Bioorganic & Medicinal Chemistry 18 (2010) 7034–7042

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

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

A fluorescein-containing, small-molecule, water-soluble receptor for cytosine free bases

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ARTICLE INFO

Article history:
Received 29 June 2010
Revised 2 August 2010
Accepted 4 August 2010
Available online 9 August 2010

Keywords:
Fluorescein
Receptor
Cytosine
5-Fluorocytosine
Binding
^{15}N NMR spectroscopy
Fluorescence quenching

ABSTRACT

In this study, we synthesized small-molecule, water-soluble, fluorescein-containing ureido compounds **6** and **8** as target receptors for cytosine free bases and then investigated the binding of cytosine free bases with the receptors using ^{15}N NMR spectroscopy and partially labeled cytosine-2,4-^{13}C-1,3,4-^{15}N-cytosine. Binding with the receptor **6a** (the disodium form of **6**) caused the chemical shift of the nitrogen atom of the amino group of cytosine to move downfield; binding of the receptor **8a** (the disodium form of **8**), which is possessing no corresponding aryl nitrogen atom, had no effect on this signal. Fluorescence spectroscopy revealed that binding of cytosine and its derivatives led to quenching of the fluorescence of receptor **6a**; in contrast, the quenching of receptor **8a** was only slightly affected by cytosine. Because the fluorescence of **6a** was not quenched by either deoxycytidine or uracil, it appears that this receptor is a specific for cytosine among the DNA bases. We used the fluorescence of **6a** to measure the apparent binding constants for various cytosine derivatives, including the anticancer prodrug 5-fluorocytosine. Receptor **6a** is the first small-molecule, water-soluble fluorescent receptor for the specific binding of cytosine free bases in aqueous solution.

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1. Introduction

Human cytosine-DNA glycosylase (CDG) is a mutant of uracil-DNA glycosylase (UDG) in which residue Asn123 has been replaced by an Asp123 unit.[1,2] Recently, thymine-DNA glycosylase (TDG), involving a residue Glu42, was found to also have the ability to remove cytosine and derivatives from DNA.[3] The common feature for CDG and TDG is that both of them possess acidic residues for binding and catalysis of the glycosylation. This common feature also exists in other cytosine-related enzymes, including activation-induced cytosine deaminase (AID) and yeast cytosine deaminase (YCD).[4–6] These two cytosine deaminases use their acidic residues Glu58 and Glu64, respectively, for binding and catalysis. Therefore, it appears that cytosine prefers to interact with these units when protonated. Indeed, this situation exists in the Watson–Crick base pairing between cytosine and guanine,[7] where cytosine donates one NH hydrogen atom for hydrogen bonding with guanine but receives two NH hydrogen atoms in return. Overall, cytosine obtains one net NH hydrogen atom donated from guanine and becomes protonated.[8] Although the binding of cytosine with guanine is common in DNA, the binding of cytosine's free base with a synthetic receptor is rarely studied.

In addition to cleaving mismatched and unwanted cytosine bases from DNA, CDG also removes healthy cytosine bases, but with less selectivity compared with that of UDG.[2,9] This cleavage is pH-dependent; CDG is most reactive at pH 6.2.[1] Notably, many human diseases are exacerbated at low pH; for example, acidic extracellular pH promotes vascular endothelial growth factor (VEGF) in human glioblastoma cells, tumor microenvironments induce metastasis of human hepatocellular carcinoma cells, and acid mediates tumor invasion.[10–12] Therefore, cancers are more likely to form under acidic conditions. A lower pH will more likely result in higher levels of the cytosine free bases, not only because cancer is more likely to deteriorate under acidic conditions but also because cytosine is more likely to be cleaved.[1] As a result, systems that recognize cytosine free bases might aid in monitoring of such diseases.

At present, the analysis of cytosine from DNA relies mainly on measuring the radioactivity of cytosine that has been pre-labeled with tritium.[2] The operation of the experiments is tedious. Although a macrobicyclic compound has been reported to bind cytosine,[13] it exhibits low selectivity. In addition to cytosine, it binds the nucleobases adenine and guanine, that is, as long as the guest compounds have pyridine nitrogen atoms. Furthermore, this molecule (molecular weight: 1812 g/mol) is much larger than the molecular weight cut-off (500 g/mol) for small-molecule drugs.[14] Recently, small-molecule ureidoquinoline and diaminonaphthyridine derivatives have been developed as intercalators for DNA-containing cytosine.[15,16] When binding with DNA, these compounds pair with cytosine units by forming three hydrogen bonds while simultaneously taking part in π–π stacking interactions with

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doi:10.1016/j.bmc.2010.08.013

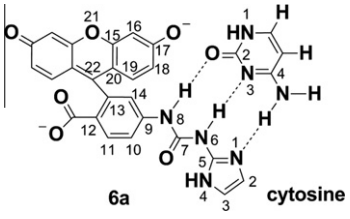
nucleobases in duplex DNA. Several receptors—based on pyridyl ureas, imidazolyl ureas, and guanine/pyrene conjugates—have been developed for the recognition of cytosine free bases and their derivatives in organic solvents.[17–19] Furthermore, a perylene-containing DNA receptor has been developed to specifically bind to cytosine in DNA.[20] To the best of our knowledge, small-molecule water-soluble receptors for cytosine free base have not been reported previously.

In order to find a convenient method to analyze cytosine, we employed an imidazolyl urea motif, which mimics the binding motifs in guanine, to develop a small-molecule (424 g/mol), water-soluble receptor **6** for cytosine that features 2-aminoimidazole and fluorescein units, both of which are water-soluble. The salient feature of the imidazolyl urea moiety is that it forms an intramolecular hydrogen bond between its imidazolyl NH with urea oxygen atom.[18] We also designed compound **6** so that it would be amiable to future in vivo studies because, when hydrolyzed, it forms 2-aminoimidazole and 6-aminofluorescein; the former occurs naturally in marine sponges and it is not toxic,[21–23] whereas the latter is used widely in the diagnosis of human diseases (e.g., through fluorescein injection).[24–27] This paper describes the synthesis of the cytosine receptor **6** using a general binding mode (Scheme 1) and a molecular recognition study of the hydrogen bonding of receptor **6a** (the disodium form of **6**) with cytosine using ^{15}N nuclear magnetic resonance spectroscopy [using partially labeled cytosine-2,4-^{13}C-1,3,4-^{15}N-cytosine (C1315)]. For comparison, we have also synthesized the receptor **8**. By taking advantage of quenching of fluorescence of **6a** by cytosine and its derivatives, including the anticancer drug 5-fluorocytosine (Chart 1), the apparent binding constants for them have been measured conveniently.

2. Results and discussion

2.1. Syntheses of 6 and 8

In one-pot, the 6-carboxyfluorescein **1** was converted through sequential reactions with *N*-hydroxysuccinimide (NHS) and NaN_3 to give the 6-carboazidofluorescein **3** in 71% yield (Scheme 2).[28] Compound **3** was then decomposed to compound **4**, which was reacted directly with 2-aminoimidazole to give compound **5** in 63% yield.[18] Compound **5** was hydrolyzed to give receptor **6** in 65% yield.[29] Likewise, we synthesized receptor **8**, which features a phenyl unit in place of the imidazole ring of **6**, from compound **3** (Scheme 3). Both **6** and **8** were converted to their disodium forms, **6a** and **8a**, respectively, using 1 N NaOH.



