Dyes and Pigments 83 (2009) 334–338

Contents lists available at ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

A water-soluble D–π–A chromophore based on dipicolinic acid: Synthesis, pH-dependent spectral properties and two-photon fluorescence cell imaging

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ARTICLE INFO

Article history:
Received 7 April 2009
Received in revised form
18 May 2009
Accepted 20 May 2009
Available online 2 June 2009

Keywords:
Dipicolinic acid
pH sensor
Spectroscopic properties
Two-photon absorption
Fluorescence imaging
Water-soluble

ABSTRACT

The presence of two carboxylic acid groups and two hydroxyl groups within the novel dye, (E)-4-(4-bis(2-hydroxyethyl)amino)styryl)pyridine-2,6-dicarboxylic acid, resulted in good water solubility. The compound's UV absorption and photoluminescence spectra were each highly pH sensitive in acidic solutions and two isobestic points were observed. The TPA cross-section coefficient and two-photon fluorescence cell imaging of the dye were investigated.

1. Introduction

The determination of pH is of great importance because it usually plays a crucial role in a variety of systems. There are many methods for pH measurement at present [1–8]. Of the several methods available to determine pH, optical methods have several advantages including rapid response time, high signal-to-noise ratio and excellent pH-sensitivity [9].

Nonlinear excitation of fluorophores is intensively used in recent years in microscopy techniques for biological applications because of several advantages [10–15]. The excitation and fluorescence emission are confined to the focal point because of the squared power dependence of the absorption with the light intensity. Excitation wavelengths are shifted to the near-infrared region, avoiding limitations of UV lasers and optics, and providing a deep penetration necessary to imagine thick biological specimens. There is no background, because Rayleigh and Raman scattering occur at much longer wavelength than the collected fluorescence, and the out-of-plane excitation of chromophores is not possible. Also, out-of-focus photobleaching is eliminated. For a decade, chromophores have been specially optimized for such applications with a high σ_2 value and good water solubility [1,16–18].

Pyridine-2,6-dicarboxylic (dipicolinic) acid and its derivatives are highly useful tridentate ligands. They form nine-coordinate luminescent complexes with lanthanides, providing high quantum yields of phosphorescence and are used as luminescent tags, labels, and barcodes [19–22]. We have previously reported a series of D–π–A chromophores containing dipicolinates [23]. Among them, compounds PASPD (trans-dimethyl-4-[4′-(N,N-diphenylamino)-styry1]-pyridin-2,6-dicarboxylate) and MASPD (trans-dimethyl-4-[4′-(N,N-dimethylamino)-styry1]-pyridin-2,6- dicarboxylate) show strong two-photon absorption. However, they are hydrophobic, thereby limiting their applications in aqueous biological environments. To solubilize MASPD into water, acetyloxyethyl groups instead of methyls were introduced at the nitrogen atom in the aniline fragment to be hydrolyzed as a hydrophilic group in the later stage of the synthesis and two carboxylic acid groups were introduced by hydrolysis of the two methoxycarbonyl groups in pyridine ring. The synthetic route to target **6** is shown in Scheme 1. As expected, compound **6** exhibits good water solubility and strong two-photon absorption. Compound **6** also exhibited absorption and PL pH sensitivity. Therefore, the potential of **6** as a pH indicator and the two-photon fluorescence (2 PF) biological imaging property were investigated and are reported herein.

