2H, CH of the anthr rings); 7.55 (dd, 2H, CH of the py rings); 7.4 (m, 2H, CH of the anthr rings); 7.3 (d, 2H, CH of the py rings); 4.70 (s, 2H, NH—$CH_2$—anthr); 3.65 (s, 4H, py—$CH_2$—N); 2.9 (t, 2H, NH—$CH_2$—$CH_2$—NH—anthr); 2.75 (t, 2H, NH—$CH_2$—$CH_2$—NH—anthr).

### Physical measurements and titration experiments

NMR spectra were recorded on a Bruker AMX 400 instrument. Mass spectra (ESI) were obtained on a Finnigan TSQ 700 instrument. UV/VIS spectra were taken with a Hewlett Packard HP8453 diode array spectrophotometer. Emission spectra were recorded with a Perkin Elmer LS-50 luminescence spectrometer (excitation wavelength 368 nm; maximum emission intensity at 424 nm) and all were uncorrected for instrumental response.

Spectrofluorimetric titrations were performed in water–acetonitrile (8 : 2 v/v) solutions (30 ml, $10^{-4}$ M, made 0.1 M in sodium perchlorate), under a nitrogen atmosphere, in a cell thermostatted at 25 °C; standard $HClO_4$, NaOH and $Zn(ClO_4)_2$ solutions were used. Potentiometric titrations were performed in water–acetonitrile (8 : 2 v/v) solutions (50 ml, made 0.1 M in sodium pechlorate) containing ligand ($5 \times 10^{-4}$ M) and an excess of standard perchloric acid, by addition of standard aqueous NaOH, under a nitrogen atmosphere, in a cell thermostatted at 25 °C. In each titration 60–70 points were recorded. The pH scale was calibrated prior to each experiment by the Gran method.[13] Refinement of the potentiometric data was performed using the Hyperquad package,[14] which mimimizes a least-squares function. The fit between calculated and experimental emf data was evaluated through the $\sigma$ and $\chi^2$ parameters,[15] whose values, in each treatment, were found to be $1 \pm 0.4$ and $<11\%$, respectively.

## Discussion

### The L + H$^+$ system

Due to the very low solubility of **L** in water, its properties as a base have been examined in a water–acetonitrile (8 : 2, v/v) mixture, using potentiometric titrations with standard base on solutions containing **L** plus an excess of acid. solvent mixtures high stability towards acidic and basic hydrolysis is required of the organic component if one wishes to calculate equilibrium constants from potentiometric data, titrations have been carried out in a restricted pH range (*i.e.*, $3 < pH < 11$). By processing titration data,[14] four stepwise protonation constants have been determined, which are reported in Table 1. The high values found for log $K_1$ and log $K_2$ allows us to associate the relative protonation equilibria to the two amino groups. The 2.5 log units difference between the first and the second constants is consistent with what is found for a number of diamines, in which the two nitrogen atoms are separated by a $CH_2$—$CH_2$ chain and bear a different number of substituents (*e.g.*, log $K_1 = 10.25$ and log $K_2 = 7.42$ for $CH_3NHCH_2CH_2NH_2$ in 1.0 M $KNO_3$ at 25 °C [16]). A larger gap is observed between the third and the second protonation constants, as the equilibrium relative to log $K_3$ must necessarily involve one of the two pyridine groups. The obtained value (4.1) is quite low for a monoalkyl-substituted pyridine (*e.g.*, log $K = 6.06$ for 2-methylpyridine in 0.1 M $NaClO_4$ at 25 °C [17]). However, it must be taken into account that protonation takes place on a doubly charged molecule, $\mathbf{LH_2}^{2+}$, and a strong repulsive effect must be expected for the incoming proton. Such an effect is even stronger for the protonation of $\mathbf{LH_3}^{3+}$ and as a consequence a very low log $K_4$ value (2.5) is found.

From the log *K* values a distribution diagram can be drawn (Fig. 1, solid lines), in which the percentage of each species as

**Table 1** Stepwise protonation constants for ligand **L** in 8 : 2 (v/v) water–acetonitrile mixtures (0.1 M in sodium perchlorate; $T = 25$ °C)

| Equilibrium | Protonation constant |
|---|---|
| $\mathbf{L} + H^+ \rightleftharpoons \mathbf{LH}^+$ | log $K_1 = 10.28 \pm 0.01$ |
| $\mathbf{LH}^+ + H^+ \rightleftharpoons \mathbf{LH_2}^{2+}$ | log $K_2 = 7.7 \pm 0.1$ |
| $\mathbf{LH_2}^{2+} + H^+ \rightleftharpoons \mathbf{LH_3}^{3+}$ | log $K_3 = 4.1 \pm 0.2$ |
| $\mathbf{LH_3}^{3+} + H^+ \rightleftharpoons \mathbf{LH_4}^{4+}$ | log $K_4 = 2.5 \pm 0.2$ |

