

Fluorescence of a Benzothienopyridopyrimidone in Solution and in Lipid Vesicles

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Fluorescence properties of a biologically active benzothienopyridopyrimidone in solution and in lipid vesicles are reported. Assays at different pH values (0.5–10) allowed the determination of $pK_a = 2.0$, showing that this compound may be useful as a pH indicator for $pH \leq 4$. In lipid vesicles, benzothienopyridopyrimidone locates in a water-rich environment, indicating that it can be carried in the hydrophilic region of liposomes for drug delivery applications.

KEY WORDS: Benzothienopyridopyrimidone; pH probe; Lipid vesicles.

INTRODUCTION

Natural tetracyclic heteroaromatic planar compounds, like ellipticine and olivacine (derivatives of carbazoles and pyridines), are known as anti-tumor compounds. Synthetic analogues are being evaluated in order to obtain more active and less toxic compounds, and they are also being studied for other types of biological activity.

A new tetracyclic benzothienopyridopyrimidone **1** (Fig. 1) was obtained by us [1], and it was subjected to some biological activity studies. Preliminary studies of antimicrobial activity evidenced selectivity against *Bacillus cereus* (gram-positive) among representative bacteria and antifungal activity against a *Candida albicans* species [1]. Recent studies on the antiproliferative activity of compound **1** in a panel of human tumor cell lines showed a remarkable cytotoxicity upon UVA irradiation [2].

In previous DNA intercalation studies, compound **1** has shown a low affinity toward the macromolecule [2] and, therefore, we turned our attention to the study of its interaction with lipid membranes. Here we report

fluorescence studies on benzothienopyridopyrimidone **1** in homogeneous solution, varying the solvent and pH, and the fluorescence properties of compound **1** when incorporated in lipid vesicles of neutral phospholipids (DPPC and DOPE) and of a cationic lipid (DODAB). The neutral phospholipids DPPC and DOPE are major components of biological membranes, while cationic liposomes based in DODAB have been used as vehicles for DNA transfection and drug delivery [3–5].

EXPERIMENTAL

Materials and Methods

All the solutions were prepared using spectroscopic grade solvents and ultrapure water (Milli-Q grade). Buffer solutions of variable pH were prepared from a mixed solution of 0.2 M boric acid/0.05 M citric acid and a 0.1 M solution of sodium phosphate, as described by Perrin and Dempsey [6]. All buffers were adjusted in the pH range 0.5–10 by addition of HCl or NaOH solution.

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanola-

ABBREVIATIONS: DPPC, dipalmitoyl phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; DODAB, dioctadecyldimethylammonium bromide.

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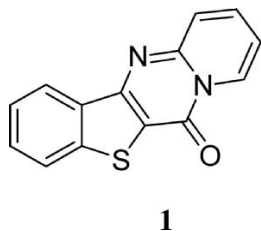


Fig. 1. Structure of the benzothienopyridopyrimidone.

mine (DOPE), both from Sigma, and dioctadecyldimethylammonium bromide (DODAB), from Tokyo Kasei, were used as received (lipid structures are shown below). Lipid/probe films were prepared from stock solutions in chloroform, by evaporation of solvent under a nitrogen stream. Vesicles were formed by hydration of lipids with an aqueous buffer solution (20 mM HEPES and 10 mM NaCl), at room temperature for DOPE and at 60°C for DODAB and DPPC (above transition temperature of both lipids, *ca.* 45°C [7] and 41°C [8], respectively), followed by bath-sonication to obtain optically clear solutions [7,9]. The final lipid concentration was 1 mM, with a probe/lipid molar ratio of 1:500. The vesicle solutions were cooled to room temperature and stored for 24 hr prior to measurements.

Spectroscopic Measurements

Absorption spectra were recorded in a Shimadzu UV-3101PC UV-Vis-NIR spectrophotometer. Fluorescence

measurements were performed using a Spex Fluorolog 2 spectrofluorimeter. For fluorescence quantum yield determination, the solutions were previously bubbled for 20 min with ultrapure nitrogen. Fluorescence spectra were corrected for the instrumental response of the system.

The fluorescence quantum yields (Φ_s) were determined using the standard method (Eq. 1) [10,11]. 9,10-diphenylanthracene in ethanol was used as reference, $\Phi_r = 0.95$ [12].

