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Synthesis, photophysical properties, and DNA-binding of novel A- π -D- π -A' two-photon absorption chromophores

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^b Department of Chemistry, Anhui Province Key Laboratory of Functional Inorganic Materials, Anhui University, Hefei 230039, PR China Graphical abstract

One of the novel A- π -D- π -A' compounds synthesized by us (**3PyB**) has good water solubility, once bound to ctDNA, the two-photon action cross-section can be increased to 45 GM, which may serve as a potential DNA fluorescent probe.

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Highlights

The highlights of this manuscript are as follows:

- 1. Three novel A- π -D- π -A' two-photon absorption chromophores were synthesized.
- 2. The photophysical properties in various solvents were systematically investigated.
- 3. The structure-property relationships were discussed aided with the DFT calculations.

4. The DNA-binding of **3PyB** was systematically investigated.

Abstract

Three novel two-photon absorption chromophores (**1BI**, **2TAZ**, and **3PyB**), which have *trans*-1,4-divinylbenzene π -bridge, methoxy donors, and different acceptors, were synthesized. Their structures were elucidated by means of hydrogen-1 nuclear magnetic resonance, carbon-13 nuclear magnetic resonance, Fourier transform infrared spectroscopy, and high-resolution mass spectrometry. Their photophysical properties, including linear absorption, single-photon excited fluorescence, two-photon absorption, and two-photon excited fluorescence, were systematically investigated in various solvents. The structure-property relationships were studied aided with the density functional theory calculations. In addition, the absorption, single- and two-photon fluorescence spectra of **3PyB**-ctDNA were examined to evaluate whether **3PyB** could be used as a DNA stain. The possible interaction mechanism of **3PyB**-ctDNA was also studied by the NaCl titration and the viscosity experiment. The results indicate that **3PyB** may serve as a potential DNA fluorescent probe.

Keywords: Two-photon absorption; DNA-binding; A- π -D- π -A' compound; Structure-property relationship

1. Introduction

In 1931, the concept of the two-photon absorption (TPA) process was first proposed by Göppert-Mayer^[1], which involves simultaneous absorption of two photons of identical or different frequencies and leads to excitation of a molecule from the ground state to the excited state. Since then, organic materials with TPA properties have developed rapidly and been applied in various research areas, such as two-photon photodynamic therapy^[2], three-dimensional optical data storage^[3], optical limiting^[4], up-converted lasing^[5], three-dimensional microfabrication^[6], and especially two-photon fluorescence microscopy and imaging^[7]. Recently, two-photon fluorescence microscopy and imaging has attracted significant interest as results of its unique advantages over conventional single-photon fluorescence microscopy and imaging, including near infrared photons as excitation source, imaging in the black background, avoidance of photodamage and photobleaching, high transverse and longitudinal resolutions, small absorption coefficient of light in tissue, lower tissue autofluorescence, etc^[8]. To achieve this application, materials must have large two-photon action cross-sections ($\Phi \times \delta$). The fluorescence quantum yield (Φ) gives the efficiency of the fluorescence process, which is defined as the ratio of the number of photons emitted to the number of photons absorbed^[9]. The TPA cross-section (δ) is defined as the probability for simultaneous absorption of two photons by an atom or a molecule to be excited from the ground state to an upper energy state^[1]. Φ and δ are the important parameters to characterize the fluorescence and TPA properties of materials. Consequently, it is significant and essential to develop TPA materials with

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large $\Phi \times \delta$.

The structure of materials is a decisive factor for their TPA properties and fluorescence properties. Only when the material can absorb light, it is possible to emit fluorescence, so the structure of fluorescent materials usually contains strong absorption groups with π -conjugated bonds^[10]. In addition, a variety of researches reveal that introduction of electron donors (D) and electron acceptors (A) to the π -conjugated system can increase the degree of intramolecular charge transfer (ICT) and improve the δ value^[11]. Therefore, in order to obtain TPA materials with large $\Phi \times \delta$, it is critical to select the appropriate π -conjugated bridge, D, and A. The *trans*-configuration of 1,4-divinylbenzene π -bridge has better planarity and conjugation effect than the *cis*-configuration, which favors the intramolecular charge delocalization. Methoxy, a relative strong electron-donating group, which has the lone pair electrons supplied by the oxygen atom, can form p- π conjugation by connecting with a benzene ring. The combination of the negative induction effect and the negative conjugation effect makes the cyano group be a strong electron-withdrawing group. Benzimidazole contains a benzene ring fused to an imidazole ring. The imidazole ring is a noncentrosymmetric electron-deficient structure, which may link to strong electron-donating groups and have excellent properties of electronic transmission. s-Triazine is a six-membered N-heterocycle, which possesses good coplanarity, good thermal stability, and strong electron affinity. Its derivatives exhibit good application prospects in many aspects of organic optoelectronic functional materials^[12]. Owing to its positive charge, pyridinium cation not only has strong electron-withdrawing ability but also can increase the water solubility of materials. Numerous researches demonstrate that many chromophores with the above-mentioned donors (methoxy^[13]) or acceptors (cyano^[14], benzimidazole^[15], s-triazine^[16], pyridinium cation^[17]) exhibit strong TPA and two-photon fluorescence properties. Therefore, in order to obtain TPA materials with large $\Phi \times \delta$, we designed and synthesized three novel asymmetric TPA chromophores with A- π -D- π -A' structure (1BI, 2TAZ, and 3PyB) (Fig. 1), which have trans-1,4-divinylbenzene as π -conjugated bridge, methoxy as D, cyano (benzimidazole, s-triazine, and pyridinium)

