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Selective Recognition of Uracil and Its Derivatives Using a DNA Repair Enzyme Structural Mimic

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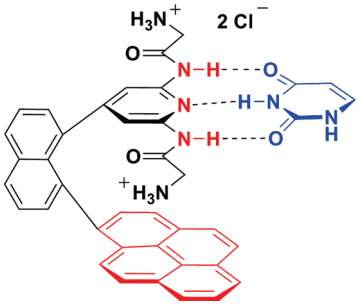

During DNA repair, uracil DNA glycosylase (UDG) pulls unwanted uracil into its active site through hydrogen bonding and $\pi-\pi$ stacking interactions. The reason why UDG binds only uracil tightly—and not its derivatives, such as thymine—remains unclear. In this study, we synthesized the stable, water-soluble receptor **1a** as a structural mimic of the active site in UDG. Compound **1a** contains a 2,6-bis(glycylamino)pyridine group, which mimics the amino acid residues of UDG that interact with uracil through a hydrogen-bonding network; it also possesses a pyrene moiety as a $\pi-\pi$ stacking interaction element and fluorescent probe that mimics the aromatic groups (phenyl and fluorescent indolyl units) found in the active site of UDG. Receptor **1a** binds selectively to uracil and derivatives (including thymine, 5-formyluracil, 5-fluorouracil, and 5-nitrouracil) and some DNA and RNA nucleosides (including thymidine and uridine) through hydrogen bonding and $\pi-\pi$ stacking interactions. Interestingly, a plot of log K_b with respect to the values of pK_a of the N(3)H units of uracil and its derivatives was linear, with a negative slope (β) of -0.24 ± 0.03. Thus, compounds featuring lower values of pK_a for their N(3)H units provided greater apparent binding constants for their complexes with receptor **1a**, suggesting acidity-dependent binding of uracil and its derivatives to this receptor; notably, uracil bound more tightly than did thymine. Our study provides some insight into how uracil and its derivatives in DNA are bound by DNA repair enzymes through hydrogen bonding and $\pi-\pi$ stacking interactions.

Introduction

Uracil DNA glycosylase (UDG) is a paradigm DNA repair enzyme that cleaves an unwanted uracil group from damaged DNA as the first step of the DNA repair process.[1] During the reaction, the enzyme uses a four-step mechanism (pinch, push, plug, and pull) to flip the uracil unit out of the DNA strand and draw it into the active site for cleavage.[2] In the active site, uracil is bound to the enzyme through hydrogen bonding and $\pi-\pi$ stacking interactions.[3] For instance, in the complex formed between *Escherichia coli* UDG and uracil (PDB, 2EUG), UDG uses its asparagine 123 and histidine 187 residues to hydrogen bond with uracil and its phenylalanine 77 residue to contact uracil through $\pi-\pi$

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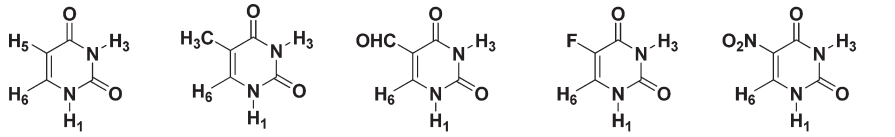
CHART 1. Uracil and Its Derivatives



stacking interactions.[3] In addition, because UDG possesses a tryptophan 164 residue, which is fluorescent and positioned adjacent to phenylalanine 77 in the active site, fluorescence quenching occurs during the binding of UDG with uracil. In contrast, quenching of this tryptophan fluorescence is not observed when titrating with thymine.[4] Thus, it appears that UDG binds uracil selectively, presumably because of the presence of a gatekeeper residue (tyrosine 66) that allows only uracil to enter the active site.[5] Thus, the steric effect of this tyrosine residue appears to be an important factor of the binding during DNA repair. It is not clear whether the acidity of uracil is also important in the binding and discrimination against thymine during DNA base flipping. Noticeably, the cleavage of uracil and its halogenated derivatives by another DNA repair enzyme, human thymine DNA glycosylase (TDG), is dependent on the acidity of the N(1)H unit of the uracil derivatives and the stability of the N-glycosidic bond.[6] Studies of this kind of acidity-dependent specificity for UDG are impossible without mutating the tyrosine residue, because many uracil derivatives, such as thymine and halogenated uracil, will not fit in the UDG active site. An alternative approach toward studying the specificity of the binding of uracil and its derivatives through hydrogen bonding and $\pi-\pi$ stacking interactions is the use of synthetic structural mimics of the active site of UDG.

Molecular recognition of uracil and thymine is particularly interesting because they are RNA and DNA bases, which are directly related to human diseases and cancers (Chart 1).[7] Uracil and 5-formyluracil are well-known damaged DNA bases; their study and detection might assist in the diagnosis of human diseases and aging problems.[8] Because the drug 5-fluorouracil (5-FU) is widely used for the treatment of cancers, studies of its binding behavior might assist cancer research.[9] 5-Nitrouracil and its derivatives exhibit antiviral effects because they are inhibitors of thymidylate synthetase.[10] Therefore, uracil and its derivatives are attractive targets for molecular recognition studies. Originally, uracil was found to bind adenine specifically in $CHCl_3$, as revealed by IR spectroscopy.[11] The pattern of hydrogen bonds between 1-methyluracil and 9-ethyladenine was discovered crystallographically; at the same time, the effect of substituent groups at the 5-position of uracil was noted, with greater acidity of the uracil derivatives facilitating their binding with the adenine derivative 9-ethyladenine.[11] Later, Hamilton and co-workers discovered that the binding between thymine and 2,6-bis(acylamino)pyridine occurred through three hydrogen bonds.[12] Recently, 2,6-bis(acylamino)pyridine receptors have been developed for the effective binding of uracil derivatives through hydrogen bonding and $\pi-\pi$ stacking interactions;[13] because these receptors are typically insoluble in water, their binding studies have been performed mostly in organic solvents, in which the molecular recognition of DNA and RNA bases and nucleosides is limited because of low solubility.[14]

Pyrene-containing receptors have been developed for the effective recognition of nucleobase derivatives, such as 9-butyladenine, through hydrogen bonding and $\pi-\pi$ stacking interactions.[15,16] By using our recently developed naphthalene scaffold, 2,6-bis(acylamino)pyridine as an element to bind uracil and its derivatives, and pyrene as a fluorescent probe and $\pi-\pi$ stacking interaction element, we have prepared a water-soluble receptor **1a** as an enzyme structural mimic of the active site of UDG (Scheme 1).[17] This paper describes a molecular recognition study of the hydrogen bonding and $\pi-\pi$ stacking interactions of receptor **1a** with uracil, its derivatives, other nucleobases, DNA and RNA nucleosides, and 2′-deoxyuridine. We also report the effects of the acidities of the N(1)H and N(3)H groups of uracil and its derivatives on their recognition behavior.