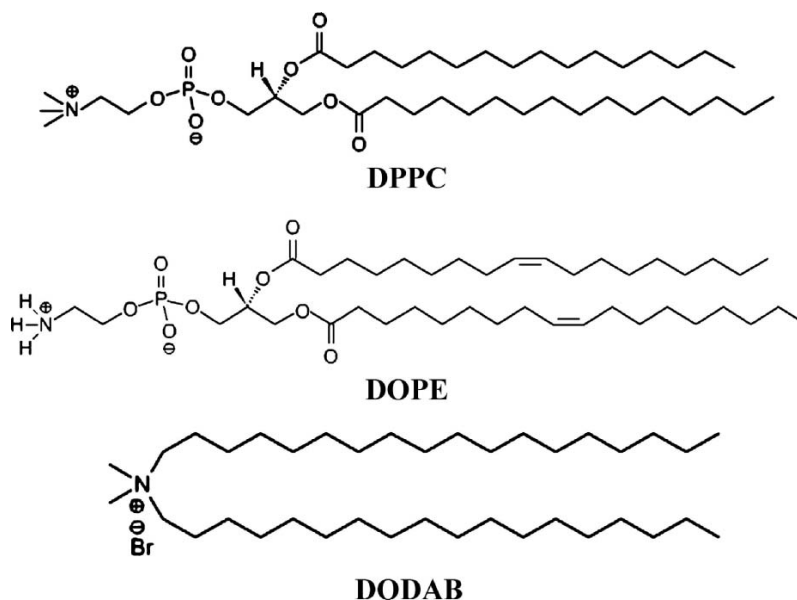
$$\Phi_s = \left[\frac{A_r F_s n_s^2}{A_s F_r n_r^2} \right] \Phi_r \quad (1)$$

where A is the absorbance at the excitation wavelength, F the integrated emission area and n the refractive index of the solvents used. Subscripts refer to the reference (r) or sample (s) compound.

RESULTS AND DISCUSSION

Absorption spectra of benzothienopyridopyrimidone **1** in different solvents are shown in Fig. 2. It can be observed that the absorption maxima shift to higher energies (blue shift) with the increase of solvent polarity, thus indicating that a $n \rightarrow \pi^*$ transition is involved [13]. Despite this, the molar extinction coefficients at absorption maxima (366–370 nm) are typical of a $\pi \rightarrow \pi^*$ transition (ϵ values are close to $10^4 \text{ M}^{-1} \text{ cm}^{-1}$), suggesting that the two electronic states are nearby in energy, resulting in state-mixing [14].

Figure 3 shows the fluorescence spectra of compound **1** in several solvents. A loss of vibrational structure is



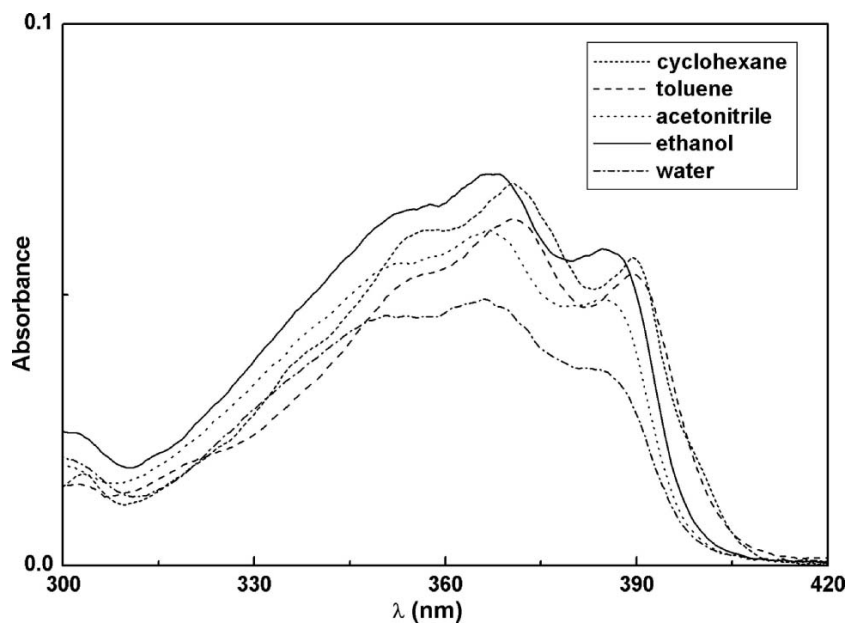


Fig. 2. Absorption spectra of compound 1 (4×10^{-6} M) in several solvents.

observed in polar organic solvents, which is a common behaviour in several solvatochromic probes [15,16]. However, in our case, the emission maximum exhibits only a small solvent dependence. This weak dependence on solvent polarity is also observed in the fluorescence quantum yields (Table I). The presence of the S atom in the

compound structure can promote the intersystem crossing process by enhancement of spin-orbit coupling interaction [14], justifying the low fluorescence quantum yields observed.