Scheme 1. Possible mode of binding between **6a** (two sodium ions are omitted for clearance) and cytosine.

Compound **6a** exhibits UV absorption maxima at 239, 276, 319, and 490 nm in aqueous phosphate buffer (50 mM) at pH 7.4. Its maximum fluorescence emission is located at 518 nm. Compound **8a** features UV absorption maxima at 239, 270, 321, and 491 nm in the same buffer, with its maximum fluorescence emission at 517 nm.

Our syntheses of compounds **6** and **8** were rather straightforward, each involving two one-pot transformations. The dipivaloyl 6-carboazidofluorescein **3** is a new building block that functions as a fluorescent probe. We suspect that it might find use in the synthesis of other ureido derivatives from such amino group-containing biomolecules as peptides, proteins, DNA, RNA, and peptide nucleic acids, thereby allowing their highly fluorescent probing.

Although a few ureidopyridine and ureidoquinoline derivatives have been reported to bind specifically to cytosine in DNA,[15,16] compound **6a** is the first water-soluble, fluorescent probe-labeled, small-molecule receptor that exhibits long-wavelength in UV absorption and fluorescence emission.

2.2. Monitoring the dimerization of receptor 6b in aqueous solution using ^{1}H NMR spectroscopy[30]

Receptor **6** was converted into its disodium form **6b** using NaOD solution (Scheme 4). The resulting solution was diluted with phosphate (50 mM) buffer (pD 7.4) in D_2O to prepare a stock solution; the pD was adjusted to 8.75 using NaOD and DCl solutions. At this pD, the receptor **6** should also exist as a form of **6b** because the fluorescein group has values of pK_a of 6.3 and 6.8.[31]

Next, we performed a ^{1}H NMR spectroscopic dilution study of **6b**, changing the concentration from 10.0 to 0.16 mM at 298 K, using a phosphate (50 mM) buffer (pD 8.75) in D_2O, to investigate its dimerization patterns and its monomer structure (Fig. 1). In D_2O, all of the active hydrogen atoms (NH, OH) were replaced by D atoms; therefore, they did not appear in the ^{1}H NMR spectra.

As revealed in Figure 1, dilution caused most of the signals of the aromatic protons of **6b**, except that for H14, to move downfield in the NMR spectra, suggesting that **6b** dimerized at high concentration. Interestingly, among all of the signals, that for H10 underwent the largest downfield movement ($\Delta\delta$ = 0.34 ppm) upon decreasing the concentration of **6b** from 10 to 0.16 mM. This behavior can be explained by considering that, at low

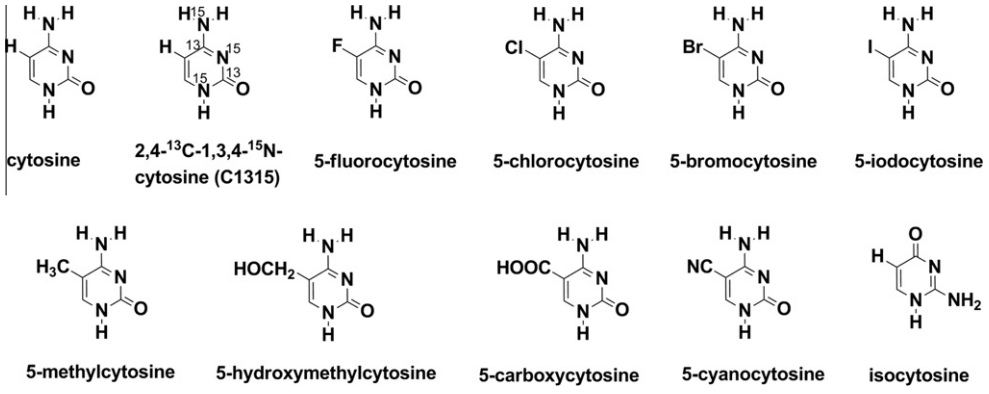
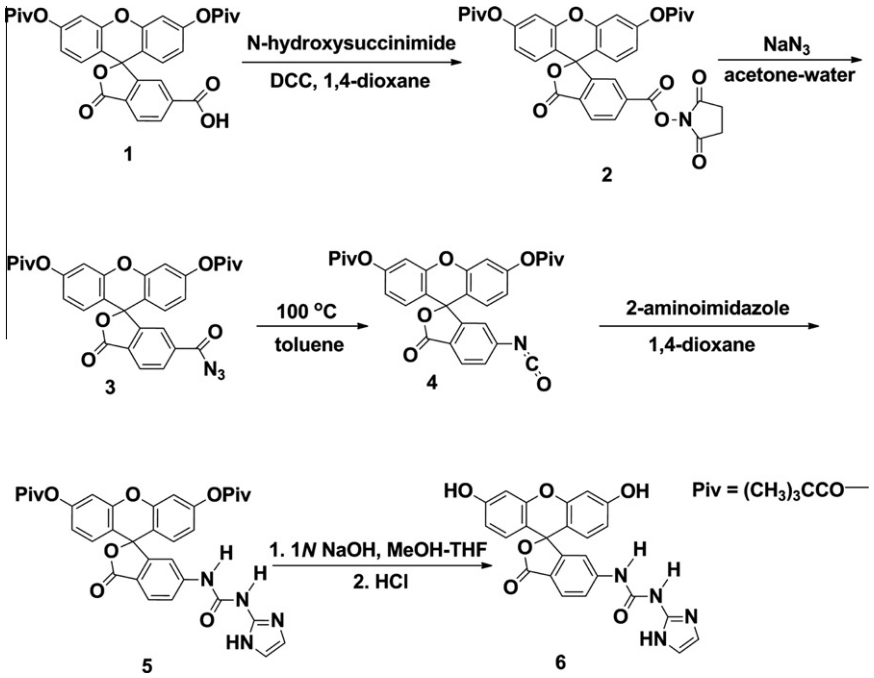
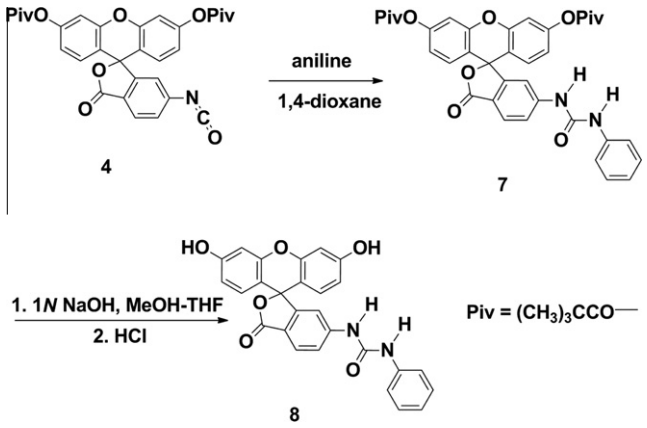


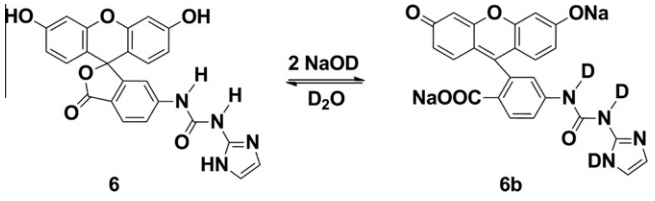
Chart 1. Cytosines.