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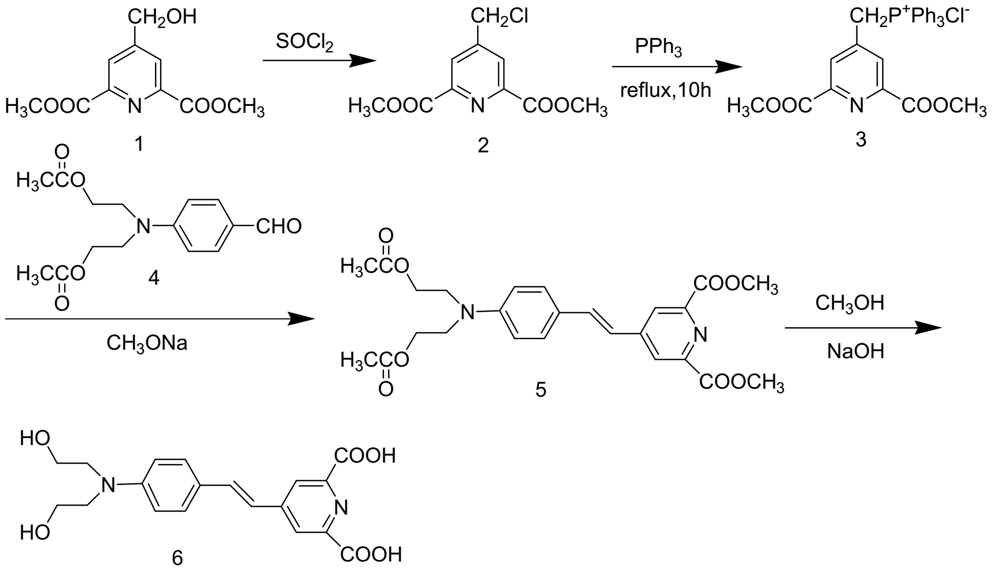
doi:10.1016/j.dyepig.2009.05.012



Scheme 1. Synthesis of compound **6**.

2. Experimental

2.1. Instruments and reagents

Melting points were determined with an XT4A apparatus and are uncorrected. Mass spectral studies were carried out using VG12-250 mass spectrometer. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker DRX 400 MHz spectrometer. Chemical shifts were reported in ppm relative to a Me_4Si standard. IR spectra were obtained on a Nicolet Avatar 370 DTGS spectrometer. Elemental analyses were performed by Atlantic Microlab. Steady-state emission and excitation spectra were recorded on Perkin Elmer LS55 instrument. Visible absorption spectra were determined on Perkin Elmer Lambda 35 spectrophotometer. All the pH values were measured with a model PHS-3C meter (Shanghai Leici Equipment Factory, China). A Ti:sapphire mode-locked fs laser system with regenerative amplifier (Spectra-Physics) was employed to investigate the TPA properties. The open-aperture Z-scan technique was used to measure the TPA cross-section. In our measurements, the laser beam with the pulse energy about 0.75 μJ was focused by a lens of 10 cm focal length on the solution filled in a 1 mm cell, and the transmitted light behind the solution was detected by a photodiode connected to a Lock-in amplifier. Two-photon excited luminescence images were taken on an Ultimal IV confocal microscope equipped with a femtosecond Ti:sapphire laser.

All reagents and solvents were commercial reagents of analytical grade and were used as received. Further purification and drying by standard methods were employed and they were distilled prior to use when necessary. Silica gel (200–300 meshes) was used for column chromatography. The pH titration was run in water. In a typical experiment, a solution of the dye containing small amounts of 1 M HCl was prepared. Diluted NaOH solution was added to achieve the appropriate pH change. The overall volume change did not exceed 2%.

2.2. Synthesis

2.2.1. 4-(chloromethyl)pyridine-2,6-dicarboxylate (**2**)

To a solution of compound 1 (0.5 g, 2.20 mmol) in anhydrous $CHCl_3$ (5 mL) and sulfuryl dichloride [ed. Note: toxic; highly corrosive; reacts violently with water; incompatible with acids, alcohols, bases, metals, amines, moisture] (0.2 mL, 2.82 mmol) was added dropwise under nitrogen atmosphere at −5 °C with continuous stirring for up to 40 min. Excess solvent was removed under reduced pressure and the crude product was purified by recrystallization from ethanol to give the solid **2** 0.4 g (1.65 mmol) after drying in vacuum. Yield: 75%; m.p. 168–170 °C. IR (KBr), ν/cm^{-1}: 3079, 2959, 2836, 1725, 1710, 1380, 1257, 1125, 798. EI-MS: *m*/*z* (M^+) 243. ^{1}H NMR (400 MHz, $CDCl_3$), δ: 8.27(s, 2H, Py–H), 4.45 (s, 2H, $-CH_2Cl$), 4.06 (s, 6H, $-OCH_3$).