The spectrum in water is different in shape and the emission maximum wavelength exhibits a blue shift

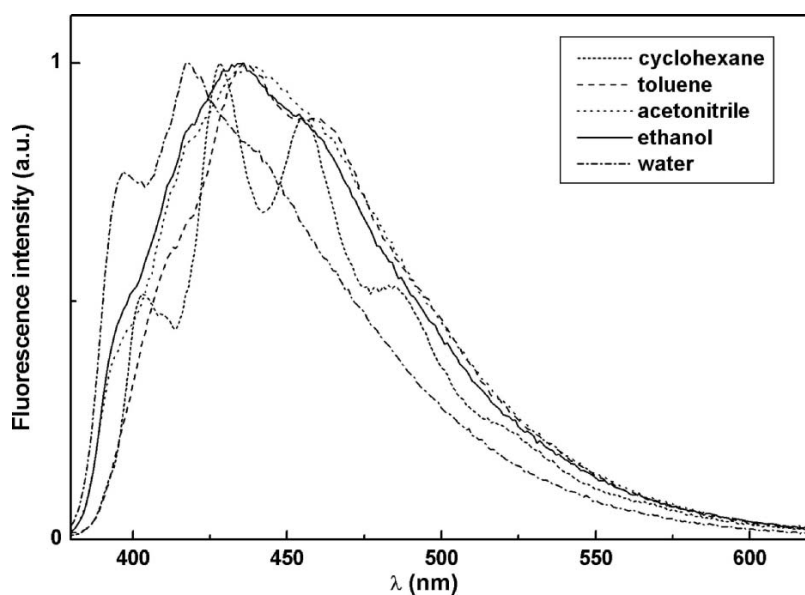


Fig. 3. Normalized fluorescence spectra of benzothienopyridopyrimidone 1 (4×10^{-6} M) in different solvents ($\lambda_{exc} = 368$ nm).

Table I. Fluorescence Quantum yields (Φ_F) and maximum Absorption and Emission Wavelengths (λ_{abs} and λ_{em}) for Benzothienopyridopyrimidone in different solvents

Solvent	λ_{abs} (nm)	λ_{em} (nm)	Φ_F^a
Cyclohexane	370	428	0.12
Toluene	370	437	0.18
Dichloromethane	369	436	0.13
Dimethylsulfoxide	368	441	0.16
Acetonitrile	367	437	0.13
Ethanol	367	436	0.12
Methanol	366	435	0.12
Water	366	418	0.14

^aRelative to 9,10-diphenylanthracene in ethanol ($\Phi_F = 0.95$ [12]).

relative to the other solvents. One possible explanation for this behaviour could be the formation of a hydrogen bond between water and compound **1** in ground state, which becomes weaker in the excited state. The possibility of compound aggregation in water was ruled out from the measurement of emission at several concentrations (5×10^{-6} M, 10^{-6} M and 5×10^{-7} M), the spectral shape being maintained.

The dependence on pH of the benzothienopyridopyrimidone **1** emission was also investigated (Fig. 4). The variation of fluorescence intensity at 418 nm with pH (inset of Fig. 4) indicates that our compound can be used as a pH indicator, sensitive to $\text{pH} \leq 4$. The protonation of

compound **1** at low pH is responsible for this behaviour. At $\text{pH} = 2$ the emission from two different species is clearly observed and spectral changes relative to $\text{pH} = 7$ can also be detected in the excitation spectrum (Fig. 5).

The $\text{p}K_a$ value of a compound can be determined using Eq. (2),

$$\text{pH} = \text{p}K_a + \log \frac{I - I_A}{I_B - I} \quad (2)$$

where I is the fluorescence intensity at a given wavelength, I_A and I_B are the fluorescence intensities (at the same wavelength) when the compound is only in the acidic form (I_A) or only in the basic form (I_B). As usually recommended, it is preferable to determine the ratio $R = I(\lambda_1)/I(\lambda_2)$ of the fluorescence intensities measured at two wavelengths (λ_1 and λ_2), applying Eq. (3) as a modified Eq. (2) [15],

$$\text{pH} = \text{p}K_a + \log \frac{R - R_A}{R_B - R} + \log \frac{I_A(\lambda_2)}{I_B(\lambda_2)} \quad (3)$$

where R_A and R_B are the R values when only the acidic form or the basic form is present.

In order to determine the $\text{p}K_a$ value of benzothienopyridopyrimidone **1**, the fluorescence measurements were extended to lower pH values (Fig. 6).