Scheme 2. Synthesis of **6**.



Scheme 3. Synthesis of **8**.



Scheme 4. Preparation of **6b**.

concentrations, the monomeric form of **6b** should exist as depicted in Scheme 4, whereas, when the concentration of **6b** is high, its tautomer **6c** might form (Scheme 5).[32,33] In its dimeric form, tautomer **6c** features hydrophilic and hydrophobic groups at its exterior and interior, respectively; the hydrophilic groups are presented to interact with the solvent (water) molecules through hydrogen bonding and electrostatic interactions, while its hydrophobic units can pack through van der Waals interactions. Furthermore, in **6b**, the hydrogen atom H10 is located at the para position of the xanthenyl ring with respect to the sp^2-hybridized carbon atom C22; the π electrons of the xanthenyl ring become conjugated with those of the phenyl ring containing H10, as indicated in Scheme 5. Because the quinonyl ring, just like a C═O group, is electron-withdrawing, it causes the C10 and H10 atoms to be slightly electron-deficient. Furthermore, H10 becomes even more electron-deficient in the form of its monomer **6b** because of possible contact with the C═O oxygen atom at position 7.[34,35] Therefore, the chemical shift of H10 appears relatively downfield. In contrast, the carbon atom C22 is sp^3-hybridized in **6c**. Thus, the chemical shift of H10 is relatively upfield.

More interestingly, only the signal of proton H14 moved upfield ($\Delta\delta$ = 0.30 ppm) when we decreased the concentration of **6b** from 10 to 0.16 mM. This behavior also can be explained by considering the tautomerization of **6b** to **6c** at high concentrations (Scheme 5). In **6b**, the hydrogen atom H14 is located above the six-membered aromatic ring containing the carbon atom C22; its signal in the ^{1}H NMR spectra was, therefore, shielded by this xanthenyl group. In **6c**, H14 sits above the nonaromatic six-membered ring containing carbon atom C22; its signal is not shielded. Notably, dilution resolved the signals of all of the CH units of receptor **6b**.

Using nonlinear fitting and Grafit 6 software, we estimated the dimerization constant of **6b** to be 604 ± 68 M^{-1} (Supplementary Fig. S1).[30,34] This relatively large value is consistent with the large changes in the chemical shifts of protons H10 and H14 (Scheme 5). Scheme 6 presents a possible structure for the dimer of **6c** in D_2O, involving four possible [N–D···N] hydrogen bonds.[18]

2.3. Monitoring the binding of receptor 6a with cytosine in aqueous solution using ^{15}N NMR spectroscopy[30]

For this study using ^{15}N NMR spectroscopy, we used solutions comprising 90% H_2O and 10% D_2O. First, receptor **6** was converted into **6a** using NaOH solution. The resulting solution was diluted with phosphate (50 mM) buffer (pH 7.4) to form the stock solution of **6a**. Likewise, we prepared a stock solution of **8a**. In addition, we

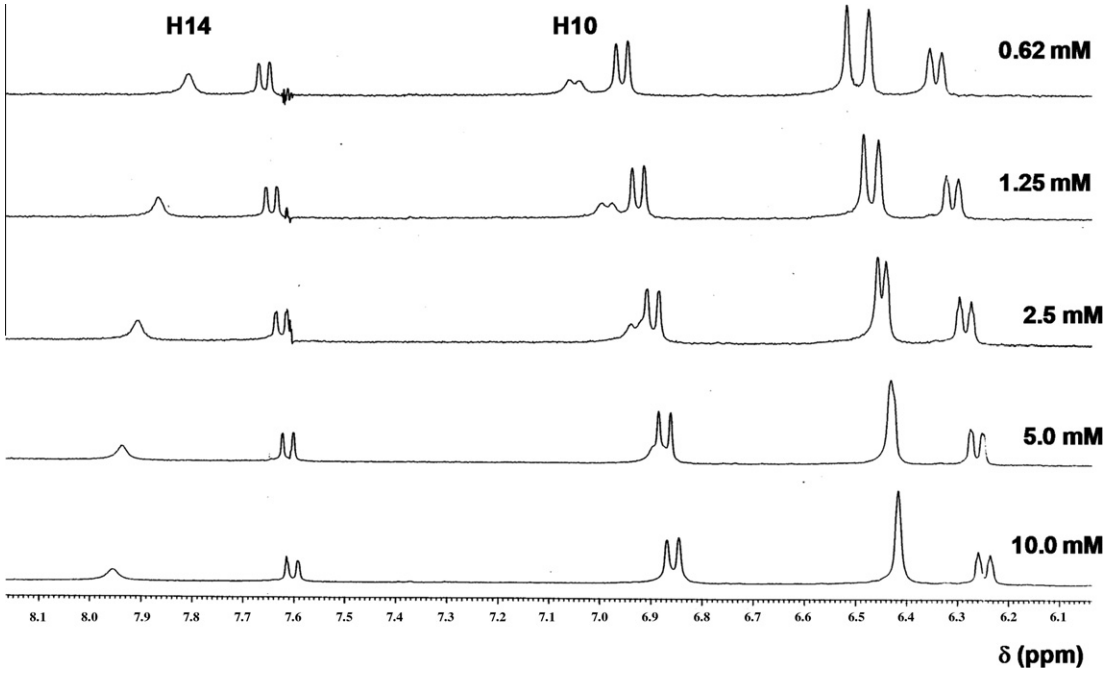
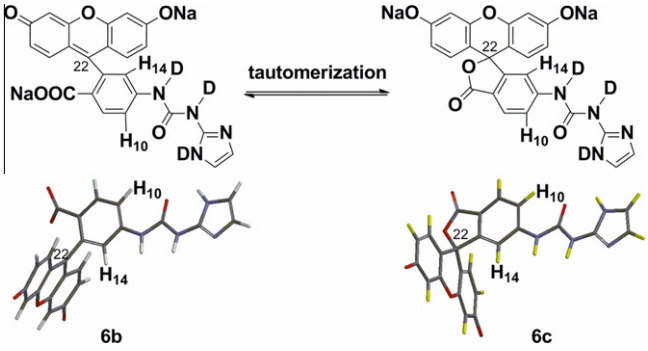
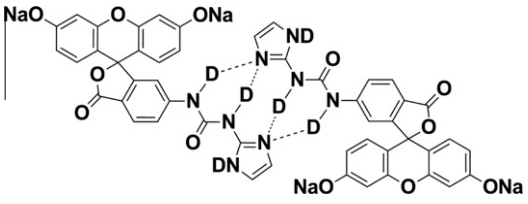


Figure 1. Partial ^{1}H NMR spectra of **6b** recorded at various concentrations in 50 mM phosphate buffer in D_2O (pD 8.75) at 293 K.