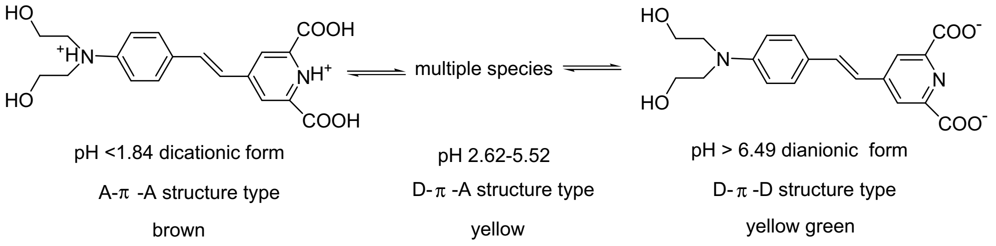
2.2.2. (E)-dimethyl 4-(4-(bis(2-acetoxyethyl)amino)styryl)pyridine-2,6-dicarboxylate (**5**)

A solution of triphenylphosphine [ed. Note: toxic; incompatible with oxidizing agents and acids] (0.5 g, 1.91 mmol) and compound **2** (0.3 g, 1.24 mmol) in benzene (10 mL) was refluxed for 10 h, then cooled to the room temperature. The reaction mixture was filtered to obtain the colourless solid **3** 0.21 g (0.415 mmol). Yield:34%.

To the absolute methanol (2 mL) solution of compound **3** (0.094 g, 0.186 mmol) and compound **4** (0.05 g, 0.171 mmol), 2 mL absolute methanol solution of sodium methanolate (12 mg, 0.222 mmol) was added dropwise and stirred up to 6 h under nitrogen atmosphere at −5 °C. The solution was concentrated under reduced pressure and a whitish solid **5** 0.06 g (0.124 mmol) was obtained by chromatography eluting from ethyl acetate and petroleum ether (1:5, v/v). Yield: 73%; m.p. 250 °C. EI-MS: *m*/*z* 484 (M^+). ^{1}H NMR ($CDCl_3$, δ ppm): 8.35 (s, 2H, Py–H), 7.50 (d, 2H, $J = 8.4$ Hz, Ph–H), 7.10 (d, 1H, $J = 8.4$ Hz, C=CH), 6.80 (d, 2H, $J = 8.8$ Hz, Ph–H), 6.60(d, 1H, $J = 8.4$ Hz, C=CH), 4.30 (m, 4H,–CH_2OAc), 4.00(s, 6H, $-OCH_3$), 3.70 (m, 4H, $-NCH_2$), 2.10(s, 6H, $-OCOCH_3$). ^{13}C NMR ($CDCl_3$, δ ppm): 171.15, 165.75, 165.48, 149.56, 148.72, 147.50, 135.89, 129.35, 127.94, 124.57, 122.37, 112.25, 111.88, 61.42, 53.36, 49.80, 21.11. Calculated for $C_{25}H_{28}N_2O_8$: C, 61.97; H, 5.83; N, 5.78. Found: C, 61.88; H, 5.85; N, 5.76.

2.2.3. (E)-4-(4-bis(2-hydroxyethyl)amino)styryl)pyridine-2,6-dicarboxylic acid (**6**)

0.021 g compound 5 (0.0433 mmol) and 10 mg NaOH were added to 10 mL methanol and the mixture was stirred at room temperature for 4 h. The solvent was then removed under reduced



Scheme 2. The acid–base equilibrium of **6** in aqueous media.

pressure and 0.0155 g of a whitish solid **6** (0.0417 mmol) was obtained by 732 cation resin column eluting from water. Yield: 96%; m.p. 250 °C. EI-MS: *m*/*z* 372(M^+). ^1H NMR($CDCl_3$, δ ppm): 8.35 (s, 2H), 7.4–7.5(m, 3H, ArH, CH=CH), 6.7–7.0(m, 3H, ArH, CH=CH), 3.6–3.9(m, 8H, $-NCH_2CH_2O-$). ^{13}C NMR(DMSO-d_6, δ ppm): 158.31, 154.69, 153.89, 140.59, 134.41, 129.61, 129.14, 126.41, 120.16, 116.42, 62.65, 58.49. Calculated for $C_{19}H_{20}N_2O_6$: C, 61.28; H, 5.41; N, 7.52. Found: C, 61.36; H, 5.40; N, 7.54.

