Redox-Dependent Binding Ability of a Flavin Cyclophane in Aqueous Solution: Hydrophobic Stacking versus Cavity-Inclusion Complexation

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Abstract: A novel water-soluble flavinophane (**1**) and a nonmacrocyclic isoalloxazine comparison compound **3** were prepared. The host-guest binding interactions and the self-association of these flavin derivatives were investigated in an aqueous borate buffer at pH 10 in concentration ranges below 5×10^{-3} mol L^{-1}. The two oxidized derivatives **1a** and **3a** undergo a very similar, strong self-association, and the experimental data are best explained by the formation of dimers, stabilized by hydrophobic $\pi-\pi$ stacking interactions. The free energies for the formation of these dimers are between 3 and 4 kcal mol^{-1}. A significant self-association is not observed with the reduced forms **1b** and **3b**. The two oxidized flavin derivatives **1a** and **3a** show very similar capabilities in host-guest binding. They both form hydrophobic $\pi-\pi$ stacking complexes with naphthalene derivatives that are stabilized by free energies of formation of 3-4 kcal mol^{-1}. The experimental data indicate that the flavinophane **1a** does not bind substrates in its cavity because external $\pi-\pi$ stacking between guest and isoalloxazine moiety occurs preferentially. Whereas no significant complexation ability is observed for the dihydroisoalloxazine **3b**, the reduced flavinophane **1b** forms cavity-inclusion complexes with naphthalene derivatives. These cavity-inclusion complexes are of similar stability to the external $\pi-\pi$ stacking complexes formed by the oxidized host. The results of the association and binding studies with the oxidized **1a** and **3a** lead to the conclusion that $\pi-\pi$ stacking interactions play a considerable role in the binding of aromatic substrates in proximity to the isoalloxazine unit of FAD or FMN at flavoenzyme active sites.

Current research efforts in our laboratories address the activation of coenzymes by the specific microenvironment of cyclophane binding sites and the influence of coenzymes on the strength of substrate binding.[1-3] A significant contribution of the coenzyme to the substrate binding interactions can especially be expected in natural[4] and artificial flavoenzymes.[5-7] Flavin coenzymes [flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD)[4,8,9] are known to be tightly bound or, in some cases, even covalently attached to the active sites of flavoenzymes.[10] The isoalloxazine unit in the oxidized form is planar[4a,11] whereas the dihydroisoalloxazine unit in the $2e^-$ reduced form, according to X-ray crystallography,[11,12] takes a butterfly shape by bending with an angle of up to ~30° around the two nitrogens N-5 and N-10 of the central ring in the tricyclic system. Depending on their function, enzymes or artificial analogues can stabilize either the planar oxidized or the bent reduced form and thus alter considerably the redox potential of the flavin.[4a,7] On the other hand, it can be expected that the different shapes of the large tricyclic isoalloxazine unit in the oxidized and $2e^-$ reduced form considerably affect the complexation ability of the molecular binding site. (Scheme I).

Scheme I

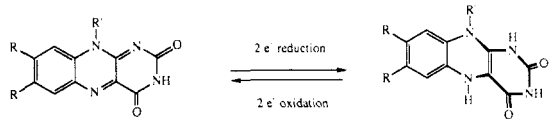
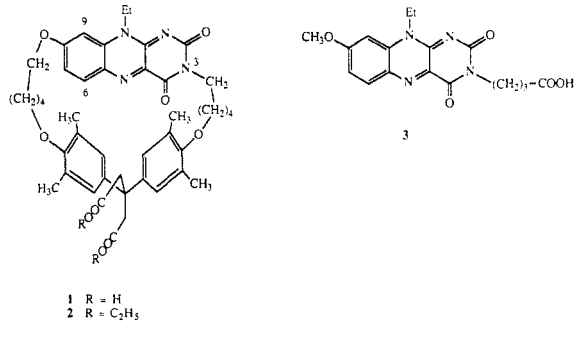


Chart I



1 R = H
2 R = C_2H_5

Previously, we had observed large, selective accelerations of the transport of arenes through aqueous solutions mediated by complexation to cyclophane hosts.[13] Since the transport from an organic source phase through the aqueous phase into an organic receiving phase was a "passive" process, driven by a concentration gradient, back transport rapidly became significant. The occurrence of back transport limits the utilization of the high transport selectivity for material separations. Therefore, the development of mediators with switchable binding properties for "active" transport in one defined direction was very desirable.[14]

