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Recognition of Guanine by a Designed Triazine-based Fluorescent Probe through Intermolecular Multiple Hydrogen Bonding

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A new fluorescent probe with a long-wavelength emission and multiple hydrogen bond sites for guanine, 3-(4-chloro-6-*p*-nitrophenoxy-1,3,5-triazinylamino)-7-dimethylamino-2-methylphenazine (CNTDP), was designed and synthesized by using cyanuric chloride as a molecular scaffold, neutral red as a fluorophore and *p*-nitrophenol as an assistant unit. The recognition behavior of CNTDP for guanine and its spectroscopic properties in different solvents were investigated. It was found that the probe's fluorescence can be selectively quenched by guanine instead of thymine, indicating that fully complementary hydrogen bonding plays a key role in such a recognition process. In addition, the fluorescence quenching mechanism of the probe by guanine and the electronic effects of neutral red, triazine ring and *p*-nitrophenol moieties on the fluorescence of the whole molecule were also discussed.

Keywords: Intermolecular hydrogen bond; Triazine fluorescent probe; Molecular recognition; Guanine

INTRODUCTION

Guanine is a purine base, which can selectively form hydrogen bonds with cytosine in nucleic acids of all living organisms. Many studies have dealt with the tautomerism of guanine [1–3], because its rare tautomeric or ionized forms are thought to be related to the formation of base mispair during polymerase-mediated DNA replication, resulting in genetic mutations. By theoretical calculation, Jang *et al.* [3] explored the relative stabilities of tautomers of guanine in various environments and their pK_a values in aqueous solution, and estimated that in the range of pH 7–8 the relative population of deprotonated guanine is 0.2–2%, a sufficiently significant amount to potentially play a role in mispair formation. On the other hand, the research on novel artificial photo-receptors for selective recognition of guanine has also attracted much attention [4,5], because of its potential help not only in furthering the understanding of the mechanism of molecular recognition and transition *in vivo* but also in developing new methods for guanine detection. In fact, this area has been a hot topic of interdisciplinary fields including supramolecular chemistry, molecular biology, bioanalytical and bio-organic chemistry [6,7]. However, owing to the similar properties (close ultraviolet absorption bands and chemical reactivities) of nucleobases the specific recognition of an individual one remains a challenge.

Hydrogen bonds are an important intermolecular action force and have been extensively applied to designing and synthesizing receptors for selective recognition of nucleobases [4,8–10]. In this regard, the most prevalent design strategy is to make use of the natural base pairing, that is, to place one nucleic acid base in the receptor to recognize another naturally matchable one [11]. Recently, other molecular skeletons have also been demonstrated elegantly as hydrogen-bonding moieties in the design of receptors [4,12–15]. Nakatani *et al.* [13,14] developed 2-amino-1,8-naphthyridine and its derivatives. These molecules can form complementary multiple hydrogen bonds with guanine and can thus be used for the detection of a guanine–guanine mismatch or a guanine bulge in duplex DNA *via* NMR analysis. In another study, a fluorescent probe Pyrrolo-dC was

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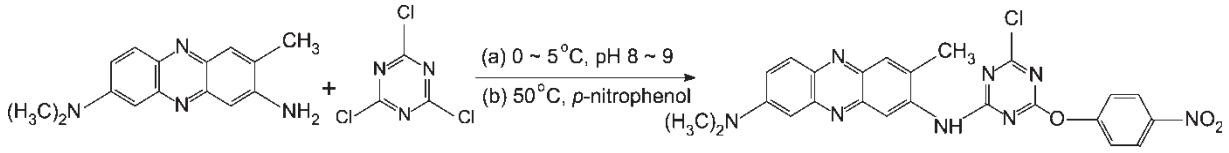

FIGURE 1 Synthesis of CNTDP.

proposed for the monitoring of local melting of the guanine–cytosine base pairs in a DNA helix by fluorescence quenching [15]. Nevertheless, among the reported photoreceptors are very few ones possessing a long excitation or emission wavelength [4], though such probes can effectively avoid the interferences of background fluorescence from biological matrix and are more useful in practice. In this work, we report such an attempt to design and synthesize a new hydrogen-bond receptor with a long-wavelength emission character for guanine by using cyanuric chloride as a molecular scaffold, neutral red as a fluorophore and *p*-nitrophenol as an assistant unit.

