Molecular recognition studies of selected isoalloxazines with 2,6-diamidopyridine derivatives

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Triple hydrogen bond formation towards the uracil moiety of an isoalloxazine ring with 2,6-diamidopyridine derivatives has been observed in chloroform. A hydrogen bonded isoalloxazine–pyridine complex has been successfully utilised in disulfide formation as a metal-free organocatalyst.

Keywords: molecular recognition, isoalloxazine, amidopyridine, disulfide

The flavoenzymes are ubiquitous redox enzymes involved in metabolic processes, electron transfer, detoxification of xenobiotics, and regulation of neurotransmitters.[1-4] The redox properties of flavoenzymes are mainly controlled by noncovalent apoenzyme–isoalloxazine interactions, which are controlled by hydrogen bonding, aromatic stacking and dipolar/multipolar and steric effects.[1-3] These enzymes exhibit considerable variation in isoalloxazine redox behaviour depending on the microenvironment of the active site.[5] The isoalloxazine moiety of the flavoenzymes, *i.e.* part of the molecule, which is involved in catalysis, offers several possibilities for interaction with various protein functions. Chemically, the xylene moiety is hydrophobic and prone to interact with a hydrophobic portion of the protein, whereas the pyrimidine ring is relatively electron-deficient as well as hydrophilic and is comparable with pyrimidine bases in its capability to form hydrogen bridges (Fig. 1).[6,7] This suggests that the two site binding strategy, which has been applied to the recognition of nucleotide bases, is also useful for modification of isoalloxazine reactivity. Incorporation of suitable amide and hydrophobic groups within a macrocyclic framework leads to receptors capable of simultaneous hydrogen bonding and aromatic stacking to a substrate.[8]

To provide enhanced versatility in the design of recognition-based sensors and devices and gain a better understanding of the factors that govern hydrogen-bonding processes, isoalloxazines and receptors have been synthesised and their interactions have been studied. The arrangement of the donor (D) and acceptor (A) groups in the order AD–DA or AA–DD or ADA–DAD shows the molecular recognition between two molecules.

Results and discussion

Synthesis: The reaction of 2-substituted aminoanilines (**1**) with alloxan monohydrate (**2**) under acidic conditions afforded 10-substituted isoalloxazines (**3**), which on further treatment with methyl iodide in the presence of DBU (1,8-diazabicyclo[5.4.0]undec-7-ene[9]) gave the corresponding N^3-methylated isoalloxazines (**4**) (Scheme 1).[10-13] Receptor **7a** was synthesised by the reaction of 2,6-diaminopyridine (**5**) with acetic acid and acetic anhydride. Receptors (**7b–7d**) were synthesised from 5 and the corresponding acid chlorides (**6**) according to the literature (Scheme 2).[14] The formations of the isoalloxazines (**3**) and receptors (**7a–d**) were confirmed by different spectroscopic data (experimental section)

Effect of receptors on the electronic absorption spectra of isoalloxazines: The hydrogen bonding at the heteroatoms of the isoalloxazine nucleus changes the absorption spectra of

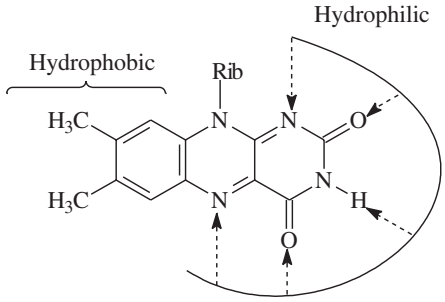
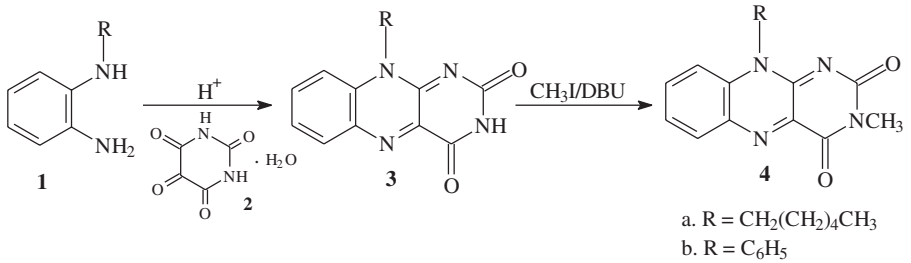
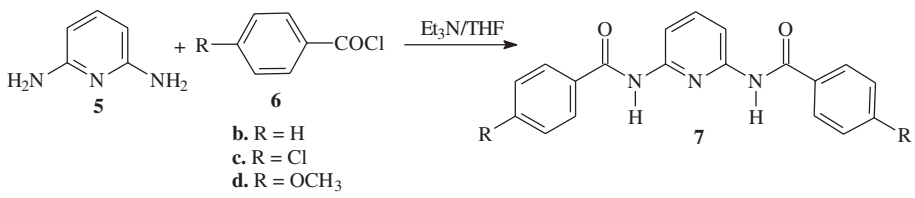


