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Synthesis of 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline and its use as DNA probe

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ABSTRACT

A novel pyrazoline derivative, named 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline, was synthesized, and its structure was confirmed by means of IR, ^{1}H NMR, and elementary analysis. The compound emits strong yellow fluorescence. Decrease of fluorescence intensity of the pyrazoline derivative in the presence of calf thymus DNA (ct DNA) is observed, and the quenching obey Stern–Volmer equation. There is a single quenching mechanism for the complex, which belongs to static quenching. KI quenching study shows that the magnitude of K_{SV} of the bound pyrazoline is lower than that of the free one. It is also found that ionic strength could affect the interaction. Binding constants for pyrazoline with ct DNA and salmon sperm DNA (ss DNA) are in the same order of 10^4 mol L^{-1}, and binding site size are about 1 per base pairs. Experimental results indicate that the new compound might insert into DNA base pairs by intercalative binding mode.

1. Introduction

Binding studies of small molecules with deoxyribonucleic acid (DNA) are important tools in the understanding of drug–DNA interactions, and they have attracted considerable interests in chemical biology for the design of new and efficient drugs targeted to DNA. The binding of small molecules to DNA is also very useful in clarifying the structure, function of DNA, and protein–nucleic acids interaction. There are three primarily binding modes concerning the interaction of small molecules with nucleic acids [1,2], intercalative binding, groove binding, and electrostatic interactions. Among the three modes, the most effective mode is intercalative binding. In general, planarity and non-negative charge are suggested to be the important features needed for efficient intercalators.

2-Pyrazolines are well known fluorescent compounds with high fluorescence quantum yields and widely used in industry as whitening or brightening reagents [3–5]. They are very efficient sensitizers in photography, biological stains, redox indicators, laser dyes and fluorescence probes. Conjugation structure and electron donation from N atom make them well known intramolecular charge transfer (ICT) compounds. An ICT process has been reported to exist in pyrazoline moiety in the excited state [6]. Pyrazoline derivatives have also been investigated in many other respects due to good photoconductivities [7–9], ready accessibility, and easily being used as carrier transporting as well as emitting materials [10]. Many 1,3,5-triaryl-2-pyrazolines have been reported as hole transporting in organic electroluminescent devices (OELDs) [11–17].

In recent years, pyrazolines have attracted many attentions from medicinal chemists and clinicians because of extensive biological activities. For example, 2-pyrazoline derivatives have been reported to exhibit antimicrobial [18–22], anti-inflammatory [18] and antihypertensive pharmacological activities [23]. Unfortunately, the mechanism of their biological activities is not very clear. Most small molecules studied in this field are organic dyes, metal complexes and drugs [24–26], no attention has been paid to pyrazoline fluorescent dyes for the interaction with DNA. Therefore, it is necessary to research and understand the modes and factors affected the binding of this kind of molecules to DNA.

Carbaozole derivatives are also fluorescent compounds with blue fluorescence. If carbazole radical is linked to pyrazoline molecule, it would bring an excellent fluorescence efficiency. Based on this target, a carbazole radical was introduced to 2-pyrazoline ring on C-5 in this paper, and a new derivative of pyrazoline was synthesized. It is named 1-phenyl-3-biphenyl-5-(N-ethyl carbazole-3-yl)-2-pyrazoline (PBEP) (**5** in Schemes 1–3), which is a good fluorescent dye emitting strong yellow light under ultraviolet light (fluorescent quantum yield is 0.48 in $CHCl_3$). The product was characterized by IR, ^{1}H NMR, elementary analysis and fluorescence techniques. Fluorescence analysis plays an important role in the study of DNA-binding [27,28]. In this paper, the

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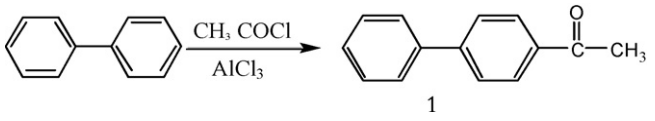


Scheme 1. Synthesis of P-phenylacetophenone.

investigation on the binding properties of PBEP with DNA was reported based on fluorescence spectroscopy. It is observed that the fluorescence of PBEP is quenched dramatically and immediately by the addition of DNA, and stable complex is formed. DNA-binding affinities and binding mode are presented. Part of PBEP's planar molecular structure, carbazole radical, is expected to facilitate the interaction of pyrazoline into the interior of the DNA helix. The compound shows promising applications in the study of DNA.

