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RESEARCH ARTICLE

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Creatinine recognition using designed synthetic receptors

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Abstract

A series of neutral nonenzymatic receptors have been synthesized for the recognition of creatinine in a nondegrative way. The receptors contain different heterocyclic moieties for better interactions between host and guest. Among these, **1**, **4**, and **5** are fluorescent receptors for creatinine. From this study, it was found that the receptors **1** and **4** containing the naphthyridine moiety have higher binding affinity to the guest creatinine than receptors containing other heterocyclic moiety. Theoretical studies for the calculation of binding energy were carried out using discrete Fourier transform (DFT) for the hosts and their complexation with creatinine in both gas phase and acetonitrile medium.

KEYWORDS

creatinine, DFT, fluorescence sensor, host-guest, molecular recognition

1 | INTRODUCTION

Creatinine is a blood metabolite of considerable importance in clinical chemistry,[1] particularly as indicator of renal function.[2] It is proved that determination of creatinine is more valuable for the detection of renal dysfunction than that of urea.[3] Generally, about 1.6% of the body's content of creatine spontaneously breaks down daily to form creatinine.[4] In medical practice, plasma and urinary creatinine levels are used on a day-to-day basis for the detection of renal diseases. Routine clinical analysis of creatinine in blood is currently conducted calorimetrically by means of the Jaffe reaction or by enzymatic method. While the Jaffe reaction is not specific for creatinine, the enzymatic methods are more expensive.[5]

Its exceptional importance inspired different research groups to be actively engaged in clinical as well as diagnostic research. Hence, recognition of creatinine and its analytical applications plays a major role in biomedical research particularly related to renal function.[6] Different synthetic receptors have been synthesized to study the recognition process of creatinine. For instance, a calix[4]pyrrole with a monophosphonate bridge receptor has been reported in the recent past, which acts as an excellent ionophore for the quantification of creatinine levels in urine or plasma.[7] Another fluorescent chemosensor has been reported that allows detection of

creatinine by visual inspection, where a dinitrophenolic moiety is responsible for the colorimetric changes.[8a] Similarly, a Pd^{2+}-coordinated naphthalimide works as an efficient fluorescent probe for creatinine in aqueous buffer at pH 7.2.[8b] Beside these types of chemical sensors, some groups designed electrochemical sensors for the detection of creatinine.[9] Recently, L-cysteine–stabilized copper nanoparticles and calix[4]arene-attached gold nanoparticle-based colorimetric sensors for creatinine were also reported.[10] Molecularly imprinted polymer (MIP) containing nickel nanoclusters is also promising electrochemiluminescence (ECL) emitters with very high detection limit for creatinine.[11]

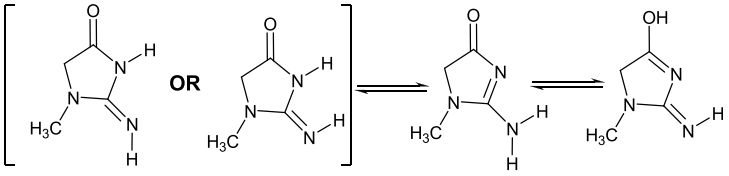
We have previously reported on the recognition of different biologically important substrates by designed synthetic receptors.[12] Here, we attempt to recognize creatinine by neutral synthetic receptors in a non-degrative protocol. Accordingly, a series of receptors were designed, synthesized, and tested for the recognition of creatinine in organic medium. Creatinine has three different tautomeric forms (Scheme 1) as **A**/**B**, **C**, and **D**, and among all these forms, **C** is the energetically more favorable tautomeric form.[7,13] At comparatively neutral environment, it can exist in either **A**/**B** or **C** as reported in literature.[7] We also have studied the donor-acceptor arrangement of creatinine and its interaction and supramolecular behavior with simple monocarboxylic acids in solid phase, where ionic complexes of the protonated tautomeric form **C** are involved instead of molecular complexes as in MeOH–$CHCl_3$.[14] Hence, interaction of synthetic neutral host and creatinine in form **D** may be ruled out in nonaqueous environment.

The synthetic receptors were designed with the binding sites of donors and acceptors being properly arranged to recognize creatinine molecule efficiently. In receptor **1**, the design is based on the concept that two hydrogen bond donors (one is isophthaloyl-butyramide N—H, and the other is isophathaloyl-naphthyridine amide N—H) should make two hydrogen bonds with the lactam oxo group of creatinine. The two naphthyridine ring "N" atoms will accept two hydrogen bonds from the lactam and imido N—H groups. Receptor **1** binds creatinine in its tautomeric form **B**. Similarly, receptor **5** was designed and synthesized where ring "N" atoms of both the pyridine and quinoline moiety act as hydrogen bond acceptors, whereas two amide protons act as hydrogen bond donors to the lactam moiety of creatinine. Other receptors may bind creatinine in form **A** or form **B** through three- or four-point interactions, as these are more flexible compared with the receptor **1**. Receptors **2** and **3** contain pyridine and imidazole moieties with the pyridine 2-amide moiety acting as acceptor to the exo imido N—H. Among the receptors **2** and **3**, one extra acceptor unit in receptor **2** is more flexible and thus stays away from the pyridine 2-amide moiety compared with the receptor **3**, which was also reflected by the lower binding constants for receptor **2** than receptor **3**. Receptors **1**, **4**, and **5** may be used as potential fluorescence sensors as they contain a fluorophore in their backbone. The receptors are shown below (Scheme 2).