Fig. 1 Representation of possible isoalloxazine–protein interactions.



Scheme 1



Scheme 2

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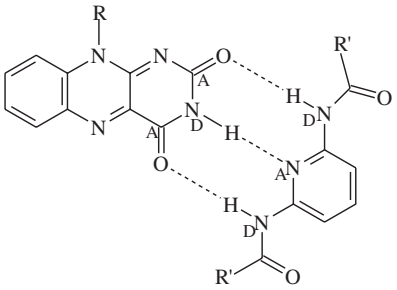


M1: R = $CH_2(CH_2)_4CH_3$; R' = CH_3
M2: R = $CH_2(CH_2)_4CH_3$; R' = C_6H_5
M3: R = $CH_2(CH_2)_4CH_3$; R' = 4'-ClC_6H_4
M4: R = $CH_2(CH_2)_4CH_3$; R' = 4'-$OCH_3C_6H_4$
M5: R = C_6H_5; R' = CH_3
M6: R = C_6H_5; R' = C_6H_5
M7: R = C_6H_5; R' = 4'-ClC_6H_4
M8: R = C_6H_5; R' = 4'-$OCH_3C_6H_4$

Fig. 2

isoalloxazines.[15] The absorption band of **3a** (λ_{max} 440.0 nm) is slightly shifted to λ_{max} 443 nm with increase of the optical density upon addition of **7a** indicating the formation of an ADA–DAD type complex M1 (Fig. 2). This observation is in agreement with the result of *ab initio* calculations of the hydrogen-bonded isoalloxazine at O(2), H(3) and O(4).[16] Similar spectral changes in the UV-visible spectra have also been observed for the other 2,6-diamidopyridine receptors **7b–d** (Table 1). There was no appreciable change observed for the N-3 alkylated isoalloxazines (**4a,b**) in the presence of receptors. The above results indicate triple hydrogen bond formation as shown in Fig. 2, and further confirmed by ^{1}H NMR spectra.

Effect of receptors on the ^{1}H NMR spectra of isoalloxazines: The ^{1}H NMR spectra of isoalloxazine, the receptor and their 1:1 mixture have been taken in a common solvent system to examine the effect of hydrogen bonds on the NH protons of both isoalloxazines and receptors. The ^{1}H NMR spectra of the NH protons of **3a**, **7a** and their 1:1 mixture are shown in Fig. 3. The N–H protons of **3a** and **7a** showed downfield shifts. However, such a chemical shift of the N–H of the receptor was not observed in the case of **4a**, which confirm that **3a** and **7a** form a molecular ADA–DAD type complex (**M1**) with three hydrogen bonds. If there was were not three hydrogen bonds, the two protons of receptor **7a** appeared at two different δ values. Similar spectroscopic changes in the ^{1}H NMR spectra have also been observed for the other 2,6-diamidopyridine receptors **7b–d** (Table 2) confirming the formation of triple hydrogen bonded ADA–DAD complexes (experimental section). The more downfield chemical shifts indicate stronger hydrogen bonds. Hence, the results for **M1** and **M5** in comparison with **M2**–**M4** and **M6**–**M8** from Table 2, may be explained by steric hindrance of the benzene rings of the receptors **7b–d** in the complex formation. The receptor **7a** forms stronger hydrogen bonds with **3**, and among receptors **7b–d**, **7c** forms stronger hydrogen bonds.