cation) as A. Their structures were characterized by ¹H NMR, ¹³C NMR, FT-IR, and HRMS. Their linear and nonlinear photophysical properties in various solvents were investigated. At the same time, the density functional theory (DFT) calculations were utilized to survey their structure-property relationships.

Among the three materials, **3PyB** has good water solubility, which is essential for the realization of two-photon fluorescence microscopy and imaging in living cells. Cells live in aqueous surroundings. The material that wants to cross the plasma membrane into the cell must have good water solubility. DNA is the hereditary information carrier for most living things, which plays an extremely important role in the life process of breeding, inheritance, and variation of organisms. DNA detection has become a hot topic for scientists of various subjects owing to its importance in gene sequencing, biomedical research, diagnosis for the virus and so on. In this paper, the linear absorption, single- and two-photon fluorescence spectra, and the interaction mechanism of **3PyB**-ctDNA were discussed to evaluate whether **3PyB** could be used as a fluorescent probe for DNA detection. The results show that **3PyB** can bind to DNA and has a moderate $\Phi \times \delta$, which is promising to be an effective two-photon DNA fluorescent probe.





Fig. 1. Molecular structures of the target compounds (1BI, 2TAZ, and 3PyB).

2. Experimental

2.1. Materials and instruments

Unless otherwise noted, all reagents and solvents were purchased from commercial suppliers and used without further purification. DNA titration experiments were performed in Tris-HCl buffer solution (10 mmol L⁻¹ Tris-HCl, 100 mmol L⁻¹ NaCl, pH 7.2). The concentration of ctDNA was estimated by measuring the UV absorbance at 260 nm ($\varepsilon = 6600 \text{ mol}^{-1} \text{ L cm}^{-1}$). All solutions were allowed to equilibrate for 5 min before making measurements.

Melting points were measured on an X-4 micromelting point apparatus without correction. ¹H and ¹³C NMR spectra were recorded on a Bruker AVANCE III 500 spectrometer using DMSO-*d*₆ or D₂O as a solvent and all shifts were referred to tetramethylsilane (TMS). FT-IR spectra were recorded on a Thermo Nicolet 6700 spectrometer using a powder sample on a KBr plate. Mass spectra were performed on a Thermo LCQ TM Deca XP plus ion trap mass spectrometry instrument.

The linear absorption spectra were measured on a Shimadzu UV-2550 UV-visible spectrophotometer. The single-photon excited fluorescence (SPEF) spectra measurements were performed using a RF-5301PC fluorescence spectrophotometer with the maximum absorption wavelengths as the excitation wavelengths. The two-photon excited fluorescence (TPEF) spectra were measured using a femtosecond Ti : sapphire laser system (Chameleon Ultra II, 680-1080 nm, 80 MHz, 140 fs).

2.2. Physical measurements

The Φ was determined using fluorescein in 0.1 mol L⁻¹ sodium hydroxide aqueous solution ($\Phi = 0.9^{[18]}$) as the standard according to the following equation:

$$\boldsymbol{\Phi}_{\rm s} = \boldsymbol{\Phi}_{\rm r} \frac{A_{\rm r}}{A_{\rm s}} \frac{n_{\rm s}^2}{n_{\rm r}^2} \frac{F_{\rm s}}{F_{\rm r}} \tag{1}$$

where the subscripts s and r designate the sample and the reference, respectively. A is the absorbance at the excitation wavelength, n is the refractive index of the relevant solution, and F is the integrated area under the corrected emission spectrum.

The δ was obtained by comparing the TPEF intensity of the sample with that of a reference compound by the following equation^[19]:

$$\delta_{\rm s} = \frac{F_{\rm s}}{F_{\rm r}} \frac{\Phi_{\rm r}}{\Phi_{\rm s}} \frac{n_{\rm r}}{n_{\rm s}} \frac{c_{\rm r}}{c_{\rm s}} \delta_{\rm r} \tag{2}$$

where the subscripts s and r denote the sample and the reference, respectively. F and Φ represent the TPEF integral intensity and the fluorescence quantum yield. n and c are the refractive index and the concentration of the solution.