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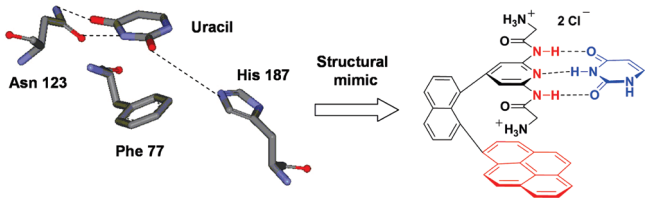
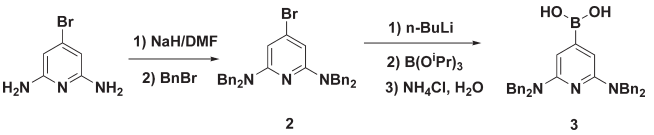
SCHEME 1. Structural Mimicry of the Truncated Complex of UDG with Uracil (PDB, 2EUG) Using a Synthetic Pyrene Receptor/Uracil Complex[3]



SCHEME 2. Synthesis of 3



Results and Discussion

Synthesis of 1a. 4-Bromo-2,6-diaminopyridine[18,19] was benzylated in the presence of NaH to afford compound **2**, which was converted into the boronic acid **3** through sequential treatment with *n*-BuLi and tri(isopropyl) borate followed by hydrolysis (Scheme 2).[20] Compound **3** was isolated as a colorless or light-yellow oil; because it turned purple after storage in a refrigerator, it was used directly, without further purification, in a standard Suzuki coupling with 1-[1′-(8′-bromonaphthyl)]pyrene (**4**) to afford compound **5**,[15] which was debenzylated through treatment with trifluoromethanesulfonic acid (TfOH) in the presence of pyrene (as a carbocation scavenger) in CH_2Cl_2 under reflux.[21] The resulting 2,6-diaminopyridine derivative **6** was then reacted with *N*-Boc-Gly-OH in the presence of HATU [O-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate] and Et_3N to afford compound **7**,[22] which was deprotected using a solution of hydrogen chloride in EtOAc for 5 min to afford the solid **1**.[23] This final product was purified through recrystallization from water/THF (Scheme 3). Elemental analysis of the carbon, hydrogen, and chlorine atom contents of solid **1** revealed a composition of $M(HCl)_{2.5}(H_2O)_4$, where M is the free amine of **1**, suggesting that the solid consisted of 50% each of compounds **1a** and **1b**. We used solid **1** for studies of the molecular recognition of uracil and its derivatives. To provide a sample suitable for NMR and IR spectroscopic characterization, we removed the residual solvent from solid **1** (< 1%) by dehydrating an aqueous solution of **1** in a spin vacuum machine.

This paper is the first to report the use of benzyl groups to doubly protect both amino groups of 2,6-diaminopyridine. All of these benzyl groups were readily removed using TfOH at low temperature (40 °C). We suspect that the organoboronic acid **3** might be a useful material for the synthesis of other bioactive receptors and druglike compounds.

Binding of Receptor 1a with Uracil and Derivatives Revealed by NMR Spectroscopy.[24] We performed an NMR spectroscopic dilution study of **1** at concentrations from 9.0 to 0.28 mM in D_2O at 298 K to investigate its dimerization patterns and monomer structure (Figure 1). In this study, we employed **1** as a mixture of **1a** and **1b**. In H_2O, **1b** is likely to dissociate to form the neutral pyridine unit because the pK_a of such a group is estimated to be 3.68 (Scheme 4).[25] As a result, the dilution of **1** with water will promote not only the formation of monomers of **1a** and **1b** but also the dissociation of **1b** to **1a**, which is more efficient in the binding of uracil because of its neutral pyridinyl moiety. At a concentration of 0.28 mM, we estimate that **1** contains 91% of **1a** and 9% of **1b**. Therefore, the truncated top NMR spectrum in Figure 1 mainly represents that of **1a**.

Dilution of a solution of **1** caused a downfield shift and splitting of the signal of the pyridinyl hydrogen atoms (from one to two peaks). This downfield chemical shift was presumably the result of diminishing $\pi-\pi$ stacking interactions between the pyrenyl groups in the dimers, suggesting their dissociation into monomers. The concentrations of **1** and the corresponding chemical shifts were fitted into a nonlinear regression curve, which provided an averaged dimerization constant for **1** of 600 ± 40 M^{-1} (Figure S1, Supporting Information). Scheme 5 summarizes the plausible dimerization patterns for a sample of **1** containing both **1a** and **1b**. The homodimer **1a**−**1a** features hydrogen bonding pairs involving the pyridinyl nitrogen and amide hydrogen atoms of **1a**; the heterodimer **1a**−**1b** features hydrogen bonding pairs involving the pyridinyl nitrogen and amide hydrogen atoms of **1a** and the amide bond hydrogen and oxygen atoms of **1b**; the homodimer **1b**−**1b** features hydrogen bonding pairs involving the amide bond hydrogen and oxygen atoms of **1b**.[26,14c]

The splitting of the signal in Figure 1 from one to two can also be attributed to the monomeric form of **1**. Because the pyrenyl group is not symmetrical in the monomer **1** and because of its restricted rotation, due to the peri-disubstituted naphthalene unit, the apparent symmetry of the pyridinyl ring in either **1a** or **1b** is destroyed in the monomer, resulting in the two pyridinyl hydrogen atoms appearing at different chemical shifts (Schemes 6 and 7). The maximum difference between chemical shifts of Hp_1 and Hp_2 of **1**, containing mostly **1a**, was 0.04 ppm (Figure 1).

Next, we used NMR spectroscopy to study the molecular recognition of uracil with receptor **1a** in D_2O at 298 K. In this binding study, we also used **1** as a mixture of **1a** and **1b**, where **1a** is more likely to bind uracil because of its more effective hydrogen bond network (Scheme 6). In H_2O, however, **1b** is likely to dissociate to form the neutral pyridine unit (Scheme 4).[26] As a result, both of the components of **1** will bind uracil (Scheme 6).