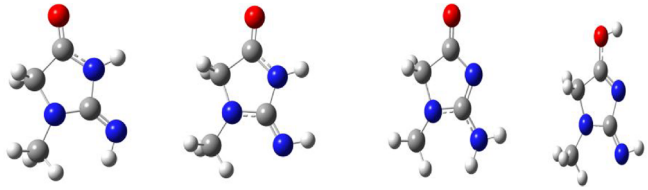
2 | SYNTHESIS

The receptors were synthesized according to the following schemes starting from commercially available compounds and fully characterized for binding studies (SI). Receptor **1** was synthesized under high-dilution conditions from 2-methyl-7-amino-1,8-naphthyridine and ^{n}butyl amine (Scheme 3). Intermediate naphthyridine was prepared according to the reported procedure.[15a]

Receptors **2** and **3** were prepared using 2-pivaloylamino-6-bromomethyl pyridine (Scheme 4); 2-Pivaloylamino-6-bromomethyl pyridine has been



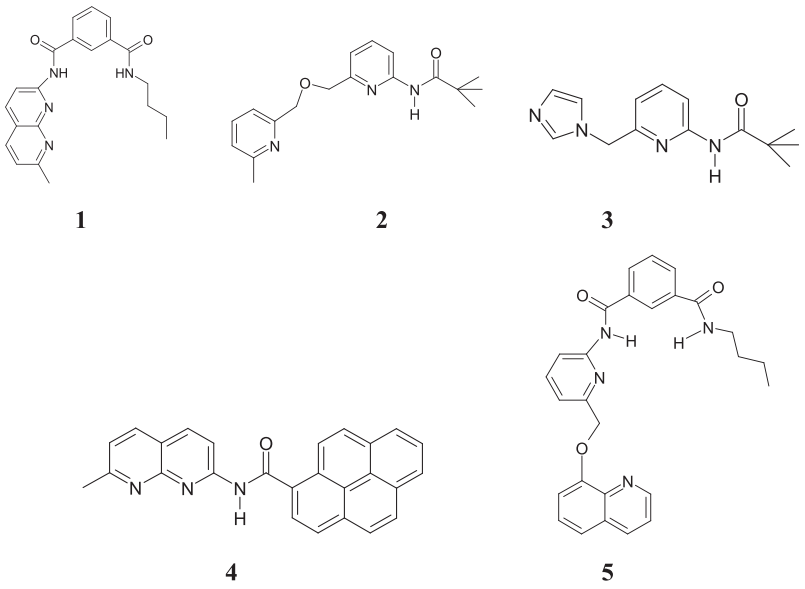
(A)

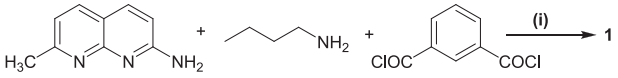


(B)

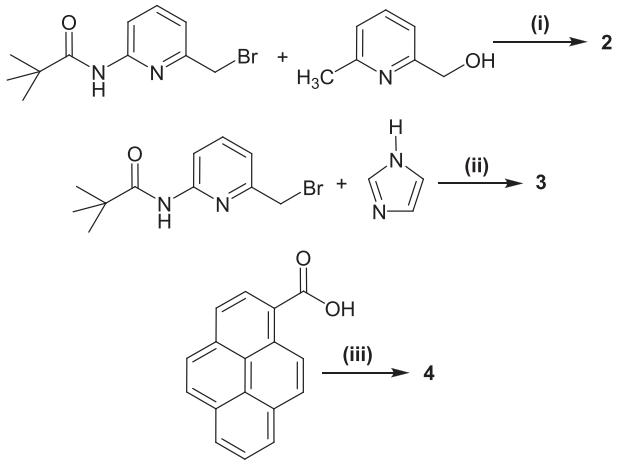
SCHEME 1 (A) Tautomeric forms of creatinine (**A** or **B**, **C**, and **D**) and (B) optimized structure of tautomers

SCHEME 2 Designed receptors (**1**-**5**) for the complexation of creatinine





SCHEME 3 Reagents and conditions: Et_3N, dry CH_2Cl_2, high dilution, 30 hours



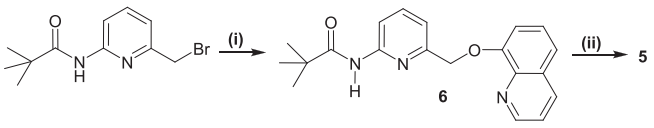
SCHEME 4 Reagents and conditions: (A) NaH, THF, r.t., 12 hours; (B) MW, 5 minutes, 450 W; (C) (a) oxalyl dichloride, DMF, dry CH_2Cl_2, r.t., 3 hours; (b) 2-amino-7-methylnaphthyridine, Et_3N, dry CH_2Cl_2, high dilution, r.t., 20 hours

synthesized using reported procedure.[15b] For receptor **2**, another pyridine moiety was attached by sodium hydride–mediated coupling of 2-hydroxymethyl-6-methylpyridine with 2-pivaloylamino-6-bromomethyl pyridine to generate the ether linkage, which makes the receptor more flexible. In contrast, in receptor **3**, one imidazole moiety was directly attached to pivaloylamino pyridine by direct coupling of 2-pivaloylamino-6-bromomethyl pyridine with imidazole under microwave irradiations. Receptor **4** was synthesized by simple amidation of the acid chloride of pyrene-1-carboxylic acid[16] and 2-amino-7-methyl-1,8-naphthyridine.

Receptor **5** was synthesized following a similar procedure as that for receptor **1** using high-dilution conditions (Scheme 5) from the intermediate isolated by hydrolysis of compound **6**. Compound **6** was prepared by the coupling of intermediate 2-pivaloylamino-6-bromomethyl pyridine and 8-hydroxyquinoline.

3 | THEORETICAL CALCULATIONS

The binding energy of the host-guest complexation was calculated using discrete Fourier transform (DFT)[17] at the B3LYP/6-31G(d,p)[18] level of theory in both gas phase and acetonitrile as solvent (see Supporting Information). Objective of this study is identification of promising receptors, which would preferably bind with which tautomer among the possible different tautomers of the guest creatinine (Scheme 1).[19] Here, binding energy of the receptors towards both anti and syn orientation of