The binding constant (k) was obtained by the single-photon fluorescence titration method according to the Scatchard equation^[20]:

$$\frac{r}{c_{\rm f}} = kn - kr \tag{3}$$

where *k* and *n* refer to the binding constant and the binding sites, respectively. $c_{\rm f}$ is the concentration of the free dye. *r* is a ratio of $c_{\rm b}$ to $c_{\rm DNA}$. $c_{\rm b}$ is the concentration of the bound dye, and $c_{\rm b}$ is calculated by the following equation^[21]:

$$c_{\rm b} = \frac{F - F_0}{F_{\rm max} - F_0} c_{\rm t}$$
(4)

where c_t is the total dye concentration. F_0 and F are the fluorescence integral intensity of the free dye and the dye-DNA complex, respectively. F_{max} is the saturated fluorescence integral intensity in DNA titration.

Viscosity experiment was carried out using an Ubbelohde viscometer maintained at a constant temperature of 25 °C in a thermostatic waterbath. Data were presented as $(\eta / \eta_0)^{1/3}$ versus the concentration of **3PyB**-ctDNA solution, where η is the viscosity of DNA in the presence of the dye, η_0 is the viscosity of DNA alone. η was calculated from the relation $\eta = (t - t_0) / t_0$, where *t* is the observed flow time of **3PyB**-ctDNA solution, t_0 is the flow time of buffer^[22].

2.3. Synthesis

2.3.1. 4-[(1*E*)-2-(4-Formyl-2,5-dimethoxyphenyl)ethenyl]benzonitrile (4b) Trimethyl orthoformate (0.58 g, 5.5 mmol) was added slowly to the mixture of
2,5-dimethoxy-1,4-benzenedicarboxaldehyde (1b) (0.97 g, 5 mmol) and methanol (30 mL). Then the mixture was stirred at room temperature for 16 h. The solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography eluted with petroleum ether / ethyl acetate (10 : 1, V / V) to give
4-(dimethoxymethyl)-2,5-dimethoxybenzaldehyde (1d) as a colorless oily liquid (0.87 g). Yield: 72.5%. ¹H NMR (DMSO-*d*₆, 500 MHz) δ: 10.34 (s, 1H), 7.25 (s, 1H), 7.21 (s, 1H), 5.57 (s, 1H), 3.89 (s, 3H), 3.81 (s, 3H), 3.31 (s, 6H).

1d (1.44 g, 6 mmol), [(4-cyanophenyl)methyl]phosphonic acid diethyl ester (4a) (1.52 g, 6 mmol), and potassium *tert*-butoxide (0.81 g, 7.2 mmol) in dry THF (25 mL) were added into a dry flask under a nitrogen atmosphere with ice bath cooling. The reaction mixture was stirred at room temperature for 16 h (monitored by TLC). Then acetic acid (3.60 g, 60 mmol) in water was added and stirred at room temperature for 6 h. After neutralization with saturated aqueous sodium hydrogen carbonate solution and extraction with CH_2Cl_2 (2 × 30 mL), the combined organic layers were washed with water (20 mL) and brine (20 mL), dried with anhydrous sodium sulfate, and then filtered. The filtrate was concentrated under vacuum. The residue was purified by silica gel column chromatography eluted with petroleum ether / ethyl acetate (15 : 1, V / V) to give 4b as bright yellow needle crystals (1.24 g). Yield: 70.5%. m.p. 159-160 °C; ¹H NMR (DMSO- d_6 , 500 MHz) δ : 10.34 (s, 1H), 7.87 (d, J = 8.5 Hz, 2H), 7.82 (d, J = 8.5Hz, 2H), 7.63 (s, 2H), 7.58 (s, 1H), 7.28 (s, 1H), 3.98 (s, 3H), 3.89 (s, 3H); FT-IR (KBr) v: 3060, 2947, 2846, 2224, 1670, 1601, 1488, 1468, 1410, 1320, 1268, 1212, 1036, 969, 890, 689, 550 cm⁻¹; HRMS (ESI): *m/z* calcd for C₁₈H₁₆NO₃: 294.1130 [M+H]⁺; found: 294.1119.

2.3.2.