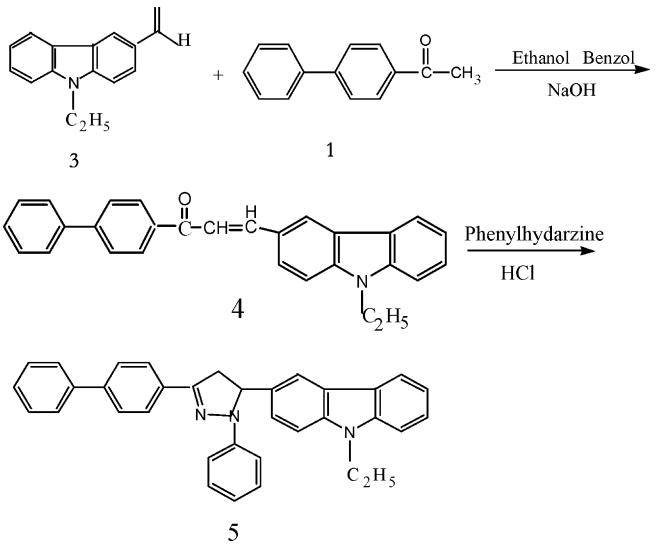
2. Experimental

2.1. Reagents and materials

Pyrazoline was newly synthesized according to Schemes 1–3. The stock standard solution with the concentration of 1×10^{-3} mol L^{-1} was prepared by dissolving in alcohol. ct DNA and ssDNA were commercially obtained from Sigma and used without further purification. They were dissolved in NaCl (0.1 mol L^{-1}) and stored at 4 °C, the concentrations had been determined by the absorption at 260 nm after establishing the absorbance ratio of A_{260}/A_{280} in the range of 1.8–1.9, and molarities of DNA were calculated based on ε_{260} = 6600 L mol^{-1} cm^{-1} [29]. All above solutions were further diluted as required. 0.05 M Tris–HCl buffer (pH 7.80) was used to control the pH of the reaction system. Different pH value of the media was controlled with Britton–Robinson buffer solutions, which were prepared by adding appropriate amounts of 0.2 mol L^{-1} NaOH into H_3BO_3, H_3PO_4 and HAc (0.04 mol L^{-1}). All other reagents were of analytical reagent grade and doubly distilled water was used throughout.

2.2. Apparatus

The fluorescence measurements were performed on a Cary Eclipse fluorescence spectrophotometer, VARIAN (USA) connected to an ultrathermostate of temperature precision ±0.1 °C with single cell peltier accessory. UV–Vis absorbance experiments were conducted on a TU-1901 UV–vis spectrophotometer (Puxi Instrument Factory, Beijing, China) with 2.0 nm spectrum bandwidth and slow speed scan. The measurement of pH value of solution was performed on a Model pHS-29A pH-meter, 2nd Analysis Instrumental Factory (Shanghai, China). Melting points were determined on x-5 melting point determinter. IR spectra were recorded with a FTIR 1730. ^{1}H NMR spectra were obtained on a Bruker 300 MHz instrument. Standard 1 cm quartz cells were used. Excitation and emission bandwidths were set at 5 and 10 nm, respectively. All experiments were performed at 20 ± 1 °C.



Scheme 3. Synthesis of biphenyl-(N-ethylcarbazole-3-yl) ethylene ketone and 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline.

2.3. Procedures

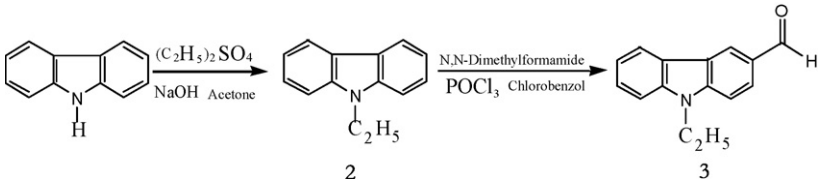
2.3.1. Synthesis of 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline

*2.3.1.1. Synthesis of P-phenylacetophenone (**1**) (Scheme 1).* A mixture containing 6 g of biphemy, 28 ml of nitrobenzene, and 5 g of $AlCl_3$ were added to a 3-necked flask and stirred with 2.8 ml of acyl chloride being dropped at ambient temperature. The reaction lasted for 4 h. Nitrobenzene was removed by steam distillation. The residue was recrystallized from ethanol, 4 g of yellow crystals was obtained. Yield: 52%. m.p. 120–121 °C. ^{1}H NMR ($CDCl_3$) δ: 2.66 (3H, $-CH_3$), 7.27–8.1 (9H, diphenyl).

