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Recognition Properties of Flavin Analogues with Bile Acid-Based Receptors: Role of Steric Effects in Hydrogen Bond Based Molecular Recognition

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The recognition properties of 7,8-dimethyl flavin analogues by bile acid-based receptors that contain 2,6-diaminopyridine and the dioctylamide of 2,6-diaminopyridine in $CHCl_3$ were determined. The results show that the bile acid-based receptors bind 7,8-dimethyl flavin analogues less effectively as compared to 7,8-unsubstituted flavins reported earlier, which is contrary to the known fact that the association constants increase with increasing electron-donating capacity of the substituents at the 7 and 8 positions of the flavin analogues.

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Introduction

Flavoenzymes that contain a coenzyme riboflavin, often as flavin mononucleotide (FMN) or flavin adeninedinucleotide (FAD), are a diverse class of biological redox catalysts, and act in cellular redox metabolism by either single electron or simultaneous two-electron transfer mechanisms.[1] In the process of catalyzing biological redox reactions, the flavin moiety itself is reduced upon accepting electrons from a substrate and reoxidized by electron transfer to another acceptor. The apoprotein is responsible for substrate selectivity and kinetic and thermodynamic controls of the electron-transfer process.

There has been a considerable argument over the reaction pathways and mechanisms used by these flavoproteins because of the complexity of the native enzymatic systems.[2] Synthetic models have a deciding role to play, as they help us to understand the individual interactions and reaction pathways, which become fairly difficult with the complex flavoenzyme systems. To mimic the function of these enzymes by chemical models for mechanistic studies, much effort has been devoted to the chemical synthesis of flavin analogues.[3]

2,6-Diaminopyridine derivatives bind flavin analogues by three hydrogen bonds at C(2)=O, N(3)–H, and C(4)=O positions in aprotic solvents (Fig. 1). Consequently, there has been considerable interest to modulate the strength of hydrogen bond interactions in the complexes of 2,6-diaminopyridine derivatives and flavin analogues to better understand or to better mimic the flavoenzyme behaviour.[4]

In a systematic study, Rotello and Cooke have extensively studied the effect of functional group variations at C7 and C8 positions of artificial flavins on hydrogen bond interactions and the redox potential of isoalloxazine derivatives in aprotic solvents using synthetic receptors based on 2,6-diaminopyridine.[3i] These studies highlighted the profound electronic effect on the hydrogen bonding. The affinity of the flavin unit to bind with a 2,6-diaminopyridine receptor increased with electron-donating substituents at the 7 and 8 positions of the isoalloxazine ring and decreased with electron-withdrawing substituents. It is also believed that enzyme-cofactor π-stacking interactions play a substantial role in the modulation of flavin reactivities by the apoenzymes. Rotello and coworkers have explored the effect of π-stacking on flavin redox chemistry. In these studies, they used a series of receptors to examine the effects of π-stacking on flavin recognition and established that aromatic stacking interactions between the receptors and their flavin guests effectively modulate hydrogen bonding and hence, the redox potential.[5] However, there have been very little studies on the role of steric effects on the binding properties of flavin with diaminopyridine model systems. Rotello and coworkers studied the binding behaviour of 7,8-dimethyl-10-isobutyl flavin with various amide and urea derivatives of 2,6-diaminopyridine. It was observed that 2,6-*N*,*N*′-di(propionylamino)pyridine bound three times stronger than did the 2,6-*N*,*N*′-di(isobutanoylamino)pyridine receptor to the 7,8-dimethyl-10-isobutyl flavin, which suggests that the increase in branching of the acyl substitutents

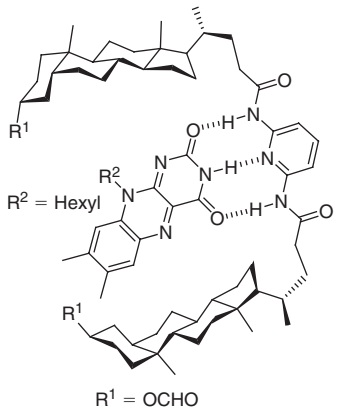

Fig. 1. Three point hydrogen bonding between bile acid-based acyclic receptor and 7,8-dimethyl-*N*(10)-hexyl flavin.

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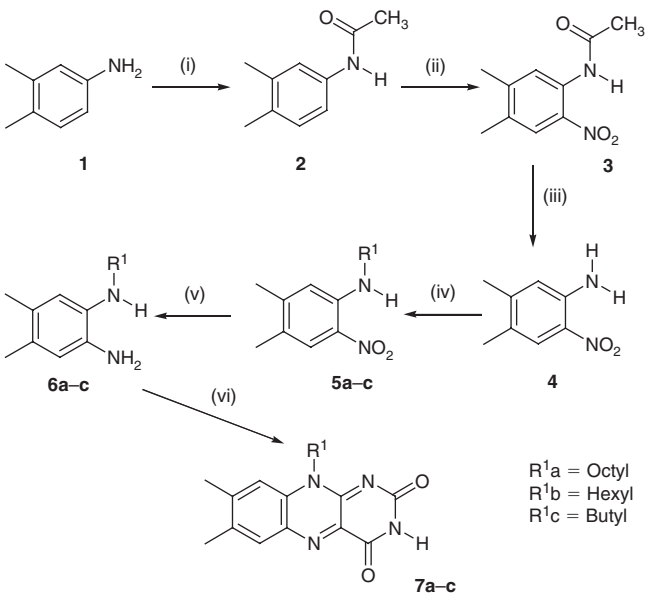