3. Results and discussion

3.1. Synthesis

Compound **1** was prepared starting from 2,6-dimethylpyridin according to the literature method [22,24]. Converting the hydroxyl group in **1** into chloride afforded precursor **3** that was easily transformed to phosphonium salt. Compound **4** was synthesized in three steps starting from aniline. Subsequent Wittig reaction of **4** with phosphonium salt **3**, using CH_3ONa as a base, gave product **5**. The hydrolysis of compound **5** afforded the final product **6** in good yield. Compounds were characterized by NMR, IR, MS and EA.

3.2. Absorption and emission spectra

Compound **6** exists, in aqueous solution, under different forms, i.e., dicationic, neutral, dianionic species (Scheme 2), making its absorption and fluorescence properties strongly pH-dependent. The sensitivity of **6** towards the pH of the medium is also accompanied by a fast and reversible change in colour from brown to yellow green (Fig. 1) [25].

The absorption and emission spectra of **6** in acidic solutions are shown in Fig. 2 and Fig. 3, respectively. It is known that the basicity of the diethylaminobenzene nitrogen is significantly higher than that of pyridine-2,6-dicarboxylic (dipicolinic) acid nitrogen.[1] But in the low pH, two nitrogen atoms in the molecules of the dye can be protonated (Scheme 2). In pH 1.09 solution, **6** under its dicationic form shows one absorption peak at 338 nm. With the increase in pH, the absorption peak at 338 nm becomes weak, and one absorption peak around 500 nm becomes stronger and finally it becomes the major band at pH 2.62. When the pH is increased from 3.66 to 5.52, the absorption maximum is blue shifted gradually. In pH 2.62–5.52 solutions, the change in the UV spectra of **6** indicates that complex ionization processes and multiple species (i.e., neutral form, cationic and anionic forms) are present. Note that compound **6** gave almost the same absorption spectra (absorption maximum at 411 nm) in pH 6.49–9.64 solutions. The result indicates that the same species (dianionic form) is present in these pH solutions. We can conclude that the absorption maximum at 411 nm is due to the dianionic form. From Fig. 2, we found that the absorption spectra present two isobestic points (at around 385 nm and around 423 nm) [26,27]. The UV pH-sensitivity is apparently due to the carboxyl and diethylamino groups of compound **6**. The protonated diethylamino group and CO_2H are electron-withdrawing groups whereas CO_2^- is electron-donating group [28], which account for the significant difference in the absorption maxima of their respective species. The structure types of dicationic and dianionic forms are A–π–A and D–π–D, respectively (Scheme 2). The absorption maximum at 500 nm can be attributed a D–π–A structure type, where the absorption wavelength is predictably shifted to longer wavelength [1,29–31].

The evolution of emission spectra of **6** with the increase of pH follows a similar tendency to that of the UV spectra. In acidic solutions (pH 1.09–2.62), excitation of **6** at 335 nm leads to an

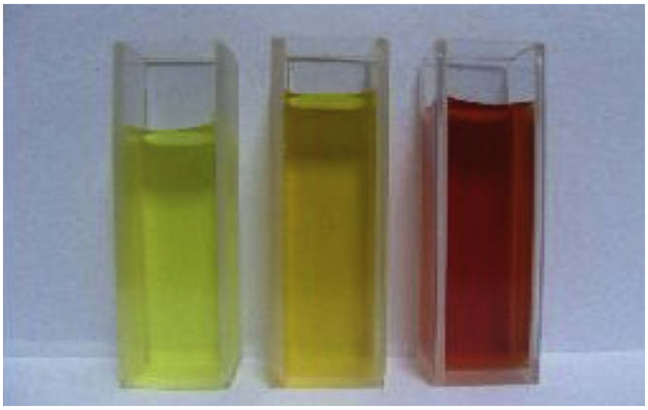


Fig. 1. Photograph of compound **6** at pH 8.24, 5.48, 2.46 respectively (from left to right).