*2.3.1.2. Synthesis of N-ethylcarbazole (**2**) (Scheme 2).* The synthesis of N-ethylcarbazole was performed as our previous work [30]. Yield: 75%. m.p. 72–74 °C. ^{1}H NMR ($CDCl_3$). δ: 1.4 (3H, $-CH_3$), 4.4 (2H, $-CH_2-$), 7.2–8.2 (8H, carbazole).

*2.3.1.3. Synthesis of N-ethyl-3-formyl carbazole (**3**) (Scheme 2).* The synthesis of *N-ethyl-3-formyl carbazole* was performed also according to our previous work [30]. Yield: 72%. m.p. 86–88 °C. ^{1}H NMR ($CDCl_3$). δ: 1.5 (3H, $-CH_3$), 4.4 (2H, $-CH_2-$), 10.1 (1H, CHO), 7.3–8.7 (7H, carbazole). IR (KBr) cm^{-1}: 1682 (–CHO).

*2.3.1.4. Biphenyl-(N-ethylcarbazole-3-yl) ethylene ketone (**4**) (Scheme 3).* Biphenyl (16 g), 30 ml of nitrobenzene, and 0.9 g of p-phenylacetophenone were dissolved in 20 ml of ethanol and 1 ml of 10% NaOH aqueous solution. The reaction solution was stirred for 24 h at ambient temperature. A yellow powder product of 0.6 g was obtained after being filtrated and recrystallized from ethanol. Yield: 33%. m.p. 76 °C. ^{1}H NMR ($CDCl_3$). δ: 1.49 (3H, $-CH_3$), 4.4 (2H, $-CH_2-$), 6.28 (1H, –CO–CH=), 7.88 (1H, =CH-(N-



Scheme 2. Synthesis of N-ethyl-3-formyl carbazole.

ethylcarbazole)), 7.3–8.6 (16H, biphenyl, N-ethylcarbazole). IR (KBr) cm^{-1}: 1610 (–CO–).

*2.3.1.5. Synthesis of 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline (**5**) (Scheme 3).* Biphenyl-(N-ethylcarbazole-3-yl) ethylene (0.6 g) was dissolved in 10 ml of ethyleneglycol monoethyl ether, then added a little hydrochloric acid and some drops of phenyl hydrazine. The mixture was stirred for 8 h at 110 °C. The reaction liquid was poured into water, which resulted in a greenish–black precipitate. Recrystallized from ethanol, filtrated, and dried, 0.3 g brown powder was obtained. Yield: 47.2%. m.p. 104–106 °C. ^{1}H NMR ($CDCl_3$). δ: 1.5 (3H, $-CH_3$). 2.4 (2H, $-CH_2-$, pyrazoline), 3.6 (1H, –CH=, pyrazoline), 4.3 (2H, $-CH_2-$), 7.2–8.8 (19H, ph + carbazole). IR (KBr) cm^{-1}: 1566 (C=N). Anal. calcd. for $C_{35}H_{29}N_3$ (%): C, 85.55; H, 5.91; N, 8.55. Found (%): C, 85.04; H, 5.83; N, 9.02.

2.3.2. Spectral behaviour studies

1.0 ml of buffer solutions with different pH and certain volume of fluorescent dye solution were transferred to a 10 ml volumetric flask, and the mixed solution was diluted to the final volume with doubly distilled water and shaken thoroughly. Record the fluorescence spectra of the mixed solution and the reagent blank on fluorescence spectrophotometer at room temperature using quartz cell of 1 cm path.

2.3.3. Binding interaction studies of PBEP with ctDNA

To the solution of 2 ml PBEP (1×10^{-5} mol L^{-1}) in Tris–HCl buffer (pH 7.80), Spectrofluorometric titrations were performed with addition of aliquots of DNA. This operation ensured that the [DNA] increased gradually. The concentration of PBEP in spectro photomctric titration is 4×10^{-5} mol L^{-1}. The mixing was achieved by stirring for 3 min. Then the corresponding fluorescence and absorption spectra were measured.