Scheme 1. Reagents and conditions (and yields): (i) $(CH_3CO)_2O$, dichloroethane, triethylamine, reflux, 12 h (98%); (ii) HNO_3, H_2SO_4, glacial acetic acid, 0–10°C, 0.5 h (85%); (iii) 10% NaOH, H_2O, reflux, 12 h (90%); (iv) NaH, alkyl bromide, DMF, rt, 12 h (~70%); (v) Pd/C, MeOH, H_2, rt, 4 h (~95%); (vi) alloxan, H_3BO_3, glacial acetic acid, 60°C, 12 h (40–50%).

on the amino groups of the 2,6-diaminopyridine receptors decreases their association constants with 7,8-dimethyl-10-isobutyl flavin.[4d] The present paper throws light on the influence of the steroidal features of bile acids as well as flavin analogues in downplaying the electronic effects with regard to their hydrogen bonding interactions.

Results and Discussion

Synthesis of 7,8-Dimethyl Flavin Analogues

7,8-Dimethyl flavin analogues **7a**–**c** were prepared using the modified method of Rotello and coworkers[3i] and is outlined in Scheme 1. The method is, in principle, applicable to the synthesis of riboflavin analogues that possess a variety of substituents at the *N*(10)-position, starting from readily available 3,4-dimethyl aniline **1**. Acetylation of **1** with acetic anhydride followed by nitration afforded **3** in good yields.[6]

Hydrolysis of **3** gave **4**, which on reaction with the corresponding alkyl halide using NaH in DMF gave **5a**–**c**. Reduction of the nitro group in **5a**–**c** was accomplished with Pd/C in methanol and the crude *N*-alkyl-1,2-benzenediamines **6a**–**c** were used without further purification for the condensation with alloxan in acetic acid to give **7a**–**c**.[7]

*Crystal Structure of Flavin Analogue **7c***

Recrystallization of **7c** from $CHCl_3$/MeOH afforded yellow single crystals, and thus, X-ray crystal analysis* was carried out to clarify the structural details of **7c**. The thermal ellipsoid diagram of **7c** is shown in Fig. 2.

The crystal structure of **7c** shows that it exists as a dimer with intermolecular hydrogen bonding giving the *trans*-geometry as shown in Fig. 3.

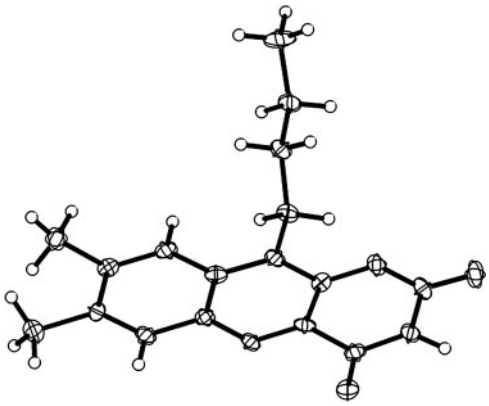


Fig. 2. Thermal ellipsoid view of compound **7c** with 30% probability factor.

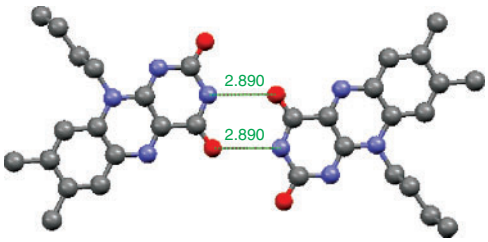


Fig. 3. X-Ray crystal structure of **7c** showing weak hydrogen bonding.