It is well known that cyanuric chloride has three chlorine atoms with different reactivities, which may be replaced sequentially, depending upon the temperature and the pH of the medium [16]. This property makes cyanuric chloride so valuable and was used some time ago as a bridging agent to prepare reactive dyes [17] and fluorescent probes [18,19]. In particular, the specific structure of triazine skeleton, together with its multiple electronegative nitrogen atoms, might be useful for the development of hydrogen-bonding probes. Based on this idea, a multiple hydrogen-bond acceptor for thymine was synthesized in our previous study by employing cyanuric chloride as the backbone of hydrogen bonds [20]. Herein we extend this strategy to develop a fluorescent probe, 3-(4-chloro-6-*p*-nitrophenoxy-1,3,5-triazinylamino)-7-dimethylamino-2-methylphenazine (CNTDP; Fig. 1), for the recognition of guanine by changing assistant moiety. Furthermore, the fluorescence response mechanism of the probe to guanine and the electronic effects of neutral red, triazine ring and *p*-nitrophenol moieties on the fluorescence of the whole molecule were also investigated.

RESULTS AND DISCUSSION

The Absorption and Fluorescence Properties of CNTDP in Different Solvents

The spectroscopic properties of fluorescent molecules usually depend on the polarity of solvents, and thus the absorption and fluorescence properties of CNTDP in different solvents were examined first. Three kinds of solvents were selected according to their polarity and hydrogen-bonding capability: an aprotic and apolar solvent, dichloromethane; an aprotic but polar solvent, *N*,*N*-dimethylformamide; and a protic and polar solvent, ethanol. The absorption spectra of CNTDP in these solvents displayed little difference, and even in the protic and polar solvent of ethanol, the redshift of its absorption spectrum was limited (Fig. 2), suggesting that the molecular conformation [21,22] or the electron distribution of the ground state of the probe was not affected significantly by the solvent's polarity and hydrogen bonding. In contrast, the fluorescence spectra of CNTDP were intensively influenced by the solvent's polarity and hydrogen-bonding capability, as shown in Fig. 3.

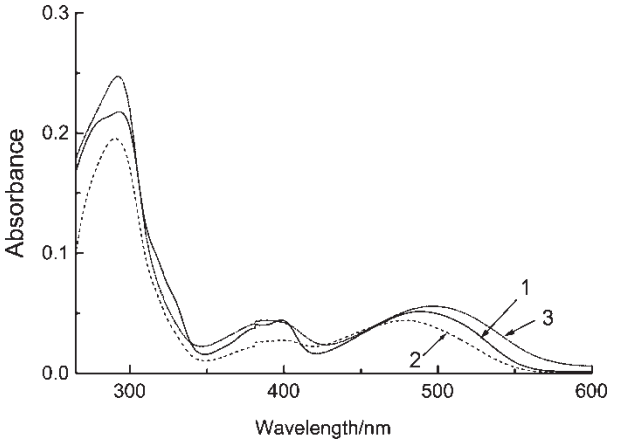

FIGURE 2 Absorption spectra of 5.0×10^{-6} mol l^{-1} CNTDP in different solvents (each against its reagent blank): dichloromethane (1), *N*,*N*-dimethylformamide (2), and ethanol (3).