From Fig. 6, the emission maxima of 418 nm and 465 nm were chosen for λ_1 and λ_2 . The values of I_A and R_A were obtained from the spectrum at $\text{pH} = 0.5$,

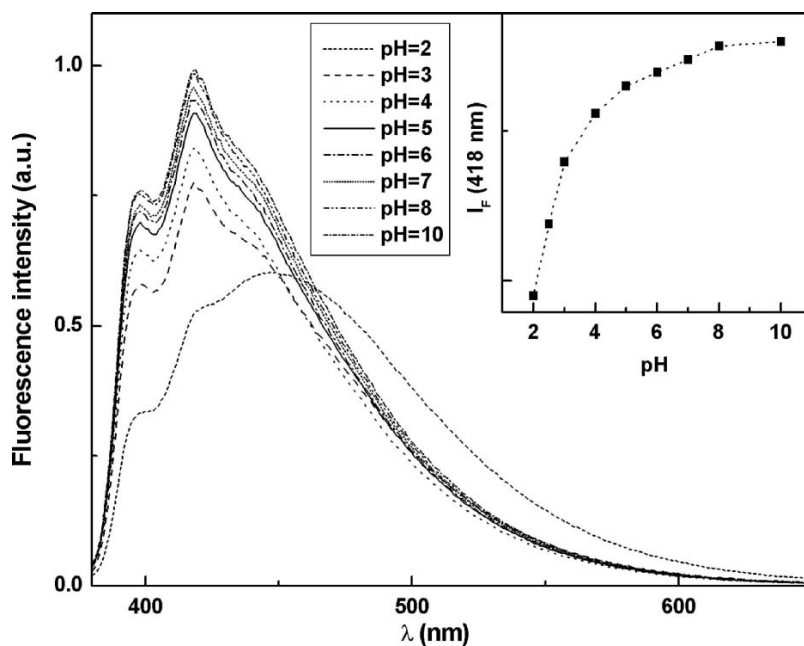


Fig. 4. Fluorescence spectra of benzothienopyridopyrimidone **1** (10^{-6} M) at $\text{pH} 2\text{--}10$ ($\lambda_{\text{exc}} = 368$ nm). Inset shows the variation of fluorescence intensity with pH ($\lambda = 418$ nm).

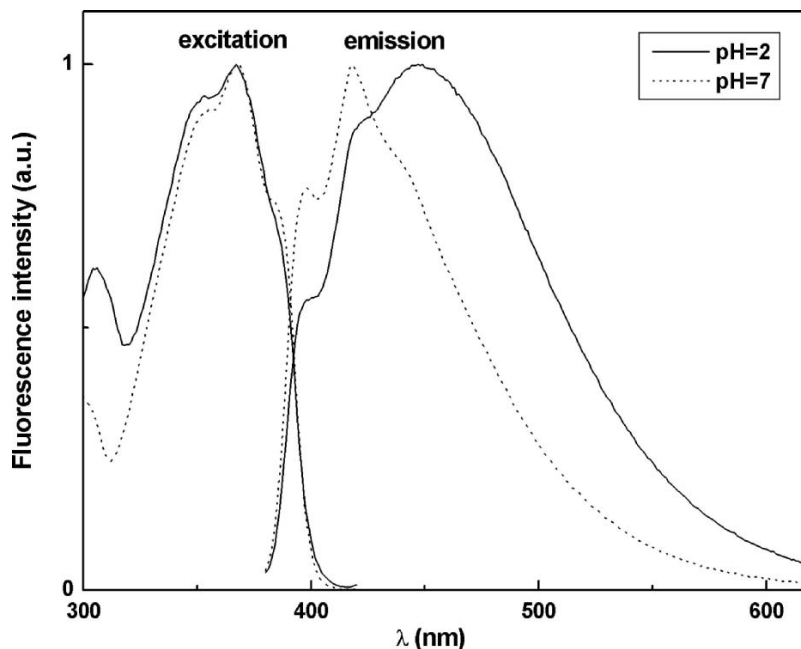


Fig. 5. Normalized excitation ($\lambda_{em} = 430$ nm) and emission ($\lambda_{exc} = 368$ nm) spectra of compound **1** at pH = 2 and pH = 7.

while I_B and R_B were taken at pH = 10 (Figs. 4 and 6). The fit of Eq. (3) to our experimental data (inset of Fig. 6) allows the determination of $pK_a = 2.0$ for compound **1**. The usual working range for a pH indicator is about 2 pH

units around the pK_a value [15], therefore our compound can be considered a pH probe for $pH \leq 4$.