Table 1. Selected bond lengths [Å] and angles [°] for compounds 7b and 7c

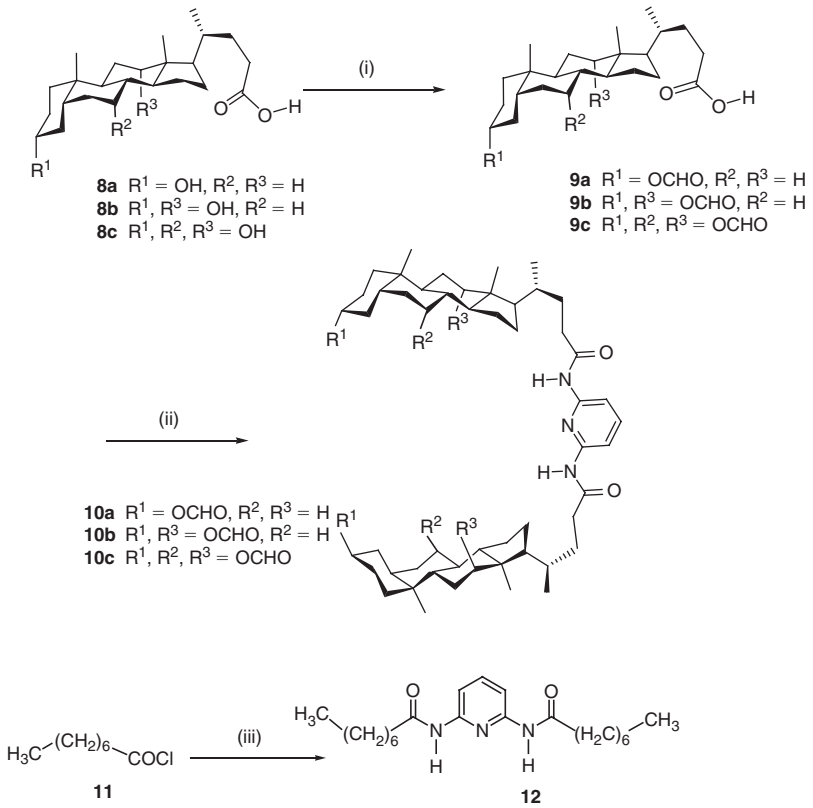
Bonds	Compound	
	7b	**7c**
	Bond lengths [Å]	
N1–C2	1.363(3)	1.359(4)
N1–C10A	1.318(3)	1.314(4)
C2–O1	1.226(3)	1.219(4)
N3–C2	1.398(3)	1.406(4)
N3–C4	1.362(3)	1.355(4)
N5–C4A	1.304(3)	1.291(4)
N5–C5A	1.369(3)	1.367(4)
N10–C9A	1.362(3)	1.385(4)
N10–C10A	1.362(3)	1.365(4)
	Bond angles [°]	
C10A–N1–C2	118.0(2)	117.8(3)
N1–C2–O1	121.5(3)	122.6(3)
O2–C4–N3	121.8(3)	123.0(3)

The crystal structure of **7b** was reported earlier.[7a] Selected bond distances and angles for **7b** and **7c** are listed in Table 1. As expected, no appreciable differences in their values are observed in these compounds.

Modified Synthesis of Bile Acid-Based Acyclic Receptors

Recently, bile acids, because of their rigid framework, facial amphiphilicity, and suitably oriented hydroxy groups, have attracted considerable interest from a molecular engineering point of view.[9] Previously, we have reported the synthesis of a

*Single-crystal diffraction study was carried out on a Bruker Smart Apex CCD diffractometer with a Mo K_α ($\lambda = 0.71073$ Å) sealed tube. The crystal structure was solved by direct methods and refined using the *SHELXTL* package.[8] All hydrogen atoms were included in idealized positions, and a riding model was used. Non-hydrogen atoms were refined with anisotropic displacement parameters.



Scheme 2. Reagents and conditions (and yields): (i) HCOOH, 60°C, 4 h (98%); (ii) ethyl chloroformate, triethylamine, rt, 4 h, 2,6-diaminopyridine, triethylamine, THF, reflux, 12 h (89%); (iii) 2,6-diaminopyridine, triethylamine, THF, 12 h (90%).

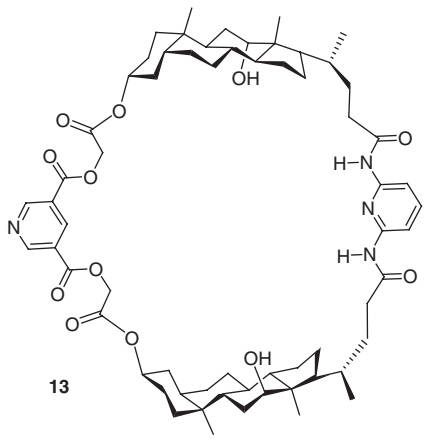


Fig. 4. Steroid-based cyclic receptor having 2,6-diaminopyridine unit.

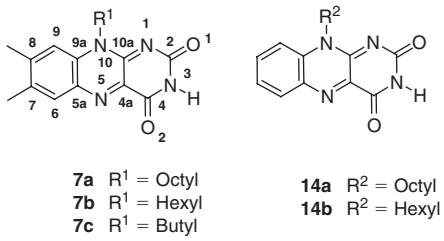


Fig. 5. Chemical structures of flavin analogues.

new class of bile acid-based acyclic as well as cyclic receptors that contain a 2,6-bis(acylamino)pyridine unit for recognition of 7,8-unsubstituted flavin and uracil analogues.[4m,10]

We have now used a modified procedure for the synthesis of the acyclic receptors that is outlined in Scheme 2 and starts with the protection of hydroxy groups of the bile acids to obtain **9a**–**c**. In the following step, formylated bile acids **9a**–**c** were treated with ethyl chloroformate in the presence of triethylamine to give the corresponding anhydrides that were subsequently condensed with 2,6-diaminopyridine to give the acyclic receptors **10a**–**c**. Compound **10b** was further used to synthesize cholaphane **13**[4m] shown in Fig. 4.