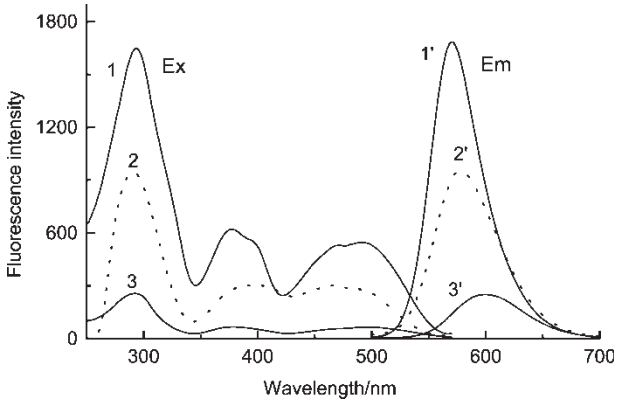

FIGURE 3 Fluorescence spectra of 2.0×10^{-5} mol l^{-1} CNTDP in different solvents: dichloromethane (curves 1 and 1′; $\lambda_{ex/em}$ = 286/571 nm), *N*,*N*-dimethylformamide (curves 2 and 2′; $\lambda_{ex/em}$ = 286/583 nm), and ethanol (curves 3 and 3′; $\lambda_{ex/em}$ = 286/603 nm). Curves 1–3 are excitation spectra; curves 1′–3′ are emission spectra. The bandpath widths were 10 nm at both the entrance and exit slits.

TABLE I Fluorescence parameters of CNTDP in different solvents

Solvent	Dielectric constant*	λ_{ex} (nm)	λ_{em} (nm)	$\Delta\lambda$ (nm)	$\Phi^{\dagger}$
Dichloromethane	9.14	286	571	285	0.052
N,*N*-Dimethylformamide	38.3	286	583	297	0.028
Ethanol	25.3	286	603	317	0.007

*Data from Dean [25]. † Quantum yields at room temperature (25°C) were determined by using fluorescein as a standard (0.1 mol l^{-1} NaOH, $\Phi = 0.95$) [26].

The excitation maximum of CNTDP was hardly altered, but the emission spectra showed redshifts and the fluorescence intensity decreased with the increase of solvent's polarity and hydrogen-bonding capability. For instance, the fluorescence intensity of the emission maximum at 571 nm in dichloromethane was about 7.4 times larger than that of a bathochromically shifted band at 603 nm in ethanol. This behavior is the experimental signature of intramolecular charge transfer [23,24], probably being active from neutral red to the electron acceptor *p*-nitrophenol. The fluorescence parameters including the Stokes' shifts ($\Delta\lambda$) of CNTDP in different solvents are summarized in Table I.

Figure 4 shows the effect of water in different solvents on the fluorescence intensity of CNTDP. Since water is a strong polar and hydrogen-bonding solvent, it is understandable that the fluorescence would be quenched when water was added into CNTDP solutions. However, the quenching was limited in dichloromethane due to the extremely poor solubility of water in this solvent. In ethanol, the fluorescence quenching was also small because ethanol itself is a strongly protic and polar solvent, and in such a solvent the fluorescence of CNTDP had already been quenched to a large extent. Considering the poor solubility of CNTDP in water, ethanol was chosen as the solvent of CNTDP in the experiments. Nevertheless, guanine has low solubility in ethanol, so its effect on the fluorescence behavior of CNTDP was investigated in this work by thoroughly mixing the aqueous solution of guanine with an ethanol solution of CNTDP.

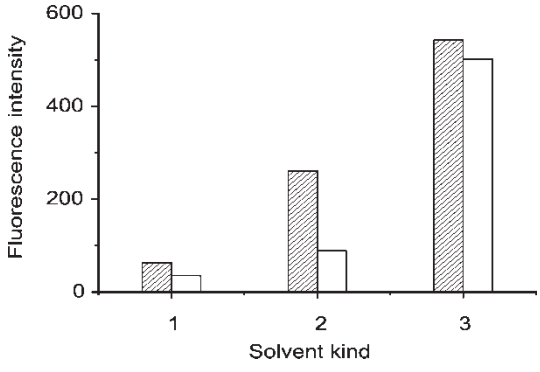

FIGURE 4 Fluorescence quenching of 5.0×10^{-6} mol l^{-1} CNTDP by water in different solvents. The fluorescence intensity was measured at $\lambda_{ex} = 286$ nm, and $\lambda_{em} = 603$ nm in ethanol (1), $\lambda_{em} = 583$ nm in *N*,*N*-dimethylformamide (2) and $\lambda_{em} = 571$ nm in dichloromethane (3), respectively. The hatched bars indicate the fluorescence intensity of CNTDP in dry solvents, and the open bars indicate the fluorescence intensity of CNTDP in solvents containing 10% (v/v) of water (except for dichloromethane, in which the solubility of water is only 0.23% (v/v) at 25°C [27]).