SCHEME 5 Reagents and conditions: (A) 8-hydroxyqinoline, K_2CO_3, TBAB, dry acetone, r.t., 2 days; (B) (a) 4M KOH solution and EtOH (1:1), reflux, 6 hours; (b) isophthaloyldichloride, $^nBuNH_2$, Et_3N, dry CH_2Cl_2, r.t., high dilution, 36 hours

lactam N—H and imido N—H was calculated along with other tautomers in gas phase as well as considering acetonitrile as solvent (Table 1). Binding energy of the receptors is further lowered by the complexation with creatinine in its different forms. All the receptors preferably bind with guest creatinine in its form **B** except receptors **1** and **2** in gas phase. But in case of acetonitrile as medium during calculation, all the receptors except **2** and **3** bind preferably with creatinine in form **B**. The trends of the binding energy show that receptor **1** binds with the tautomeric form **C** of creatinine with maximum efficiency as reveled by lowest energy (**1C**: −24.84 kcal/mol) in comparison with other tautomeric forms. Whereas in case of receptor **1**, the binding energies with the tautomeric forms **B** and **C** are very close in gas phase, the binding energy with form **B** of creatinine in acetonitrile is higher than all other forms. Receptor **1** binds strongly with **A**, **B**, and **C** of creatinine as compared with all other receptors in both gas phase and acetonitrile due to the four-point interaction. Similarly, receptors **4** and **5** also bind form **B** of creatinine with highest efficiency in both phases. In case of receptors **2**, **3**, and **5**, ring "N" atoms of imidazole, pyridine, and quinoline moieties, respectively, represent hydrogen bond acceptor sites for the guest creatinine, separated by either ether linkage or methylene moiety from the core binding region of pyridine amide and isothaloyl diamide moiety. The highest binding energy (**3B**: −17.65 kcal/mol) was observed for receptor **3** in the gas phase, whereas in acetonitrile, this receptor prefers to bind form **D** of creatinine due to polarity of the solvent. Receptor **4** binds more strongly with form **B** in gas phase (−18.16 kcal/mol) than in acetonitrile (−12.17 kcal/mol). This is due to the perfect orientation of the hydrogen bonding array between host and guest. The binding energy of receptor **5** with tautomeric form **B** (**5B**: −19.99 kcal/mol) is higher in comparison with all the tautomeric forms in gas phase, whereas it is −12.70 kcal/mol in case of acetonitrile. The binding behavior of receptor **2** is completely different with respect to all the other receptors. It prefers to bind the tautomeric form **D** in both gas and acetonitrile medium, possibly due to basic nature of the alkyl pyridine moiety of the receptor. The binding energy with the tautomeric form **D** (**2D**: −14.55 kcal/mol) is higher in the gas phase than in acetonitrile (−11.12 kcal/mol). The optimized structure of receptors and their mode of binding with guest creatinine in different forms in gas phase are as follows (Figure 1). All the theoretical calculations are carried out by G09[20] software.

4 | BINDING STUDIES

4.1 | UV-Vis studies

The binding behavior of the receptors with creatinine was studied by means of UV-Vis titrations. The titration experiments were carried out with known solutions of receptors **1**, **2**, **3**, **4** (1×10^{-6}M), and **5** (1×10^{-5}M) in CH_3CN. The solution of guest creatinine was also prepared in either approximately 1×10^{-4}M or 1×10^{-5}M order in CH_3CN. Each titration was performed using 2 mL of the stock solution of receptors and the solution of guest substrates by judicious choice
(see Supporting Information). Receptor **1** showed a strong absorption at approximately 335 nm, which gradually decreased upon addition of the guest solution during titration. The other absorption peak appeared at

TABLE 1 Binding energy[a] (kcal/mol) of the complexes in gas phase and in acetonitrile between receptors and creatinine in different forms

	Receptors				
Tautomeric Forms of Creatinine	**1**	**2**	**3**	**4**	**5**
Binding energy of the complexes in gas phase					
A	−21.23	−12.99	−11.35	−15.44	−17.14
B	−24.16	−12.58	−17.65	−18.16	−19.99
C	−24.84	−14.07	−17.02	−6.02	−17.64
D	−14.07	−14.55	−14.35	−15.00	−14.67
Binding energy of the complexes in acetonitrile					
A	−14.23	−8.91	−5.86	−10.48	−12.57
B	−16.65	−7.98	−10.78	−12.70	−13.67
C	−15.60	−9.34	−7.72	−5.38	−12.34
D	−10.86	−11.12	−10.94	−11.24	−10.82

[a]Binding energy ($E_{Complex} - [E_{Host} + E_{Guest}]$).