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(24) All dilution experiments and binding constant measurements were performed in water without using any buffer, because receptor **1** became cloudy and precipitated in phosphate buffer at pH 7.
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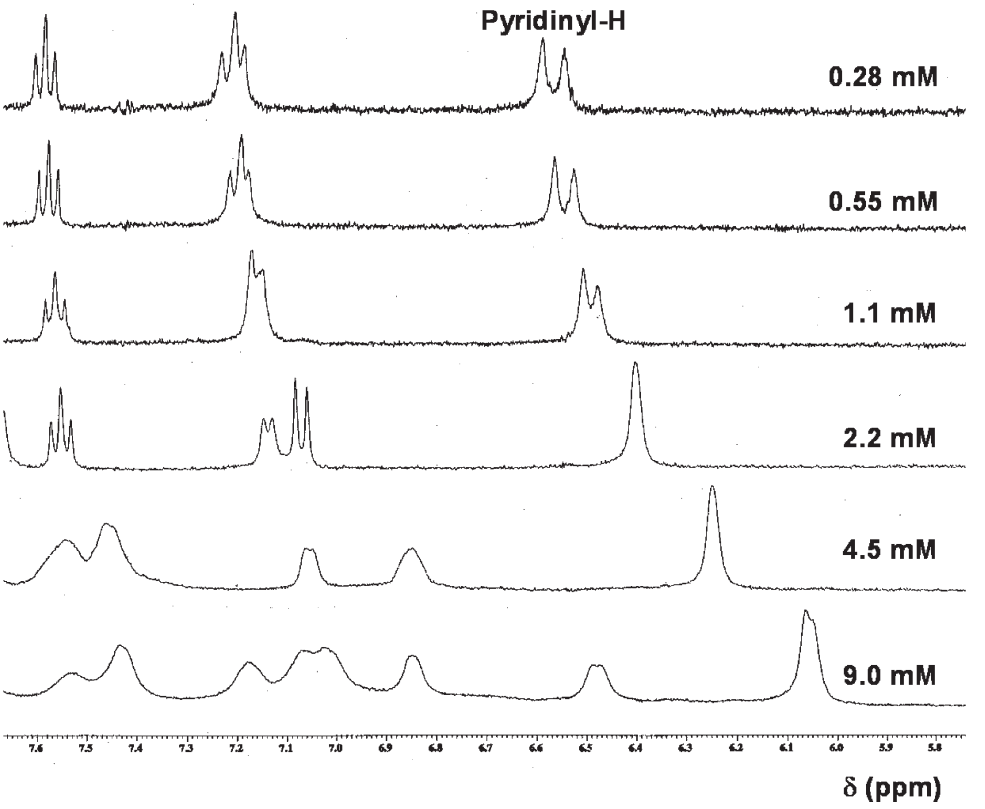
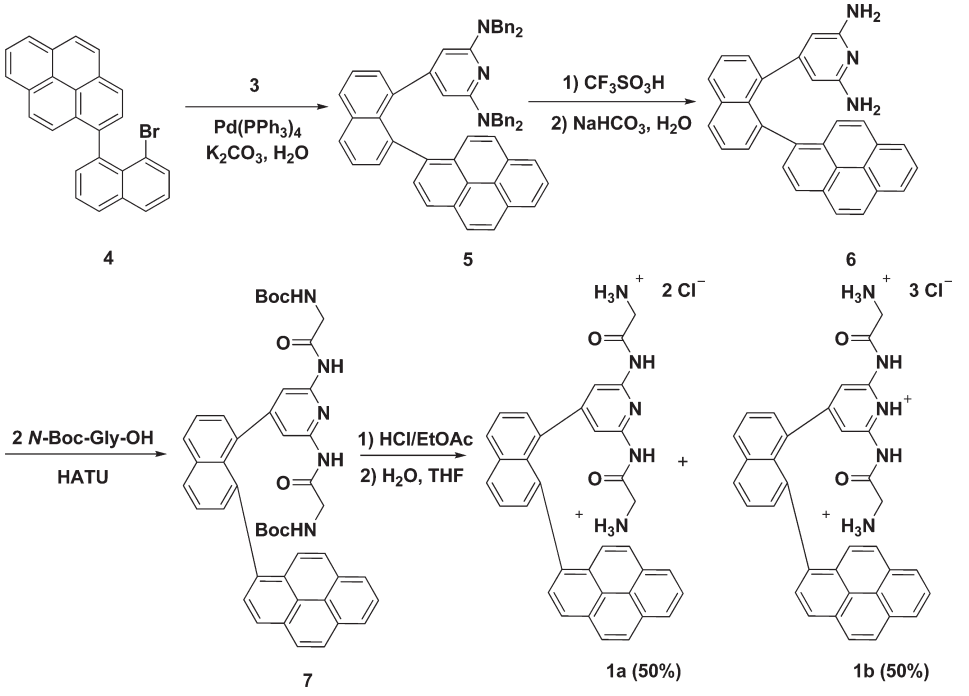


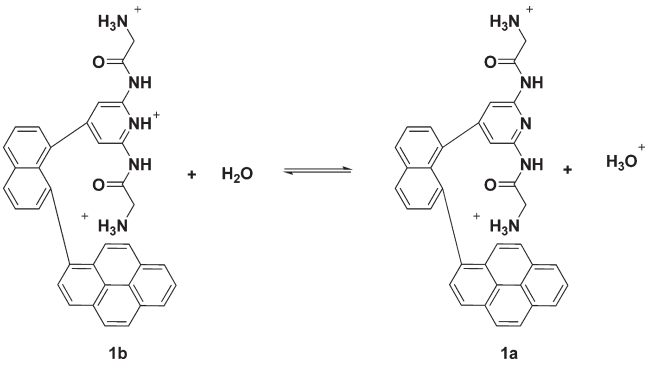
FIGURE 1. Partial ^{1}H NMR spectra of **1** at various concentrations in D_2O at 298 K.

SCHEME 3. Synthesis of 1

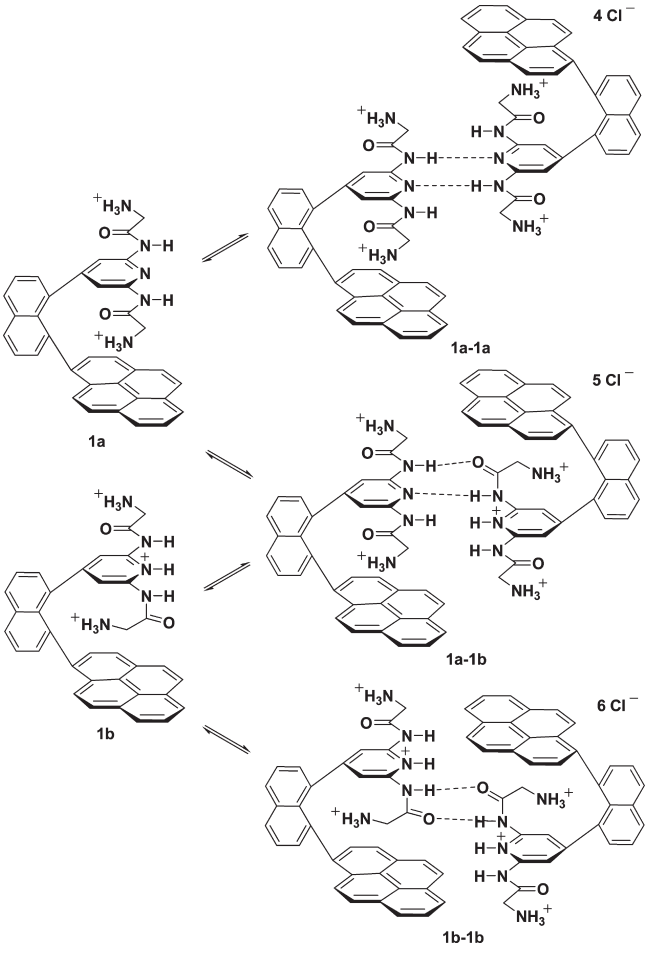


Job's plots, generated to identify the binding stoichiometry between uracil and receptor **1a**, revealed 1:1 binding (Figure S2, Supporting Information).[15] The variation in the chemical shifts of the CH protons of uracil (10.0 mM) were determined on the basis of NMR spectra recorded in the presence and absence of **1** (8.7 mM). In the presence of **1** (Figure 2), the signals of the C(5)H and C(6)H protons both moved upfield by 0.13 ppm, suggesting that uracil was bound to receptor **1a** through $\pi-\pi$ stacking interactions. The binding of uracil to the receptor also resulted in splitting of the signal for the pyridinyl hydrogen atoms Hp_1 and Hp_2 [difference in chemical shift: 0.03 ppm (similar to that of