In the complexes **M2–M4** and **M6–M8**, slight upfield shifts of the benzene ring protons were also observed (materials and methods), which suggest the π–π stacking between the benzene ring of isoalloxazine and receptors.[14]

Effect of receptors on the oxidation-activity of isoalloxazines: The hydrogen bonding towards heteroatoms of an isoalloxazine ring plays an important role at active sites of flavoenzymes.[17] The oxidation of thiols by isoalloxazine involves nucleophilic attack of a thiol anion at the C(4a) position to form a covalent adduct followed by nucleophilic attack of the second thiol anion to afford the corresponding disulfide and 1,5-dihydroisoalloxazine (Scheme 3).[18,19] The hydrogen bonding at the N(5)-position of an isoalloxazine ring facilitates the reactions proceeding via C(4a)-attack by stabilizing the negative charge generated on the N(5)-atom. Quantum mechanical considerations have suggested that the hydrogen bonds occurring at C(2)=O, N(3)–H and C(4)=O of an isoalloxazine ring are one of the important factors regulating the catalytic activity of flavoproteins.[16]

The reaction of benzyl thiol (**8**) with 10-hexylisoalloxazine (**3a**) and DBU in chloroform gave the corresponding disulfide **10** in poor yield whereas the same reaction with the isoalloxazine-receptor complex **M1** gave **10** in appreciable yield (Table 3). The reaction of **8** with DBU in the absence of isoalloxazine in chloroform gave no product. A similar result has also been obtained with **M6**. The formation of the

Table 1 Comparison of shoulder band in the UV-visible spectra (λ_{max}/nm)

S. No.	Isoalloxazines		Receptors			
			7a	**7b**	**7c**	**7d**
1	**3a**	440.0	443.0(**M1**)	442.9(**M2**)	443.4(**M3**)	442.6(**M4**)
2	**3b**	441.0	442.8(**M5**)	443.3(**M6**)	442.9(**M7**)	442.9(**M8**)
3	**4a**	439.2	439.6	439.0	439.8	438.3
4	**4b**	438.9	439.0	438.9	439.5	439.0

Table 2 Comparison of chemical shifts (δ) of NH protons in the ^{1}H NMR spectra

Isoalloxazine (FI)	Receptors (R)	NH(δ_1) (R)	N(3)H (δ_2) (FI)	Complex	1:1(δ_3)[a] R/FI	$\delta_3 - \delta_1$ (R)	$\delta_3 - \delta_2$ (FI)
3a			8.50				
	7a	7.56		**M1**	9.06/11.46	1.5	2.96
	7b	8.95		**M2**	9.28/11.28	0.33	2.78
	7c	8.99		**M3**	9.37/11.39	0.38	2.89
	7d	8.80		**M4**	9.11/11.01	0.31	2.51
3b			8.55				
	7a	7.56		**M5**	8.89/11.26	1.33	2.71
	7b	8.95		**M6**	9.20/11.22	0.25	2.67
	7c	8.99		**M7**	9.27/11.23	0.28	2.68
	7d	8.80		**M8**	9.09/11.00	0.29	2.20

[a]1:1 mixture of isoalloxazine (FI) **3** and receptor (R) **7** in $CDCl_3$.