2-[[4-[(1*E*)-2-(1*H*-Benzimidazol-2-yl)ethenyl]-2,5-dimethoxyphenyl]methylene] propanedinitrile (**1BI**)

The mixture of

4-[(1*E*)-2-(1*H*-benzimidazol-2-yl)ethenyl]-2,5-dimethoxybenzaldehyde (**1c**) (1.23 g, 4 mmol), propanedinitrile (0.29 g, 4.4 mmol), piperidine (0.34 g, 4 mmol), and methanol (30 mL) was refluxed for 6 h. After cooling to room temperature, the reaction mixture was filtered. The filter cake was recrystallized from DMF / water to give **1BI** as red needle crystals (1.04 g). Yield: 73.1%. m.p. 161-163 °C; ¹H NMR (DMSO-*d*₆, 500MHz) δ : 12.97 (s, 1H), 8.42 (s, 1H), 7.92 (d, *J* = 16.6 Hz, 1H), 7.72 (s, 1H), 7.60 (d, *J* = 16.6 Hz, 1H), 7.58 (s, 1H), 7.56-7.58 (m, 2H), 7.20-7.23 (m, 2H), 4.00 (s, 3H), 3.91 (s, 3H); ¹³C NMR (DMSO-*d*₆, 125 MHz) δ : 154.24, 153.59, 150.64, 138.30, 132.76, 127.24, 122.46, 120.11, 114.68, 113.80, 110.61, 110.36, 80.20, 56.64, 56.11; FT-IR (KBr) *v*: 3356, 3050, 2937, 2834, 2227, 1607, 1563, 1495, 1469, 1404, 1361, 1291, 1228, 1035, 966, 848, 742, 611 cm⁻¹; HRMS (ESI): *m/z* calcd for C₂₁H₁₇N4O₂: 357.1352 [M+H]⁺; found: 357.1364.

2.3.3.

4-[(1*E*)-2-[4-[(1*E*)-2-(4,6-Dimethyl-1,3,5-triazin-2-yl)ethenyl]-2,5-dimethoxyphenyl] ethenyl]benzonitrile (**2TAZ**)

4b (0.88 g, 3 mmol) dissolved in methanol (30 mL) was added dropwise to the mixture of 2,4,6-trimethyl-*s*-triazine (**2a**) (0.55 g, 4.5 mmol), potassium hydroxide (0.3 g), and methanol (30 mL). The reaction mixture was refluxed for 12 h. After the solvent was removed under reduced pressure, the residue was purified by silica gel column chromatography eluted with petroleum ether / ethyl acetate (8 : 1, V / V) to give **2TAZ** as yellow crystalline powder (0.78 g). Yield: 65.3%. m.p. 186-188 °C; ¹H NMR (DMSO-*d*₆, 500MHz) δ: 8.42 (d, *J* = 16.1 Hz, 1H), 7.84 (d, *J* = 8.4 Hz, 2H), 7.79 (d, *J* = 8.4 Hz, 2H), 7.61 (d, *J* = 16.5 Hz, 1H), 7.51 (d, *J* = 16.5 Hz, 1H), 7.43 (s, 1H), 7.31 (d, *J* = 16.1 Hz, 1H), 3.96 (s, 3H), 3.93 (s, 3H), 2.55 (s, 6H); ¹³C NMR (DMSO-*d*₆, 125 MHz) δ: 175.55, 170.60, 152.21, 151.28, 141.99, 135.42, 132.63, 128.66, 127.77, 127.15, 126.16, 126.08, 124.15, 118.95, 110.82, 109.82, 109.57, 56.25, 56.18, 25.16; FT-IR (KBr) *v*: 3053, 2922, 2852, 2221, 1628, 1602, 1531, 1464, 1411, 1375, 1211, 1042, 968, 872, 675, 551 cm⁻¹; HRMS (ESI): *m/z* calcd for C₂₄H₂₃N₄O₂: 399.1821 [M+H]⁺; found: 399.1833.

2.3.4. 4-[(1*E*)-2-[4-[(1*E*)-2-(4-Cyanophenyl)ethenyl]-2,5-dimethoxyphenyl]ethenyl] -1-ethylpyridinium bromide (**3PyB**)

The mixture of 1-ethyl-4-methylpyridinium bromide (**3a**) (0.61 g, 3 mmol), **4b** (0.88 g, 3 mmol), piperidine (0.26 g, 3 mmol), and methanol (30 mL) was refluxed for 10 h. After the solvent was removed under reduced pressure, the residue was purified by silica gel column chromatography eluted with dichloromethane / methanol (15 : 1, V / V) to give **3PyB** as red crystalline powder (0.87 g). Yield: 60.8%. m.p. 225-227 °C; ¹H NMR (D₂O, 500MHz) δ : 8.37 (d, *J* = 6.6 Hz, 2H), 7.56 (d, *J* = 6.6 Hz, 2H), 7.28 (d, *J* = 8.5 Hz, 2H), 7.24 (d, *J* = 8.5 Hz, 2H), 7.21 (d, *J* = 16.4 Hz, 1H), 6.79 (d, *J* =16.5 Hz, 1H), 6.75 (s, 1H), 6.70 (s, 1H), 6.60 (d, *J* = 16.4 Hz, 1H), 6.54 (d, *J* = 16.5 Hz, 1H), 4.24 (q, 2H), 3.82 (s, 3H), 3.80 (s, 3H), 1.44 (t, *J* = 7.4 Hz, 3H); ¹³C NMR (DMSO-*d*₆, 125 MHz) δ : 152.99, 152.52, 151.28, 143.96, 141.94, 134.92, 132.70, 129.14, 128.29, 127.25, 125.94, 124.42, 124.18, 123.74, 118.97, 111.01, 110.11, 109.70, 56.36, 56.31, 55.17, 16.16; FT-IR (KBr) *v*: 3055, 2927, 2856, 2218, 1641, 1598, 1495, 1468, 1413, 1323, 1212, 1174, 1041, 965, 861, 686, 550 cm⁻¹; HRMS (ESI): *m/z* calcd for C₂₆H₂₅N₂O₂: 397.1916 [M-Br]⁺; found: 397.1907.