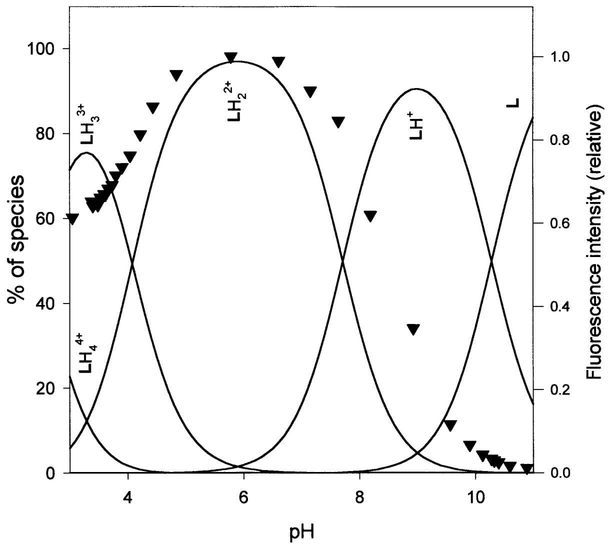


**Fig. 1** (———) Distribution diagram (percent of species *vs.* pH) for ligand **L**, in 8 : 2 (v/v) water–acetonitrile. The species relative to each curve are indicated on the diagram. (▼) Relative fluorescence intensity of ligand **L**, as a function of pH

a function of pH can be readily visualized. It can be seen that, over large pH interval (5–7), $\mathbf{LH_2}^{2+}$ (featuring two protonated amines and two free pyridine groups) is the predominant species. On going to lower pH values (<5), species containing also one (or two) protonated pyridines begin to form, while at high pH values (>7) species containing free amino groups become predominant.

The fluorescence instensity of ligand **L** has been examined in the 3–11 pH range and the obtained $I_f$ *vs.* pH profile is shown in Fig. 1 (black triangles), superimposed on the distribution diagram profiles. The predictable 'off-on-off' behaviour for $I_f$ is found, with maximum intensity between pH 5 and 7. Comparision with the % of species *vs.* pH diagram clearly shows that high $I_f$ values are found in a pH interval in which $\mathbf{LH_2}^{2+}$ is the only species present, or at least is by far the predominant one. On going to low pH values, the pyridinium-containing species $\mathbf{LH_3}^{3+}$ (and $\mathbf{LH_4}^{4+}$) begin to form and, accordingly, $I_f$ decreases. However, at the lower pH limit (pH = 3), $I_f$ reaches 60% of its highest value (which is found at pH 5.9); thus fluorescence is not completely 'switched off'. This is due in part to the fact that a residual quantity of $\mathbf{LH_2}^{2+}$ is still present at this pH value, but, in addition, it must noted that $\mathbf{LH_3}^{3+}$ (identical arguments can be put forward for $\mathbf{LH_4}^{4+}$) contains both a pyridinium group and two protonated amines: the positively charged pyridinium unit is thus kept far away from the ammonium groups, and consequently from the anthracene unit, by an electrostatic repulsive effect. The increased average distance between pyridinium and anthracene makes the quenching PET process in $\mathbf{LH_3}^{3+}$ less efficient[18] and, in this regard, it should be remembered that a direct correlation between PET efficiency and the distance separating anthracene and a pyridinium quencher in a multicomponent system has already been observed.[1] Finally, it must be also pointed out that single experiments made at high $H^+$ concentration (*e.g.* $10^{-1}$–$10^{-2}$ M) showed that $I_f$ can further decrease to about 50% of its highest value, due to disappearance of the residual $\mathbf{LH_2}^{2+}$ and to formation of a significant percentage of $\mathbf{LH_4}^{4+}$, a species that contains two pyridinium groups, which can make the PET process more favourable, at least from a statistical point of view.

On the other side, with increasing basicity $I_f$ starts to decrease above pH 7, corresponding to the formation of $\mathbf{LH}^+$ from $\mathbf{LH_2}^{2+}$, as can be seen in Fig. 1; this is due to the PET quenching process taking place in $\mathbf{LH}^+$, which involves the amino group that in this species is not protonated. However, it must be pointed out that the $I_f$ profile, in the basic zone, is not exactly superimposable on the $\mathbf{LH_2}^{2+}$ distribution curve, but is somewhat shifted to higher pH values. Complete fluorescence quenching is obtained at pH ⩾ 10, at which value $\mathbf{LH_2}^{2+}$ no longer exists and a small but significant quantity of **L** forms: this may be connected to some residual fluorescence (of the $\mathbf{LH}^+$ species) that is completely quenched in **L**, which contains two free amines.