This synthetic route is much cleaner, easy to handle, and most importantly gives good yields. The dioctyldiamide of 2,6-diaminopyridine[11] **12** was prepared from the reaction of the corresponding acid chloride **11** with 2,6-diaminopyridine.

Complexation Studies

7,8-Dimethyl flavin analogues **7a**–**c** and 7,8-unsubstituted flavin analogues **14a**,**b** employed in the complexation studies are shown in Fig. 5.

The binding behaviour of the flavin analogues were determined with 2,6-diaminopyridine-based receptors using ^{1}H NMR titration in $CDCl_3$. As a typical example of a NMR titration, addition of flavin derivative **7a** to the solution of bile acid-based acyclic receptor **12** in $CDCl_3$ resulted in the downfield shift in the resonances of the amidic protons of the receptor. The change in the chemical shift of the amidic protons was followed as a function of increasing guest concentration until saturation of the chemical shift values was reached. Analysis of the saturation data

Table 2. Binding constants K_a (M^{-1}) for complexation of flavin analogues with 2,6-diaminopyridine-based receptor 12[A]

Flavin derivative	K_a [M^{-1}]
7a	280
7b	300
7c	300
14a	145
14b	150

[A]Determined in $CDCl_3$, at 25°C, errors estimated to be ≤10%.

Table 3. Binding constants K_a (M^{-1}) for complexation of flavin analogues with bile acid-based receptors[A]

Receptor	Flavin derivatives				
	7a	**7b**	**7c**	**14a**	**14b**
10a	240	400	150	250[B]	600[B]
10b	230	350	200	240[B]	400[B]
10c	230	310	200	260	400
13	95	100	100	110[B]	110[B]

[A]Determined in $CDCl_3$, at 25°C, errors estimated to be ≤10%. [B]Data from reference [4m].

with *WINEQNMR* software,[12] a non-linear regression curve fitting program, revealed a 1:1 complexation, which was further confirmed by Job's plot. The titration experiment was carried out at least twice. The observed binding constants for the complexation of receptor **12** with flavin analogues **7a**–**c** and **14a**,**b** are listed in Table 2.

The ^1H NMR data in Table 2 show that the association constants are low (K_a 145–150 M^{-1}) for 7,8-unsubstituted flavin analogues **14a**,**b** and bis(octanoylamino)pyridine **12**. Upon introducing a methyl substituent at the C7 and C8 positions of the flavin analogues **7a**–**c**, the association constants increase to 280–300 M^{-1}. Complexation of **12** with 7,8-dimethyl flavin analogues **7a**–**c** is stronger than 7,8-unsubstituted flavin analogues **14a**,**b** because the electron donating capacity of the methyl groups at the C7 and C8 positions increases the electron density throughout the flavin system, which subsequently increases the hydrogen bonding capability, because there are two hydrogen bond acceptors C(2)=O and C(4)=O against one hydrogen bond donor N(3)–H. This observation concurs with the earlier works of Rotello and coworkers[3i] and Hasford and Rizzo.[13] The results of the titration experiments for the complexation of bile acid-based receptors with flavin analogues **7a**–**c** and **14a**,**b** are summarized in Table 3.

Surprisingly, the electronic effects of the methyl substituents at the C7 and C8 positions of the flavin analogues do not seem to play a prominent role in the complexation of the flavin analogues with bile acid-based acyclic receptors. The binding constants of the complexes of the acyclic receptors **10a**–**c** with 10-hexylisoalloxazine (7,8-unsubtituted flavin analogue) **14b** is in the range of 400–600 M^{-1},[4m] whereas with 7,8-dimethyl-*N*(10)-hexylisoalloxazine (7,8-dimethyl flavin analogue) **7b**, they are in the range of 310–400 M^{-1}. A similar trend was observed for the complexation of 7,8-unsubstituted and substituted-*N*(10)-octyl flavin with bile acid-based acyclic receptors **10a**–**c**. Although the strength of multiple hydrogen bonds in complexes is related to the individual hydrogen-bonding acidity of the donor group and the basicity of the acceptor group involved, there are additional factors that also influence the stability of the complex. The manifestation of this comes from the fact that 7,8-unsubstituted flavin analogues **14a**,**b**, as compared to the 7,8-dimethyl flavin analogues **7a**–**c**, show a higher affinity towards acyclic bile acid-based receptors, which is in contrast with the earlier works of the groups of Rotello and Rizzo. The unexpected decrease in the binding constant, presumably, is a result of steric effects arising from the interaction of the methyl groups at the 7 and 8 positions of the flavin with the rigid steroidal framework, which outplay the electronic effects of the flavin system. Moreover, the difference in the binding affinity between 7,8-dimethyl-*N*(10)octyl flavin **7a** and *N*-10-octylisoalloxazine **14a** for acyclic bile acid receptors **10a**–**c** is small compared to the difference in the binding affinity between 7,8-dimethyl-*N*(10)hexyl flavin **7b** and *N*-10-hexylisoalloxazine **14b**. This might be attributed to the large chain length of the octyl group that creates more hindrance in binding and decreases the influence of the 7,8-dimethyl groups. However, the binding affinity of 7,8-dimethyl-*N*(10)-butyl flavin, which can have better binding because of the smaller size of the butyl group, shows a lower binding affinity compared to **7a** and **7b**, which may be a result of the dominant steric interactions of the 7,8-dimethyl groups with the steroidal framework. Hence, it seems that among **7a**–**c**, 7,8-dimethyl-*N*(10)hexyl flavin **7b** has the most suitable chain length at *N*(10) for the formation of a supramolecular structure with acyclic bile acid-based receptors **10a**–**c**. In the case of the cyclic receptor cholaphane **13**, which is already a poor receptor because of its constrained geometry, there is no significant change in the binding constant with respect to 7,8-unsubstituted and substituted flavin analogues.