SCHEME 4. Equilibrium between 1b and 1a in Aqueous Solution



SCHEME 5. Plausible Dimerization of 1 in Water Involving Both Hydrogen Bonding and $\pi-\pi$ Stacking Interactions[a]

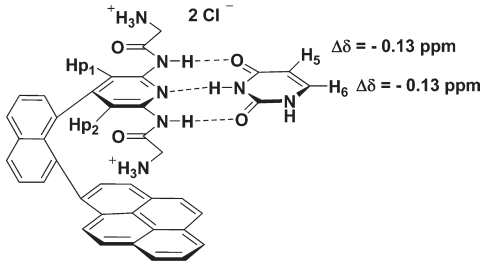


[a]The structures **1a**−**1a** and **1b**−**1b** are formed through homodimerization of **1a** and **1b**, respectively; **1a**−**1b** is the heterodimer formed from **1a** and **1b**.

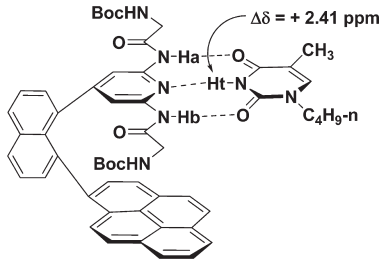
0.04 ppm for the monomer of **1a**)], suggesting that they experienced different chemical environments after dissociation of the dimer and formation of the complex with uracil (Scheme 6).

To study the hydrogen bonding between uracil and receptor **1a**, we recorded ^{1}H NMR spectra of mixtures of their derivatives 1-butylthymine and **7**, respectively. After mixing

SCHEME 6. Binding of Uracil (10.0 mM) and 1 (8.7 mM) in D_2O at 298 K Resulted in Upfield Shifts of the Signals of the C(5)H and C(6)H Protons and Splitting of the Signal of Hp1 and Hp2 by a Chemical Shift Difference of 0.03 ppm



SCHEME 7. Binding of 1-Butylthymine (10 mM) and 7 (10 mM) in $CDCl_3$ at 298 K Resulted in Downfield Shifts of the Signals for the Protons Ht (by a Chemical Shift Difference of 2.41 ppm) and Ha and Hb (by Chemical Shift Differences of 1.08 and 1.24 ppm, Respectively)



1-butylthymine (10 mM) with **7** (10 mM) in $CDCl_3$, the signals of the protons Ht (of 1-butylthymine) and Ha and Hb (of **7**) all moved downfield by 2.41, 1.08, and 1.24 ppm, respectively (Scheme 7), suggesting strong hydrogen bonding between 1-butylthymine and the 2,6-bis(glycylamino)-pyridinyl group in organic solvent[27] and, hence, the possibility of a strong hydrogen bond network between the receptor **1a** and uracil in water.

Binding of 1a with Uracil and Derivatives Revealed by Fluorescence Spectroscopy. The fluorescence emission spectrum of **1** in water reveals a signal maximum at 472 nm. Therefore, we investigated the binding of **1a** with uracil using fluorescence spectroscopy with water as a solvent at room temperature (298 K). In the binding study, we still used **1** as a mixture of **1a** and **1b**, with a total concentration of 0.87 μM. In this dilute solution, the majority of **1b** is likely to dissociate to form **1a** (Scheme 4). The calculated concentration of **1a**, which was available for binding, was 0.869 μM.[28]

During the measurements, we monitored the change in fluorescence intensity during the titration of the receptor with uracil (Figure 3). The molecular recognition of uracil by **1a** resulted in a decrease in the fluorescence intensity at 472 nm, providing further evidence for the binding of these

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(28) The pH of the solution of **1** was not controlled using any pH buffer. The calculated value of the pH for **1** (0.87 μM) was 6.4, determined using the estimated pK_a (3.68) of **1b** and the concentrations of **1a** and **1b** (both 0.435 μM). At these concentrations, 99.8% of **1b** will dissociate to form **1a**. Because the proton was not involved in the binding, the concentration of protons from **1b** should remain constant. Although the most acidic uracil derivative that we investigated (5-nitrouracil) would decrease the pH of the solution to a calculated value of pH 5.4 during the measurement, the concentration of **1a** would barely change because of the decrease in pH.

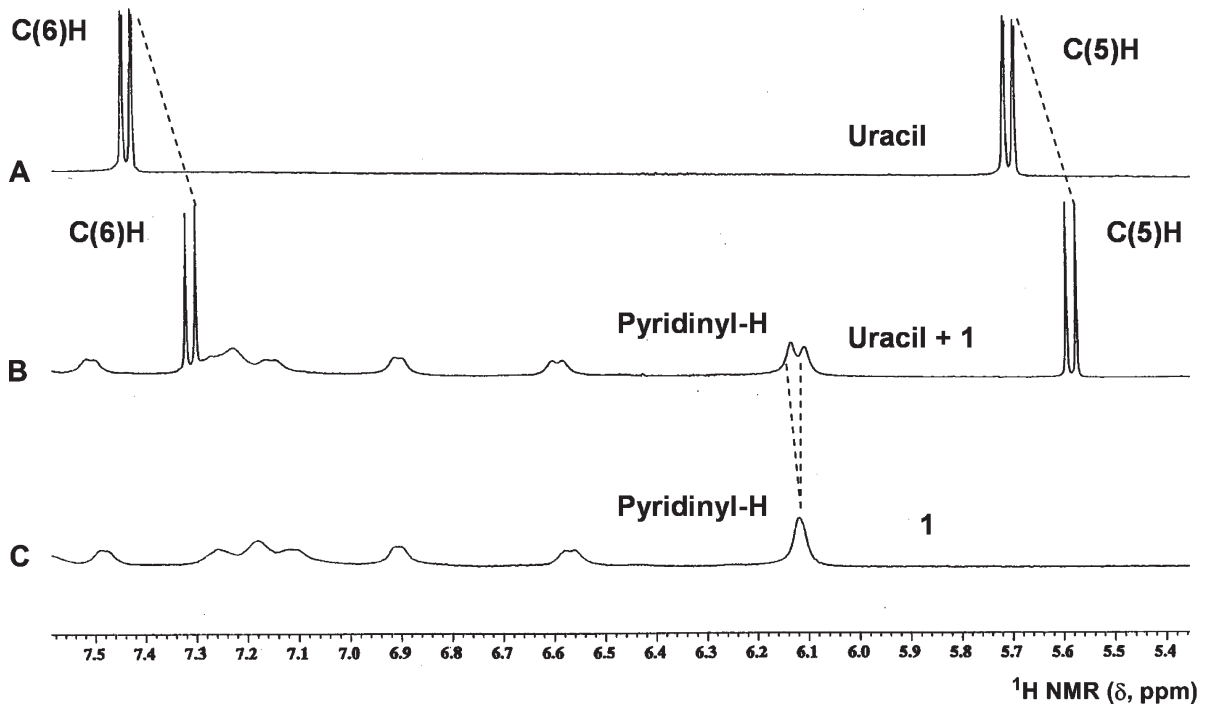

FIGURE 2. Partial ^{1}H NMR spectra (D_2O, 298 K) of uracil (10 mM, A), a mixture of uracil (10.0 mM) and **1** (8.7 mM, B), and **1** (8.7 mM, C).