Effect of Acidity on the Spectroscopic Properties of CNTDP

In order to find a suitable reaction medium for the present system, the effects of acidity on the absorption and fluorescence spectra of CNTDP were investigated. As shown in Figs. 5 and 6, the decrease of acidity caused noticeable blueshifts of both the absorption bands and the fluorescence emission peak, accompanying a large fluorescence enhancement. For example, addition of 0.1 ml of 0.04 mol l^{-1} NaOH into 3 ml of ethanol solution of CNTDP shifted the absorption band in the visible region from 525 nm to 470 nm (Fig. 5) and the fluorescence emission from 603 nm to 579 nm (Fig. 6), respectively. The appearance of an isosbestic point at 505 nm in the absorption spectra reflected the existence of an acid–base equilibrium; furthermore, the two excitation peaks in the wavelength range of 350–550 nm coalesced nearly to a single one in basic media. The above indicates that CNTDP can give a stronger fluorescence in basic media. On the other hand, guanine is soluble in basic aqueous solutions, but stronger basic media may cause serious deprotonation of guanine. So in this work it is appropriate to use a moderately basic medium such as 0.008 mol l^{-1} of NaOH.

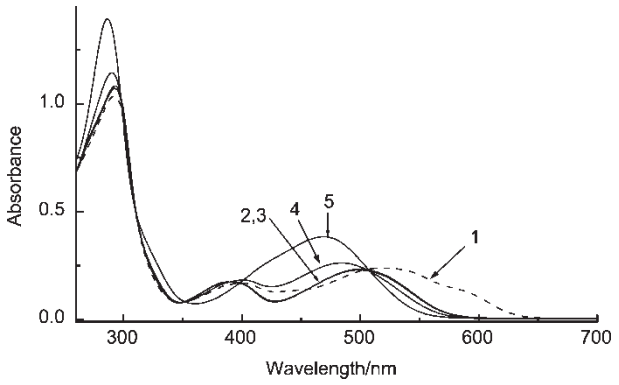

FIGURE 5 Absorption spectra of 2×10^{-5} mol l^{-1} CNTDP in ethanol solutions containing 3.3% (v/v) of 0.01 mol l^{-1} HCl (1), H_2O (2), 0.006 mol l^{-1} NaOH (3), 0.008 mol l^{-1} NaOH (4), and 0.04 mol l^{-1} NaOH (5), respectively. Corresponding reagent blank as reference.

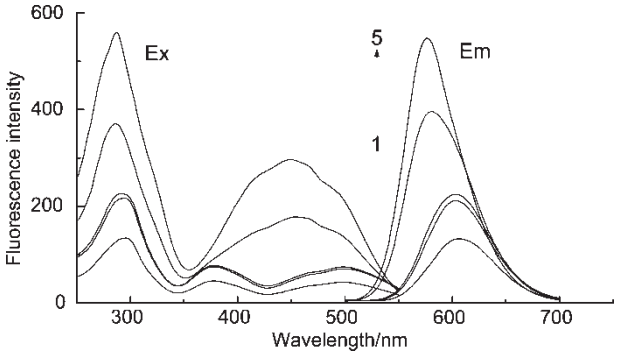

FIGURE 6 Fluorescence spectra of 2×10^{-5} mol l^{-1} CNTDP in ethanol solutions containing 3.3% (v/v) of 0.01 mol l^{-1} HCl (1; $\lambda_{ex/em} = 296/603$ nm), H_2O (2; $\lambda_{ex/em} = 295/603$ nm), 0.006 mol l^{-1} NaOH (3; $\lambda_{ex/em} = 291/603$ nm), 0.008 mol l^{-1} NaOH (4; $\lambda_{ex/em} = 286/582$ nm), 0.04 mol l^{-1} NaOH (5; $\lambda_{ex/em} = 286/577$ nm), respectively.