Scheme 5. Plausible tautomerization of **6b** and **6c** in pD (8.75) buffer. The low-energy, three-dimensional structures **6b** and **6c** were calculated using Spartan 06 at RM1 level.[36]



Scheme 6. Plausible structure for the dimer of **6c**, stabilized through hydrogen bonding interactions.

also prepared a stock solution of the partially labeled cytosine-2,4-^{13}C-1,3,4-^{15}N-cytosine (C1315) in phosphate buffer (pH 8.75). We used these stock solutions to prepare the samples for NMR spectroscopic analysis, adjusting their values of pH to 8.75 using NaOH and HCl solutions.

Next, we performed an ^{15}N NMR spectroscopic study of the binding between the receptors **6a** and **8a** and C1315 in 50 mM phosphate buffer (pH 8.75) at 293 K.[37] We obtained NMR spectra for C1315 (10.0 mM) alone and for its mixtures with the receptors **6a** and **8a**, individually (each concentration: 10.0 mM). Figure 2 displays three corresponding partial NMR spectra; Figure S2 provides calculated chemical shift changes for the nitrogen atoms at the N1 and N4 positions of C1315 upon binding. In the presence of receptor **6a**, these signals for C1315 underwent downfield shifts of +0.008 and +0.053 ppm, respectively. In the Watson–Crick base pair between cytosine and guanine, guanine transfers a proton to cytosine. In the binding between cytosine and receptor **6a**, we would also expect receptor **6** to donate a proton to cytosine.[8] As reported, protonation of a cytosine base in an oligonucleotide will result in downfield shifts of the signals of N1 and N4 in ^{15}N NMR spectra.[38] The formation of a DNA base pair involving cytosine also causes a downfield shift of the signal for N4 in ^{15}N NMR spectra.[39] Therefore, our observation of downfield chemical shifts of the signals of the nitrogen atoms N1 and N4 in cytosine (C1315) in the presence of **6a** is consistent with a protonated cytosine unit that is bound to the receptor. Therefore, these spectra reveal that Watson–Crick-type hydrogen bonding exists between the receptor **6a** and the cytosine free base under these conditions.

Figure S2B reveals that the shifts in the signals of the nitrogen atoms N1 and N4 in the NMR spectra of C1315 were −0.008 and 0.000 ppm, respectively, in the presence of **8a**, suggesting that little or no hydrogen bonding occurred to the receptor. This behavior can be explained by considering that receptor **8a** lacks the aryl nitrogen atom that receptor **6a** possesses for additional hydrogen bonding with cytosine. Any possible interaction between receptor **8a** and cytosine will also evoke steric repulsion between the phenyl group of **8a** and the amino group of cytosine (Scheme 7). Thus, receptor **8a** is a suitable control that suggests positive binding occurs between receptor **6a** and cytosine.

^{15}N NMR spectroscopy is used widely to study the hydrogen bond-mediated base pairing of cytosine units in DNA and RNA. These cytosine bases are usually also involved in π–π stacking interactions. In this study, we found that ^{15}N NMR spectroscopy is also useful for investigating the binding between a cytosine free base and a synthetic receptor, mediated through hydrogen bonding in aqueous solutions.

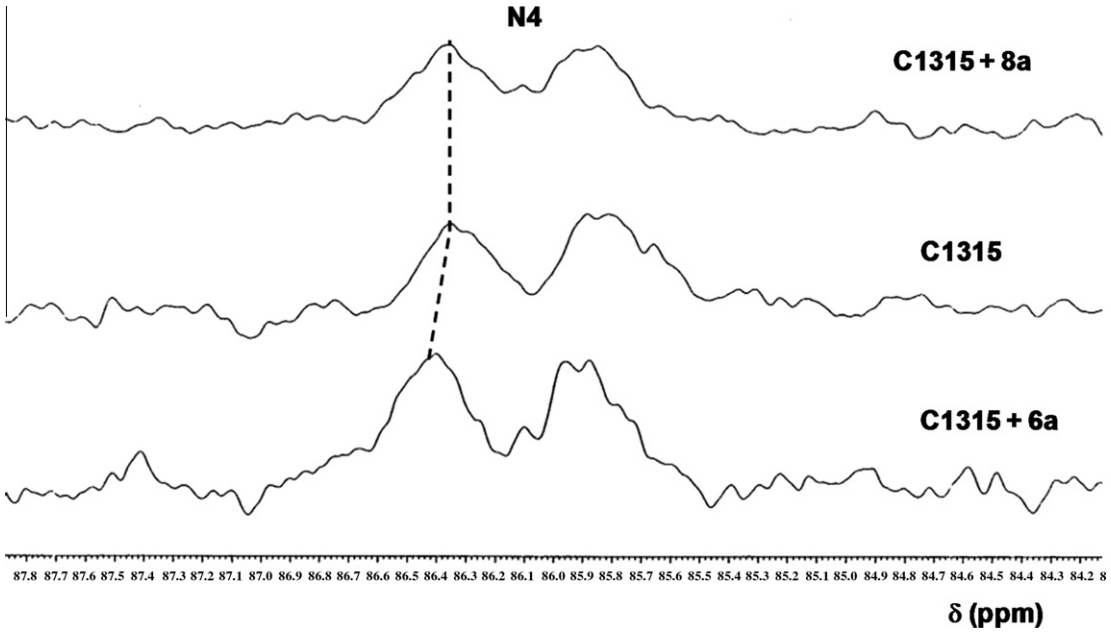
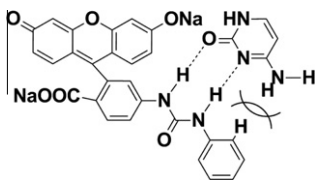


Figure 2. Partial ^{15}N NMR spectra of (top) cytosine (C1315, 10.0 mM) in the presence of receptor **8a** (10.0 mM), (middle) cytosine (C1315, 10.0 mM) only, and (bottom) cytosine (C1315, 10.0 mM) in the presence of receptor **6a** (10.0 mM); spectra were recorded from solutions in 50 mM phosphate buffer (pH 8.75) in 10% D_2O at 293 K.



Scheme 7. Plausible structure of the sterically encumbered complex formed between **8a** and cytosine.

2.4. Binding of 6a with cytosine quenches its fluorescence[30,34,35,40]

For this study, we used a dilute solution of **6a** (50 μM) in phosphate buffer (50 mM, pH 7.4) in water. At this pH, the receptor **6** would exist in the form of **6a** because the values of pK_a of the fluorescein group are ca. 6.3 and 6.8.[31] Although receptor **6a** is a fluorescent molecule exhibiting a fluorescence maximum at 518 nm, we performed this study of the binding between receptor **6a** and cytosine using the weaker excitation signal at 300 nm at 298 K.