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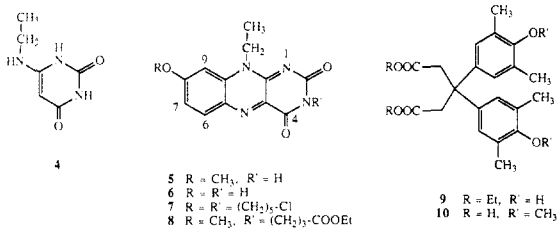
Chart II



4

5 R = CH_3, R' = H
6 R = R' = H
7 R = R' = $(CH_2)_5$-Cl
8 R = CH_3, R' = $(CH_2)_3$-COOEt

9 R = Et, R' = H
10 R = H, R' = CH_3

We designed the water-soluble flavinophane **1**[6,15,16] to investigate the influence of isoalloxazine units in their different oxidation states on substrate binding and to explore their utilization in the development of synthetic receptors with complexing properties that can be reversibly switched by a redox process.

In this paper, we describe the synthesis and the structural and electrochemical characterization of the flavinophane **1** in its oxidized and $2e^-$ reduced state. A comparative study of self-aggregation properties provides strong evidence for the formation of dimers by both the oxidized cyclophane **1** and isoalloxazine **3**. Host–guest-complexation studies demonstrate efficient binding via hydrophobic $\pi-\pi$ stacking of aromatic substrates by both oxidized **1** and **3**. These investigations show that, in the $2e^-$ reduced state, only the flavinophane **1** forms molecular complexes. The nonmacrocyclic **3** lacks a cavity binding site and does not undergo complexation. (Chart I).

Synthesis

The synthesis of the isoalloxazine moiety in **1** and **3** was accomplished by the method of Yoneda et al.[17] and began with 6-(*N*-ethylamino)uracil (**4**),[18a] prepared in 70% yield by the treatment of 6-chlorouracil with ethylamine in a sealed tube at 120 °C for 3 h. Condensation of **4** with *p*-nitrosoanisole[18b] in a mixture of acetic acid and acetic anhydride afforded the isoalloxazine **5** (60% yield). The demethylation to **6** was accomplished in 99% yield with boron tribromide in 1,2-dichloroethane. Treatment of the crude phenol with an excess of 1,5-dichloropentane (Cs_2CO_3, DMF) gave the dichloride **7** in 69% yield. The diphenylmethane unit **9** was prepared in 75% yield by the condensation of 2,6-dimethylphenol with diethyl-1,3-acetonedicarboxylate in 80% sulfuric acid. Cyclization of **7** and **9** (Cs_2CO_3, DMF) provided the flavinophane diester **2** in 12% yield. Attempts to generate **1** by alkaline hydrolysis of **2** (K_2CO_3 in ethanol/water or CsOH in water/Me_2SO) failed and led, instead, to the hydrolysis of the isoalloxazine ring.[19] Therefore, the ester groups in **2** were hydrolyzed under acidic conditions using methanesulfonic acid in 88% formic acid under reflux to yield the bright yellow target host **1** in 90% yield. The comparison compound **3** was obtained by reacting **5** with ethyl 4-bromobutanoate (K_2CO_3, DMF, 74%) to give **8** and subsequent hydrolysis (89%) as described for **1**. (Chart II).

Structural Characterization of the Oxidized and Reduced Flavinophanes 1a and 1b

The oxidized flavinophane is readily soluble in alkaline deuterated borate buffer (D_2O, D_3BO_3/NaOD, pD = 10.4). Figure 1A shows a 500-MHz ^1H NMR spectrum of the dicarboxylate **1a** ($c = 5 \times 10^{-4}$ mol L^{-1}) in this buffer. Reduction of the bright yellow solution of **1a** under argon was achieved (i) chemically with sodium borohydride or (ii) by irradiating with a 220-W daylight lamp in the presence of ethylenediaminetetraacetate (EDTA) or (iii) chemically with sodium dithionite. The ^1H NMR spectrum in Figure 1B shows that, upon reduction, a solution containing exclusively the colorless reduced trianionic[20] host **1b** is obtained. The quantitative character of the reduction is also fully supported by electronic absorption (see below) and emission spectroscopy. The bright greenish fluorescence (λ_{exc} = 450 nm, λ_{em} = 492 nm) of **1a** disappeared completely upon reduction. By introduction of oxygen into the solution of **1b**, the oxidized flavin-host **1a** is regenerated quantitatively. The redox cycles can be repeated numerous times without affecting the molecular structure of the host, and similar results are obtained with the isoalloxazine **3**. (Scheme II).

