



# From visible to far-red excitable chromophores derivatives of vitamin B6. Evaluation as pH-responsive probes and solvatochromic study



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## ABSTRACT

Owing to the great potential and application of pH probes in biological and environmental sciences, four aqueous soluble vitamin B6 derivatives **1–4** bearing visible excitable chromophores as coumarin, fluorescein, cresyl violet and nile blue were successfully synthesized and characterized. Compounds **2–4** are highly pH dependent revealed significant spectral changes in the ground and excited states, with exception of **1** whereas no changes in the excited state were visualized. In the same way, at pH 6–7 probes **1** and **3** exhibited the highest fluorescent quantum yield, with values of 0.65 and 0.51. Concerning the solvatochromic behaviour, a positive effect was witnessed for compound **4**. Moreover, in the excited state were observed different colours ranging from *red* (CH<sub>2</sub>Cl<sub>2</sub>) to *orange* (CHCl<sub>3</sub>, CH<sub>3</sub>CN), to *pink* (EtOH), to *green* (DMSO) and to *blue* (H<sub>2</sub>O). The dipole moments in the ground and excited states were determined for compound **4**, whereas values of  $\mu_{g,\text{imineE}} = 3.62$  D,  $\mu_{e,\text{imineE}} = 12.35$  D ( $\Delta\mu = 8.73$  D),  $\mu_{g,\text{imineZ}} = 3.38$  D and  $\mu_{e,\text{imineZ}} = 11.54$  D, ( $\Delta\mu = 8.16$  D), revealing that **4** is more polar in the excited state.

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## 1. Introduction

Fluorescent organic dyes, visible excitable have been target of several studies, mainly due to their wide use in biochemistry, cellular biology and imaging, clinical diagnosis and drug discovery [1–3]. However, one limitation of the synthesized organic dyes is their low aqueous solubility. In order to overcome this issue, several modifications in the molecular design, such as, introduction of aqueous soluble groups as amino acids, vitamins and other biological molecules, have been performed to increase the solubility [4,5].

Aqueous soluble organic dyes could penetrate in cells, exhibiting fluorescent changes with the pH or upon other analyte detection. Particularly, pH probes have a great potential in the diagnosis of many diseases, which are related with changes in the pH media [6]. More specifically, clinical inflammation [7], lung related-

pathologies [8], ischaemia [9], kidney diseases [10] and cancer [11] are diseases, which can be detected by changes in the pH media. For example, the interstitial pH of malignant tumours is lower than in normal tissues, being this parameter used for diagnosis and therapies [12].

On the other hand, fluorescence spectroscopy is a high sensitive technique, with very low detection limits, but *in vivo*, this emission could be highly reduced [13]. Thus, the research on the design of dyes that emit in the far-red (above 600–700 nm) has increased, because they have a low background, and far red light can penetrate deeper in living cells, facilitating the *in vivo* studies [14]. In this work are presented four compounds, **1–4**, derivatives from vitamin B6 bearing signalling units that emit from the UV–visible light to the far red light. The signalling units coumarin, fluorescein, cresyl violet and nile blue are employed.

Coumarins are in the class of benzopyrones and are known to exhibit remarkable anti-tumoral, anti-inflammatory and antiviral effects [15–18]. They also present high fluorescent quantum yields, and due to their great optical properties can be applied as laser dyes, nonlinear optical dyes and as fluorescent probes [19,20]. As an example, Liu et al. [21] published coumarin probes bearing the benzothiazole or indole units, which have optical responses in

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acid pH. Their studies revealed that these coumarin derivatives could be used for quantitative fluorescence imaging in the detection of intracellular pH by the formation of an intramolecular charge transfer (ICT) platform in acidic environments, leading to colour changes. In the same way, Zhu et al. [22] design a new ratiometric fluorescent pH coumarin–quinoline derivative probe, whereas in acidic pH exhibits a strong fluorescence, being very useful for monitoring pH variations from neutral to acidic conditions in living cells.