3. Results and discussion

3.1. Synthesis and characterization

The synthetic routes of **1BI**, **2TAZ**, and **3PyB** are detailedly showed in scheme 1. **1a**^[23], **1b**^[24], **1c**^[25], **2a**^[26], **3a**^[27], and **4a**^[28]were prepared with reference to the reported methods. The important intermediate **4b** was reported here for the first time, which was synthesized by the Horner-Wadsworth-Emmons (HWE) reaction of **4a** with **1d**, followed by hydrolysis with aqueous acetic acid. **1BI** was obtained by the Knoevenagel condensation of **1c** with propanedinitrile. **2TAZ** and **3PyB** were synthesized in a similar way as **1BI**.

In the ¹H NMR spectra of **1BI**, **2TAZ**, and **3PyB**, the *trans*-configurations of the C-C double bonds are evidenced by the coupling constants J = 16.1-16.6 Hz for the olefinic protons. The chemical shifts (δ) at 3.91 and 4.00 ppm (**1BI**), 3.93 and 3.96 ppm (**2TAZ**), 3.80 and 3.82 ppm (**3PyB**) correspond to the methoxy protons. For **1BI**,

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the singlet at 12.97 ppm can be attributed to the NH proton. In the FT-IR spectra of **1BI**, **2TAZ**, and **3PyB**, the characteristic absorption peaks at 2218-2227 cm⁻¹ indicate the presence of CN group. The peaks at 1211-1228 cm⁻¹ are attributed to the C-O-C asymmetric stretching vibration, and the peaks at 1035-1042 cm⁻¹ are attributed to the C-O-C symmetric stretching vibration. The peaks at 965-968 cm⁻¹ correspond to the =C-H deformation vibration which belongs to the *trans*-configurations of the C-C double bonds.





Scheme 1. Synthesis of the target compounds (1BI, 2TAZ, and 3PyB).

3.2. Linear and nonlinear photophysical properties

The linear and nonlinear photophysical properties of the target compounds are summarized in Tables 1 and 2, respectively. The spectra of linear absorption and SPEF in CH₂Cl₂ are depicted in Figs. 2 and 3. The $\Phi \times \delta$ spectra of the target compounds are depicted in Figs. 4 and 5.

As shown in Fig. 2 and Table 1, the target compounds exhibit two absorption bands. The band occurring in the 335-389 nm region ascribed to the π - π * transition of the *trans*-1,4-divinylbenzene moiety, while the other band, namely the maximum linear absorption peak, occurring in the 411-470 nm region is assigned to the ICT transition. The maximum absorption peak positions (λ_{max}^{abs}) of **1BI** and **2TAZ** change slightly (±5 nm) as the solvent polarity increases from THF to C₂H₅OH, which indicates that the surrounding solvent molecules have little effect on the transition energy of the target compounds. Whereas the λ_{max}^{abs} of **3PyB** changes considerably in different solvents and exhibits the minimum value in water. This phenomenon is attributed to a relatively high polar mesomeric form, which is predominant in the ground state. As a result, the hydrogen bond donating solvent decreases the energy of the ground state,

leading to a blue shift of the absorption band. The λ_{max}^{abs} values can be sequenced as

1BI > 3PyB > 2TAZ (except in CH₂Cl₂). This might be attributed to that the number of cyano acceptors increases (1BI *cf.* 2TAZ and 3PyB) and pyridinium cation has stronger electron-withdrawing ability than *s*-triazine (3PyB *cf.* 2TAZ).