Recognition of Guanine by CNTDP

Following the above procedure, 2 ml of ethanol solution of CNTDP was mixed with a test solution containing a variable concentration (e.g., 0, 0.91×10^{-5}, 1.8×10^{-5}, 2.7×10^{-5}, 3.6×10^{-5}, 4.5×10^{-5}, 5.5×10^{-5} and 7.3×10^{-5} mol l^{-1}) of guanine to examine the recognition behavior of CNTDP. The results are shown in Fig. 7. It can be seen that the fluorescence intensity at 579 nm decreased with the increase in guanine concentration, and meanwhile two isoemissive points appeared at 529 nm and 627 nm, respectively, indicating that a 1:1 complex of CNTDP with guanine may be formed through hydrogen bonding [24,28]. However, an apparent stoichiometric ratio of 2:1 (guanine to CNTDP) was obtained for the complex by the molar method (the inset of Fig. 7). The reason for this disagreement is unclear, but a possible explanation is that some of the guanine molecules might associate with CNTDP through stacking interaction, causing an apparent binding ratio of 2:1. Another possible explanation may be that a small part of the deprotonated guanine in basic media cannot form the three complementary hydrogen bonds with CNTDP, which however has been counted in the total guanine concentration, also leading to an apparent binding ratio of 2:1. From the inset of Fig. 7, it is also seen that the fluorescence quenching (ΔF) is linear to the guanine concentration of not more than 3.6×10^{-5} mol l^{-1}, with a linear regression equation of $\Delta F = 2.4 \times 10^6\,C\,(\text{mol l}^{-1}) + 0.36$ ($n = 5, r = 0.9997$). This may constitute the basis of quantitative recognition of guanine.

To confirm the important role of the fully complementary hydrogen bonding in recognition, the interaction between CNTDP and thymine was also investigated under the same conditions. The fluorescence quenching of 2×10^{-5} mol l^{-1} CNTDP was not more than 10% when thymine up to a concentration of 4.5×10^{-5} mol l^{-1} was added (Fig. 9), since it cannot form the fully complementary hydrogen bonds with thymine (Fig. 8). Further, some common species (Table II) did not interfere with the recognition of guanine either, indicating that the present system has good selectivity.

Mechanism of Fluorescence Quenching

In order to investigate the mechanism of fluorescence quenching of the probe, the electronic effects of molecular skeletons or substituents such as neutral red, triazine ring, *p*-nitrophenol and methylamine moieties were explored by comparing the absorption and fluorescence spectra

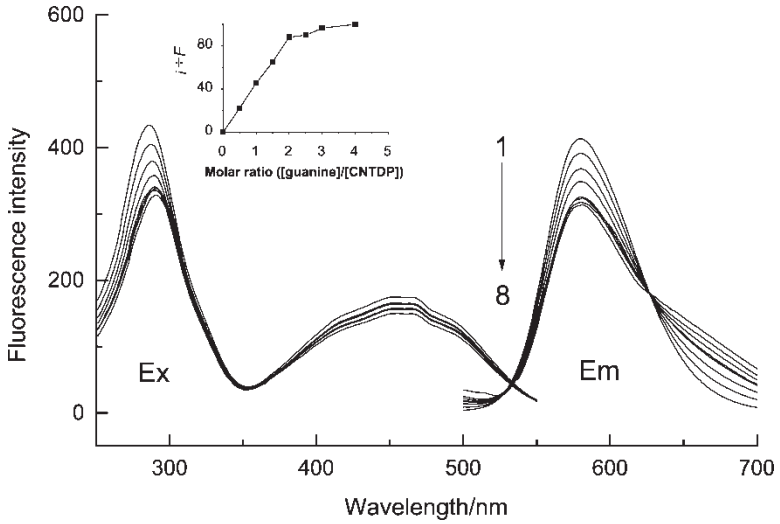

FIGURE 7 Fluorescence quenching of 2×10^{-5} mol l^{-1} CNTDP by $(0–7.3) \times 10^{-5}$ mol l^{-1} guanine (from up to down) at $\lambda_{ex/em} = 286/579$ nm. The inset shows the profile of ΔF versus molar ratio of guanine to CNTDP; $\Delta F = F_0 - F$, where F_0 and F were the fluorescence emission intensities at 579 nm before and after guanine was added, respectively.