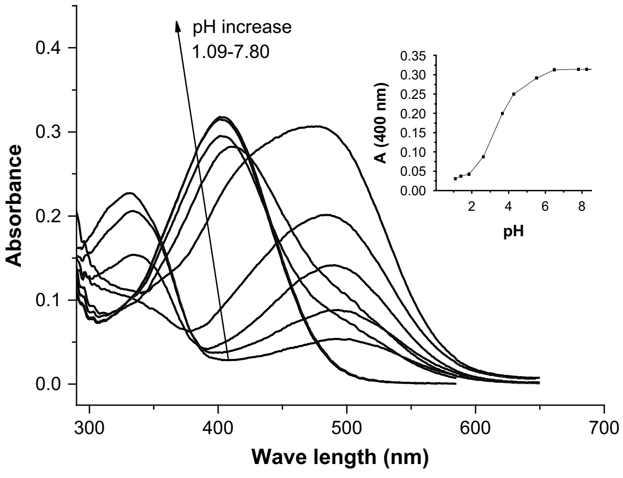


Fig. 2. The UV spectral changes of **6** (1×10^{-5} M) in different aqueous pH solutions. From bottom to top: 1.09, 1.39, 1.84, 2.62, 3.66, 4.48, 5.52, 6.49, 7.80. Inset: The absorbance of λ_{400} as a function of pH.

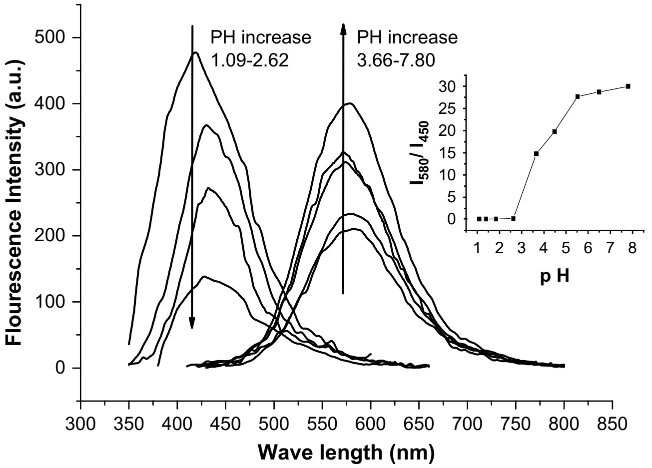


Fig. 3. The PL spectral changes of **6** (1×10^{-5} M) in different aqueous pH solutions. Inset: The intensity ratio of 580 and 450 nm as a function of solution pH.

emission band centred at around 430 nm. However, when the pH reached 3.66, a new emission band appeared in the range of 500–700 nm (excitation at 400 nm). The emission maximum is blue shifted gradually and the PL intensity increases with an increase of pH from 3.66 to 9.64. In acidic solutions, an analysis of I_{580}/I_{450} (Fig. 3, inset) versus pH shows a similar trend to that of A_{400} (Fig. 2, inset) [28].

3.3. *Two-photon fluorescence cell imaging*

The TPA cross sections of molecule **6** have been measured by open-aperture Z-scan experiments performed with a femtosecond (fs) laser source. Fig. 4 shows the Z-scan data of dyes **6** in water (pH = 7.0), measured in a 1 mm cell, with 0.749 μJ pulse. The TPA cross-section coefficient of **6** is 38 GM.