As shown in Fig. 3 and Table 1, the SPEF maxima (λ_{max}^{SPEF}) of **1BI** and **2TAZ** undergo red shifts with increasing the solvent polarity. For example, the λ_{max}^{SPEF} of **1BI** (**2TAZ**) is located at 526 (481) nm in THF and red-shifted to 544 (519) nm in C₂H₅OH. One reasonable interpretation is that the polarity of the excited state is larger than that of the ground state, and the enhanced dipole-dipole interactions caused by increasing the polarity of the solutes and / or solvents will lead to a more significant energy level decrease for the excited state^[29]. On the contrary, the λ_{max}^{SPEF} of **3PyB** changes slightly with increasing the solvent polarity, which is always located in the orange light region (570-578 nm). Analyzing the data collected in Table 1, it follows that the Φ of **1BI** is very low and almost independent of the solvent polarity, the Φ of **2TAZ** significantly decreases with increasing the solvent polarity, and the ϕ of **3PyB** in the medium polar solvents (THF, CH₂Cl₂) is larger than that in the highly polar solvents (DMF, CH₃CN, C₂H₅OH, and H₂O), especially in water (Φ = 0.04). The Φ of target compounds can be sequenced as **2TAZ** > **3PyB** > **1BI**. The low Φ values indicate that the emitting state of **1BI** might facilitate the nonradiative pathways^[30].

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			λ_{\max}^{abs}	$10^{-4}\varepsilon^{b}$	$\lambda_{\max}^{\text{SPEF}\ c}$	Δv^{d}	${\pmb \Phi}^{{ m e}}$
	Compound	Solvent	(nm)	$(\text{mol}^{-1} \text{ L cm}^{-1})$	(nm)	(cm^{-1})	
	1BI	THF	458	3.64	526	2823	0.08
		CH ₂ Cl ₂	460	4.12	529	2836	0.07
		DMF	459	3.96	537	3165	0.05
		CH ₃ CN	456	3.23	541	3446	0.05
		C ₂ H ₅ OH	455	3.74	544	3596	0.06
	2TAZ	THF	415	7.36	481	3306	0.72
		CH_2Cl_2	416	8.42	492	3713	0.68
		DMF	415	3.44	496	3935	0.53
		CH ₃ CN	411	7.39	499	4291	0.41
		C ₂ H ₅ OH	416	4.41	519	4771	0.28
	3PyB	THF	446	3.97	571	4908	0.37

 Table 1. Linear photophysical properties of the target compounds (1BI, 2TAZ, and 3PvB).

CH ₂ Cl ₂	470	3.55	578	3976	0.31
DMF	449	4.93	574	4850	0.12
CH ₃ CN	443	5.60	574	5152	0.11
C ₂ H ₅ OH	453	4.02	574	4653	0.13
H ₂ O	434	3.82	570	5498	0.04

^a Maximum linear absorption wavelength, $c = 1 \times 10^{-5}$ mol L⁻¹.

^b Maximum molar absorption coefficient.

^c Maximum single-photon excited fluorescence wavelength, $c = 1 \times 10^{-6} \text{ mol } \text{L}^{-1}$.

^d Stokes shift.

^e Fluorescence quantum yield, measured using fluorescein in 0.1 mol L⁻¹ sodium

hydroxide aqueous solution as the standard.



Fig. 2. Linear absorption spectra of the target compounds (**1BI**, **2TAZ**, and **3PyB**) in CH₂Cl₂ with a concentration of 1×10^{-5} mol L⁻¹.



Fig. 3. Single-photon excited fluorescence spectra of the target compounds (**1BI**, **2TAZ**, and **3PyB**) in CH₂Cl₂ with a concentration of 1×10^{-6} mol L⁻¹.

There is no linear absorption in the wavelength range of 600-920 nm for **1BI**, **2TAZ**, and **3PyB**, which indicates that there are no energy levels corresponding to an electron transition in this spectral range. Therefore, if frequency up-converted fluorescence appears excited by a tunable laser in this spectral range, it should be attributed to TPEF. Meanwhile, the log-log plot of the excited fluorescence signal *versus* the exciting powers is depicted in Fig. 6b (with a slope of 2.01 for **3PyB**), which provides the direct evidence for a two-photon excitation process.

As shown in Table 2, the maximum δ values of **1BI**, **2TAZ**, and **3PyB** are 607 (CH₂Cl₂), 264 (THF), and 713 GM (CH₂Cl₂), respectively, which follows the order: **3PyB > 1BI > TAZ**. In addition, it is found that the peak positions of TPEF are red-shifted by 28-38 nm compared with those of SPEF, which can be explained by the effect of reabsorption in highly concentrated solutions^[31]. As shown in Table 2, Figs. 4 and 5, for **1BI**, **2TAZ** and **3PyB**, their maximum $\Phi \times \delta$ are 42 (CH₂Cl₂), 190 (THF), and 221 GM (CH₂Cl₂), respectively. Meanwhile, due to its good water solubility, the $\Phi \times \delta$ of **3PyB** in H₂O is 11 GM, which is much larger than that of the commercial DNA stain DAPI (0.16 GM)^[32].