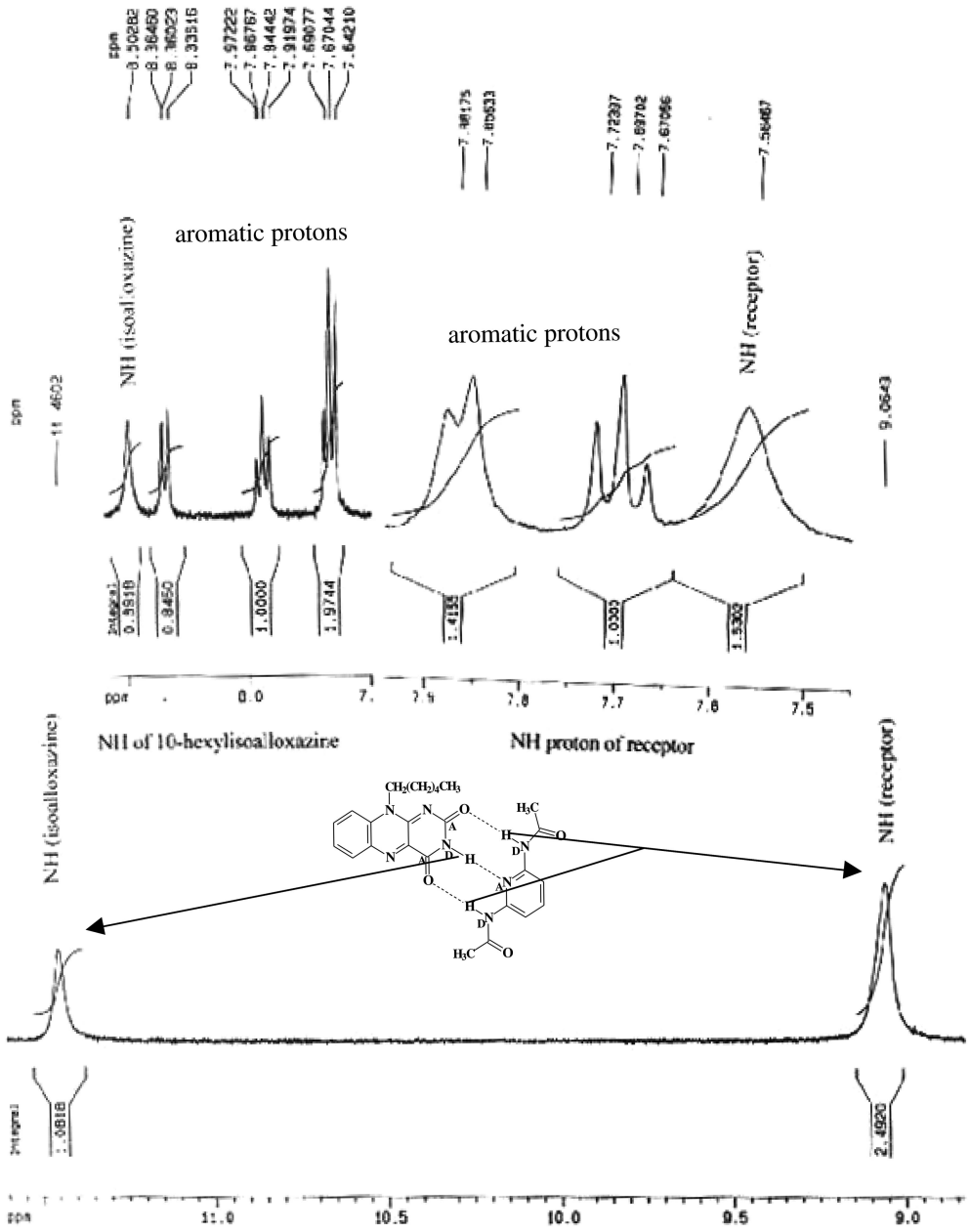


Fig. 3 Comparative ^{1}H NMR spectra of NH's {1:1 mixture of 10-hexylisoalloxazine (**3a**) and receptor (**7a**)}.

Table 3 % yield of disulfide 10 under different reaction condition

Entry	Reaction system[a]	Yield/%[b]
1	8/DBU	Nil
2	8/DBU/**3a**	21
3	8/DBU/**M1**	73
4	8/DBU/**3b**	18
5	8/DBU/**M6**	68

[a]**8** (0.5 mmol), DBU (0.5 mmol), **3** (0.5 mmol), **M** (0.5 mmol); [b]Isolated yield.

disulfide has been confirmed by comparison with an authentic sample on TLC and IR and mass spectrometry. The oxidation reaction with other complexes is in progress.