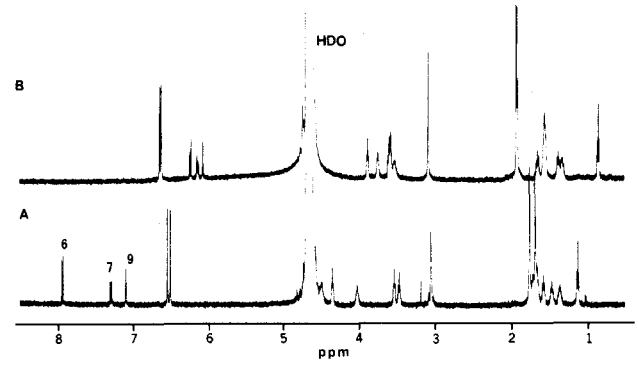
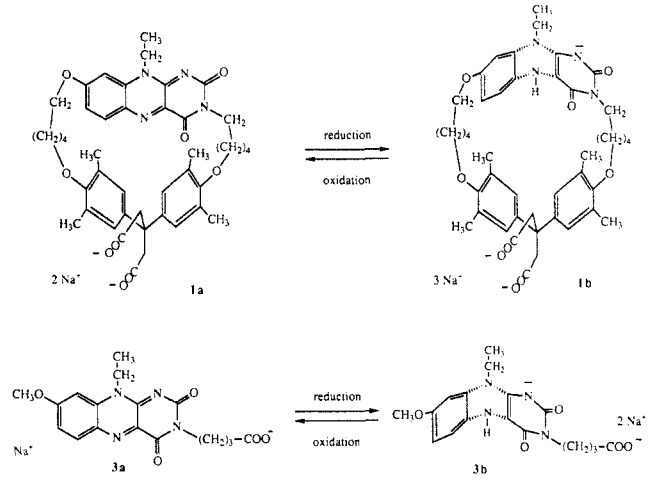


Figure 1. 500-MHz ^1H NMR spectra of the oxidized flavinophane **1a** (A) and of the reduced **1b** (B) in deuterated borate buffer, pD = 10.4, T = 295 K, $c = 5 \times 10^{-4}$ mol L^{-1}.

Scheme II



The determination of ^1H NMR cyclization shifts $\Delta\delta_{cycl}$ = [δ(protons in the cyclophane) − δ(corresponding protons in the cyclization components)][21a] provides an experimental method for evaluating whether the aromatic rings in a cyclophane are collapsed on each other or whether they organize a macrocyclic cavity with the potential for inclusion complexation. Weak cyclization shifts ($\Delta\delta \leq 0.1$ ppm) are calculated from the resonances observed for the flavinophane **2** and the cyclization components **7** and **9** in $CDCl_3$. This, in agreement with CPK model examinations, indicates that the diphenylmethane and the isoalloxazine units in the oxidized flavinophane are located at considerable distance from one another, thus creating a macrocyclic cavity as schematically shown in **1a**.

The model examinations suggest that steric interactions prevent the dihydroisoalloxazine unit in **1b** from bending inward and

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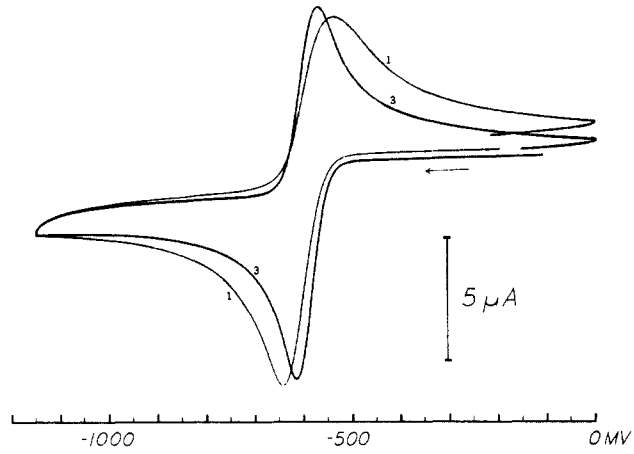

Figure 2. Cyclic voltammogram of **1** (c = 8.5 × 10^{-4} mol L^{-1}) and **3** (c = 8.76 × 10^{-4} mol L^{-1}) at a glassy carbon electrode in aqueous borate buffer, pH 10, T = 298 K, scan rate 20 mV s^{-1}.