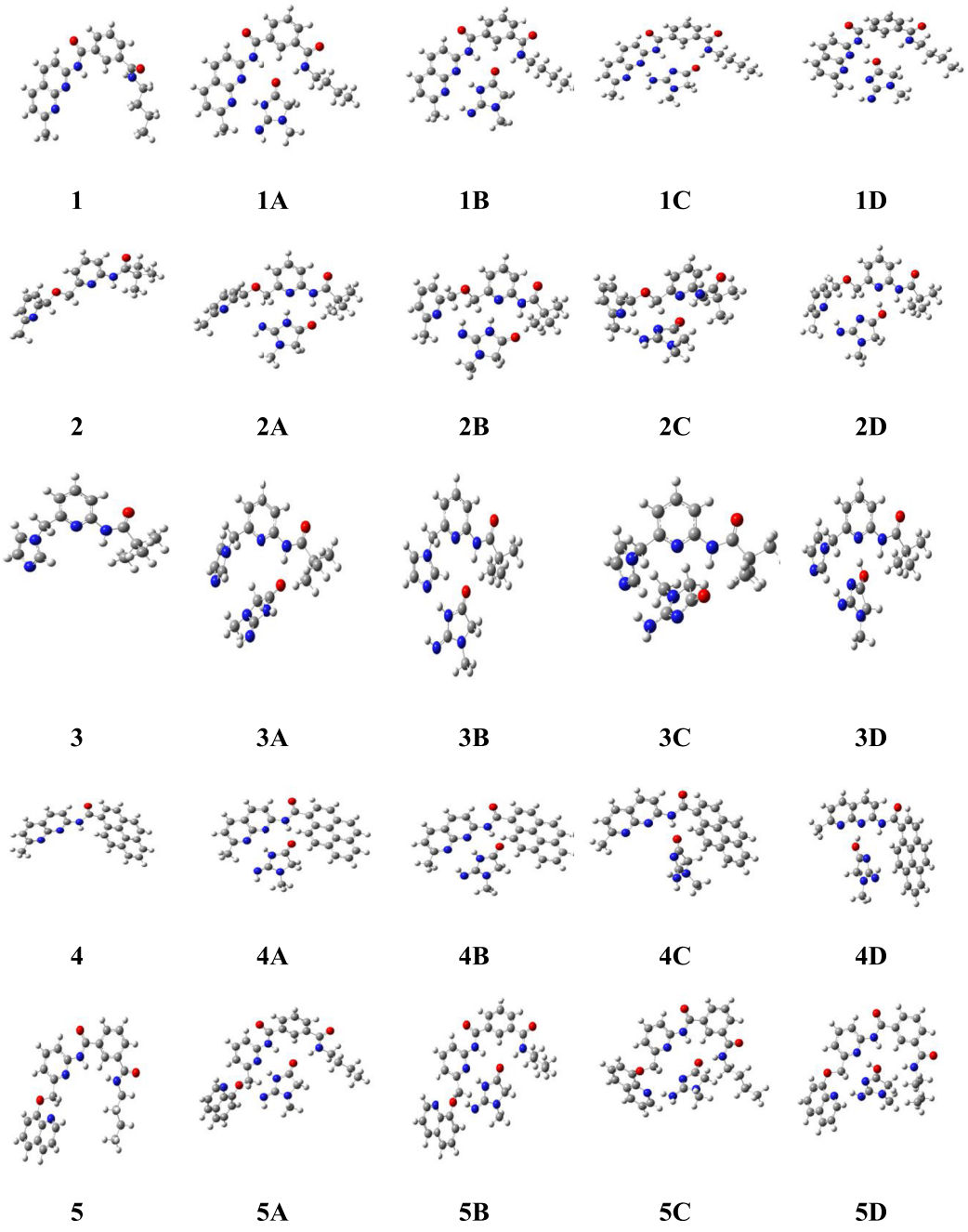


FIGURE 1 Calculated energy minimized structure in gas phase of receptors and their different mode of complexation with tautomeric forms **A**/**B**, **C**, and **D** of creatinine using B3LYP/6-31+G(d,p) method

approximately 321 nm with reduced intensity. Receptor **2** showed absorption at approximately 273 nm, which also gradually decreased upon addition of guest solution. The presence of the imidazole instead of a pyridine moiety slightly changed the absorption spectra of receptor **3**, where strong absorption was observed at approximately 279 nm. In this case, the spectral change upon addition of creatinine solution was more prominent with respect to the other two receptors. The absorption spectra of receptor **4** contained two humps (λ_{max} = 341, 276 nm) of very close intensity. In this case, decrease of intensity at 341 nm was more prominent than at 276 nm during titration with creatinine. The absorption spectra of the receptor **5** (λ_{max} = ~287 nm) also gradually decreased upon addition of creatinine solution.

The binding constant values were calculated using the data of UV-Vis titration experiments (Figure 2A).[21] The binding constant values depend on the donor-acceptor array of the receptors. The receptors **1** and **4** show higher association constant values due to the presence of the naphthyridine moiety in which two ring "N" atoms act as hydrogen bond acceptors. The other

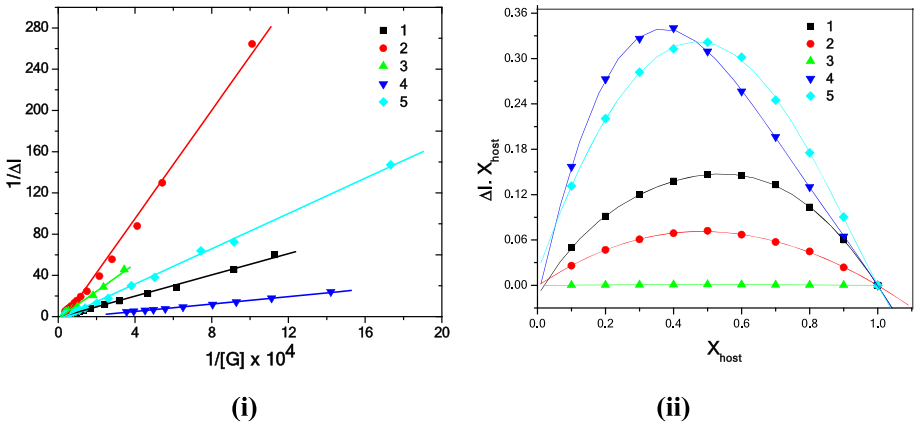


FIGURE 2 (A) Binding constant calculation curves and (B) JOB plots of the receptors with creatinine in CH_3CN by UV-Vis method

two receptors **2** and **3** bind weakly with guest creatinine. The association constant of receptor **3** with creatinine is higher due to the presence of two ring "N" atoms in close proximity compared with receptor **2**. Receptor **5** shows a lower association constant with respect to receptors containing naphthyridine moiety (**1** and **4**) due to the higher flexibility of the quinoline moiety.

The stoichiometry of the host-guest interaction has been derived from JOB plots of the receptors with creatinine in CH_3CN (Figure 2B). A 1:1 stoichiometry in case of the receptors **1**, **2**, **3**, and **5** was observed, whereas in case of receptor **4**, it slightly deviated due to the dynamic nature of complex.

4.2 | Fluorescence studies

The binding behavior of the receptors **1**, **4**, and **5** was also studied by the fluorescence titration method at the same concentration as for the UV-Vis titration except for receptor **4**. The solution of receptor **4** was further diluted 10 times for the measurement of emission intensity due to the presence of strong fluorescent pyrene moiety. Receptors **1** and **4** showed emission due to the presence of naphthyridine moiety, which acted as a binding motif for creatinine as well as a fluorophore to study the photophysical behavior.[15] The emission spectra of receptor **1** showed one peak at approximately 402 nm, when excited at 335 nm. Upon addition of creatinine, the emission intensity was gradually decreased due to the formation of host-guest complex. But the emission spectra of receptor **4** are different due to the presence of both naphthyridine and pyrene moieties. In this case, the emission spectra consisted of two bands (λ_{max} = 392, 409 nm) when excited at 341 nm. Between these two bands, the peak at the higher wavelength is of higher intensity. The receptor **5** contains one quinoline moiety attached through an ether linkage, which acts as both acceptor to the imido N—H of creatinine and fluorophore to study the binding behavior.[22] The receptor **5** was excited at 287 nm during binding studies, and the emission intensity was decreased (λ_{max} = ~393 nm) regularly upon addition of increasing amount of guest solution (SI).