All measurements fluorescence intensity were taken at the corresponding maxima of excitation and emission wavelength and the fluorescence widths of $slit_{ex}$ and $slit_{em}$ were set at 5/10 nm.

3. Results and discussion

3.1. Spectral behaviour studies

Big conjugation system makes compound present strong fluorescence emission in aqueous solution. It is obvious that pH value greatly affects the luminescence emission of PBEP (Fig. 1). Under neutral media, the fluorescence emission spectrum of the compound is a single peak at 372 nm, while there are three peaks at 230, 260, and 280 nm in the excitation spectrum. Among them, the first peak is maximum, and others are relative weak. The fluorescence intensity was measured at 230 nm, which is lower in acidic and neutral medium than in basic solution. It increases sharply with the increase of pH (<8) of the sample solution and reaches to the maximum in weak alkaline solution of pH 8.97, and remains strong in the whole range of pH selected. This illustrates that the pH can affect the molecular configuration and the distribution of charges. A pH of 7.80 is recommended in the subsequent investigations.

3.2. Interaction of PBEP with DNA

3.2.1. Study on the binding conditions

The factors affected the fluorescence of PBEP and DNA complex were investigated in detail. The effect of incubation time shows that upon addition of DNA into pyrozaline solution (7.5×10^{-6} mol L^{-1}), the maximum decrease of the fluorescence intensity can be observed immediately and remains constant for more than 2 h. Therefore, in this paper the fluorescence intensity was directly measured after mixture, and an additional incubation time was not needed.

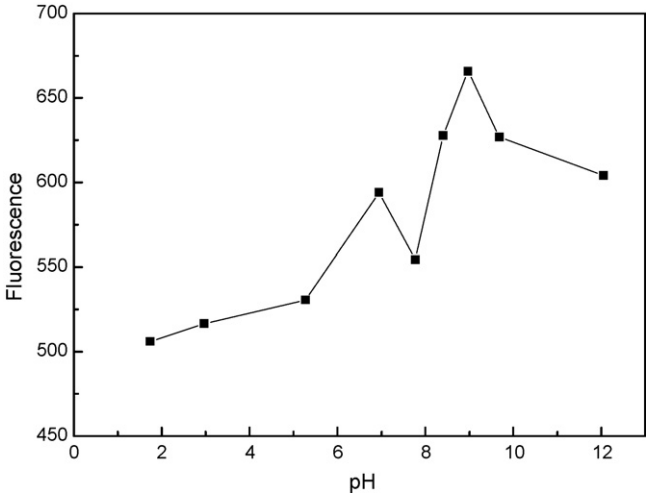


Fig. 1. Effect of pH on the fluorescence intensity of 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline.

Effect of the concentration of pyrazoline on binding was performed also. Remain the concentration of DNA at 1.27×10^{-5} mol L^{-1}, change the concentration of pyrazoline from 0 to 2×10^{-5} mol L^{-1}, the value of F_0/F increases greatly and reaches to maximum at 7.0×10^{-6} mol L^{-1}. This concentration is chosen as the optimum concentration.

3.2.2. Absorption study of PBEP in the presence of DNA

Binding mode of PBEP to DNA was studied by spectrophotometric titration. Upon complex with DNA, characteristic changes to the absorption spectra of PBEP are evident. A hypochromic effect in the region of the absorption and red shifts was detected as shown in Fig. 2. Since the hypochromicity of chromophore generally results from p-electron transfer by stacking interaction with nucleic bases, the spectral change appears to reflect the contribution of intercalation into the nucleic base pair.