Conclusion

In conclusion, an efficient synthetic procedure for the synthesis of 7,8-dimethyl-*N*(10)alkyl flavins has been developed. Binding studies of these flavins with bile acid-based diaminopyridine (DAP) systems shows a prominent influence of the steric factors that is the opposite to electronic effects. Hence, it seems likely that the steric effect also plays an important role in the regulation of binding and electrochemical properties of flavoenzymes.

Experimental

Melting points are uncorrected. IR spectra were recorded on a Nicolet Protégé 460 FTIR Spectrometer, using potassium bromide pellets. ^1H and ^{13}C NMR spectra were recorded on a Bruker Spectrospin DPX 300. Tetramethylsilane was used as internal reference and the chemical shifts are expressed as displacement (δ) in ppm downfield from tetramethylsilane. High-resolution mass spectra (ES) were recorded on a VG-Fisons 'Autospec' spectrometer. Column chromatography was carried out using Spectrochem silica gel 230–400 mesh for flash chromatography. The solid compounds were dried under vacuum in the presence of P_2O_5.

N-*(3,4-Dimethylphenyl)acetamide 2*

3,4-Dimethylaniline **1** (5.0 g, 41.1 mmol) was dissolved in dry 1,2-dichloroethane (25 mL) and to this acetic anhydride (6.3 g, 61.8 mmol) and triethylamine (6.2 g, 61.8 mmol) were added. The reaction mixture was refluxed in an oil bath for 12 h. TLC was used to monitor the course of the reaction and to confirm the complete consumption of starting material. The

solvent was then evaporated under reduced pressure and the residue was dissolved in chloroform (30 mL) and washed with 1 N hydrochloric acid (10 mL), saturated sodium bicarbonate solution (10 mL), and brine (10 mL), respectively, dried over sodium sulfate and concentrated. The crude product was purified by flash chromatography on silica gel (elution with ethyl acetate/hexane, 3/7) to give 6.59 g of **2**. Yield 98%, mp 97–100°C. ES-HRMS calc. for $(C_{10}H_{13}NONa)^+$ 186.0895, found 186.0894. ν_{max} (KBr)/cm^{-1} 3291, 3190, 2967, 1540, 1663, 1607, 1313. δ_H (300 MHz, $CDCl_3$, TMS) 7.27 (s, 1H, –NH), 7.20 (m, 2H, Ar-H), 7.05 (d, 1H, *J* 8.10, Ar-H), 2.23 (s, 3H, CH_3), 2.21 (s, 3H, CH_3), 2.14 (s, 3H, $–COCH_3$). δ_C (75 MHz, $CDCl_3$, TMS) 168.92, 137.04, 135.82, 132.55, 129.84, 121.65, 117.82, 29.71, 24.30, 19.82, 19.15.

N-*(4,5-Dimethyl-2-nitrophenyl)acetamide* ***3***

To a solution of *N*-(3,4-dimethylphenyl)acetamide **2** (2 g, 12.3 mmol) in glacial acetic acid (10 mL) was added concentrated nitric acid (0.73 mL) over 15 min while maintaining the reaction temperature below 10°C, followed by the dropwise addition of sulfuric acid (0.46 mL) over 15 min. The resulting solution was stirred at room temperature for 30 min. The reaction mixture was added dropwise on to crushed ice and the yellow precipitate obtained was filtered off and washed generously with water. The crude product was purified by flash chromatography on silica gel (elution with ethyl acetate/hexane, 1/9) to give 2.17 g of **3**. Yield 85%, mp 101–104°C. ES-HRMS calc. for $(C_{10}H_{12}N_2O_3Na)^+$ 231.0746, found 231.0744. ν_{max} (KBr)/cm^{-1} 3341, 2959, 1705, 1620, 1583, 1502. δ_H (300 MHz, $CDCl_3$, TMS) 10.35 (s, 1H, –NH), 8.54 (s, 1H, Ar-H), 7.98 (s, 1H, Ar-H), 2.34 (s, 3H, $–COCH_3$), 2.28 (s, 3H, CH_3), 2.27 (s, 3H, CH_3). δ_C (75 MHz, $CDCl_3$, TMS) 167.91, 145.78, 133.12, 131.73, 131.30, 124.83, 121.61, 24.57, 19.47, 18.08.