All the receptors (**1**, **4**, and **5**) behave as fluorescent probes towards creatinine.[23] The emission intensity in all the cases was quenched during complexation. The receptors **1** and **4** differ slightly probably due to the strength of the host-guest interaction as well as the change of order of the concentration of the receptors during titration experiment. The higher extent of quenching of receptor **1** during titration also indicates the higher interaction with creatinine. But in the case of receptor **5**, the extent of quenching is higher than that of the other receptors due to the presence of different fluorophore as well as different mode of interaction with creatinine.

The binding constants (Figure 3) were determined by the fluorescence titration method.[24] The association constants of the receptors **1** and **4** having naphthyridine moiety as binding motif are higher with respect to those of receptors with the quinoline moiety. The association

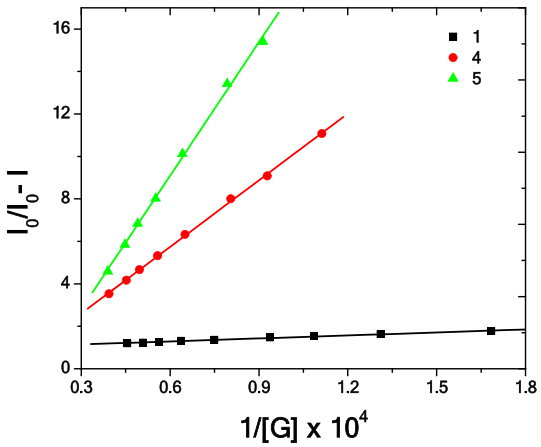


FIGURE 3 Binding constant calculation curves of the receptors **1**, **4**, and **5** with creatinine in CH_3CN by fluorescence titration method

constants determined by fluorescence method are almost double with respect to the association constants determined by UV-Vis method in case of the receptor **5**.

5 | DISCUSSION

The association constant values determined by UV-Vis and fluorescence methods are summarized (Table 2). All the receptors are of mainly three types. Receptors **1** and **4** are of the same type where naphthyridine amide moiety is the basic binding motif and this type of receptors binds better compared with other type of receptors due to matching of the donor-acceptor array between receptors and creatinine of form **B**. The association constant value in case of receptor **1** is higher than receptor **4** in both the methods due to the presence of one extra donor site. This may be attributed by the arrangement of two donor amide protons directed inward to the cavity of the receptor **1**, which enhance the binding efficiency to the guest.

The other types of receptors contain one pyridine unit (receptor **2**) and one imidazole unit (receptor **3**) as extra hydrogen acceptor motifs, which are attached with the same pyridine 2-amide moiety. In this case, receptor **2** shows higher association constant than receptor **3** due to flexible ether linkage by which the pyridine ring "N" interacts with the guest more predominantly, whereas the imidazole moiety is oriented away from the pyridine amide binding moiety due to repulsive forces. The receptor **5** contains a quinoline moiety linked to the pyridine amide unit through an ether linkage. The association constant of this receptor is lower as that of the receptors **1** and **4** but comparable with that of receptors **2**, **3**, and **5**. This is probably due to the distance among the two hydrogen bond acceptors ring "N," which is higher and also due to the free movement of the quinoline moiety in solution phase.

TABLE 2 Association constants (K_a [M^{-1}])[a] and the free energy changes (ΔG [kcal/mol]) at 25°C for receptors determined by UV-Vis (**1**-**5**) and fluorescence titration methods (**1**, **4**, and **5**)

	Methods			
	UV-Vis Method[b]		Fluorescence Method[c]	
Receptors	K_a	ΔG	K_a	ΔG
1	1.47×10^4	−5.75	2.14×10^4	−6.03
2	3.79×10^3	−4.32	-	-
3	1.32×10^3	−5.36	-	-
4	1.24×10^4	−6.62	1.74×10^4	−6.92
5	3.03×10^3	−5.10	1.66×10^3	−5.54

[a]All the errors are ±15%.
[b]For receptor **1**: λ_{max} = 335 nm; receptor **2**: λ_{max} = 273 nm; receptor **3**: λ_{max} = 279 nm; receptor **4**: λ_{max} = 341 nm; receptor **5**: λ_{max} = 287 nm.
[c]For receptor **1**: λ_{max} (ex) = 335 nm, λ_{max} (em) = 402 nm, emission slit width = 5.5 nm, excitation slit width = 8.0 nm. For receptor **4**: λ_{max} (ex) = 341 nm, λ_{max} (em) = 409 nm, emission slit width = 2.7 nm, excitation slit width = 14.0 nm. For receptor **5**: λ_{max} (ex) = 287 nm, λ_{max} (em) = 393 nm, emission slit width = 4.0 nm, excitation slit width = 10.0 nm. Scan rate is 500 nm/min in all cases.

TABLE 3 Association constants (K_a [M^{-1}]) for receptors (**1** and **5**) determined by UV-Vis method under different water concentration in acetonitrile

Receptors	15% H_2O in CH_3CN	10% H_2O in CH_3CN	5% H_2O in CH_3CN
1	1.25×10^4	2.39×10^4	1.14×10^4
5	ND	3.87×10^4	1.131×10^4

Abbreviation: ND, not determined.