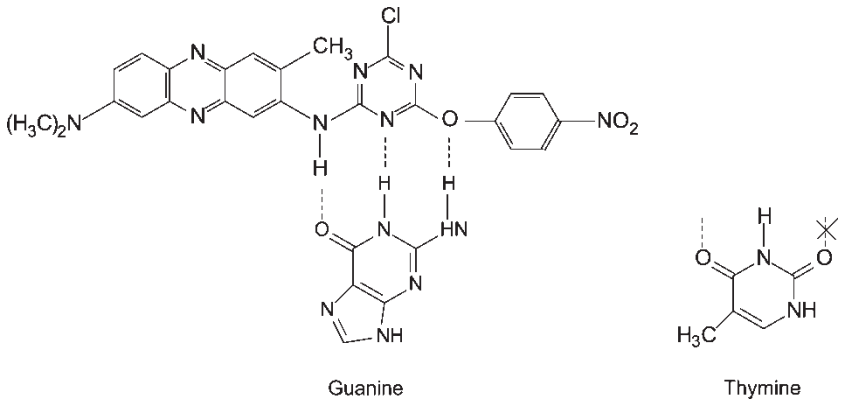

FIGURE 8 Hydrogen bonding between CNTDP and guanine or thymine.

of compounds neutral red, DTDP [29], CMTDP [20] and CNTDP (Figs. 10 and 11).

The absorption spectrum of DTDP is quite different from that of neutral red (Fig. 10). Neutral red has two strong absorption bands at 275 and 537 nm; while DTDP has three, located at 292, 391 and 480 nm, respectively. Similar to DTDP, both CMTDP and CNTDP also have three absorption bands, situated at 291, 410, 487 nm and 291, 386, 497 nm, respectively. These results indicate that the introduction of triazine ring dramatically changed the electron distribution of neutral red skeleton. The further introduction of methylamine and *p*-nitrophenol moieties did not produce any obvious effect on the absorption spectrum of DTDP, but caused a large increase and decrease in the fluorescence intensity of the resulting derivatives CMTDP and CNTDP (Fig. 11), respectively. This behavior may be ascribed to the difference in electron-donating ability of the introduced groups. The methylamino substituent is a strong electron donor, which can donate electron to the fluorophore; in contrast, the *p*-nitrophenoxy moiety is an electron-deficient group, which can withdraw electron on the triazine ring to itself. Therefore it can be concluded that the fluorescence of CNTDP will be quenched when an electron acceptor is bound to the probe. The recognition reaction of CNTDP with guanine led to the fluorescence quenching, suggesting that intermolecular electron charge transfer occurred from the probe to guanine, that is, the fluorescence quenching mechanism of CNTDP by guanine can be attributed to the electron-withdrawing action of guanine (serving as an electron acceptor) through the formation of three complementary hydrogen bonds with the probe.

In conclusion, a novel fluorescence probe that has a long-wavelength emission and can form the fully complementary multiple hydrogen bonds with guanine has been synthesized by using cyanuric chloride as a molecular scaffold. The electron-withdrawing action of guanine was proved to be responsible for the fluorescence quenching of the probe. This finding would be useful for developing multiple hydrogen-bond receptors for the recognition of other guest molecules by employing a triazine skeleton.