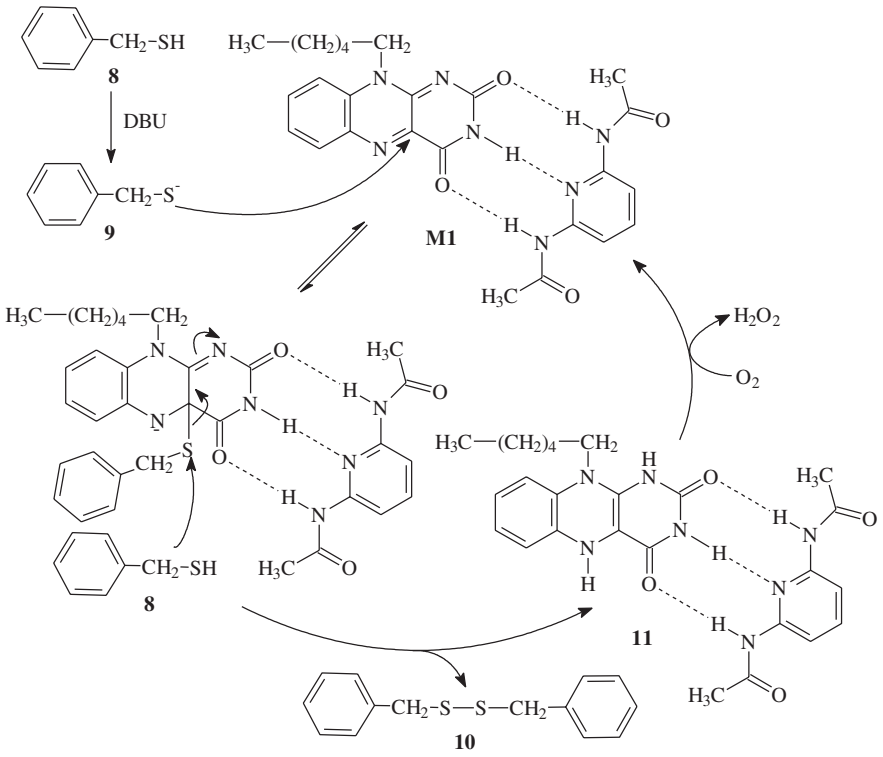
Conclusion

The 2,6-diamidopyridine derivatives act as isoalloxazine receptors *via* triple hydrogen bond formation at the O(2), N(3)H and O(4) atoms of the isoalloxazine ring. Since the study employs both the isoalloxazine and the receptor dissolved in a solvent of relatively low dielectric constant, the situation resembles more closely the enzyme-bound cofactor, which is present in the hydrophobic interior of the protein.

The UV-visible and ^{1}H NMR data indicated the hydrogen bond formation between the isoalloxazine and the receptors clearly. There was a direct correlation between the electronic properties of the receptors and the efficiency of the recognition. The triple hydrogen-bonded isoalloxazine shows increased reactivity towards benzyl thiol oxidation depending upon the 2,6-diamidopyridine derivatives and is found to be a potential metal-free organocatalyst.

Experimental

Melting points were determined on a Thomas Hoover Unimelt capillary melting apparatus and are uncorrected. Electronic spectra were recorded on a Shimadzu UV-260 spectrophotometer and absorption maxima have been expressed in nanometres. IR spectra were recorded on a Perkin Elmer 1710 FTIR spectrophotometer and the ν_{max} are expressed in cm^{-1}. ^{1}H NMR spectra were recorded on



Scheme 3

a Bruker Avance-300 spectrometer (300 MHz) and Perkin Elmer spectrometer (60 MHz) and the chemical shifts were expressed in ppm. The abbreviation s, d, t, q, m and bs stand for singlet, doublet, triplet, quartet, multiplet and broad singlet respectively.

2,6-Diaminopyridine and alloxan monohydrate were obtained from Acros (Belgium). Benzoic acid, 4-chlorobenzoic acid and 4-methoxy benzoic acid were obtained from s.d. Fine Chemicals, India. 10-Substituted isoalloxazines (**3**) were synthesised according to the literature procedure,[10-12] by acidic cyclocondensation of 2-substituted aminoanilines (**1**)[13] with alloxan monohydrate (**2**) (Scheme 1).