3.2.3. Fluorescence study of PBEP in the presence of DNA

PBEP molecule features a planar carbozole ring, which is expected to facilitate intercalation into the DNA helix. The interac-

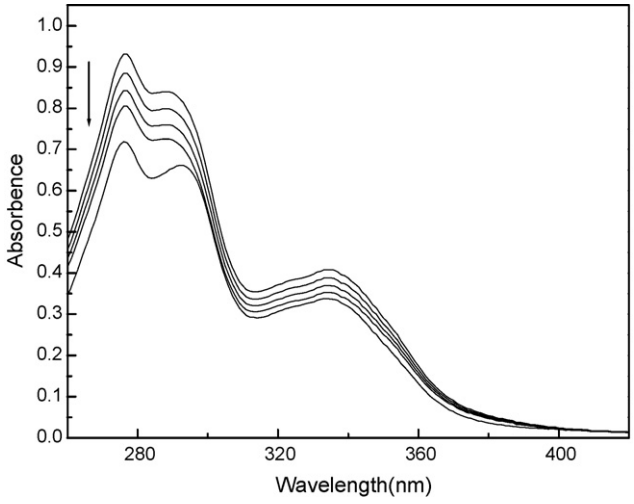


Fig. 2. Absorption titration of ct DNA on 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline. The arrow presents concentration of DNA $\times 10^{-5}$ mol L^{-1}: 0, 0.98, 2.50, 4.78, 5.76.

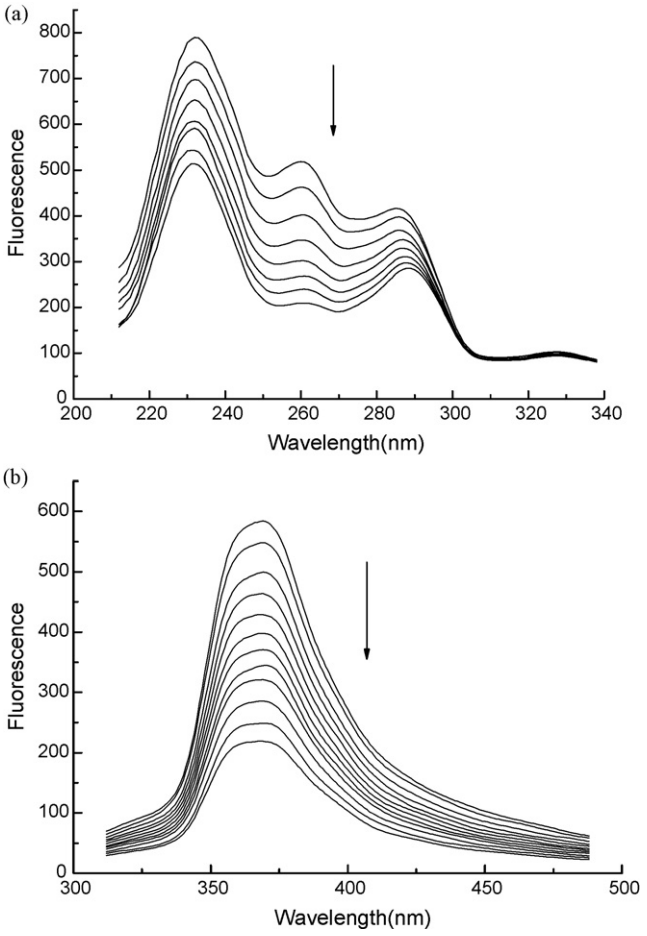


Fig. 3. (a) Excitation spectra of 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline (λ_{em} = 372 nm, 5/10 nm). The arrow presents concentration of DNA × 10^{-5} mol L^{-1}: 0, 1.27, 2.54, 3.81, 5.8, 6.35, 7.62, 8.89. (b) Fluorescence titration of ct DNA on 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline (λ_{ex} = 260 nm, 5/10 nm). The arrow presents concentration of DNA × 10^{-5} mol L^{-1}: 0, 0.633, 1.27, 1.90, 2.54, 3.17, 4.43, 5.08, 5.69, 6.35, 7.62, 8.89.

tion was investigated by means of fluorescence. It is observed that the addition of DNA leads to efficient decrease in the fluorescence intensity of pyrazoline, with almost no shifts in excitation and emission wavelengths. All three excitation peaks can be quenched, and the intensity at 260 nm is decreased more obviously than the other two peaks when the concentration of DNA increasing (Fig. 3a). Fig. 3b shows the emission spectra of fluorimetric titration of PBEP with DNA, which strongly indicates the interaction of PBEP with DNA.