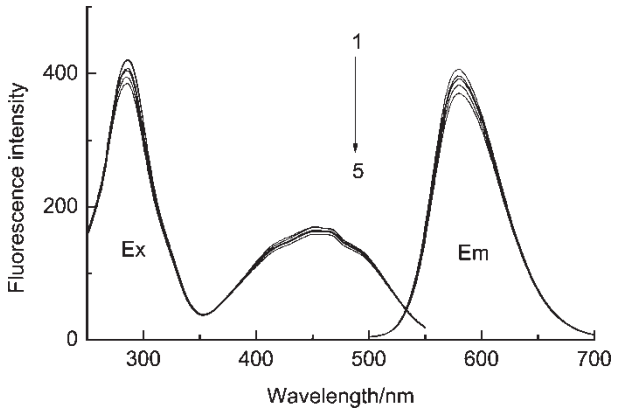

FIGURE 9 Fluorescence quenching of 2×10^{-5} mol l^{-1} CNTDP by $(0–4.5) \times 10^{-5}$ mol l^{-1} thymine (from up to down) at $\lambda_{ex/em} = 286/579$ nm.

TABLE II Effects of some common species on the recognition of 4.5×10^{-5} mol l^{-1} guanine by CNTDP

Species	Molar ratio (species/guanine)	Recovery (%)*
Phenylalanine	1	109
Tryptophan	1	97.5
Tyrosine	1	107
$CaCl_2$	2.5	99.4
$MgCl_2$	1	99.5
K_2SO_4	2.5	91.1
$CuCl_2$	1	101.6
$ZnSO_4$	1.25	92.7
Na_3PO_4	0.5	90.0
Na_2CO_3	2.5	101
$NaNO_3$	0.25	101
Egg albumin	1	107.5

*Recovery was calculated by comparing the concentration of guanine found [based on equation $\Delta F = 2.4 \times 10^6$ C (mol l^{-1}) + 0.36] with that (4.5×10^{-5} mol l^{-1}) added.

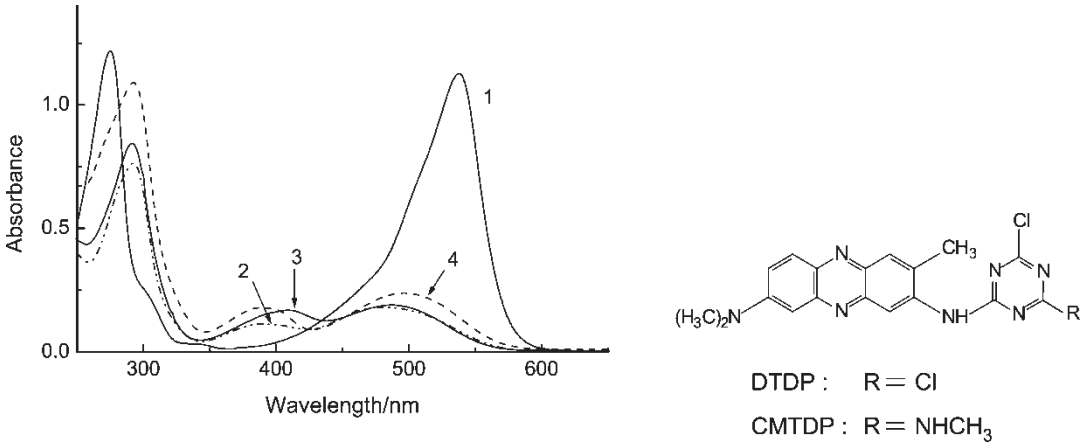

FIGURE 10 Comparison of absorption spectra of neutral red (1), DTDP (2), CMTDP (3), and CNTDP (4) in ethanol. The concentrations of all of them were 2.0×10^{-5} mol l^{-1}. The reagent blank was used as reference. The right shows the structures of DTDP and CMTDP.

EXPERIMENTAL

Apparatus and Materials

The fluorescence spectra were measured with a Hitachi F-2500 spectrofluorimeter. Absorption spectra were recorded on a UV–vis 8500 spectrophotometer. Electron impact (EI) mass spectra were recorded on an MS-50 DS90 instrument, and ^{1}H NMR spectra were measured on a Varian Unity 200 spectrometer in $CDCl_3$ with tetramethylsilane as the internal standard. A model 25 pH meter was used for pH measurements.