10-Hexylisoalloxazine **(3a):** M.p.: 254–256 °C (lit.[20] m.p. 254–256 °C); UV-visible ($CHCl_3$) λ_{max} (ε_{max} mM): 266 (18.4), 335 (4.8) 421 (5.2), 440 (8.7) and 463 (5.3); IR (KBr): 3466, 3220, 2925, 1723, 1674, 1549, 1503, 1404, 990 and 771 cm^{-1}; ^1H NMR ($CDCl_3$): 0.92 (t, 3H, CH_3), 1.36–1.49 and 1.85–2.18 (m, 8H, 4 × CH_2), 4.71 (t, 2H, $N^{10}CH_2$), 7.64–7.69 (m, 2H, H-7 and H-9), 7.97 (dd, 1H, H-8, *J* = 7.66 and 7.40 Hz), 8.36 (d, 1H, H-6, *J* = 7.88 Hz) and 8.50 (bs, 1H, N^3H).

10-Phenylisoalloxazine **(3b):** M.p.>300 °C (lit.[10] m.p. 335–336 °C); UV-visible ($CHCl_3$) λ_{max} (ε_{max} mM): 270 (54.4), 335 (13.1), 420 (15.3), 441 (17.2) and 465 (14.5); IR (Nujol): 3433, 3200, 1710, 1660, 1570, 1426, 1280, 1260, 1180, 1090, 880 and 860 cm^{-1}; ^1H NMR (DMSO-d_6): 6.82 (d, 1H, H-9, *J* = 8.20 Hz), 7.42 (d, 2H, H-3' and H-5', *J* = 7.47 Hz), 7.57–7.73 (m, 5H, H-7, H-8, H-2', H-4' and H-6'), 8.19 (dd, 1H, H-6, *J* = 8.05 and 1.18 Hz) and 8.55 (bs, 1H, N^3H).

Synthesis of N,N'-Pyridine-2,6-diylbis(acetylamide) **(7a):** A solution of 2,6-diaminopyridine (**5**) (0.04 mol), acetic acid (0.5 mmol) and acetic anhydride (0.5 mmol) was refluxed for 4 h. The reaction mixture was cooled to room temperature and ice was added to precipitate out *N,N'*-pyridine-2,6-diylbis(acetylamide) **7a**. The solid was filtered and dried at a pump.

Yield: 6.65 g (86%); M.p.: 204 °C (lit.[14] m.p. 205–206 °C); ^1H NMR ($CDCl_3$): 2.19 (s, 6H, 2 × CH_3), 7.56 (bs, 2H, 2 × NH), 7.69 (t, 1H, H-4, *J* = 8.085 Hz), 7.86 (d, 2H, H-3 and H-5, *J* = 7.6Hz) and elemental analysis for $C_9H_{11}N_3O_2$ calculated: C, 55.95; H, 5.7; N, 21.75; found: C, 55.8; H, 5.7; N, 21.7.

General procedure for the preparation of 2,6-diamidopyridine derivatives **(7b–d):** Thionyl chloride (2 mmol) was added to the corresponding acid (1 mmol) and left overnight after fitting the flask with $CaCl_2$ guard tube. The reaction mixture was refluxed for half an hour on a water bath, the excess of thionyl chloride was removed under reduced pressure on an oil-bath and the residual liquid was distilled under reduced pressure at the respective boiling point to get the acid chlorides **6** (Scheme 2).

A solution of 2,6-diaminopyridine (**5**) (0.04 mol) and triethylamine (80 mmol) in dry tetrahydrofuran (THF) (100 ml) was added to a solution of the corresponding acid chloride (**6**) (80 mmol) in dry THF with cooling (ice bath) and the mixture was stirred overnight at room temperature. The reaction mixture was concentrated and dissolved in dichloromethane (100 ml). The organic layer was washed with water (2 × 100 ml) and dried over anhydrous sodium sulphate. The solvent was removed under reduced pressure to get the desired compounds.

N,N'-Pyridine-2,6-diylbis(phenylamide) **(7b):** Yield: 9.65 g (76%); M.p.: 275–277 °C (lit.[21] m.p. 278 °C); IR (KBr): 3275, 1673, 1660, 1594 and 1550 cm^{-1}; ^1H NMR ($CDCl_3$): 7.45–7.64 (m, 6H, *m* and *p*-H), 7.86 (t, 1H, H-4), 7.99 (d, 1H, H-2'/6', *J* = 7.47 Hz), 8.12 (d, 1H, H-2'/6', *J* = 7.67 Hz), 8.18 (d, 2H, H-3 and H-5, *J* = 8.13 Hz) and 8.95 (bs, 2H, NH) and elemental analysis for $C_{19}H_{15}N_3O_2$ calculated: C, 71.9; H, 4.8; N, 13.2; found: C, 71.9; H, 4.8; N, 13.2.