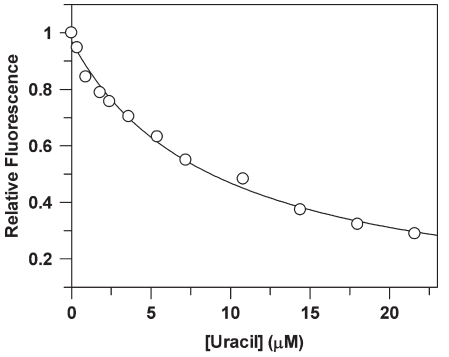

FIGURE 3. Titration of receptor **1** (0.87 μM) with uracil (0−24.00 μM), monitored using fluorescence spectroscopy at 298 K.

two species, with $\pi-\pi$ stacking interactions between the two molecules leading to quenching of the fluorescence of **1a**. The apparent binding constant for the complex formed between the receptor **1a** and uracil was 110 ± 18 M^{-1}, determined from the fluorescence intensities and nonlinear square curve fitting (Table 1). The binding constant is close to a reported value of 714 ± 51 M^{-1} for uracil and the DNA repair enzyme UDG.[29] The binding constant for the complex formed between the receptor **1a** and uracil would be higher if there were no energetic penalty for breaking the dimers of **1a**, because we estimated the dimerization constant to be 600 ± 40 M^{-1}. Nevertheless, the receptor **1a** is an effective binding partner for uracil, as revealed from the NMR and fluorescence spectroscopy studies.

Table 1 lists the apparent binding constants for receptor **1a** with the uracil derivatives thymine, 5-formyluracil, 5-fluorouracil, and 5-nitrouracil, determined through fluorescence titrations. Thymine was the poorest guest for receptor **1a**; 5-nitrouracil was the best. Thus, receptor **1a** can distinguish between uracil and thymine; the apparent binding constant for uracil was about 1.8-fold greater than that for thymine (60 M^{-1}).

TABLE 1. Apparent Binding Constants (K_b) of Uracil and Its Derivatives, Nucleosides of DNA and RNA, and 2′-Deoxyuridine with Receptor 1a in Water at 298 K

substrate	K_b (M^{-1})	pK_a of N(3)H[32,b]
thymine	61 ± 14	10.04
uracil	110 ± 18	9.34
5-formyluracil	195 ± 22	7.58
5-fluorouracil	315 ± 31	7.26
5-nitrouracil	446 ± 29	6.91
thymidine	154 ± 21	
2′-deoxycytidine	14 ± 2	
2′-deoxyadenosine	n.d.[a]	
2′-deoxyguanosine	n.d.[a]	
uridine	273 ± 21	
cytidine	n.d.[a]	
adenosine	n.d.[a]	
guanosine	n.d.[a]	
2′-deoxyuridine	352 ± 97	

[a]Not determined because of the small fluorescence change. [b]Calculated using the Poisson−Boltzmann continuum−solvation model.

A plot of the value of log K_b with respect to the value of pK_a of the N(3)H unit of uracil and its derivatives gave a straight line having a negative slope (β) of −0.24 ± 0.03 (Figure 4). We also plotted the values of pK_b of uracil and its derivatives with respect to the values of pK_a of the N(1)H units (Chart 1), but the plots had a much greater errors and a smaller absolute negative slope, −0.16 ± 0.05 (Figure S3, Supporting Information). These findings indicate that only the N(3)H units of uracil and its derivatives were involved in significant hydrogen bonding with receptor **1a**, providing

(29) Jiang, Y. L.; Cao, C.; Stivers, J. T.; Song, F.; Ichikawa, Y. *Bioorg. Chem.* **2004**, *32*, 244–262.

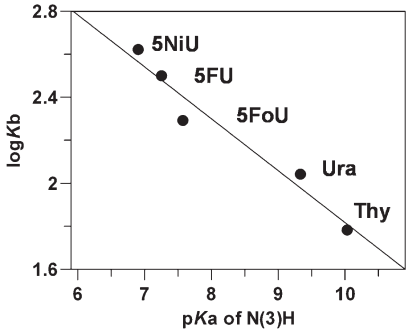

FIGURE 4. Values of log K_b for the complexes of **1a** with uracil derivatives plotted with respect to the values of pK_a of the N(3)H units of uracil, thymine (Thy), uracil (Ura), 5-formyluracil (5FoU), 5-fluorouracil (5FU), and 5-nitrouracil (5NiU).[32]

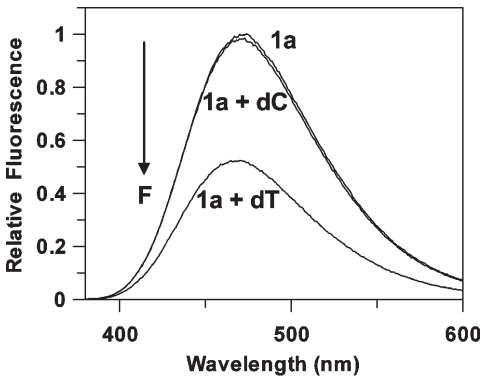

FIGURE 5. Significant quenching of the fluorescence of receptor **1** or **1a** (0.87 μM)[28] by the DNA nucleoside thymidine (dT, 8 mM) and small quenching by 2′-deoxycytidine (dC, 8 mM) in water at 298 K. No fluorescence quenching was induced by other DNA nucleosides (2′-deoxyadenosine and 2′-deoxyguanosine); for clarity, their spectra are not displayed.

further evidence for the binding mode presented in Scheme 6. These results are also consistent with the fact that only the N(3)H unit of uracil is a hydrogen-bonding donor in the complex with UDG (Scheme 1). Our results also reveal that a smaller value of pK_a for the N(3)H unit of a uracil derivative will provide a complex with receptor **1a** having a greater apparent binding constant, suggesting acidity-dependent binding of uracil and its derivatives by the receptor **1a** through the N(3)H group. This result is consistent with Rich's earlier observation of uracil derivatives of greater acidity exhibiting superior binding with 9-ethyladenine.[11] It also suggests that uracil is bound more tightly by the receptor **1a** than is thymine because the N(3)H unit of uracil has a greater value of pK_a for than that of thymine.

It is possible that a similar phenomenon may explain why UDG binds uracil more tightly than it binds thymine.[1b] A slight difference between uracil and thymine in the binding with UDG may contribute to the discrimination of uracil from thymine by UDG during the early stages of DNA base flipping.[30] Furthermore, the greater efficiency in the hydrogen bonding of uracil will eventually facilitate DNA base flipping by pulling a uracil moiety from a DNA base stack to the active site of UDG.[31]

Next, we investigated the molecular recognition of DNA and RNA nucleosides and 2′-deoxyuridine with receptor **1a**. Significant binding occurred between receptor **1a** and 2′-deoxyuridine, thymidine, and uridine, but there was little binding with 2′-deoxycytidine. The apparent binding constant for thymidine was ca. 11-fold greater than that for 2′-deoxycytidine, presumably because 2′-deoxycytidine does not possess the hydrogen bonding donor N(3)H. In addition, we observed no binding of receptor **1a** with the other nucleosides, suggesting that it can selectively recognize thymidine among the DNA nucleosides and uridine among the RNA nucleosides (Table 1 and Figure 5).