As regards $\mathbf{LH}^+$, it is not easy to assert whether the free amine is that closer to anthracene (*i.e.*, $-CH_2-CH_2-NH-CH_2-$anthr) or the more distant one (*i.e.* $(-CH_2)_2-N-CH_2-CH_2-$). Comparison of available literature data on the related, separate fragments does not help, as similar $pK_a$ values are found, in water–organic solvent mixtures, for trialkyl- and benzylamines (*e.g.*, $pK_a = 9.40$ for trimethylamine, in 50 : 50 dioxane–water[19] and $pK_a = 9.36$ for benzylamine in 50 : 50 ethanol–water[20]). However, the fact that $I_f$ steeply decreases on going from $\mathbf{LH_2}^{2+}$ to $\mathbf{LH}^+$ and that with a further increase of pH (*e.g.* to $[OH^-] = 10^{-3}$–$10^{-2}$ M) the fluorescence does not further dramatically decrease, may indicate that it is the amine closer to anthracene that loses its proton.

## The L + H⁺ + Zn²⁺ system

Ligand **L** is a tetradentate ligand that can be related to either tren or tpa, as regards the coordination geometry that it imposes on metal centres. For tren and tpa the formation of 1 : 1 ligand–metal complexes is the rule.[9,11] However, in water or aqueous mixtures 1 : 1 ligand–metal complexes of the $[M(L')]^{2+}$ type (L′ = tren or tpa) will form only in a restricted pH range (typically with one or two solvent molecules completing the coordination sphere), as mixed ligand–metal–proton(s) and ligand–metal–hydroxide anion(s) species may easily form.[9,11b,c] In the case of $Zn^{2+}$, the literature data available for both tren and tpa suggest that in water $[Zn(L')]^{2+}$ is five-coordinated, with trigonal bipyramidal geometry; one water molecule completes the coordination sphere in the apical position. In basic solution, the coordinated water loses a proton to form the hydroxide-containing species $[Zn(L')OH]^+$.[9]

In this work, attempts have been made to determine complexation constants from potentiometric titration experiments with standard base on solutions containing **L** and $Zn^{2+}$ (1 : 1 molar ratio) plus an excess of acid, but calculations[14] failed to give constants that fit the experimental potentiometric data.[21] However, examinations of the $I_f$ *vs.* pH profiles (Fig. 2, empty squares) obtained from fluorimetric titrations with standard base on solutions containing **L** and zinc perchlorate in a 1 : 1 molar ratio plus an excess of acid, allowed us to disclose the solution behaviour of the system.

An 'off-on-off' profile is found also in this case, but comparison with the $I_f$ *vs.* pH profile in the absence of added metal (filled triangles in Fig. 1 and filled circles in Fig. 2) shows that when $Zn^{2+}$ is present $I_f$ begins to rise at a lower pH. This indicates that, as expected, in the presence of $Zn^{2+}$ complexation of the pyridine groups to the metal cation favours the release of protons from the pyridinium nitrogens. Moreover, on going to high pH values, $I_f$ decreases with a less steep profile then in the absence of $Zn^{2+}$, reaching not full quenching, but *ca.* 60% of its highest value. This is consistent with the formation of species containing one (or more) $OH^-$ anions, such as $[Zn(L)(OH)_n]^{(2-n)+}$. As a matter of fact, the $d^{10}$ $Zn^{2+}$ cation itself is not capable of any quenching effect, but a partial quenching of the anthracene emission has been observed in comparable Zn–ligand systems,[10] due to the formation of complexes containing the electron-rich anion $OH^-$. This is the case also for our system, as confirmed by redrawing titration data as $I_f$ *vs.* B/LG, where B/LG is the added base/ligand ratio, which can be calculated at each point

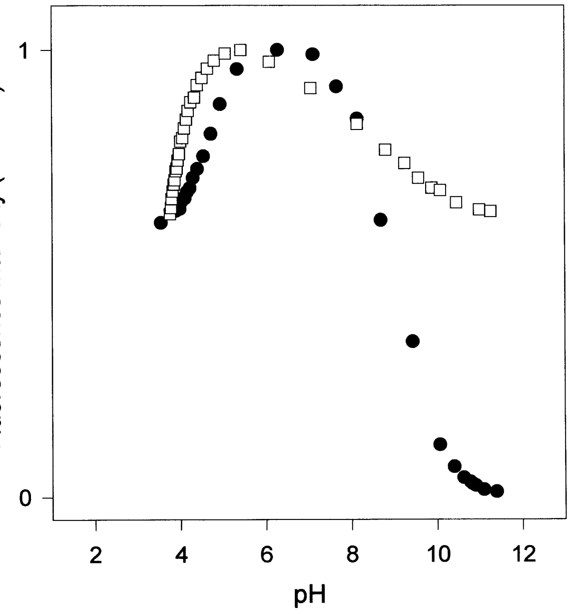


**Fig. 2** (□) Relative fluorescence intensity for 8 : 2 (v/v) water–acetonitrile solutions containing **L** and zinc perchlorate (1 : 1 molar ratio), as a function of pH. (●) Relative fluorescence intensity of ligand **L**, as a function of pH