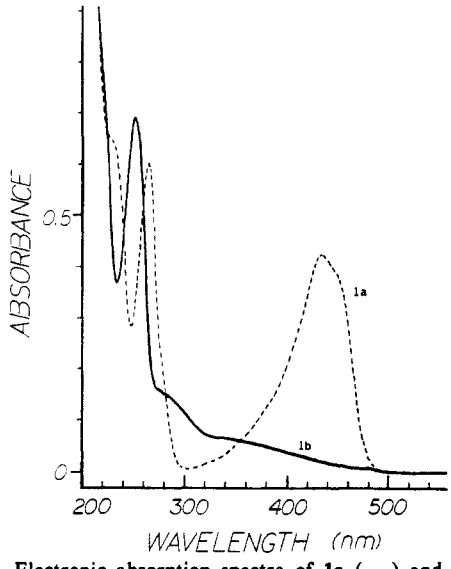

Figure 3. Electronic absorption spectra of **1a** (---) and **1b** (—) in aqueous borate buffer, pH 10, c = 1.05 × 10^{-3} mol L^{-1}, recorded in a spectroelectrochemical cell.

closing a possible binding site. Upon reduction of **1a** to **1b**, the ^{1}H NMR resonances of the methyl and aromatic protons of the diphenylmethane unit move downfield (Figure 1). This provides additional experimental support for an outward bending of the dihydroisoalloxazine unit yielding a more elongated cavity as schematically shown in **1b**.

Electrochemical Investigations of the Flavinophane 1

Since the flavinophane **1** was of interest as a potential redox mediator host,[22-24] its electrochemical properties were studied. The cyclic voltammograms (CVs) in Figure 2 show the similar electrochemical behavior of the flavinophane **1** and the nonmacrocyclic **3**.[10] In an aqueous borate buffer at pH 10, they possess practically the same formal standard potential $E^{\circ\prime}$ (−581 mV), which was determined by the relationship $(E_p^c + E_p^a)/2$.[25,26] The isoalloxazine **3** shows smaller separations in peak potential, higher current ratios, $-i_p^a/i_p^c$, and faster charge-transfer kinetics. The electron-donating alkoxy substituent in the 8-position of **1** and **3** stabilizes the oxidized form of the isoalloxazine and shifts the redox potential to more negative values as compared to flavin derivatives without such a substituent.[10] The CV investigations of **1** at the glassy carbon electrode at different scan rates (5–500 mV/s) showed quasi-reversible behavior, with increased peak potential separations and decreased peak current ratios at increased scan rates.

The absorption spectra of the oxidized flavinophane **1a** and the reduced **1b** in the aqueous borate buffer are shown in Figure 3. The spectra were measured in a spectroelectrochemical cell, and constant potential electrolysis (−730 mV) was applied to generate the reduced form. Upon reduction, the characteristic flavin band at λ = 427 nm disappeared, while weaker absorptions became visible at 344 and 280 nm. The peak in **1a** at λ = 262 nm shifted upon reduction to 253 nm. Similar electronic absorption spectra were observed for the two oxidation states in **3** as well as in other flavin derivatives.[27,28] The spectroelectrochemical experiments support that 2e$^-$ transfer steps occurred in the cyclic voltammetric studies of **1** and **3** described above.

Table I. Self-Association of **1a** and **3a**[a]

Upfield Shifts of the Isoalloxazine ^{1}H NMR Resonances in the Concentration Range from 3.0 × 10^{-4} to 4.0 × 10^{-3} mol L^{-1}

flavin derivative	isoalloxazine protons, Δδ (ppm)				
	C(8)-$OCH_{2(3)}$	N(3)-CH_2	6-H	7-H	9-H
1a	0.038	0.055	0.121	0.064	0.094
3a	0.020	0.052	0.139	0.080	0.104

Calculated Dimerization Constants, K_{dim} (L mol^{-1}), and Free Energies of Dimer Formation, ΔG° (kcal mol^{-1})

method	**1a**		**3a**	
	K_{dim} L mol^{-1}	ΔG° kcal mol^{-1}	K_{dim} L mol^{-1}	ΔG° kcal mol^{-1}
ref 35	936	−4.0	476	−3.6
ref 36	450	−3.6	270	−3.3
ref 32b	255	−3.3	134	−2.9

[a] Borate buffer, pD = 10.4, T = 295 K, 500-MHz NMR.