Fluorescein dyes have great photochemical properties, as high molar extinction coefficients, are pH dependent, whereas at basic pH, the fluorescein ring opens being strongly emissive, with high fluorescent quantum yield, and showing a very intense greenish-yellow colour [23,24]. E. Oliveira et al. [25] published a fluorescein derivative containing as building blocks the amino acid alanine, which it was pH dependent, and at pH 8 presented an unprecedented selectivity towards the  $\text{Hg}^{2+}$  toxic metal ion, with a colorimetric change from yellow to red, in aqueous media.

Concerning the Cresyl violet, it has been often used as staining agent, employed to study modifications in DNA and RNA rich compounds in tissues, because it can be easily distinguished due to their photophysical and photochemical properties. These cresyl violet properties are widely influenced by electrostatic, acid–base interaction in solution and hydrogen-bonds [26].

Furthermore, Nile blue is a phenoxazine dye fluorescent probe with many applications in therapeutic and scientific areas, due to their high stability and brightness [27,28]. Additionally, Nile blue derivatives are used in photodynamic therapy once have high affinity to cancerous cells than normal ones, and they are oxygen photosensitizers [29–31].

Therefore, Nile blue has been widely applied to monitor events that depend on solvent polarity, once in polar media its absorption and emission maxima shift to red, revealing a stabilized charge separation in the excited state [32,33]. However, one of the limitation of Nile blue is the low solubility in aqueous media [34,35]. Thus, it has been published several papers on Nile blue derivatives aiming their water solubility [36].

Herein, it is presented the synthesis of four vitamin B6 derivatives, **1–4**, bearing visible excitable chromophores, as coumarin, fluorescein, cresyl violet and Nile blue, respectively. The compounds **1–4** were characterized by  $^1\text{H}$  NMR, elemental analysis, infrared spectroscopy, MALDI-TOF-MS spectrometry, lifetime, UV–visible absorption and emission fluorescence spectroscopy in milliQ water. Their ability as potential pH responsive probes were also evaluated, as well as, the solvatochromic behaviour of **4** in different solvents.

## 2. Experimental

### 2.1. Chemicals and starting materials

NaOH (sodium hydroxide), HCl (Hydrochloric Acid), triethylamine have been purchased from Panreac. Pyridoxal 5'-phosphate hydrate (vitamin B<sub>6</sub>), 7-Amino-4-methyl-coumarin and 6-Amino fluorescein were from Sigma Aldrich. Cresyl Violet 670 perchlorate, Nile Blue 690 perchlorate were from Exciton. The water used in all the experiments was Milli-Q grade.

All chemicals were used without further purification.

### 2.2. Physical measurements

Elemental analyses were carried out on a Thermo Finnigan-CE Instruments, Flash EA 1112 model, CHNS series, and the  $^1\text{H}$  NMR in a Bruker Avance III 400 at an operating frequency of 400 MHz at REQUIMTE, Chemistry Department from Faculty of Sciences and

Technology of University NOVA of Lisbon. Infrared spectra were recorded in KBr windows using JASCO FT/IR-410 spectrophotometer. Lifetime measurements were recorded in TEMPRO Fluorescence Lifetime System, from HORIBA JOBIN YVON.

The MALDI-MS analyses have been performed in a MALDI-TOF-TOF-MS model Ultraflex II Bruker, Germany, equipped with nitrogen, from BIOSCOPE group, REQUIMTE, Chemistry Department from Faculty of Sciences and Technology of University NOVA of Lisbon.

The MALDI mass spectra of the soluble samples (1 or 2 mg/mL), such as the ligands were recorded using the conventional sample preparation method for MALDI-MS. 1  $\mu\text{L}$  of ligand was put on the sample holder. In all spots in the holder, a sinapic acid matrix was added. Afterthought the sample holder was inserted in the ion source.