N,N'-Pyridine-2,6-diylbis[(4-chlorophenyl)amide] **(7c):** Yield: 11.43 g (74%); M.p.: 250 °C (lit.[21] m.p. 248 °C); IR (KBr): 3295, 1683, 1660, 1593 and 1550 cm^{-1}; ^1H NMR ($CDCl_3$): 7.55–7.64 (m, 6H, *m*-H), 7.80 (t, 1H, H-4), 7.89 (d, 1H, H-2'/6', *J* = 7.47 Hz), 8.10 (d, 1H, H-2'/6', *J* = 7.67 Hz), 8.13 (d, 2H, H-3 and H-5, *J* = 8.13 Hz) and 8.99 (bs, 2H, NH) and elemental analysis for $C_{19}H_{13}N_3O_2Cl_2$ calculated: C, 59.1; H, 3.4; N, 10.9; found: C, 59.0; H, 3.3; N, 10.9.

N,N'-Pyridine-2,6-diylbis[(4-methoxyphenyl)amide] **(7d):** Yield: 13.98 g (78%); M.p.: 280 °C (lit.[21] m.p. 281 °C); IR (KBr): 3279, 1671, 1660, 1590 and 1555 cm^{-1}; ^1H NMR ($CDCl_3$): 3.92 (s, 6H, 2 × OCH_3) 7.54–7.58 (m, 4H, Ph-*m*H), 7.77 (t, 1H, H-4), 7.93–7.98 (m, 4H, Ph-*o*H), 8.14 (t, 2H, H-3 and H-5) and 8.80 (bs, 2H, NH) and elemental analysis for $C_{21}H_{19}N_3O_4$ calculated: C, 56.3; H, 4.3; N, 9.4; found: C, 56.3; H, 4.2; N, 9.35.

^1H NMR study for the interaction of synthetic isoalloxazines **(3)** *with 2,6-diamidopyridines* **(7)** *in 1:1 ratio recorded in $CDCl_3$*

M1: ^1H NMR ($CDCl_3$): 0.92 (t, 3H, CH_3), 1.32–1.38 and 1.84–1.95 (m, 8H, 4 × CH_2), 2.29 (m, 8H, 2 × CH_3), 4.77 (t, 2H, $N^{10}CH_2$), 7.67–7.74 (m, 4H, H-7, H-9 of isoalloxazine and H-3, H-5 of receptor), 7.94–8.00 (m, 2H, H-8 of isoalloxazine and H-4 of receptor), 8.39 (dd, 1H, H-6, *J* = 1.287 and 7.26 Hz) and 9.06 (bs, 1H, NH of receptor), 11.46 (s, 1H, N^3H of isoalloxazine).

M2: ^1H NMR ($CDCl_3$): 0.93 (t, 3H, CH_3), 1.37–1.40 and 1.84–2.01 (m, 8H, 4 × CH_2), 4.76 (t, 2H, $N^{10}CH_2$), 6.99 (d, H-9, *J* = 8.01 Hz), 7.47–7.74 (m, 9H, H-7 of isoalloxazine and H-3, H-5, *m* and *p*-H of receptor), 7.88 (t, 1H, H-8 of isoalloxazine), 7.98–8.19 (m, 6H,

H-6 of isoalloxazine and H-4, H-2', H-6' of receptor), 9.28 (bs, 1H, N–H of receptor), 11.28 (s, 1H, N^3–H of isoalloxazine).

M3: ^{1}H NMR ($CDCl_3$): 0.91 (t, 3H, -CH_3), 1.33–1.40 and 1.74–2.01 (m, 8H, 4 × CH_2), 4.73 (t, 2H, $N^{10}CH_2$), 7.01 (d, H-9, *J* = 8.01 Hz), 7.57–7.78 (m, 7H, H-7 of isoalloxazine and H-3, H-5, *m*-H of receptor), 7.87–7.98 (m, 2H, H-8 of isoalloxazine and H-4 of receptor), 8.12–8.33 (m, 5H, H-6 of isoalloxazine and 2', 6' of receptor), 9.37 (bs, 1H, N–H of receptor), 11.39 (s, 1H, N^3–H of isoalloxazine).