Binding of Receptor 1a with Uracil and Derivatives Revealed by Structural Calculations. We used Spartan'06 software at the RM1 level to calculate the structure of the complex formed between uracil with receptor **1a** (parts A and B of Figure 6).[33] Table 2 lists the distances and bond angles for the hydrogen bonds. The hydrogen atoms of the amide bonds of receptor **1a** formed two strong hydrogen bonds with the carbonyl oxygen atoms of uracil, with distances (bond angles) of 1.754 (159.5) and 1.766 Å (163.9°), respectively. The nitrogen atom of the pyridinyl group of **1a** formed a hydrogen bond with the N(3)H unit of uracil, with a distance of 1.841 Å and a bond angle of 175.4° (Figure 6A). All the bond lengths and angles satisfy the criteria for strong hydrogen bonding, suggesting that uracil is bound by receptor **1a** tightly.[34] Furthermore, the pyrenyl group is aligned almost parallel to both the pyridinyl group in receptor **1a** and the bound uracil, indicating that the complex features $\pi-\pi$ stacking interactions (Figure 6B). The vertical distance from the pyrenyl ring to the pyridinyl ring was ca. 3.78 Å. Although the uracil moiety is not positioned exactly above the pyrenyl ring in Figure 6A, it eclipses the edge of the pyrenyl group, sitting above the H4 and H5 atoms of the pyrenyl ring (Figure S4, Supporting Information). Taken together, our results suggest that the

TABLE 2. Hydrogen-Bonding Distances and Angles in the Complex Formed between Uracil and the Receptor 1a, Calculated Using the Spartan'06 Software Package at the RM1 Level[33]

hydrogen bond	distance (Å)	bond angle (deg)
N−Ha···O=C	1.754	159.5
N···H−N	1.841	175.4
N−Hb···O=C	1.766	163.9

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(31) (a) Parikn, S. S.; Walcher, G.; Jones, G. D.; Slupphaug, G.; Krokan, H. E.; Blackburn, G. M.; Tainer, J. A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 5083–5088. (b) Krosky, D. J.; Song, F.; Stivers, J. T. *Biochemistry* **2005**, *44*, 5949–5959.

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(34) Zhao, J.; Khalizov, A.; Zhang, R.; McGraw, R. *J. Org. Chem.* **2009**, *113*, 680–689.

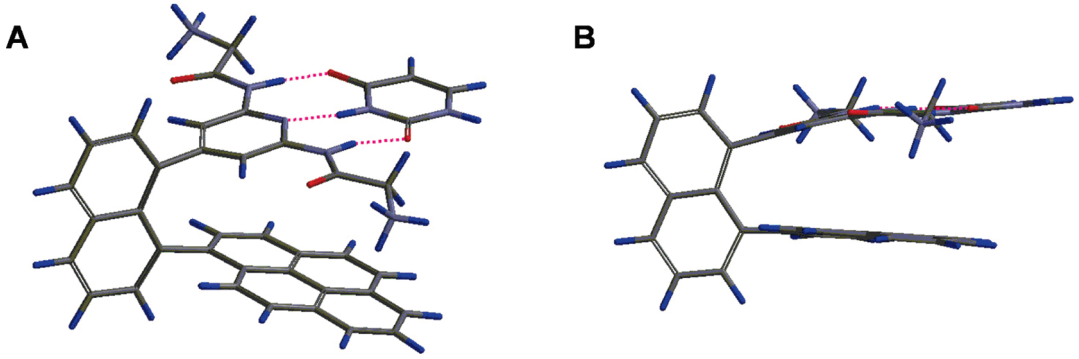

FIGURE 6. Two views of the calculated structure of the complex formed between the receptor **1a** and uracil. Dotted lines: hydrogen bonding between **1a** and uracil (two chloride ions have been omitted for clarity).

binding of uracil by receptor **1a** is stabilized through both hydrogen bonding and $\pi-\pi$ stacking interactions.

Conclusions

To determine why UDG binds uracil tightly, but not thymine, we prepared a novel fluorescent receptor **1a** that structurally mimics the active site of UDG, which cleaves uracil from DNA strands. Compound **1a** features a 2,6-bis(glycylamino)pyridinyl group that mimics the amino acid residues in UDG that participate in hydrogen bonding; it also possesses a pyrenyl group, mimicking the phenyl group that undergoes $\pi-\pi$ stacking interactions in the active site of UDG. The pyrenyl group also mimics the fluorescent indolyl group of the tryptophan residue in the active site of UDG; therefore, it undergoes fluorescence quenching during binding. We obtained **1a** in six steps from 4-bromo-2,6-diaminopyridine. During this synthesis, we employed two benzyl groups to protect each amino group in 4-bromo-2,6-diaminopyridine. We suspect that one of the intermediates in our synthesis, the pyridinylboronic acid **3**, might be a useful compound for the synthesis of biologically active molecules, such as receptors and drug-like candidates.

We performed a dilution experiment for **1** to investigate its dimerization. During the dilution experiment, the downfield shift and the splitting pattern of the signal of the pyridinyl hydrogens were consistent with dissociation of the dimers into monomers of receptor **1**.

^{1}H NMR spectroscopy revealed that $\pi-\pi$ stacking interactions occurred between the receptor **1a** and uracil in D_2O. The hydrogen bonding network formed between the 2,6-bis(glycylamino)pyridinyl group of **1a** and uracil was revealed in the NMR spectra of the complex formed between their organic-soluble congeners **7** and 1-butylthymine, respectively, in $CDCl_3$. We used fluorescence titrations to determine the apparent binding constants (K_b) of receptor **1a** with uracil and its derivatives (thymine, 5-formyluracil, 5-fluorouracil, 5-nitrouracil) and related nucleosides (uridine, 2′-deoxyuridine, thymidine, 2′-deoxycytidine). We found that receptor **1a** recognizes uracil with some selectivity. A plot of the values of $\log K_b$ of uracil and its derivatives with respect to the values of pK_a of the N(3)H unit of these uracil derivatives was highly linear, with a slope of -0.24 ± 0.03; i.e., the molecular recognition process was dependent on the acidity of the substrate. This binding study also revealed that the receptor **1a** selectively binds thymidine relative to other DNA nucleosides (2′-deoxycytidine, 2′-deoxyadenosine, 2′-deoxyguanosine) and uridine relative to other RNA nucleosides (cytidine, adenosine, guanosine). We also investigated the binding between the receptor **1a** and uracil through molecular modeling using Spartan'06 at the RM1 level; we observed effective hydrogen bonding and $\pi-\pi$ stacking interactions between the two molecules. Receptor **1a** is, therefore, an example of a small molecule that might be able to detect and recognize damaged DNA and bases, and thereby assist in the better understanding of DNA repair enzymes.