In lipid vesicles (Fig. 7), the emission spectra of benzothienopyridopyrimidone **1** are very similar to the

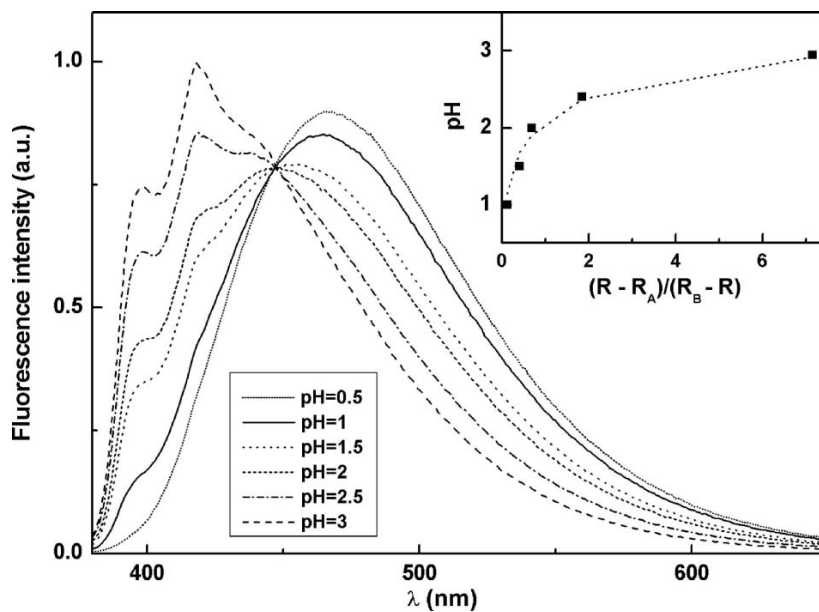


Fig. 6. Fluorescence spectra of benzothienopyridopyrimidone **1** (10^{-6} M) at pH 0.5–3 ($\lambda_{exc} = 368$ nm). Inset shows the fit (dotted line) of Eq. (3) to experimental data.

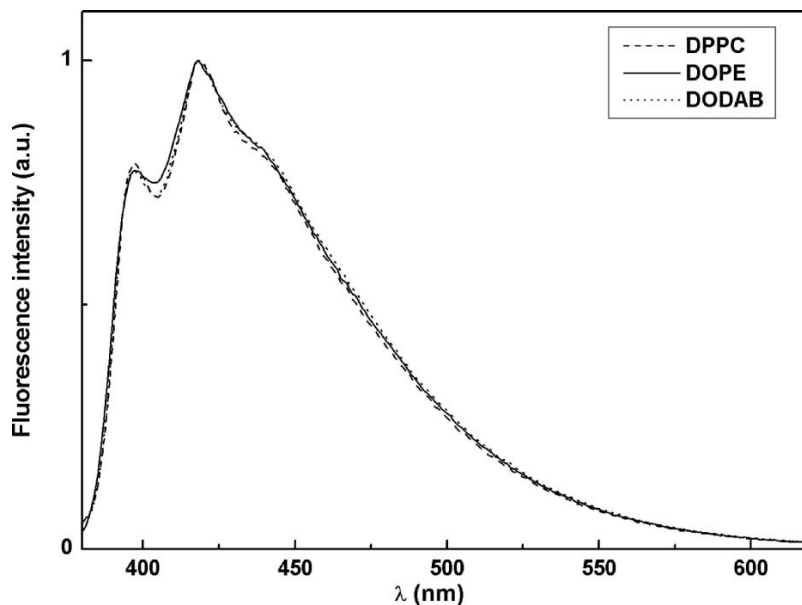


Fig. 7. Normalized fluorescence spectra of compound **1** (2×10^{-6} M) in lipid vesicles of DOPE, DPPC and DODAB ($\lambda_{\text{exc}} = 368$ nm).

emission spectrum in water (Fig. 3) and are also almost insensitive to the lipid type molecule of the vesicles. This behaviour indicates that the compound is in a water-rich environment. To clarify the location of the compound, the vesicle solutions were passed through filters of $0.1 \mu\text{m}$ diameter (Millex). The fluorescence emission of the filtered solutions was negligible, indicating that the compound is in the vesicle aqueous interior or located at the interface, near the polar lipid head groups. These studies may be important for the incorporation of our compound in liposomes for controlled drug release assays.

CONCLUSIONS

Fluorescence studies on a benzothienopyridopyrimidone show that it may be useful as a pH indicator for $\text{pH} \leq 4$. Its fluorescence in lipid vesicles shows that the compound prefers a water-rich environment, indicating that it can be incorporated in the hydrophilic region of liposomes for controlled drug release studies. These studies may be important to develop its use as an antimicrobial agent or as an anti-tumoral drug.

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