Here, we monitored the change in fluorescence intensity of **6a** during the titration of cytosine into the mixture (Fig. 3), observing a decrease in the fluorescence intensity at 518 nm to almost complete quenching (80%). From the fluorescence intensities of cytosine and nonlinear square curve fitting, we estimated the apparent binding constant for the complex formed between receptor **6** and cytosine to be $60 \pm 2\ M^{-1}$ (Fig. 4).[30] Using the control, receptor **8a** exhibited less-dramatic fluorescence quenching in the presence of cytosine, with an estimated apparent binding constant of $6.9 \pm 0.2\ M^{-1}$. Our finding of the apparent binding constant for **6a** being about 8.7-fold greater than that for **8a** reveals the importance of the imidazole unit's nitrogen atom in the formation of the complex. This result is consistent with the chemical shift of the amino nitrogen atom in the ^{15}N NMR spectra of cytosine C1315 undergoing a significant downfield shift upon binding with **6a**, whereas it underwent no change in the presence of **8a**.

The binding constant for the complex formed between receptor **6a** and cytosine would be higher if there were no energetic penalty

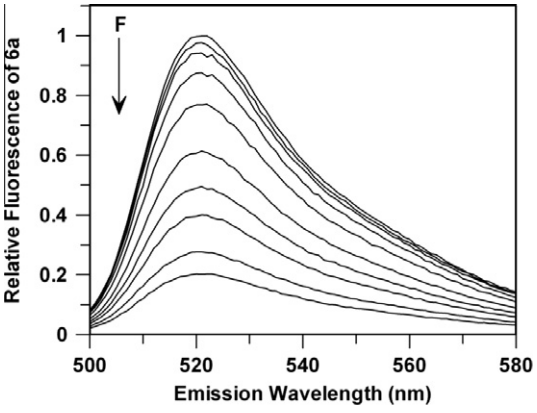


Figure 3. Incremental quenching of the fluorescence of **6a** (50 μM) at 518 nm upon the addition of cytosine at concentrations of 0.0, 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, 16.0, 24.0, 28.0, and 32.0 mM in phosphate buffer (50 mM, pH 7.4) at 298 K.

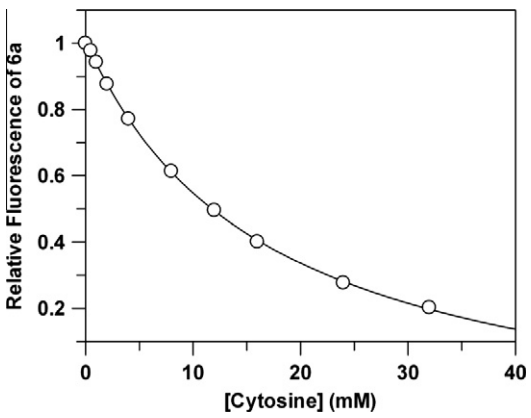


Figure 4. Relative fluorescence of **6a** plotted as a function of the concentration of cytosine in phosphate buffer (50 mM, pH 7.4) in H_2O at 298 K.

for breaking the dimers of **6a** (dimerization constant: 604 ± 68 M^{-1}). Nevertheless, the receptor **6a** is an effective binding partner for cytosine, as revealed in both the ^{15}N NMR and fluorescence spectroscopy studies. The apparent binding constant of 60 ± 2 M^{-1} for the complexation of receptor **6a** with cytosine is similar to that (110 ± 18 M^{-1}) reported for a pyrene-containing receptor with uracil;[30] notably, both of these complexes are stabilized by three hydrogen bonds in aqueous solution.

The binding of cytosine free base with receptor **6a** in water must also overcome the very high polarity of the solvent, which forms hydrogen bonds with all of the active hydrogen atoms and nitrogen and oxygen atoms in both cytosine and **6a**. Our observation of downfield chemical shifts for the signals of the nitrogen atoms in the ^{15}N NMR spectra suggests that there is binding between receptor **6a** and cytosine, presumably because three hydrogen bonds are involved in this complex. Although a couple of water-soluble, fluorescent receptors for cytosine have been reported, they were applied to studies of the binding of cytosine unit within DNA.[15,16] Neither the direct contact nor hydrogen bonding between the receptors and cytosine was investigated in those studies. Here, we demonstrate that direct contact and hydrogen bonding between receptors and cytosine can be revealed using both NMR and fluorescence spectroscopy.

To explore the specificity of the receptor **6a** in the binding of cytosine free base, we examined its behavior toward the water-soluble nucleobase uracil and the nucleoside deoxycytidine. We found that neither compound quenched the fluorescence of receptor **6a**, indicating that little or no binding occurs between **6a** and either uracil or deoxycytidine. The absence of binding between **6a** and uracil suggests that this receptor is specific for the nucleobase cytosine; the absence of binding between **6a** and deoxycytidine suggests that the sugar ring in deoxycytidine impedes the binding through a steric effect (Scheme 1). Therefore, **6a** is a specific receptor for cytosine free base, and not for cytosine-containing nucleosides.

As in other fluorescein-containing compounds, we expect the xanthenyl unit and the phenyl ring of **6a** to be aligned perpendicular to each other.[41,42] Therefore, in the complex formed between **6a** and cytosine, it is unlikely that cytosine will undergo efficient π–π stacking with the xanthenyl ring. The quenching of the fluorescence of receptor **6a** by cytosine is probably due to photoinduced electron transfer from cytosine, which is located in close proximity to the fluorophore of **6a** after binding.[43–46] Our observation of little or no fluorescence quenching of **6a** by uracil is consistent with their poor association. The relatively dramatic quenching of the fluorescence of **6a** by cytosine suggests that these molecules exist in close proximity in solution; in other words, cytosine and **6a** form a complex.

We also used 5-fluorocytosine, an anticancer prodrug of 5-fluorouracil, as a component for a study of its molecular recognition with **6a**.[5,6] Using fluorescence spectroscopy and nonlinear least-squares fitting, we estimated the apparent binding constant to be 560 ± 40 M^{-1} (Table 1)—approximately 9.3-fold better than that for cytosine. We used the same methodology to determine the binding constants for the complexes formed between **6a** and other halogenated cytosines: 5-chlorocytosine (1690 ± 122 M^{-1}), 5-bromocytosine (1806 ± 143 M^{-1}), and 5-iodocytosine (4366 ± 345 M^{-1}). Interestingly, the larger the halogen atom, the greater the apparent binding constant to receptor **6a**; this trend might be related to the acidity of the amino hydrogen atoms of the halogenated cytosines.[30]

The large difference between the apparent binding constants of **6a** for cytosine and the anticancer drug 5-fluorocytosine shows that the receptor **6a** can distinguish cytosine from 5-fluorocytosine using fluorescence quenching experiments. The significance of the experiments is that the receptor **6a** may be used in medical application such as therapeutic-drug-monitoring of 5-fluorocytosine. The advantage of the method is convenience, compared to traditional methodology based on the radioactivity of cytosine that has been pre-labeled with tritium.[2]

Many 5-substituted cytosines, such as 5-methylcytosine, 5-hydroxymethylcytosine, and 5-carboxycytosine, are related to epigenetics and DNA damage.[47,48] Therefore, we also measured, monitoring the quenching of the fluorescence of **6a** (Table 1), the apparent binding constants for the cytosines 5-methylcytosine (334 ± 10 M^{-1}), 5-hydroxymethylcytosine (151 ± 4 M^{-1}), and 5-carboxycytosine (913 ± 88 M^{-1}). The apparent binding constant of **6a** to 5-methylcytosine is 5.6-fold greater than that of **6a** to cytosine. The apparent binding constant of **6a** to 5-hydroxymethylcytosine is also greater (by ca. 2.5-fold) than that of **6a** to cytosine. Therefore, receptor **6a** can bind cytosine, 5-methylcytosine, and 5-hydroxymethylcytosine with different strengths. Receptor **6a** is the first water-soluble, fluorescent receptor to tell differences between these three cytosine free bases using binding constants. The apparent binding constant for 5-carboxycytosine is about 2.7- and 6.0-fold of those for 5-methylcytosine and 5-hydroxymethylcytosine, respectively, suggesting that the receptor **6a** can bind 5-carboxycytosine more tightly than cytosine, 5-methylcytosine, and 5-hydroxymethylcytosine.