As creatinine is an important renal function indicator and usually determined from urine samples, hence, recognition behavior of the receptors in aqueous environment has been tested. For this purpose, receptors **1** and **5** were used that have shown solubility up to 15% and 10% H_2O in acetonitrile, respectively. The association constant for receptor **1** is maximum at 10% H_2O in acetonitrile, which is even twofold higher than the value in pure acetonitrile (Tables 2 and 3). In all other cases, association constants are almost close to each other. An interesting feature for receptor **5** is that the association constant is enhanced by approximately 10-fold in the 10% H_2O in acetonitrile solvent system in comparison with pure acetonitrile. For receptor **5**, the association constant in the acetonitrile with more than 10% H_2O was not determined due to insolubility. Overall, receptor **1** showed slight enhancement in binding effect in water-acetonitrile system in comparison with the pure acetonitrile solvent, whereas receptor **5** showed prominent enhancement in water-acetonitrile system over pure acetonitrile system.

6 | CONCLUSION

In this work, a series of different receptors including fluorescent ones were designed and synthesized, and their binding behavior with creatinine was studied. All the experimental binding results were compared with theoretical calculation values. In the receptors, one pyridine amide moiety is fixed to bind the lactam moiety of creatinine, whereas the other acceptor unit of the receptors was carefully changed for better binding. The receptors contain fused pyridines (**1** and **4**), isolated pyridine (**2**), imidazole (**3**), and quinoline (**5**) with the basic pyridine amide moiety. Among all the receptors, **1** and **5** contain one extra hydrogen bond donor site to enhance the

interaction between receptors and creatinine. From the detailed study of the recognition process of creatinine by different methods, it is found that receptors containing naphthyridine moiety have a higher tendency to interact with the guest creatinine. Another important aspect from this study is that creatinine molecule binds better in water-acetonitrile than in pure acetonitrile. Receptors **1** and **5** are not able to recognize creatinine at higher concentration of water than 15% in acetonitrile due to solubility problems. Besides, the binding behavior of the receptors with different forms of creatinine has been studied theoretically using DFT at the B3LYP/6-31G(d,p) level of theory in both gas phase and acetonitrile as solvent. The overall trend remains the same with higher binding in the gas phase than in acetonitrile, but no explicit solvent molecules were used in this study. From the trend of the calculated data, the experimental results can be justified by the nature of binding.

7 | EXPERIMENTAL

7.1 | General

Chromatographic separations were performed on silica gel (100-200 meshes). For preparative thin-layer chromatographic (TLC) (PTLC) purification, the layer was formed on a glass plate using water gel–GF 254 silica gel. The petroleum ether used has a boiling range of 40°C to 60°C. All the melting points were determined on hot-coil stage melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded on 500- or 300-MHz spectrometers. ^{13}C NMR spectra were recorded on a 500-MHz spectrometer. For NMR spectra, $CDCl_3$ and DMSO-d_6 were used as solvents using transcranial magnetic stimulation (TMS) as an internal standard. Chemical shifts are expressed in δ units and ^{1}H—^{1}H, ^{1}H—C coupling constants in hertz. Infrared (IR) spectra were recorded using KBr disks.

7.2 | General procedure for UV-Vis titration

Stock solution of the receptors (**1**, **2**, **3**, and **5**) was prepared in the order of 1×10^{-5}M in CH_3CN (receptor **4** was prepared in the order of 1×10^{-6}M). The solution of guest creatinine was prepared in either 1×10^{-4}M or 1×10^{-5}M order in CH_3CN by adding individual host solutions. Then, the guest solution is added to the receptor solution (taking 2 mL in the UV cell), and continuous decreases of absorbance in UV spectra were recorded each time. Titration curves were determined by plotting ΔI vs [G]/[H] (ΔI is change of absorbance; [G] and [H] are the concentration of creatinine and receptors, respectively). Association constants were calculated by plotting 1/ΔI vs 1/[G].[21,24]

7.3 | General procedure for fluorescence titration

The same stock solutions of receptors and guest creatinine in CH_3CN were used for fluorescence titration experiment. Then, the guest solution is added to the receptor solution (taking 2 mL in the cell), and continuous decrease of intensity of emission spectra was recorded for each time. Titration curves were obtained by plotting ΔI vs [G]/[H]. Association constants were calculated by plotting $I_0/I_0 - I$ vs 1/[G] (I_0 and I are the initial and final intensity of the receptor solution after each addition during titration).[24]

7.4 | *N*-Butyl-*N*′-(7-methyl-[1,8] naphthyridin-2-yl)-isophthalamide (1)

2-Amino-7-methyl-1,8-naphthyridine was synthesized according to the literature procedure.[15] 2-Amino-7-methyl-1,8-naphthyridine (200 mg, 1.26 mmol) in dry CH_2Cl_2 (15 mL) and ^{n}butylamine (92 mg, 1.26 mmol) in dry CH_2Cl_2 (15 mL) containing Et_3N (0.1 mL) were added with isophthaloyl dichloride (255 mg, 1.26 mmol) in dry CH_2Cl_2 (20 mL) using high-dilution technique for 4 hours under N_2 atmosphere. The reaction was continued for another 30 hours. CH_2Cl_2 was distilled out, and the gummy crude product was extracted with CH_2Cl_2 (20 mL × 4) after washing with saturated $NaHCO_3$ solution, which was dried over anhydrous Na_2SO_4 and concentrated under vacuum. The crude compound was purified by preparative TLC using $CHCl_3$ (2% MeOH) as eluant and afforded an off-white solid substance (100 mg, 22%).

Mp. 149°C-151°C.

^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm): 9.12 (bs, 1H), 8.63 (d, 1H, J = 8.9 Hz), 8.33 (s, 1H), 8.23 (d, 1H, J = 8.8 Hz), 8.11-8.05 (m, 3H), 7.63 (t, 1H, J = 7.7 Hz), 7.32 (d, 1H, J = 8.2 Hz), 6.31 (bs, 1H), 3.52 (q, 2H, J = 7.1 Hz), 2.78 (s, 3H), 1.65 (qnt, 2H), 1.45 (sxt, 2H), 0.99 (t, 3H, J = 7.4 Hz).

FT-IR (KBr): 3420, 1683, 1636, 1508, 1324, 1025, 713 cm^{-1}.

MS (ESI): m/z (%): 385 ($(M + Na)^+$, 27), 363 ($(M + H)^+$, 100).