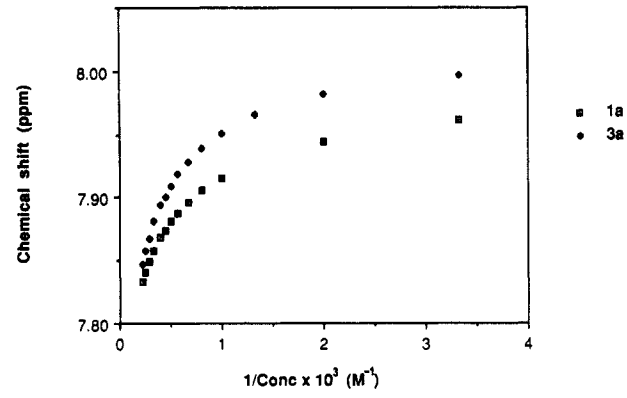

Figure 4. Change in chemical shift of the isoalloxazine proton 6-H in **1a** and **3a** due to self-association (500-MHz ^{1}H NMR, T = 295 K, deuterated borate buffer, pD = 10.4).

Self-Association Behavior of 1 and 3 in Their Oxidized States

In the analysis of the aggregation behavior of the oxidized flavinophane **1a**, we observed a specific behavior previously not encountered in our studies of water-soluble cyclophane hosts consisting of *two* diphenylmethane units. In the aqueous borate buffer above [1] ≈ 4.5 × 10^{-3} mol L^{-1} (T = 295 K), upfield shifts and strong broadening of all macrocyclic resonances indicated the formation of larger aggregates similar to the ones observed above the critical aggregation concentrations (cac) of the bis(diphenylmethane) hosts.[29] In the range of <1.0 × 10^{-4} to 4.5 ×

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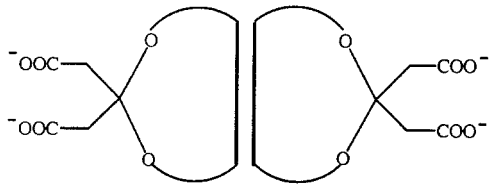


Figure 5. Schematic representation of the geometry of the flavinophane dimer.

10^{-3} mol L^{-1}, however, the concentration dependency of the ^1H NMR spectra revealed a specific self-association of the isoalloxazine moiety in the oxidized flavinophane. The resonances of the isoalloxazine protons as well as those of the methylenes units immediately adjacent to the tricyclic unit move increasingly upfield with increasing concentration of **1a** (Table I and Figure 4). The magnitude of the change in chemical shift encountered by the residual proton resonances in **1a** decreases sharply with increasing distance of these protons from the isoalloxazine unit. Thus, all protons of the diphenylmethane spacer in **1a**, located at largest distance from the isoalloxazine unit, display almost no change in position of their resonances until the cac of 4.5×10^{-3} mol L^{-1} is reached.

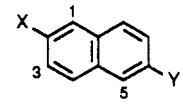
The structure of **1a**, in which one face of the isoalloxazine unit is blocked, and the observed pattern of changes in chemical shift (Figure 4) strongly suggest that, below the cac, two flavinophanes associate with their isoalloxazine units in a $\pi-\pi$ stacking array as schematically shown in Figure 5. Such a $\pi-\pi$ stacking interaction is also supported by fluorescence quenching and the large hypochromicity, not accompanied by a wavelength shift, observed at increasing concentration of **1a** for the characteristic flavin band at $\lambda_{max} = 427$ nm.

A very similar concentration dependency, indicative of stacking self-association, was seen for the proton resonances of the oxidized model compound **3a** (Table I and Figure 4). As an example, over the concentration range of 3.0×10^{-4} to 4.0×10^{-3} mol L^{-1}, the resonance of the isoalloxazine proton 6-H shifts upfield by 0.14 ppm while the signal of the methylene group α to the carboxylate, which is remote from the tricyclic system, displays an upfield shift of less than 0.01 ppm. In contrast to the flavinophane, the model **3a** can stack on both sides and form higher stacking aggregates.