M4: ^{1}H NMR ($CDCl_3$): 0.93 (t, 3H, -CH_3), 1.37–1.40 and 1.84–2.01 (m, 8H, 4 × CH_2), 4.09 (s, 6H, 2 × CH_3), 4.72 (t, 2H, $N^{10}CH_2$), 7.01 (d, H-9, *J* = 8.01 Hz), 7.59–7.88 (m, 7H, H-7 of isoalloxazine and H-3, H-5, *m*-H of receptor), 7.92–7.95 (m, 2H, H-8 of isoalloxazine and H-4 of receptor), 8.02–8.33 (m, 5H, H-6 of isoalloxazine and H-2', H-6' of receptor), 9.11 (bs, 1H, N–H of receptor), 11.01 (s, 1H, N^3–H of isoalloxazine).

M5: ^{1}H NMR ($CDCl_3$): 2.31 (s, 6H, 2 × CH_3), 6.79 (dd, 1H, H-9, *J* = 8.12 and 1.2 Hz), 7.40–7.56 (m, 3H, H-7 of isoalloxazine and H-3, H-5 of receptor), 7.67–7.89 (m, 7H, H-8 of isoalloxazine, H-3, H-5 of receptor and N-Ph protons), 8.21 (dd, 1H, H-6, *J* = 7.88 and 2.0 Hz), 8.89 (bs, 2H, NH of receptor) and 11.26 (bs, 1H, N^3H of isoalloxazine).

M6: ^{1}H NMR ($CDCl_3$): 6.81 (dd, 1H, H-9, *J* = 8.11 and 2.2 Hz), 7.45–7.71 (m, 9H, H-7 of isoalloxazine and H-3, H-5, *m*- and *p*-H of receptor), 7.78–7.93 (m, 8H, H-8, N^{10}-Ph protons of isoalloxazine and H-3, H-5 of receptor), 8.09–8.23 (m, 5H, H-6 of isoalloxazine and H-2', H-6' of receptor), 9.20 (bs, 2H, NH of receptor) and 11.22 (bs, 1H, N^3H of isoalloxazine).

M7: ^{1}H NMR ($CDCl_3$): 6.84 (d, 1H, H-9, *J* = 8.11 Hz), 7.48–7.72 (m, 8H, H-7 of isoalloxazine and H-3, H-5, *m*-H of receptor), 7.78–7.93 (m, 8H, H-8, N^{10}-Ph protons of isoalloxazine and H-3, H-5 of receptor), 8.12–8.33 (m, 5H, H-6 of isoalloxazine and H-2', H-6' of receptor), 9.27 (bs, 2H, NH of receptor) and 11.23 (bs, 1H, N^3H of isoalloxazine).

M8: ^{1}H NMR ($CDCl_3$): 6.89 (dd, 1H, H-9, *J* = 8.10 and 2.0 Hz), 7.43–7.75 (m, 8H, H-7 of isoalloxazine and H-3, H-5, *m*-H of receptor), 7.79–7.96 (m, 8H, H-8, N^{10}-Ph protons of isoalloxazine and H-3, H-5 of receptor), 8.16–8.29 (m, 5H, H-6 of isoalloxazine and H-2', H-6' of receptor), 9.09 (bs, 2H, NH of receptor) and 11.00 (bs, 1H, N^3H of isoalloxazine).

General method for the oxidation of benzyl thiol by isoalloxazine-receptor complex

To a stirred solution of isoalloxazine-receptor complex **M1** (0.5 mmol each) in chloroform (25 ml), DBU (0.5 mmol) and benzyl thiol (**8**) (0.5 mmol) was added under nitrogen atmosphere and the stirring was continued for 3 h. The reaction mixture was washed with H_2O (25 ml) and dried over anhydrous sodium sulphate. The product formation was confirmed by TLC using an authentic sample and purified by preparative TLC.

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