### 2.3. Spectrophotometric and spectrofluorimetric measurements

UV/Vis absorption spectra were recorded with a JASCO V-650 spectrophotometer and a fluorescence emission by a HORIBA Scientific FLUOROMAX-4 spectrofluorimeter. The linearity of the fluorescence emission versus the concentration was checked out in the concentration used ( $10^{-4}$ – $10^{-6}$  M). A correction for the absorbed light was performed when necessary. The photophysical characterization was performed by a stock solution of the compounds **1–4** (ca.  $10^{-3}$  M) dissolving an appropriated amount of compound in a 10 mL volumetric flask and diluting it to the mark with milliQ water. The stock solutions were then diluted still  $10^{-5}$ – $10^{-6}$  M. In order to perform the solvatochromic studies compound **4** was dissolved in chloroform, dichloromethane, absolute ethanol, methanol, acetonitrile, dimethylsulfoxide and water. The spectroscopic polarity parameters, physical properties and polarity functions of the solvent used are indicated in Table 2.

All measurements were performed at 298 K. Luminescence quantum yields were measured using a solution of quinine sulfate in sulfuric acid (0.1 M) as standard [ $\phi_F = 0.54$ ] for compound **1**; a solution of fluorescein in ethanol [ $\phi_F = 0.79$ ] for compound **2**; a solution of cresyl violet in ethanol [ $\phi_F = 0.54$ ] for compound **3**; a solution of Nile blue in methanol [ $\phi_F = 0.27$ ] for compound **4** [37,38]. All solvents used were of highest purity from Merck.

### 2.4. Synthesis of compounds **1–4**

#### 2.4.1. General synthesis

7-Amino-4-methyl-coumarin (**B**) ( $0.040\text{ g}$ ,  $2.28 \times 10^{-4}\text{ mol}$ ) or 6-Amino fluorescein (**C**) ( $0.080\text{ g}$ ,  $2.30 \times 10^{-4}\text{ mol}$ ) was dissolved in absolute ethanol, followed by the addition of pyridoxal-5'-phosphate (**A**) ( $0.056\text{ g}$ ,  $2.28 \times 10^{-4}\text{ mol}$ ;  $0.057\text{ g}$ ,  $2.30 \times 10^{-4}\text{ mol}$ ). The solution was stirred and kept under refluxing for 4 h. At the end, light orange (compound **1**) and orange (compound **2**) powders were obtained, and were washed several times with cold ethanol, diethylether and dried under vacuum.

Cresyl violet 670 perchlorate (**D**) ( $0.067\text{ g}$ ,  $1.85 \times 10^{-4}\text{ mol}$ ) or Nile blue 690 perchlorate (**E**) ( $0.070\text{ g}$ ,  $1.67 \times 10^{-4}\text{ mol}$ ) was dissolved in absolute ethanol followed by the addition of 1 equivalent of triethylamine and kept under stirring for 30 min. Thereafter, pyridoxal-5'-phosphate ( $0.042\text{ g}$ ,  $1.85 \times 10^{-4}\text{ mol}$ ;  $0.046\text{ g}$ ,  $1.67 \times 10^{-4}\text{ mol}$ ) was added. The solution was stirred and kept under refluxing for 4 h. At the end the mixture was evaporated under reduce pressure, and further purified by precipitation with cold ethanol, diethylether, whereas at the end a green (compound **3**) and a dark blue powder (compound **4**) were obtained.

Compound **1**: Orange powder, yield 81%, FW = 422.3. Anal. Calc. For  $\text{C}_{18}\text{H}_{17}\text{N}_2\text{O}_7\cdot\text{P}\cdot\text{H}_2\text{O}$ : C, 51.2; H, 4.5; N, 6.6. Found: C, 50.9; H, 4.7; N, 6.5, %. IR(KBr  $\text{cm}^{-1}$ ):  $\nu$ , 1640 (C=N),  $^1\text{H}$  NMR ( $\text{D}_2\text{O}$ , 400 MHz):  $\delta_{\text{H}}$

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