Cyanuric chloride was purchased from Acros (Belgium), and neutral red and *p*-nitrophenol were obtained from Beijing Chemical Reagent, Co. Guanine and thymine were purchased from Sigma, and both of their stock solutions (4×10^{-3} mol l^{-1}) were prepared in 8×10^{-3} mol l^{-1} NaOH solution. Deionized–distilled water was used to prepare all solutions.

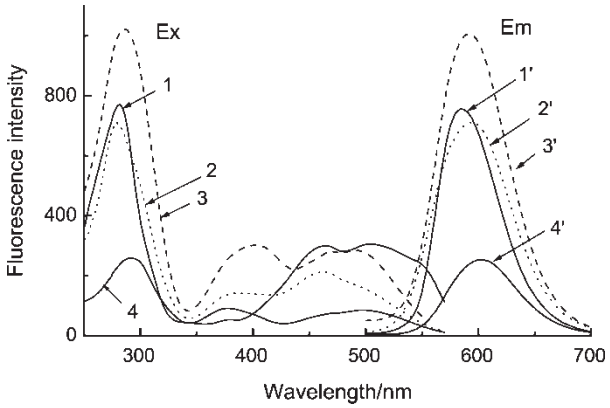

FIGURE 11 Comparison of fluorescence spectra of neutral red (1,1′; $\lambda_{ex/em} = 281/582$ nm), DTDP (2,2′; $\lambda_{ex/em} = 280/593$ nm), CMTDP (3,3′; $\lambda_{ex/em} = 284/592$ nm) and CNTDP (4,4′; $\lambda_{ex/em} = 290/603$ nm) in ethanol. The concentrations of all of them were 2.0×10^{-5} mol l^{-1}.

Syntheses

CNTDP was synthesized by subsequently reacting neutral red and *p*-nitrophenol with cyanuric chloride (Fig. 1). The first-step reaction of neutral red with cyanuric chloride was performed according to the procedure reported previously [29]. The preparation of CNTDP was then carried out by reacting the resulting dichlorotriazine derivative with *p*-nitrophenol as follows: to a 50 ml stirred acetone solution containing 150 mg of the dichlorotrizaine derivative (0.38 mmol), a mixture solution of *p*-nitrophenol (53 mg, 0.38 mmol) in 10 ml acetone and Na_2CO_3 (20 mg, 0.19 mmol) in 4 ml water was gradually added at *ca.* 50°C. After refluxing for about 1.5 h, the reaction mixture was then poured into water. The red precipitate formed was filtered off and washed twice with water. The product was purified by column chromatography (200–300 mesh silica gel, ethyl acetate as eluent), giving 153 mg of CNTDP (81% yield). The product is soluble in ethyl acetate, chloroform, dichloromethane, dioxane, acetone, *N*,*N*-dimethylformamide and ethanol, but not in water. MS (EI, 70 eV), *m*/*z*: 502 $[M]^+$ (% relative intensity 32.5); 504 $[M + 2]^+$ (11.1). ^{1}H NMR (200 MHz, 293 K, $CDCl_3$), δ, ppm: 8.5–8.4 (m, 2H), 8.1–7.9 (m, 2H), 7.7–7.4 (m, 5H), 3.5 (s, 1H), 3.4 (s, 6H), 2.6 (d, 3H).

Procedure for Recognition of Guanine

A stock solution (2.0×10^{-4} mol l^{-1}) of CNTDP was prepared by dissolving the requisite amount of it in ethanol. The recognition reaction was performed in a 15 ml test tube. Typically, 2 ml of a 2.0×10^{-5} mol l^{-1} ethanol solution of CNTDP was mixed with 0.2 ml of an aqueous test solution containing 8×10^{-3} mol l^{-1} NaOH and different concentrations of guanine. After standing for 30 min at room temperature, the absorption and fluorescence

spectra were measured against a corresponding reagent blank prepared under the same conditions.

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