In order to better understand the structure-property relationships of the target

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compounds, DFT calculations were performed using the Gaussian 09 program and B3LYP 6-31G (d, p) basis sets were used for the calculations. The optimized geometrical structures, HOMO and LUMO distributions of the target compounds are shown in Fig. 7. The HOMO-LUMO energy gaps (ΔE) are calculated and listed in Table 3. For **1BI** and **3PyB**, the electron clouds of the HOMO are mainly localized on the methoxy donor, the 1,4-divinylbenzene π -bridge, and the benzimidazole (or benzonitrile) acceptor, whereas in the LUMO, most of the electrons transfer from the benzimidazole (or benzonitrile) acceptor to the more strongly electron-withdrawing cyano (pyridinium cation) acceptor. However, for **2TAZ**, the electron clouds of the HOMO are mainly distributed on the methoxy donor and the 1,4-divinylbenzene π -bridge, whereas in the LUMO, the electron cloud density of the terminal acceptors (*s*-triazine and benzonitrile) increases significantly. The δ value shows negative correlation with $\Delta E^{[33]}$. The ΔE of **1BI**, **2TAZ**, and **3PyB** are 2.69, 2.99, and 2.15 eV, respectively, which is consistent with the sequence of δ (**3PyB** > **1BI** > **2TAZ**).

 Table 2. Nonlinear photophysical properties of the target compounds (1BI, 2TAZ, and 3PyB).

		$\lambda_{\max}^{\text{TPEF}}$ a	$\delta_{\max}{}^{\mathrm{b}}$	$\Phi imes \delta_{\max}^{c}$
Compound	Solvent	(nm)	(GM)	(GM)
1BI	THF	564	459	37
	CH ₂ Cl ₂	565	607	42
2TAZ	THF	518	264	190
	CH ₂ Cl ₂	524	194	132
ЗРуВ	THF	600	530	196
	CH ₂ Cl ₂	606	713	221
	H ₂ O	594	285	11

^a Maximum two-photon excited fluorescence wavelength, $c = 1 \times 10^{-3} \text{ mol } \text{L}^{-1}$.

^b Two-photon absorption cross-section, $1 \text{ GM} = 1 \times 10^{-50} \text{ cm}^4 \text{ s photon}^{-1}$.

^c Two-photon action cross-section.



Fig. 4. Two-photon action cross-sections of **1BI**, **3PyB** in CH₂Cl₂ and **2TAZ** in THF in the 690-910 nm range.



Fig. 5. Two-photon action cross-sections of **3PyB** in different solvents in the 690-910 nm range.



Fig. 6. (a) Two-photon excited fluorescence spectra of **3PyB** in CH₂Cl₂ ($c = 1 \times 10^{-3}$ mol L⁻¹) at 850 nm under different input powers. (b) Logarithmic plot of the output fluorescence integral (I_{out}) of **3PyB** *versus* the input laser powers (I_{in}).



Fig. 7. Optimized geometrical structures, HOMO and LUMO distributions of the target compounds (**1BI**, **2TAZ**, and **3PyB**).

 Table 3. Calculated HOMO, LOMO energy and HOMO-LOMO energy gaps of the

Compound	HOMO (eV)	LUMO (eV)	HOMO-LUMO energy gaps (eV)
1BI	-5.64	-2.95	2.69
2TAZ	-5.47	-2.48	2.99
ЗРуВ	-7.83	-5.68	2.15

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target compounds (1BI, 2TAZ, and 3PyB).
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3.3. DNA-binding studies

In order to check the possibility of 3PyB as a DNA stain, the changes in the absorption, single- and two-photon fluorescence spectra with the addition of ctDNA were investigated. The results are shown in Figs. 8, 9, and 10. With the addition of ctDNA, the absorbance values decrease when the ratio of c_{ctDNA} to c_{3PyB} is lower than 10, then increase and level off after the ratio of 65 : 1. Meanwhile, the maximum

absorption peaks are marginally red-shifted (from 432 nm to 441 nm). In general, the hypochromism and red shift are associated with the intercalative binding of the dye to DNA, due to strong stacking interaction between the dye and the base pairs of DNA, while the hyperchromism and red shift can be attributed to external contact (surface binding) with DNA^[34].

As shown in Fig. 9, the fluorescence intensity increases sharply (about 10 fold), then levels off after the ratio of 85 : 1. However, the maximum emission peaks remain steady at around 567 nm. Moreover, the binding constant between **3PyB** and ctDNA was confirmed on the basis of the single-photon fluorescence titration according to the Scatchard equation. The result shows that the binding constant is 3.05×10^5 mol L⁻¹.