Furthermore, we also measured the apparent binding constant for the complex formed between 5-cyanocytosine and **6a** (1100 ± 79 M^{-1}; Table 1). This apparent binding constant is similar to that for 5-carboxycytosine—not unexpected because both carbon atoms of the substituents at the 5-position have the same oxidation level and both are strongly electron-withdrawing.[49] The binding constant for isocytosine was 55 ± 5 M^{-1}, similar to that for cytosine (Table 1). Again, this situation is predictable because both isocytosine and cytosine are unsubstituted and have similar structures. Thus, **6a** is a versatile fluorescent indicator for various cytosine derivatives.

Table 1
Apparent binding constants (K_b) of cytosine and its derivatives to receptor **6a** in phosphate buffer (50 mM, pH 7.4) at 298 K

Substrate	K_b (M^{-1})
Cytosine	60 ± 2
5-Fluorocytosine	560 ± 40
5-Chlorocytosine	1690 ± 122
5-Bromocytosine	1806 ± 143
5-Iodocytosine	4366 ± 345
5-Methylcytosine	334 ± 10
5-Hydroxymethylcytosine	151 ± 4
5-Carboxycytosine	913 ± 88
5-Cyanocytosine	1100 ± 79
Isocytosine	55 ± 5

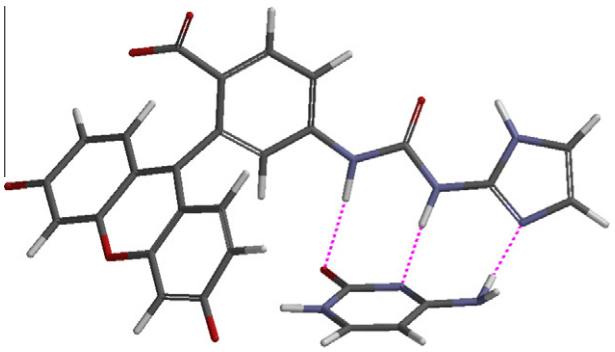


Figure 5. Calculated structure of the complex formed between the receptor **6a** and cytosine. Dotted lines: Hydrogen bonds. The two Na^+ ions have been omitted for clarity.

Table 2
Hydrogen bonding distances and angles in the complex formed between cytosine and receptor **6a**, calculated using the Spartan'06 software package at the RM1 level[36]

Hydrogen bond	Distance (Å)	Bond angle (°)
O···H–N	1.942	164.5
N···H–N	1.933	161.3
N–H···N	1.936	167.4

2.5. Binding of receptor 6a with cytosine revealed through structural calculations[30,34,35]

We used Spartan'06 software at the RM1 level to calculate the structure of the complex formed between **6a** with cytosine (Fig. 5).[36] Table 2 lists the distances and bond angles for the hydrogen bonds. The two ureido NH hydrogen atoms of receptor **6a** formed two strong hydrogen bonds with the oxygen and pyridine nitrogen atoms of cytosine, with distances (bond angles) of 1.942 (164.5) and 1.933 Å (161.3°), respectively. The pyridine nitrogen atom of the receptor **6a** formed one hydrogen bond with the amino group NH unit of cytosine, with a distance of 1.936 Å and a bond angle of 167.4° (Fig. 5). All the bond lengths and angles satisfy the criteria for strong hydrogen bonding, suggesting that cytosine binds tightly to receptor **6a**.[50] Furthermore, the xanthenyl ring of receptor **6a** is positioned near the hydrogen atom H1 of cytosine with a distance of 3.215 Å; this close proximity between the xanthenyl unit and cytosine would permit quenching of the fluorescence of receptor **6a** through photoinduced electron transfer (Scheme 1).[43–46]

3. Conclusions

We have synthesized the water-soluble, small-molecule, fluorescein-containing ureido compounds **6** and **8**, each through two one-pot transformations, using 6-carboxyfluorescein as a starting material. ^{1}H NMR spectroscopic dilution experiments revealed evidence for strong π–π stacking in the dimer formed from **6a** in phosphate buffer (pD 8.75) in D_2O. We then used ^{15}N NMR spectroscopy to investigate the binding of compounds **6a** and **8a** with cytosine at pH 8.75. In the presence of compound **6a**, but not **8a**, the signals of nitrogen atoms N1 and N4 in cytosine underwent downfield shifts, suggesting that **6a** binds cytosine, but **8a** does not. We also observed that the binding of **6a** to cytosine resulted in quenching of the fluorescence of **6a** at pH 7.4, allowing us to determine the apparent binding constant using nonlinear least-squares fitting. Uracil and deoxycytidine did not quench the fluorescence of **6a** in related binding studies, indicating that receptor **6a** is a specific receptor for cytosine free base. Measurements of the apparent binding constants of **6a** to other cytosine derivatives, such as 5-fluorocytosine, indicated that **6a** is a versatile fluorescent indicator for various cytosine free bases. Overall, receptor **6a** is the first water-soluble, small-molecule fluorescent receptor that binds specifically to cytosine free bases in aqueous solution.

4. Materials and methods

4.1. Materials and instruments

Chemicals were used as purchased without further purification. Flash chromatography was performed over silica gel (70–230 mesh) and monitored through thin layer chromatography (TLC) on silica gel plates. ^{1}H and ^{13}C NMR spectra were recorded using a Varian 400 (400 MHz) instrument with $CDCl_3$, D_2O, DMSO-d_6, and CD_3OD as solvents. Chemical shifts of protons are given in ppm relative to the signal of TMS as the internal standard; chemical shifts of carbon nuclei are reported in ppm relative to solvent signals used as internal standards. Fluorescence spectroscopy measurements were performed using a FluoroMax-3 spectrometer (HORIBA JOBIN YNON). Melting points were determined using a MEL-TEMP apparatus. IR spectra were recorded using a Genesis II FTIR spectrometer. UV–vis absorption spectra were recorded using a Shimadzu UV-1700 UV–vis spectrophotometer. Buffer pH was adjusted using an Accumet 925 pH meter (Fisher Scientific). The structure of the complex formed between cytosine and receptor **6a** was calculated using Spartan'06.[36]

4.2. Synthesis of 3′,6′-di(trimethylacetyloxy)-3-oxospiro [isobenzofuran-1(3*H*),9′-[9*H*]xanthene]-6-carbonyl azide (3)

NHS (0.53 g, 4.6 mmol) and *N*,*N*′-dicyclohexylcarbodiimide (DCC, 0.96 g, 4.8 mmol) were added sequentially to a solution of 6-carboxyfluorescein (**1**, 1.68 g, 3.1 mmol) in 1,4-dioxane (20 mL). The mixture was stirred under N_2 for 25 min. The white precipitate, *N*,*N*′-dicyclohexylurea, was removed through vacuum filtration. The filtrate was concentrated in vacuo to afford compound **2**, which was used directly for next step without purification.