The interaction pattern of the fluorescence probe with DNA can be deduced from the variation of K_{SV} according to the Stern–Volmer equation:

$$\frac{I_0}{I} = 1 + K_{SV}[Q] = 1 + K_q\tau_0[Q]$$

where I_0 and I are the fluorescence intensities in the absence and in the presence of quencher (Q), respectively. Fluorescence was measured when excited at 230 or 260 nm, and the K_{SV} obtained are 6.57×10^3 and 1.85×10^4 L mol^{-1}, respectively. 260 nm is chosen as the optimum wavelength. The linearity of the Stern–Volmer dependence means that there is only a single quenching mechanism for the complex.

The dynamic quenching will be acute with the increasing of temperature, while the static quenching will be decreased. The Stern–Volmer quenching plots from the fluorescence titration data

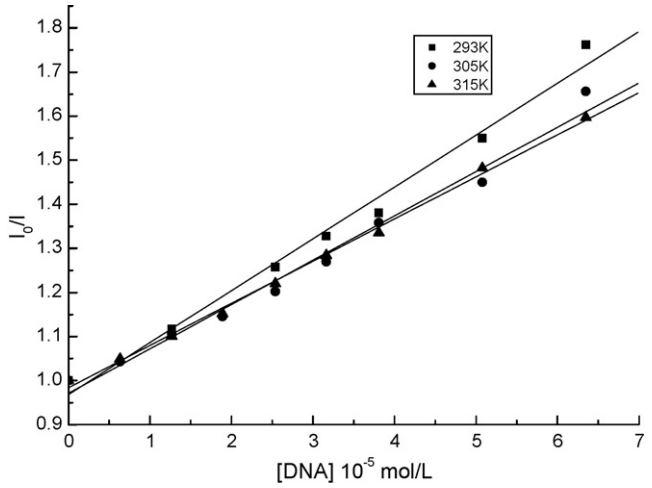


Fig. 4. Stern–Volmer plots for fluorescence quenching of 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline by DNA at different temperatures.

under other two temperatures 35 and 45 °C were investigated also (see Fig. 4). The results show that the Stern–Volmer plots are both linear, the *K*sv decrease from 1.18×10^4 to 0.96×10^4 L mol^{-1} when temperature increase from 35 to 45 °C. It means that the fluorescence quenching type in this experiment belongs to static quenching.

In addition, the Stern–Volmer analysis can determine that the quenching type should be static or dynamic. K_q is quenching rate constant, τ_0 is the average lifetime of fluorophore and its value is 10^{-9} to 10^{-7} s. For PBEP, the value of K_q is about $1.85 \times 10^{(11-13)}$ (at 20 °C), which is far greater than 2.0×10^{10} L s^{-1} mol^{-1}, the maximum diffusion collision quenching rate constant of various quenchers with the biopolymer [31]. This also suggests that the binding of PBEP with DNA is a static quenching process.

3.2.4. Characterization of binding modes

3.2.4.1. Fluorescence quenching studies. The fluorescence quenching studies were performed to determine the accessibility of the fluorescent dye to anionic quenchers and interaction pattern with DNA. If small molecules intercalate into DNA base pairs, the double helix of DNA would protect the bound molecules from the anionic quencher, owing to the base above and below the intercalator [32]. On the other hand, groove binding exposes the bound molecules to the solvent surrounding the helix [33].

Iodide ion can effectively quench the fluorescence of small molecule. To further establish the DNA-binding affinity, the fluorescence quenching experiments were chosen. I^- with increasing concentration was added into pyrazoline or pyrazoline–DNA solution, respectively. Fluorescence quenching was observed for the free PBEP and the PBEP–DNA complex. It is found from the quenching curves (not shown) that K_{SV} values of the free pyrazoline and the bound pyrazoline with ct DNA are 232.8 L mol^{-1} (r = 0.9938) and 194.4 L mol^{-1} (r = 0.9906), respectively. The magnitude of K_{SV} of the bound small molecule is lower than that of the free one, which suggests the intercalation of PBEP fluorophore into DNA bases.