As shown in Fig. 10, the two-photon fluorescence intensity first increases quickly, then levels off after the ratio of 10 : 1. This trend is similar to that of the single-photon fluorescence titration. The following factor can explain the fluorescence enhancement. When the dye is in a twisted intramolecular charge transfer (TICT) state, the nonradiative process quenches the fluorescence. Inversely, the dye will become highly luminescent, for the formation of a TICT state is restricted. For **3PyB**, the TICT can occur between the electron donor and the electron acceptors. In water, the dye in the excited state interacts with the highly polar solvent more strongly than that in the ground state, resulting in the larger dipole moment in the excited state, which can lead to charge separation and finally results in the formation of a TICT state. However, the formation of a TICT state is restricted when **3PyB** has a low $\Phi \times \delta$ value in Tris-HCl buffer solution (6.6 GM), but once bound to ctDNA, the $\Phi \times \delta$ value can be increased to 45 GM, which is far higher than the commerical DNA stain DAPI (2.18 GM)^[36]. These demonstrate that **3PyB** is a potential DNA fluorescent probe.

(a)

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Fig. 8. (a) Linear absorption spectra of **3PyB** with the addition of ctDNA. (b) Change of absorbance (•) and peak position (•). $c_{3PyB} = 1 \times 10^{-5}$ mol L⁻¹, Ratio: c_{ctDNA} / c_{3PyB} .



(b)



Fig. 9. (a) Single-photon excited fluorescence spectra of **3PyB** with the addition of ctDNA. (b) Change of SPEF intensity (•) and peak position (•). $c_{3PyB} = 1 \times 10^{-5}$ mol L⁻¹, Ratio: c_{ctDNA} / c_{3PyB} .



(b)



Fig. 10. (a) Two-photon excited fluorescence spectra of **3PyB** with the addition of ctDNA. (b) Two-photon fluorescence enhancement curves. $c_{3PyB} = 5 \times 10^{-5}$ mol L⁻¹. Ratio: c_{ctDNA} / c_{3PyB} .

In order to further explore the possible interaction mechanism of **3PyB**-ctDNA, the fluorescence of **3PyB**-ctDNA under different NaCl concentrations and the viscosity experiment were investigated. The concentrations of **3PyB** and ctDNA are 1×10^{-5} mol L⁻¹ and 5×10^{-5} mol L⁻¹, respectively. The concentrations of NaCl vary from 0.1 to 0.6 mol L⁻¹. The changes in fluorescence intensity with adding NaCl are shown in Fig. 11. Generally, Na⁺ can be partially close to phosphate of the nucleotide skeleton. If the dye-DNA interaction is an electrostatic mode, the changes in fluorescence intensity will be disturbed with the increase of Na⁺ concentrations. As we can see, the fluorescence intensity of **3PyB**-ctDNA changes slightly with the addition of NaCl, which indicates that the electrostatic interaction does not play a major role.

The hydrodynamic measurements that are sensitive to the length change are, in the absence of crystallographic data, regarded as the most critical tests of a DNA-binding mode. The intercalation mode is expected to lengthen the DNA helix as the base pairs are pushed apart to accommodate the bound dye, leading to an increase in the viscosity of DNA. In contrast, the partial, non-classical intercalation mode can bend

(or kink) the DNA helix and reduce its effective length and, concomitantly, its viscosity. When the outside binding mode occurs, the viscosity of DNA would not change basically^[37]. As shown in Fig. 12, the viscosity of ctDNA increases remarkably with increasing the concentration of **3PyB**, as expected for the lengthening of the DNA double helix resulting from intercalation. The result is consistent with the hypochromism and red shift in the absorption titration and fluorescence enhancement in the fluorescence titration. Based on the above analysis, it can be inferred that the interaction of **3PyB**-ctDNA should mainly be the intercalation binding at a low ratio of c_{ctDNA} to c_{3PyB} .



Fig. 11. Effect of NaCl concentration. $c_{3PyB} = 1 \times 10^{-5} \text{ mol } L^{-1}$, $c_{ctDNA} = 5 \times 10^{-5} \text{ mol } L^{-1}$.



Fig. 12. Effect of increasing amounts of **3PyB** on the relative viscosity of ctDNA. $c_{\text{ctDNA}} = 1 \times 10^{-4} \text{ mol } \text{L}^{-1}$, Ratio: $c_{3PyB} / c_{\text{ctDNA}}$.

Conclusions

In summary, three novel A-π-D-π-A' compounds (**1BI**, **2TAZ**, and **3PyB**) were synthesized and characterized. Their linear and nonlinear photophysical properties in different solvents have been systematically studied. Meanwhile, the spectral properties of **3PyB** in the DNA titratiion experiments were investigated. The possible interaction mechanism of **3PyB**-ctDNA was also studied by the NaCl titration and the viscosity experiment. The results show that **3PyB** may serve as a potential fluorescent probe for DNA detection.

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