4,5-Dimethyl-2-nitroaniline ***4***

N-(4,5-Dimethyl-2-nitrophenyl)acetamide **3** (2 g, 9.62 mmol) was added to a 10% sodium hydroxide solution (25 mL). The mixture was refluxed for 12 h. The reaction mixture was allowed to cool to room temperature and the rust colored precipitate was filtered off, dissolved in chloroform (30 mL), and washed thoroughly with water. The organic layer was dried over anhydrous sodium sulfate and concentrated. The crude product was purified by flash chromatography on silica gel (elution with ethyl acetate/hexane, 1/9) to give 1.43 g of **4**. Yield 90%, mp 137–139°C. ES-HRMS calc. for $(C_8H_{10}N_2O_2H)^+$ 167.0821, found 167.0822. ν_{max} (KBr)/cm^{-1} 3488, 3374, 1638, 1583, 1487. δ_H (300 MHz, $CDCl_3$, TMS) 7.87 (s, 1H, Ar-H), 6.59 (s, 1H, Ar-H), 5.90 (br s, 2H, $–NH_2$), 2.23 (s, 3H, CH_3), 2.18 (s, 3H, CH_3). δ_C (75 MHz, $CDCl_3$, TMS) 146.65, 143.08, 130.25, 126.15, 125.64, 119.04, 20.12, 18.60.

General Procedure for N-*Alkylation*

In a typical example, 4,5-dimethyl-2-nitroaniline **4** (1 g, 6.02 mmol) was dissolved in dry DMF (30 mL) and to this sodium hydride (60% suspension in mineral oil, 0.24 g) was added under a nitrogen atmosphere. Octyl bromide (1.16 g, 6.02 mmol) was then added dropwise and the reaction mixture was stirred at room temperature for 12 h. The solution was diluted with ethyl acetate (40 mL) and washed four times with water, dried over sodium sulfate, and concentrated. The crude product was purified by flash chromatography on silica gel (elution with ethyl acetate/hexane, 1/99) to give 1.17 g of a rust colored viscous oil **5a** (70%).

(4,5-Dimethyl-2-nitrophenyl)octylamine ***5a***

ES-HRMS calc. for $(C_{16}H_{26}N_2O_2Na)^+$ 301.1892, found 301.1891. ν_{max} (KBr)/cm^{-1} 3379, 3068, 2926, 2855, 1628, 1571, 1509. δ_H (300 MHz, $CDCl_3$, TMS) 7.86 (br s, 1H, –NH), 7.79 (s, 1H, Ar-H), 6.50 (s, 1H, Ar-H), 3.18–3.12 (m, 2H, $–NCH_2$), 2.15 (s, 3H, CH_3), 2.05 (s, 3H, CH_3), 1.65–1.58 (m, 2H, $–CH_2$), 1.40–1.17 (m, 10H, $–(CH_2)_5$), 0.78 (t, 3H, *J* 6.9, CH_3). δ_C (75 MHz, $CDCl_3$, TMS) 147.10, 144.23, 129.47, 126.26, 124.03, 114.02, 42.97, 31.71, 29.20, 29.06, 28.97, 27.01, 22.57, 20.61, 18.43, 13.99.

(4,5-Dimethyl-2-nitrophenyl)hexylamine ***5b***

Starting material **4** (1 g, 6.02 mmol) and hexyl bromide (0.99 g, 6.02 mmol) were used. The product was isolated as 1.05 g of a red-orange solid **5b**. Yield 70%, mp 45–48°C. ES-HRMS calc. for $(C_{14}H_{22}N_2O_2Na)^+$ 273.1579, found 273.1579. ν_{max} (KBr)/cm^{-1} 3375, 2925, 2857, 1627, 1568, 1505. δ_H (300 MHz, $CDCl_3$, TMS) 7.97 (br s, 1H, –NH), 7.92 (s, 1H, Ar-H), 6.61 (s, 1H, Ar-H), 3.30–3.24 (m, 2H, $–NCH_2$), 2.26 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 1.76–1.69 (m, 2H, $–CH_2$), 1.53–1.31 (m, 6H, $–(CH_2)_3$), 0.90 (t, 3H, *J* 7.0, CH_3). δ_C (75 MHz, $CDCl_3$, TMS) 147.24, 144.32, 129.55, 126.35, 124.13, 114.10, 43.06, 31.49, 29.02, 26.76, 22.56, 20.70, 18.52, 13.99.