Anal. Calcd. for $C_{21}H_{22}N_4O_2$: C, 69.59; H, 6.12; N, 15.46. Found: C, 69.73; H, 6.05; N, 15.71.

7.5 | 2,2-Dimethyl-*N*-[6-(6-methyl-pyridin-2-ylmethoxymethyl)-pyridin-2-yl]-propionamide (2)

(6-Methyl-pyridin-2-yl)-methanol was obtained from the corresponding aldehyde by the reduction with $NaBH_4$ in dry EtOH. The conversion was almost quantitative, and it was directly used for the next step without further purification. (6-Methyl-pyridin-2-yl)-methanol (320 mg, 2.6 mmol) was taken in a two-neck r.b. and dried well under vacuum and filled with N_2. NaH (216 mg, 10 mmol) was added carefully and stirred for 30 minutes in dry THF (5 mL) at r.t. Then, *N*-(6-bromomethyl-pyridin-2-yl)-2,2-dimethyl-propionamide (704 mg, 2.6 mmol) in dry THF (5 mL) was added dropwise for 30 minutes, and the reaction was continued by stirring overnight at r.t. THF was distilled off under reduced pressure, and the brown gummy crude was extracted with EtOAc (20 mL × 5) after washing with water, which was dried over anhydrous Na_2SO_4 and concentrated under vacuum. This crude product was purified by column chromatography using silica gel (100-200 meshes) and EtOAc (15%) in petroleum ether as eluant to afford light yellow dense liquid (530 mg, 65%).

^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm): 8.18 (d, 1H, J = 8.3 Hz), 8.16 (bs, 1H), 7.75-7.69 (m, 2H), 7.41 (d, 1H, J = 7.6 Hz), 7.20 (d, 1H, J = 7.5 Hz), 7.14 (d, 1H, J = 7.7 Hz), 4.85 (s, 2H), 4.66 (s, 2H), 2.58 (s, 3H) 1.33 (s, 9H).

^{13}C NMR ($CDCl_3$, 125 MHz): δ (ppm): 177.5, 158.1, 157.7, 156.7, 151.5, 139.3, 137.3, 122.5, 118.8, 117.8, 113.0, 74.1, 73.6, 40.2, 27.8, 24.7.

FT-IR (KBr): 3435, 1684, 1578, 1522, 1456, 1153, 1026, 798 cm^{-1}.

MS (ESI): m/z (%): 336 ((M + Na)$^+$, 95), 314 ((M + H)$^+$, 17), 207 (100), 191 (57.5), 122 (67.5).

Anal. Calcd. for $C_{18}H_{23}N_3O_2$; C, 68.98; H, 7.40; N, 13.41. Found: C, 68.75; H, 7.43; N, 13.59.

7.6 | *N*-(6-Imidazol-1-ylmethyl-pyridin-2-yl)-2,2-dimethyl-propionamide (3)

N-(6-Bromomethyl-pyridin-2-yl)-2,2-dimethyl-propionamide (270 mg, 1.0 mmol) and imidazole (70 mg, 1.0 mmol) were taken in an open-mouth conical flask and were mixed thoroughly. This mixture was irradiated at 450 W for 5 minutes in a microwave oven. Then, this mixture was directly used for purification by column chromatography (100-200 meshes) and EtOAc (15%) in petroleum ether as eluant to afford an off-white solid compound (210 mg, 81%).

Mp. 114°C-116°C.

^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm): 8.19 (d, 1H, J = 8.3 Hz), 7.97 (bs, 1H), 7.82 (s, 1H), 7.67 (t, 1H, J = 7.9 Hz), 7.14 (s, 1H), 6.99 (s, 1H), 6.77 (d, 1H, J = 7.5 Hz), 5.14 (s, 2H), 1.33 (s, 9H).

^{13}C NMR ($CDCl_3$, 125 MHz): 177.5, 154.5, 151.9, 139.9, 138.1, 130.0, 119.9, 117.3, 113.6, 52.5, 40.2, 27.9.

FT-IR (KBr): 3440, 2970, 1672, 1577, 1541, 1457, 1081, 794 cm^{-1}.

MS (ESI): m/z (%): 281 ((M + Na)$^+$, 25), 259 ((M + H)$^+$, 100), 191 (87).

Anal. Calcd. for $C_{14}H_{18}N_4O$; C, 65.09; H, 7.02; N, 21.69. Found: C, 65.21; H, 6.96; N, 21.75.

7.7 | Pyrene-1-carboxylic acid (7-methyl-[1,8]naphthyridin-2-yl)-amide (4)

2-Amino-7-methyl-1,8-naphthyridine and pyrene carboxylic acid were synthesized according to the literature procedure.[16] This pyrene carboxylic acid (155 mg, 0.63 mmol) was converted to its acid chloride with oxaloyl dichloride (0.15 mL, 1.6 mmol) and a catalytic amount of DMF in dry CH_2Cl_2 at r.t. After stirring for 3 to 4 hours, excess CH_2Cl_2 was distilled out, and dry CH_2Cl_2 (4 mL) was added. This solution was added with 2-amino-7-methyl-1,8-naphthyridine (100 mg, 0.63) in dry CH_2Cl_2 (5 mL) dropwise under N_2 atmosphere. This reaction was continued for 20 hours. After completion of the reaction, CH_2Cl_2 was distilled out and extracted with $CHCl_3$ (20 mL × 3) after washing with saturated $NaHCO_3$ solution, which was dried over anhydrous Na_2SO_4 and concentrated under vacuum. This crude product was purified by column chromatography using silica gel (100-200 meshes) and EtOAc (15%) in petroleum ether as eluant to afford a deep yellow solid (173 mg, 71%).

Mp. 194°C-197°C.

^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm): 8.89 (d, 1H, J = 8.6 Hz), 8.48 (d, 1H, J = 9.2 Hz), 8.39 (d, 1H, J = 7.9 Hz), 8.33-8.18 (m, 2H), 8.13 (d, 1H, J = 7.9 Hz), 8.10-8.05 (m, 2H), 7.99-7.92 (m, 1H), 7.84 (d, 1H, J = 9.3 Hz), 7.76-7.66 (m, 2H), 7.48 (d, 2H, J = 8.9 Hz), 2.71 (s, 3H).