Experimental Section

4-Bromo-*N*,*N*,*N*′,*N*′-tetrabenzyl-2,6-diaminopyridine (2). A solution of 4-bromo-2,6-diaminopyridine (1.07 g, 5.69 mmol) in DMF (5 mL) was added over 15 min to a mixture of NaH (60% purity, 1.95 g, 44.2 mmol) in DMF (10 mL) in a 50-mL round-bottom flask cooled to 0 °C in an ice−water bath. Benzyl bromide (4.0 mL, 5.75 g, 33.6 mmol) was added, and then the mixture was warmed to room temperature and stirred for an additional 1.5 h. Water was added, and then the aqueous phase was extracted with EtOAc. The combined extracts were concentrated, and the residue was purified through column chromatography [eluent: hexane and then a mixture of CH_2Cl_2, EtOAc, and hexane (17:17:66, v/v/v)] to afford compound **2**, which was recrystallized from EtOAc to give a white solid (1.47 g, 47%): mp 144−146 °C; IR (neat, cm^{-1}) 3029, 2911, 1556, 1494, 1473, 1450, 1360, 1200, 1172, 964, 951, 777, 730, 694; ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 4.66 (s, 8 H), 6.04 (s, 2 H), 7.16−7.29 (m, 20 H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ 50.9, 73.9, 96.8, 127.0, 127.2, 128.6, 138.5, 158.1; HR-ESI-TOF calcd for $C_{33}H_{31}BrN_3^+$ (M + H) 548.170, found 548.170.

4-(1′-[8′-(1″-Pyrenyl)naphthyl])-*N*,*N*,*N*″,*N*″′,*N*″′-tetrabenzyl-2,6-diaminopyridine (5). *n*-BuLi (1.52 mL of 1.6 M in hexane, 2.44 mmol) was added to a solution of **2** (1.20 g, 2.21 mmol) in anhydrous THF (8 mL) in a 25-mL round-bottom flask that had been cooled to −78 °C in an dry ice/acetone bath. The reaction mixture was stirred for 1.5 h at −78 °C before triisopropyl borate (0.77 mL, 3.33 mmol) was added; the mixture was then stirred at this temperature for an additional 0.5 h. After the mixture was warmed to room temperature and stirred for an additional 1.5 h, saturated aqueous ammonium chloride (8 mL) was added and the resulting mixture stirred for 2 h. The aqueous phase was extracted with CH_2Cl_2, dried ($MgSO_4$), and concentrated in vacuo to afford **3** (1.27 g) as a colorless oil, which turned purple under storage in a refrigerator. This compound was used without further purification: IR (neat, cm^{-1}) 3391, 2934, 1595, 1541, 1425, 1358, 1240, 1204, 1045, 1028, 970, 729,

694; ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 4.58 (s, 8 H), 4.64 (s, 2 H), 7.10−7.18 (m, 20 H); HR-ESI-TOF calcd for $C_{33}H_{33}BN_3O_2^+$ (M + H) 514.266, found 514.266.

1-[1′-(8′-Bromonaphthyl)]pyrene (**4**, 0.664 g, 1.3 mmol), compound **3** (0.97 g, 1.4 mmol), and 1,2-dimethoxyethane (20 mL) were added to a three-neck round-bottom flask under a N_2 stream. $Pd(PPh_3)_4$ (48 mg, 0.042 mmol) was added to the suspension, followed by a degassed solution of K_2CO_3 (0.618 g, 4.48 mmol) in water (2.93 mL). The resulting mixture was heated under reflux for 13 h under N_2. After being cooled to room temperature, the mixture was extracted with CH_2Cl_2, and the combined organic phases were dried ($MgSO_4$). After filtration, the solvents were evaporated in vacuo, and the residue was purified through column chromatography (eluent: 0−2% EtOAc/hexane) to afford **5** as an orange oil, which solidified after storage in a refrigerator (0.86 g, 83%): mp 102−105 °C; IR (neat, cm^{-1}) 3038, 2905, 1587, 1571, 1549, 1493, 1467, 1451, 1355, 1200, 1172, 969, 830, 810, 723, 694; ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 3.08 (d, J = 16.4 Hz, 2 H), 3.44 (d, J = 16.4 Hz, 2 H), 4.30 (d, J = 16.4 Hz, 2 H), 4.72 (d, J = 16.4 Hz, 2 H), 4.88 (s, 1 H), 5.58 (s, 1 H), 6.46 (d, J = 6.0 Hz, 4 H), 7.00−7.02 (m, 6 H), 7.12 (d, J = 6.8 Hz, 5 H), 7.23−7.26 (m, 2 H), 7.30−7.34 (m, 4 H), 7.42−7.47 (m, 2 H), 7.53 (d, J = 9.2 Hz, 1 H), 7.60 (t, J = 7.2 Hz, 1 H), 7.67 (d, J = 7.6 Hz, 1 H), 7.82 (d, J = 9.6 Hz, 1 H), 7.91 (d, J = 7.6 Hz, 1 H), 7.94−8.01 (m, 3 H), 8.07−8.12 (m, 3 H), 8.17 (d, J = 7.6 Hz, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ 50.0, 50.4, 96.1, 96.3, 123.7, 124.2, 124.6, 124.9, 125.1, 125.16, 125.23, 125.9, 126.0, 126.3, 126.9, 127.1, 127.4, 127.7, 127.9, 128.3, 128.78, 128.85, 128.9, 129.7, 130.0, 130.1, 130.6, 131.1, 131.5, 132.0, 134.9, 138.5, 138.7, 139.0, 139.5, 140.8, 152.6, 155.79, 155.84; HR-ESI-TOF calcd for $C_{59}H_{46}N_3^+$ (M + H) 796.369, found 796.369.

4-(1′-[8′-(1″-Pyrenyl)naphthyl])-2,6-diaminopyridine (6). TfOH (0.67 mL, 7.58 mmol) was added dropwise to a mixture of **5** (0.212 g, 0.277 mmol) and pyrene (1.00 g) in anhydrous CH_2Cl_2 (7 mL) in a 25-mL round-bottom flask, and then the resulting mixture was heated under reflux (oil bath) for 6 h. After the mixture had cooled to room temperature, it was poured slowly onto saturated $NaHCO_3$ (80 mL) with constant stirring, causing a color change from green to deep red. The aqueous phase was extracted with CH_2Cl_2, the combined organic phases were concentrated, and the residue was purified through flash column chromatography (eluents: hexane, 50% EtOAc/hexane, EtOAc, and then 0−2.5% MeOH/EtOAc). The fractions containing compound **6** were combined and concentrated; the residue was subjected again to column chromatography (eluents: $CHCl_3$ and then 10−50% acetone/$CHCl_3$) to afford **6** as a deep-red oil (73 mg, 67%): IR (neat, cm^{-1}) 3468, 3383, 3184, 3040, 1612, 1554, 1432, 1398, 1244, 1178, 907, 847, 828, 775, 723; ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 2.28 (s, 2 H), 3.81 (s, 2 H), 4.75 (s, 1 H), 5.19 (s, 1 H), 7.15 (d, J = 6.8 Hz, 1 H), 7.50 (t, J = 8.0 Hz, 1 H), 7.59 (d, J = 9.2 Hz, 1 H), 7.62−7.70 (m, 3 H), 7.85 (d, J = 9.2 Hz, 1 H), 7.94−8.08 (m, 7 H), 8.15 (d, J = 7.6 Hz, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ 100.3, 100.8, 123.6, 124.3, 124.5, 124.9, 125.1, 125.2, 125.3, 125.9, 126.2, 126.8, 127.3, 127.6, 128.6, 129.1, 129.3, 129.4, 129.6, 130.5, 130.7, 130.8, 131.4, 131.6, 135.1, 138.4, 139.2, 153.6, 154.8, 155.0; HR-ESI-TOF calcd for $C_{31}H_{22}N_3^+$ (M + H) 436.181, found 436.181.