The crude product obtained above was diluted with acetone (50 mL) and then a solution of NaN_3 (0.78 g, 12 mmol) in water (20 mL) was added. The resulting mixture was then stirred at room temperature for 3.5 h. After evaporation of the solvent in vacuo, the residue was purified through column chromatography (eluent: 0–20% EtOAc/hexane) to afford compound **3** as a white foam (1.25 g, 71%). R_f = 0.44 [EtOAc/hexane, 1:4 (v/v)]. Mp 158–160 °C. IR (neat, cm^{-1}) 2978, 2145, 1760, 1695, 1611, 1495, 1481, 1423, 1274, 1249, 1205, 1154, 1111, 1078, 995, 908, 730. ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 1.35 (s, 18H), 6.78 (s, 4H), 7.07 (s, 2H), 7.79 (s, 1H), 8.12 (d, J = 8.0 Hz, 1H), 8.30 (dd, J = 8.0, 1.5 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ 27.16, 39.31, 82.21, 110.6, 115.3, 118.0, 125.2, 125.8, 128.8, 130.4, 131.1, 137.0, 151.6, 152.9, 153.5, 168.0, 171.1, 176.6. HRMS: calcd for $C_{31}H_{27}N_3O_8Na^+$ (M+Na) 592.169, found 592.169.

4.3. Synthesis of *N*-(3′,6′-di(trimethylacetyloxy)-3-oxospiro [isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-5-yl)-*N*′-(2-imidazoyl)urea (5)

A solution of compound **3** (0.121 g, 0.20 mmol) in toluene (1 mL) was heated at 100 °C for 1 h. The solvent was then evaporated under a N_2 stream to afford compound **4**, which was used directly in the next step without purification.

The residue obtained above was dissolved in 1,4-dioxane (2 mL) and then 2-aminoimidazole (0.017 g, 0.21 mmol) was added. The mixture was then stirred at 80 °C for 1.5 h. After evaporation of the solvent, the residue was purified through column chromatography (eluent: 0–4% MeOH/CH_2Cl_2) to afford **5** as a white solid in 65% yield. R_f = 0.26 [MeOH/CH_2Cl_2, 1:20 (v/v)]. Mp >217 °C (dec). IR (neat, cm^{-1}) 3362, 2934, 2847, 1756, 1608, 1557, 1495, 1423, 1216, 1158, 1114, 759. ^{1}H NMR (400 MHz, $CDCl_3$/DMSO-d_6, ppm) δ 1.31 (s, 18H), 6.65 (s, 2H), 6.92–6.99 (m, 4H), 7.24 (s, 2H), 7.55 (d, J = 8.4 Hz, 1H), 7.79 (s, 1H), 7.89 (d, J = 8.4 Hz, 1H), 9.67 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$/DMSO-d_6, ppm) δ 27.26, 39.10, 80.39, 110.8, 117.1, 117.4, 119.1, 120.4, 126.2, 129.7, 148.5, 151.2, 152.7, 155.4, 168.9, 176.6. HRMS: calcd for $C_{34}H_{33}N_4O_8^+$ (M+H) 625.229, found 625.230.

4.4. Synthesis of *N*-(3′,6′-dihydroxy-3-oxospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-5-yl)-*N*′-(2-imidazoyl)urea (6)

1 N NaOH (0.47 mL) was added to a solution of compound **5** (0.050 g, 0.080 mmol) in MeOH (1 mL) and THF (1 mL). The

resulting solution was stirred at room temperature for 1 h. After evaporation of the solvents, the residue as dissolved in water (2 mL). The solution was filtered and acidified to pH 2–3 using 6 N HCl. The resulting solid was filtered off, washed with water, and dried to yield as a deep-red solid (23 mg, 65%). Mp >216 °C (dec). UV–vis (disodium salt in 50 mM phosphate buffer, pH 7.4, nm) λ_{max} 239, 276, 319, 490. IR (neat, cm^{-1}) 3094, 2942, 1717, 1586, 1459, 1386, 1209, 1111, 846, 737, 690. ^{1}H NMR (400 MHz, D_2O, ppm) δ 6.21–6.24 (m, 2H), 6.39 (s, 4H), 6.84 (d, J = 9.2 Hz, 3H), 7.59 (d, J = 8.4 Hz, 1H), 7.95 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD/DMSO-d_6, ppm) δ 82.39, 102.5, 110.4, 111.4, 113.0, 118.9, 120.0, 125.8, 129.6, 147.2, 152.2, 155.6, 159.7, 169.1. HRMS: calcd for $C_{24}H_{17}N_4O_6^+$ (M+H) 457.114, found 457.114.

4.5. Synthesis of *N*-(3′,6′-di(trimethylacetyloxy)-3-oxospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-5-yl)-*N*′-phenylurea (7)

A solution of compound **3** (0.107 g, 0.18 mmol) in toluene (1 mL) was heated at 100 °C for 1 h. The solvent was then evaporated under a N_2 stream to afford compound **5**, which was used directly in the next step without purification.

The residue obtained above was dissolved in 1,4-dioxane (2 mL) and then aniline (0.017 g, 0.017 mL, 0.19 mmol) was added. The mixture was stirred at 80 °C for 2 h. After evaporation of the solvent, the residue was purified through column chromatography (eluent: 0–60% EtOAc/hexane) to afford **7** as a white solid in 90% yield. R_f = 0.46 [EtOAc/hexane, 1:1 (v/v)]. Mp 182–184 °C (dec). IR (neat, cm^{-1}) 3369, 2978, 2927, 1756, 1709, 1597, 1539, 1495, 1423, 1289, 1220, 1154, 1111, 995, 893, 752, 690. ^{1}H NMR (400 MHz, $CDCl_3$/DMSO-d_6, ppm) δ 1.31 (s, 18H), 6.93–6.99 (m, 5H), 7.21–7.25 (m, 4H), 7.40 (d, J = 8.0 Hz, 2H), 7.52 (dd, J = 8.4, 1.4 Hz, 1H), 7.60 (d, J = 1.2 Hz, 1H), 7.95 (d, J = 8.4 Hz, 1H), 8.8 (s, 1H), 9.4 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$/DMSO-d_6/CD_3OD ppm) δ 27.11, 39.20, 80.62, 110.8, 111.3, 117.0, 118.3, 119.0, 119.1, 119.2, 120.4, 120.5, 122.9, 126.5, 129.2, 129.5, 139.3, 147.3, 147.4, 151.3, 152.4, 152.5, 152.8, 155.2, 168.7, 176.5. HRMS: calcd for $C_{37}H_{34}N_2O_8Na^+$ (M+Na) 657.221, found 657.222.