3.2.4.2. Ionic strength effect on binding. DNA is an anionic polyelectrolyte with phosphate groups. The ionic environment must be considered in the research on DNA. In this work, NaCl is used to control the ionic strength of the solutions. When NaCl exists in the system, the electrostatic repulsion between the negatively charged phosphate skeletons on adjacent nucleotides is reduced with increasing concentration of Na^+. This will tighten the DNA chains. Therefore, it is not easy for the organic molecules to inter-

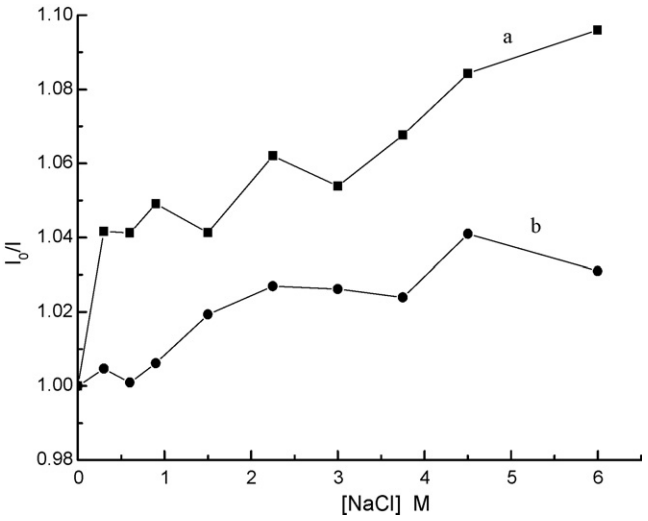


Fig. 5. The effect of NaCl on the fluorescence intensity ratio (I_0/I) of 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline: (a) in absence of DNA and (b) in presence of DNA (2.35×10^{-5} mol L^{-1}).

calate into the binding sites of DNA base pairs but to be repulsed dissociating in the solution.

The effect of the ionic strength on the fluorescence in the presence and absence of DNA was tested (Fig. 5). In the absence of DNA, adding of NaCl leads to the decreased fluorescence intensities of PBEP, and increased I_0/I value occurs. It is apparent that in the presence of DNA, fluorescence intensity of PBEP also decreases gradually and I_0/I values are greater than 1, which is smaller than those in the absence of DNA at the same concentration of NaCl. This phenomenon may be because the free of the bound PBEP from the DNA helix with the increase of NaCl, then fluorescence intensity increases. It suggests that high concentration of NaCl is not benefit for the binding of PBEP with DNA.

3.2.5. Determination of binding constant

The significant fluorescence change was used to determinate the binding constant. The fluorescent titration data (see Fig. 3) are analyzed according to modified Scatchard equation [34]:

$$\frac{r}{C_F} = K(1 - nr)$$

where r is the number of moles of bound PBEP per mol of DNA base pair, K is the binding constant and n is the binding site size in base pairs.

Binding constants and binding sites for PBEP with dsDNA and ssDNA were determined. For dsDNA, the binding constant and binding site size are 1.13×10^4 L mol^{-1} and 1.19 per base pair, respectively. For ssDNA, binding constant and binding site size are 1.45×10^4 L mol^{-1} and 0.96 per base pair, respectively (20 °C). The binding constant values suggest that PBEP forms stable complexes with both dsDNA and ssDNA with almost equal binding affinities and similar behaviors, and they have both 1:1 binding stoichiometries. It is expected that planar structure of carbozone heteroaromatic moiety in pyrazoline slides into DNA base pairs due to the twist of phenyl and biphenyl radicals.

4. Conclusions

In this paper, we described the synthesis of a novel fluorescent dye 1-phenyl-3-biphenyl-5-(N-ethylcarbazole-3-yl)-2-pyrazoline (PBEP), which appears good fluorescent characteristic with fluorescent quantum yields of 0.48. At pH 7.80, it has been proven to be a DNA-binder with high affinity. Only one type of quenching process occurs and the fluorescence quenching type belongs to static quenching. The intercalative binding mode is proven by fluorescence quenching experiment in that the fluorescence is protected from the quenching by anionic quencher after the addition of DNA. PBEP binds to dsDNA with a binding constant of 1.13×10^4 L mol^{-1} and the corresponding binding site size is 1.19 at 20 °C. In addition, it binds to ssDNA with a binding constant of 1.45×10^4 L mol^{-1} and binding site size of 0.96. From the experiments, we conclude that carbozone heteroaromatic moiety may slides into DNA base pairs, because the planarity of carbozone group is better, and other benzo groups could rotate. In the further study, we plan to improve the planarity of whole pyrazoline molecule, and discuss the effects of substituents on the pyrazoline ring to its interaction affinity with DNA. Better probe with higher affinity could be found.

Acknowledgements

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