4-(1′-[8′-(1″-Pyrenyl)naphthyl])-2,6-diaminopyridine Bis(Boc-glycamide) (7). A 25-mL round-bottom flask was charged sequentially with **6** (0.095 g, 0.232 mmol), anhydrous THF (2.5 mL), Et_3N (0.224 mL, 1.62 mmol), *N*-Boc-Gly-OH (0.123 g, 0.703 mmol), and HATU (0.263 g, 0.692 mmol), and then the mixture was heated under reflux for 4 h. After the mixture was concentrated in vacuo, the residue was purified through flash column chromatography (SiO_2; 0−50% EtOAc/hexane). The fractions containing **7** were concentrated and the residue subjected again to column chromatography (SiO_2; 0−5% acetone/$CHCl_3$) to afford a yellow oil, which solidified after refrigeration (127 mg, 73%): mp 120−122 °C; IR (neat, cm^{-1}) 3305, 2978, 1690, 1560, 1506, 1417, 1366, 1220, 1166, 848, 776, 731; ^{1}H NMR (400 MHz, $CDCl_3$, ppm) δ 1.48 (s, 9 H), 1.55 (s, 9 H), 3.10 (s, 2 H), 3.95 (ddd, J = 32, 16, 6 Hz, 2 H), 4.49 (s, 1 H), 5.28 (s, 1 H), 6.42 (s, 1 H), 6.87 (s, 1 H), 7.10 (s, 1 H), 7.16 (d, J = 6.2 Hz, 1 H), 7.42 (d, J = 9.2 Hz, 1 H), 7.49 (t, J = 7.2 Hz, 1 H), 7.64−7.70 (m, 2 H), 7.72 (s, 1 H), 7.78 (t, J = 9.2 Hz, 2 H), 7.90−7.97 (m, 4 H), 8.03−8.11 (m, 4 H); ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ 28.5, 28.6, 44.1, 45.1, 80.2, 80.6, 109.6, 111.2, 123.7, 124.3, 124.5, 124.7, 125.1, 125.5, 125.7, 126.2, 127.0, 127.5, 127.6, 129.4, 129.5, 129.9, 130.1, 130.3, 130.7, 131.2, 131.3, 131.4, 134.9, 138.0, 138.5, 146.1, 154.5, 155.7, 156.3, 166.4, 167.8; HR-ESI-TOF calcd for $C_{45}H_{44}N_5O_6^+$ (M + H) 750.329, found 750.329.

4-(1′-[8′-(1″-Pyrenyl)naphthyl])-2,6-diaminopyridine Bis-glycamide Bishydrochloride (1a) and Trishydrochloride (1b). A hydrogen chloride-saturated solution of EtOAc (2 mL) was added to a solution of **7** (65 mg, 0.087 mmol) in EtOAc (0.5 mL) in a 25-mL round-bottom flask. After 5 min, the precipitate was filtered off, washed with EtOAc (2 × 2 mL), and dried in vacuo to give a crude product (55 mg), which was then dissolved in water (0.30 mL) and mixed with THF (5 mL). The precipitate was filtered off, washed with THF (2 × 1 mL), and dried in vacuo. The residual solvent (< 1%) was removed by dissolving the solid in water (0.5 mL) and evaporating the solvent in a spin-vac system to afford **1** as a light-yellow solid (35 mg, 64%): mp > 260 °C; IR (neat, cm^{-1}) 2861, 1652, 1595, 1558, 1456, 1198, 855, 833, 773, 725, 684; ^{1}H NMR (400 MHz, D_2O, ppm) δ 2.91 (d, J = 16.4 Hz, 1 H), 3.20 (d, J = 16.8 Hz, 1 H), 3.94 (d, J = 16.4 Hz, 1 H), 4.02 (d, J = 16.4 Hz, 1 H), 6.38 (s, 2 H), 7.02 (d, J = 9.2 Hz, 1 H), 7.11 (d, J = 7.2 Hz, 1 H), 7.51 (t, J = 7.6 Hz, 1 H), 7.61 (d, J = 9.2 Hz, 1 H), 7.65−7.75 (m, 2 H), 7.78 (d, J = 7.6 Hz, 1 H), 7.93−7.99 (m, 3 H), 8.03−8.10 (m, 4 H), 8.21 (d, J = 7.2 Hz, 1 H); ^{13}C NMR (100 MHz, D_2O, ppm) δ 40.8, 41.5, 107.8, 110.1, 122.8, 123.5, 124.9, 125.2, 125.6, 126.3, 126.9, 127.3, 128.1, 128.3, 128.7, 129.2, 130.2, 130.4, 130.6, 131.0, 133.8, 136.5, 137.0, 141.0, 141.7, 157.3, 165.4, 166.7; HR-ESI-TOF calcd for $C_{35}H_{28}N_5O_2^+$ (M + H) 550.224, found 550.223; $C_{35}H_{27}N_5O_2Na^+$ (M + Na) 572.206, found 572.204. Anal. Calcd for $C_{35}H_{37.5}Cl_{2.5}N_5O_6$ [$M(HCl)_{2.5}(H_2O)_4$]: C, 58.97; H, 5.30; Cl, 12.43. Found: C, 58.68; H, 4.91; Cl, 12.67.

NMR Spectroscopic Dilution Experiment. Solutions of **1** were prepared in D_2O at concentrations of 9.0, 4.5, 2.2, 1.1, 0.55, and 0.28 mM. The ^{1}H NMR spectra of these samples were then recorded at room temperature.

Fluorescence Titration. Fluorescence spectra were recorded using a spectrofluorometer [general settings: increment, 1; integration, 0.3; slit widths, 3 (excitation) and 3 (emission); equilibration time, 2 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 350 nm. During the titrations and measurements of apparent binding constants, a solution of **1** (0.87 μM) was prepared first and then increasing amounts of the binding substrates were added to the solution using gastight syringes. The intensities of the fluorescence titration curves at 472 nm were analyzed using a one-site binding model equation and curve fitting software to evaluate the apparent binding constants.[35]

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Supporting Information Available: Improved synthesis of compound **4**; Job plots and a plot of $\log K_b$ values with respect to the values of pK_a of the N(1)H units for uracil, thymine, 5-formyluracil, 5-fluorouracil, and 5-nitrouracil; ^1H and ^{13}C NMR spectra of compounds **1**, **2**, and **5**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.