^{13}C NMR ($CDCl_3$ + 2 drops DMSO-d_6, 125 MHz): δ (ppm): 196.9, 139.6, 138.2, 136.8, 132.0, 131.4, 130.9, 130.6, 129.5, 129.3, 129.1, 127.5, 126.9, 126.7, 126.5, 126.4, 126.3, 125.3, 124.8, 124.5, 124.2, 124.1, 123.9, 122.0, 114.7, 27.2.

FT-IR (KBr): 3420, 3047, 2921, 1668, 1558, 1539, 1506, 1278, 1224, 847, 713 cm^{-1}.

MS (HRMS-FAB): m/z (%): Calculated for $C_{26}H_{18}N_3O$ is 388.1450 (M + H)$^+$; found 388.1447.

Anal. Calcd. for $C_{26}H_{17}N_3O$; C, 80.60; H, 4.42; N, 10.85. Found: C, 80.48; H, 4.45; N, 10.78.

7.8 | 2,2-Dimethyl-*N*-[6-(quinolin-8-yloxymethyl)-pyridin-2-yl]-propionamide (6)

N-(6-Bromomethyl-pyridin-2-yl)-2,2-dimethyl-propionamide (500 mg, 2.5 mmol), 8-hydroxyquinoline (336 mg, 2.5 mmol), K_2CO_3 (860 mg, 6.25 mmol), and TBAB (80 mg, 0.25 mmol) were taken in an r.b. Dry acetone (7 mL) was added to it and stirred for 2 days at r.t. Acetone was distilled out, and the crude product was extracted with $CHCl_3$ (20 mL × 4) after washing with saturated brine and with water, which was then dried over anhydrous Na_2SO_4 and concentrated under vacuum. This crude product was purified by column chromatography using silica gel (100-200 meshes) and EtOAc (15%) in petroleum ether as eluant to afford an off-white solid (561 mg, 67%).

Mp. 67°C-69°C.

^{1}H NMR ($CDCl_3$, 300 MHz): δ (ppm): 8.99 (d, 1H, J = 4.0 Hz), 8.18-8.13 (m, 2H), 8.08 (bs, 1H), 7.67 (t, 1H, J = 7.9 Hz), 7.48-7.38 (m, 3H), 7.31 (d, 1H, J = 7.5 Hz), 7.00 (d, 1H, J = 6.6 Hz), 5.42 (s, 2H), 1.35 (s, 9H).

FT-IR (KBr): 3359, 2950, 2916, 2870, 1695, 1589, 1558, 1455, 1114, 795 cm^{-1}.

MS (ESI-HRMS): m/z (%): Calculated for $C_{20}H_{22}N_3O_2$ is 336.1707 $(M + H)^+$; found 336.1710 (100), 358.1530 $((M + Na)^+$, 84).

7.9 | *N*-Butyl-*N*′-[6-(quinolin-8-yloxymethyl)-pyridin-2-yl]-isophthalamide (5)

Compound **6** was hydrolyzed to obtain the corresponding amine by refluxing in 4M KOH (1:1) in ethanol for 6 hours, which was directly used for the mixed amide synthesis after usual workup. These amine (200 mg, 0.8 mmol) in dry CH_2Cl_2 (20 mL) and ^{n}butylamine (58 mg, 0.8 mmol) in dry CH_2Cl_2 (20 mL) containing Et_3N (0.12 mL) were added with isophthaloyl dichloride (160 mg, 0.8 mmol) in dry CH_2Cl_2 (25 mL) using high-dilution technique for 5 hours under N_2 atmosphere. The reaction was continued with stirring for another 36 hours at r.t. CH_2Cl_2 was distilled out, and the gummy crude product was extracted with CH_2Cl_2 (20 mL × 4) after washing with saturated $NaHCO_3$ solution, which was dried over anhydrous Na_2SO_4 and concentrated under vacuum. The product was purified by preparative TLC using $CHCl_3$ (2% MeOH) as eluant to afford an off-white solid substance (112 mg, 31%).

Mp. 84°C-86°C.

^{1}H NMR ($CDCl_3$, 500 MHz): δ (ppm): 8.96 (d, 1H, J = 4.1 Hz), 8.89 (bs, 1H), 8.45 (s, 1H), 8.25 (d, 1H, J = 8.3 Hz), 8.15 (d, 1H, J = 8.3 Hz), 8.09 (d, 1H, J = 7.8 Hz), 8.06 (d, 1H, J = 7.8 Hz), 7.73 (t, 1H, J = 7.9 Hz), 7.59 (t, 1H, J = 7.8 Hz), 7.45 (dd, 1H, J = 8.3, 4.2 Hz), 7.43-7.38 (m, 2H), 7.32 (d, 1H, J = 7.5 Hz), 7.01 (dd, 1H, J = 7.0, 1.8 Hz), 6.64 (bs, 1H), 5.34 (s, 2H), 3.46 (q, 2H), 1.57 (qnt, 2H), 1.38 (sxt, 2H), 0.92 (t, 3H, J = 7.4 Hz).

^{13}C NMR ($CDCl_3$, 125 MHz): δ (ppm): 166.9, 165.3, 155.2, 154.5, 151.5, 149.8, 140.7, 139.6, 136.6, 135.5, 134.4, 131.9, 130.9, 129.0, 129.6, 127.1, 125.5, 122.3, 120.7, 118.6, 113.8, 109.9, 71.4, 40.4, 31.9, 20.5, 14.1.

FT-IR (KBr): 3326, 3063, 2952, 2920, 2868, 1684, 1668, 1573, 1558, 1539, 1506, 1457, 1378, 1307, 1261, 1107, 820, 784 cm^{-1}.

MS (ESI-HRMS): m/z (%): Calculated for $C_{27}H_{26}N_4O_3Na$ is 477.1897; found 477.1905.

Anal. Calcd. for $C_{27}H_{26}N_4O_3$; C, 71.35; H, 5.77; N, 12.33. Found: C, 71.18; H, 5.73; N, 12.42.

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