of the titration experiment as B/LG = (mol $H^+$ − mol $OH^-$)/mol **L** (negative values indicate an excess of $OH^-$ anion). The obtained profile is shown in Fig. 3, from which it can be seen that $I_f$ rises until B/LG reaches the value of 0, which corresponds to the formation of the $[Zn(\mathbf{L})]^{2+}$ complex.[22] Moreover, $I_f$ decreases sharply when B/LG reaches negative values, that is, in the presence of an excess of $OH^-$. In particular, it reaches its minimum value when B/LG = −2 and further addition of base does not make $I_f$ vary significantly.

These data are thus consistent with the formation of $[Zn(\mathbf{L})OH]^+$ and $[Zn(\mathbf{L})(OH)_2]$. Even if the latter, six-coordinated species has not been observed in water for zinc complexes with both tren and tpa, it must be remembered that in our experiments the medium is a water mixture with a poorly polar solvent (acetonitrile) and that ligand **L** bears a bulky and hydrophobic substituent, anthracene. This could reduce the coordinating ability of the amine or pyridine groups (thus making the binding of small external ligands more favourable) and favour the release of protons from coordinated ligands $(H_2O)^{23}$ and the formation of species with a low (or zero) overall charge.

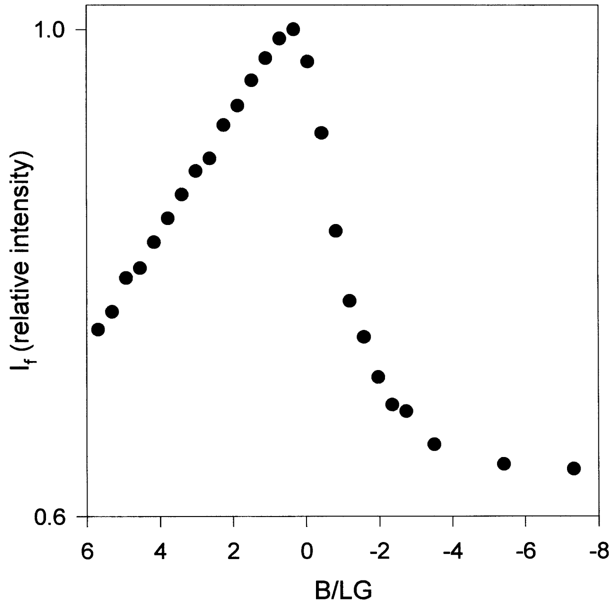


**Fig. 3** Relative fluorescence intensity for 8 : 2 (v/v) water–acetonitrile solutions containing **L**, zinc perchlorate (1 : 1 molar ratio) and an excess of perchloric acid, as a function of B/LG. B/LG = (mol $H^+$ − mol $OH^-$)/mol **L** (negative values indicate an excess of base)

Finally, it is noteworthy that comparison of the $I_f$ *vs.* pH profiles for ligand **L** alone and in the presence of one molar equivalent of $Zn^{2+}$ shows how an 'off-on-off' profile may be obtained in both cases, but that the presence of the zinc cation can help to fine-tune the position of the fluorescence window, as it shifts the value at which $I_f$ reaches its maximum value from pH = 5.9 to pH = 5.3.

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21 The identification of the species really present in solution (and calculation of the relative formation constants) is based on the fitting between experimental emf data and emf values calculated for each titration point on the basis of a hypothesized set of species. When the **L**–Zn–$H^+$–$OH^-$ system is taken into consideration, the hypothesized set of species must reasonably be much larger than in the case of the simple **L**–$H^+$ system. Thus, uncertainty of the experimental emf values at the edges of the considered pH range (due to the presence of $CH_3CN$) could make it impossible to calculate formation constants for this more complex equilibria system.
22 This is confirmed by independent spectrophotometric experiments. In an 8 : 2 water–acetonitrile solution, containing **L** at the pH value corresponding to B/LG = 0 and to the $I_f$ maximum for the

**L**–$Zn^{2+}$ system ($pH = 5.3$, maintained through the experiment with microadditions of standard base), $Zn^{2+}$ was added (as an aqueous solution of its perchlorate salt) and the complexation process followed by observing the variations of the amine charge transfer bands in the UV region. What was observed was a process with a sharp endpoint at a 1 : 1 **L** : $Zn^{2+}$ molar ratio.

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