Two-photon scanning microscopy experiments were carried out using leukemic cells fixed with ethanol and loaded with **6** in PBS (phosphate buffered saline) solution. Compound **6** possess a remarkably high water solubility. It was accordingly possible to directly employ PBS water solution of the dyes without the use of any solubilizing agent such as CrEL (polyoxyethylene castor oil) [30]. From Fig. 5, it was clear the dye had good permeativity and dispersed well in the cytosol. In addition, bright spots are observed in the nucleus, indicating that the compound preferentially targeted small organelles called nucleoli. This promising result implies that **6** may also serve as a precursor of 2 PF amine reactive probes that can be readily activated by esterification of the carboxylic acid group using succinimide hydroxide [1].

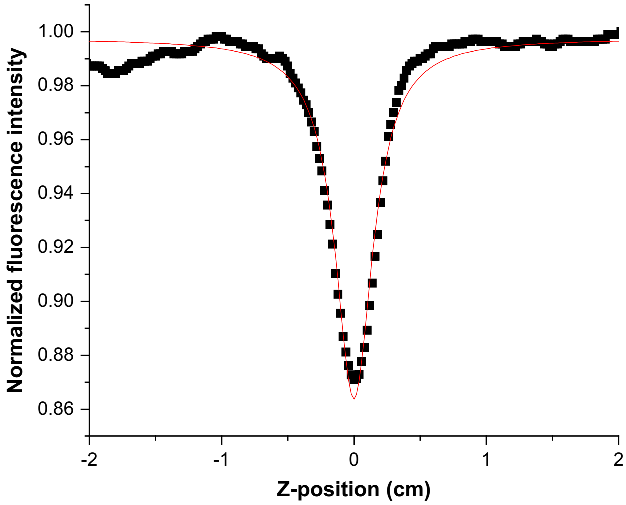


Fig. 4. Z-scan experimental data of compounds **6** in water (0.0095 M).

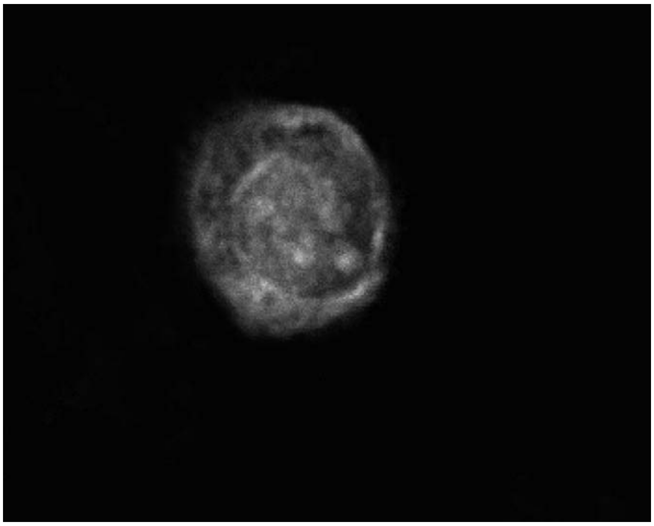


Fig. 5. Two-photon excited luminescence ($\lambda_{ex\ 800nm}$) image of leukemic cell fixed in ethanol and loaded with **6**. Images were taken on an Ultimal IV confocal microscope equipped with a femtosecond Ti:sapphire laser.

4. Conclusions

The novel, water-soluble **6** was synthesized and its absorption and fluorescence properties were investigated as a function of pH. The UV and photoluminescence spectra of compound **6** are highly sensitive in acidic solutions. Variation of pH from acidic to basic values leads to a modification of its colour. Compound **6** exhibits strong two-photon absorption and the TPA cross-section coefficient of **6** is 38 GM. According to the two-photon fluorescence cell imaging of compound **6**, it was found that compound **6** has good cell permeativity and has potential in 2 PF imaging techniques.

Acknowledgment

This work was financially supported by Innovation Program of Shanghai Municipal Education Commission (No. 09YZ160) and Shanghai Normal University (No. SK200839). Haibo Xiao thanks Prof. Fude Huang (Institute of neuroscience, The Chinese Academy of Sciences, Shanghai/China) for his help in the measurements of the two-photon fluorescence cell imaging.

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