4.6. Synthesis of *N*-(3′,6′-dihydroxy-3-oxospiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-5-yl)-*N*′-phenylurea (8)

1 N NaOH (0.40 mL) was added to a solution of compound **7** (0.042 g, 0.065 mmol) in MeOH (1 mL) and THF (1 mL). The resulting solution was stirred at room temperature for 1 h. After evaporation of the solvents, the residue as dissolved in water (2 mL) and the solution filtered and acidified to pH 2–3 using 6 N HCl. The resulting solid was filtered off, washed with water, and dried to yield **8** as deep-red solid (29 mg, 95%). Mp >216 °C (dec). UV–vis (disodium salt in 50 mM phosphate buffer, pH 7.4, nm) λ_{max} 239, 270, 321, 491. IR (neat, cm^{-1}) 3290, 3072, 1688, 1601, 1546, 1495, 1445, 1386, 1267, 1209, 1180, 1111, 850, 748, 690. ^{1}H NMR (400 MHz, CD_3OD, ppm) δ 6.54–6.68 (m, 6H), 7.00 (t, J = 7.2 Hz, 1H), 7.24 (t, J = 8.4 Hz, 2H), 7.36 (d, J = 8.4 Hz, 2H), 7.44 (s, 1H), 7.63 (dd, J = 8.4, 1.8 Hz, 1H), 7.90 (d, J = 8.4 Hz, 1H), 8.4 (s, 1H), 8.9 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD, ppm) δ 102.1, 110.3, 112.1, 112.4, 119.2, 119.7, 120.0, 123.0, 125.5, 128.6, 129.0, 138.5, 146.6, 152.8, 153.1, 160.1, 170.2. HRMS: calcd for $C_{27}H_{19}N_2O_6^+$ (M+H) 467.124, found 467.123.

4.7. Synthesis of 5-chlorocytosine[51]

N-Chlorosuccinimide (NCS, 0.801 g, 6.00 mmol) was added to a solution of cytosine (0.55 g, 5.0 mmol) in a mixture of water (2 mL) and AcOH (20 mL) and then the resulting solution was stirred for 72 h at room temperature. The precipitate was filtered off, washed with AcOH (2 mL), dried in air, and then recrystallized from water (15 mL) to afford 5-cholorcytosine (0.46 g, 62%). Mp >288 °C (dec). ^{1}H NMR (400 MHz, DMSO-d_6, ppm) δ 10.84 (s, 1H), 7.70 (s, 1H), 7.07 (s, 2H).

4.8. Determination of dimerization constants of 6b through ^{1}H NMR spectroscopic dilution experiments

During the preparation of the buffer for the NMR spectroscopy experiments, pre-weighed Na_2HPO_4 was exchanged with D_2O (99.5%) three times under a lyophilizer. The resulting Na_2DPO_4 was then dissolved in D_2O to provide a total phosphate concentration of 50 mM. Buffers having values of pD of 7.4 and 8.75 were prepared by adjusting the pD of the Na_2DPO_4 solution using NaOD and DCl solutions and a pH meter.

A stock solution of **6b** was prepared by dissolving **6** into 0.33 N NaOD (2 equiv) and adjusting the volume to the required concentrations (usually 40 mM) using phosphate buffer (pD 7.4). The solution of **6b** (10 mM) for the NMR spectroscopic dilution experiment was then prepared by diluting the stock solution with the phosphate buffer (pD 7.4) and adjusting the pD to 8.75. In other dilution experiments, the phosphate buffer was applied at a pD of 8.75.

The dimerization constant for **6b** was determined by diluting a sample from a high concentration (10.0 mM) to the minimum concentration (0.16 mM) required for detection of a signal by ^{1}H NMR spectroscopy at 293 K. The chemical shift of proton H14 at 7.956 ppm at high concentration was followed; it decreased gradually to 7.656 ppm upon decreasing the concentration of **6b**. The data were then fitted to a nonlinear regression curve on a PC using the following Eq. 1:

$$\delta = \delta_m - [(\delta_m - \delta_d)/c][c + 0.25/K_d - (0.5c/K_d + 0.0625/K_d)^{1/2}] \quad (1)$$

where δ, δ_m, δ_d, and c are the observed chemical shift, the chemical shift of the monomer, the chemical shift of the dimer, and the total concentration of the receptor; K_d is the dimerization constant.

4.8.1. ^{15}N NMR spectroscopic study of the binding between receptor 6a and cytosine

In this experiment, partially labeled cytosine-2,4-^{13}C-1,3,4-^{15}N-cytosine (C1315, Sigma) was used. The stock solution (usually 40 mM) of C1315 was prepared by dissolving C1315 into phosphate buffer in water at pH 7.4. Stock solutions of **6a** and **8a** were prepared using a similar method as described above, except that NaOH was used in place of NaOD.

Three samples for NMR spectroscopy (0.6 mL) were prepared having a D_2O content of 10% each. The first sample, which contained C1315 (10 mM) only, was prepared by diluting the stock solution of C1315 with phosphate buffer (pH 8.75) and adding D_2O. The second sample, which contained C1315 (10 mM) and **8a** (10.0 mM), was prepared by mixing the stock solutions of C1315 and **8a**, phosphate buffer (pH 8.75), and D_2O. The third sample, which contained C1315 (10 mM) and **6a** (10.0 mM), was prepared in a manner similar to for the second sample, except that the stock solution of **6a** was applied. The final pH of each sample was adjusted to 8.75 using NaOH and HCl solutions.

^{15}N NMR spectra were recorded using a Varian 400 spectrometer; the chemical shifts of the nitrogen atoms are reported in ppm. The samples were scanned 3000–6000 times for 4–8 h.

4.9. Fluorescence titration

Stock solutions of **6a** and **8a** were prepared as described in Section 4.8. Stock solutions of cytosine (0.200 M) and 5-fluorocytosine

(0.100 M) were prepared in phosphate buffer (50 mM, pH 7.4) and kept warm on an oil bath during titration.

Fluorescence spectra were recorded using a spectrofluorometer [general settings: increment, 1; integration, 0.3; slit widths, 1 (excitation) and 1 (emission); equilibration time, 4 min]. All fluorescence spectra were recorded at 298 K; the temperature was maintained using a circulating water bath. The 4-mL quartz cuvette contained solutions at a final volume of 2 mL. The excitation wavelength was 300 nm. During the titrations and measurements of apparent binding constants, a solution of **6a** (50.0 μM) form the above stock solution (40 mM) in water was prepared first and then increasing amounts of the binding substrates were added to the solution using gas-tight syringes. The intensities of the fluorescence titration curves at 518 nm were analyzed using a one-site binding model equation and curve fitting software to evaluate the apparent binding constants.[52–55]

Acknowledgments

This study was supported by the Office of Research and Sponsored Programs (RD09010, E82016, RS0026) and SFRA Grants (for P.P. and Y.L.J.) from the Honors College at East Tennessee State University.

Supplementary data

Supplementary data (^{1}H and ^{13}C NMR spectra of compounds **3** and **5**–**8**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.08.013.

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