Butyl-(4,5-dimethyl-2-nitrophenyl)amine ***5c***

Starting material **4** (1 g, 6.02 mmol) and butyl bromide (0.83 g, 6.02 mmol) were used. The product was isolated as 0.95 g of a red solid **5c**. Yield 71%, mp 54–56°C (lit.[8b] mp 55–57°C). ES-HRMS calc. for $(C_{12}H_{18}N_2O_2Na)^+$ 245.1266, found 245.1265. ν_{max} (KBr)/cm^{-1} 3372, 2925, 2862, 1626, 1569, 1507. δ_H (300 MHz, $CDCl_3$, TMS) 7.98 (br s, 1H, –NH), 7.92 (s, 1H, Ar-H), 6.62 (s, 1H, Ar-H), 3.31–3.25 (m, 2H, $–NCH_2$), 2.26 (s, 3H, CH_3), 2.17 (s, 3H, CH_3), 1.75–1.66 (m, 2H, $–CH_2$), 1.51–1.41 (m, 2H, $–CH_2$), 0.98 (t, 3H, *J* 7.5, CH_3). δ_C (75 MHz, $CDCl_3$, TMS) 144.21, 129.48, 126.23, 124.01, 114.00, 42.64, 147.11, 31.04, 20.58, 20.12, 18.40, 13.69.

General Procedure for Reduction

In a typical example, to a stirred suspension of **5a** (1 g, 3.59 mmol) in dry methanol (30 mL), 10% Pd/C (0.2 g) was added in a single portion. The resulting reaction mixture was stirred at room temperature for 4 h under balloon pressure of hydrogen. The catalyst was removed by filtration and washed with dry methanol (10 mL). The solvent was evaporated under reduced pressure. The resulting residue was extracted with chloroform and dried over anhydrous sodium sulfate. The organic layer on evaporation gave 0.85 g of a colourless viscous oil **6a**, which was used without further purification.

General Procedure for the Synthesis of 7,8-Dimethyl-10-alkyl Isoalloxazines

In a typical example, 1-(*N*-octylamino)-2-amino-4,5-dimethylbenzene **6a** (0.50 g, 2.01 mmol) was dissolved in glacial acetic acid (10 mL), and the contents were heated to 60°C. To this, a preheated (60°C) mixture of alloxan monohydrate (0.32 g, 2.01 mmol) and boric acid (0.18 g, 3.02 mmol) in acetic acid were added. The reaction mixture was stirred overnight at 60°C. Acetic acid was removed under vacuum, and the solid obtained was flashed chromatographed on a silica gel column (elution with methanol/chloroform, 3/97) to give 0.31 g of a bright yellow solid **7a**.

7,8-Dimethyl-N(10)-octylisoalloxazine ***7a***

Yield 43%, mp 230–233°C. ES-HRMS calc. for $(C_{20}H_{26}N_4O_2Na)^+$ 377.1953, found 377.1958. ν_{max} (KBr)/cm^{-1} 3456, 3163, 2925, 2856, 2811, 1716, 1659, 1577. δ_H (300 MHz, $CDCl_3$, TMS) 9.03 (br s, 1H, –NH), 8.15 (s, 1H, Ar-H), 7.38 (s, 1H, Ar-H), 4.78–4.68 (m, 2H, $–NCH_2$), 2.68 (s, 3H, CH_3), 2.56 (s, 3H, CH_3), 2.01–1.79 (m, 2H, $–CH_2$), 1.56–1.28 (m, 10H, $–(CH_2)_5$), 0.87 (t, 3H, *J* 6.6, CH_3). δ_C (75 MHz, $CDCl_3$, TMS) 159.55, 155.34, 150.00, 148.16, 136.99, 136.08, 134.92, 132.77, 131.04, 115.29, 45.31, 31.68, 29.20, 29.08, 27.10, 26.80, 22.55, 21.65, 19.47, 14.01.

7,8-Dimethyl-N(10)-hexylisoalloxazine ***7b***

Starting material **6b** (0.5 g, 2.27 mmol), alloxan monohydrate (0.36 g, 2.27 mmol), and boric acid (0.21 g, 3.40 mmol) were used. The product was isolated as 0.33 g of a bright yellow solid **7b**. Yield 45%, mp > 240°C (lit.[8a] mp 267°C). ES-HRMS calc. for $(C_{18}H_{22}N_4O_2Na)^+$ 349.1640, found 349.1643. ν_{max} (KBr)/cm^{-1} 3437, 3220, 2926, 1712, 1659, 1540. δ_H (300 MHz, $CDCl_3$, TMS) 8.50 (s, 1H, –NH), 8.07 (s, 1H, Ar-H), 7.39 (s, 1H, Ar-H), 4.65–4.72 (m, 2H, $–NCH_2$), 2.57 (s, 3H, CH_3), 2.46 (s, 3H, CH_3), 1.88–1.81 (m, 2H, $–CH_2$), 1.59–1.37 (m, 6H, $–(CH_2)_3$), 0.91 (t, 3H, *J* 6.9, CH_3). δ_C (75 MHz, $CDCl_3$, TMS) 159.63, 155.64, 150.00, 148.26, 137.09, 136.07, 134.96, 132.76, 131.05, 115.34, 45.37, 31.41, 27.11, 26.49, 22.51, 21.69, 19.50, 13.95.

7,8-Dimethyl-N(10)-butylisoalloxazine ***7c***

Starting material **6c** (0.5 g, 2.60 mmol), alloxan monohydrate (0.42 g, 2.60 mmol), and boric acid (0.24 g, 3.90 mmol) were used. The product was isolated as 0.37 g of a bright yellow solid **7c**. Yield 48%, mp > 240°C (lit.[8b] mp > 240°C). ES-HRMS calc. for $(C_{16}H_{18}N_4O_2Na)^+$ 321.1327, found 321.1320. ν_{max} (KBr)/cm^{-1} 3451, 3153, 3100, 3023, 2959, 2866, 2815, 1713, 1656. δ_H (300 MHz, $CDCl_3$, TMS) 8.51 (s, 1H, –NH), 8.05 (s, 1H, Ar-H), 7.39 (s, 1H, Ar-H), 4.72–4.67 (m, 2H, $–NCH_2$), 2.56 (s, 3H, CH_3), 2.44 (s, 3H, CH_3), 1.89–1.79 (m, 2H, $–CH_2$), 1.60–1.49 (m, 2H, $–CH_2$), 1.02 (t, 3H, *J* 7.2, CH_3). δ_C (75 MHz, $CDCl_3$, TMS) 159.56, 155.19, 150.05, 148.27, 137.05, 136.05, 134.97, 132.82, 131.08, 115.28, 45.15, 29.12, 21.74, 20.16, 19.54, 13.78.

General Procedure for the Modified Synthesis of Acyclic Bile Acid-Based Receptors

In a typical example, to a solution of **9a** (4 g, 9.9 mmol) and triethylamine (1.4 mL) in dry tetrahydrofuran (THF) under a nitrogen atmosphere was added ethyl chloroformate (1.07 g, 9.9 mmol) dropwise over a period of 10 min. The reaction mixture was stirred at room temperature for 4 h. A solution of 2,6-diaminopyridine (0.44 g, 4.03 mmol) in dry THF (15 mL) was added and the reaction mixture was refluxed for 12 h. THF was evaporated and the residue was extracted with chloroform. The solution was washed with 1 N HCl, saturated sodium bicarbonate solution, and brine, dried over sodium sulfate and evaporated. The residue was purified by flash chromatography (elution with EtOAc/hexane, 1/6) to give 3.88 g of **10a** (89%). All the compounds **10a**–**c** gave identical melting points and spectroscopic values similar to the compounds reported earlier.[4m]

Crystallographic Data

A yellow crystal with dimensions of $0.37 \times 0.27 \times 0.09$ mm^3 was obtained by slow evaporation of compound **7c** in methanol/chloroform solution. Molecular formula $C_{16}H_{18}N_4O_2$, M_w 298.34, triclinic, space group P-1, *a* 8.152(6) Å, *b* 10.051(8) Å, *c* 10.325(8) Å, α 64.562(12)°, β 68.541(13)°, γ 78.667(14)°, *V* 710.2(10) Å^3, *T* 298 K, D_{calcd} 1.408 g cm^{-3}, μ 0.095 mm^{-1}, *Z* 2, λ 0.71073 Å, ω and φ scans, R_1/wR_2 ($I \geq 2\sigma(I)$) 0.0871/0.1593. Crystallographic data for the structure have been deposited with the Cambridge Crystallographic Database Centre as supplementary publication number CCDC 645150.

^{1}H NMR Titrations and Determination of Stoichiometry

The binding tendency of receptors based on 2,6-diaminopyridine for flavin analogues has been investigated by ^{1}H NMR titration experiments in $CDCl_3$ on a Bruker DPX300 (300 MHz) spectrometer at 298 K. In particular, a solution [$(8–12) \times 10^{-3}$ M] of the receptor in $CDCl_3$ was titrated with aliquots from a flavin stock solution [$(40–60) \times 10^{-3}$ M] in the same solvent. The changes in the chemical shift of the amide protons were monitored as a function of increasing flavin concentration until saturation of the chemical shift values were reached. The association constants K_a were calculated by the analysis of the saturation data with *WINEQNMR* software,[13] a non-linear regression curve fitting program, which also revealed a 1:1 complexation. Every titration was repeated at least once until consistent results were obtained. The complex stoichiometry was determined by using Job's method of continuous variations. A plot of the product of the host concentration and difference in the change of the chemical shift of the host upon addition of the guest versus the guest mole fraction was made. In each case, this peaked at a guest mole fraction of 0.5, which indicated the 1:1 stoichiometry of the complex formation.

Accessory Publication

X-ray data (CIF), NMR spectra, Job's plot figures and binding isotherms are available from the author or, until March 2013, the *Australian Journal of Chemistry*.

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