The self-association of flavin derivatives[11a,30] and of similar polycyclic systems such as acridine dyes,[31] nucleosides,[32a] and others[33] has been previously studied. The application of ^1H NMR spectroscopic methods to estimate self-association constants has recently been reviewed.[34] One of the serious limitations to the estimation of self-association constants by ^1H NMR is the estimation of the value of the proton chemical shifts, δ_n, in the aggregates containing n molecules. Most commonly, the dimer ($n = 2$) is of interest. Simple extrapolation techniques are for many systems rough estimates at best, due to the possibility of aggregates higher than $n = 2$ contributing increasingly to the observed chemical shift, δ_{obs}, at the higher concentrations. In this case, the determination of δ_2 by extrapolating plots of chemical shift versus concentration to very high concentrations will lead to erroneous values.

With the flavinophane **1a**, a bridged isoalloxazine, self-association below the cac (which characterizes the formation of structurally ill defined aggregates) can only lead to the formation of a hydrophobically $\pi-\pi$ stacking dimer. We take the similarity of the plots of δ_{obs} versus concentration for **1a** and **3a** (Figure 4) as strong evidence that the dimer is also the major aggregate formed by **3a** over the considered concentration range. In the previous work on the aggregation of flavin derivatives, such information on the state of association was not obtained.[30]

Table II. Association Constants, K_a (L mol^{-1}), and Free Energies of Formation, $\Delta G°$ (kcal mol^{-1}), for Complexes of **1a**, **3a**, and **1b** in Deuterated Borate Buffer, pD = 10.4, $T = 295$ K[ab]



11: X = CN, Y = OH
12: X = OH, Y = H
13: X = OH, Y = OMe
14: X = COOH, Y = OMe

naphthalene guest		flavin receptor			
		1a[c]	**1b**[d]	**3a**[c]	**3b**[d]
11	K_a	163	228	153	–
	$\Delta G°$	−3.0	−3.2	−3.0	
12	K_a	145	200	190	–
	$\Delta G°$	−2.9	−3.1	−3.1	
13	K_a	283[e]	327	499	–
	$\Delta G°$	−3.2	−3.4	−3.6	
14	K_a	934	288	589	–
	$\Delta G°$	−4.0	−3.2	−3.7	

[a] Determined from 500 MHz ^1H NMR titrations. [b] Error in K_a ±25%. [c] Apparent association constants due to competition of self-association. [d] Solutions under argon contain $[Na_2S_2O_4] = 0.2$ mol L^{-1}. [e] Accuracy reduced due to spectral broadening.

We estimated the dimerization constants K_{dim} (M^{-1}) for both **1a** and **3a** by three methods described in literature (Table 1).[32b,35,36] The determination of the dimerization constant in each case relies on the extrapolation of meaningful δ_2-values (chemical shift of the pure dimer) and of δ_1-values (chemical shift of the pure monomer). The chemical shift for pure monomeric **1a** and **3a** could not be directly determined since dimerization starts at concentrations below the NMR concentration range ($<1.0 \times 10^{-4}$ mol L^{-1}). The above-mentioned difficulties, especially with the accurate extrapolation of δ_2, are reflected in the considerable divergence of the thermodynamic data evaluated by the graphic extrapolation methods of Menger and Whitesell,[35] of Jentschura and Lippert,[36] and by the nonlinear curve-fitting procedure described by Bangerter and Chan.[32b] Despite the differences in calculated dimerization constants and energies, it is obvious that the hydrophobic $\pi-\pi$ stacking between two oxidized isoalloxazines in aqueous buffers is driven by a considerable gain in free energy of ≥ 3 kcal mol^{-1} (Table I). As an additional strong support for the formation of dimers by *both* **1a** and **3a**, very similar aggregation numbers (2.13 and 2.11, respectively) were calculated for the two flavin derivatives by the method of Menger and Whitesell.[35]

In studies with the reduced derivatives **1b** and **3b**, no ^1H NMR indications of any significant self-association were obtained.

Host–Guest Complexation Analysis

The complexation of the flavin derivatives **1** and **3** in both oxidation states with several substituted naphthalene guests was investigated by ^1H NMR titrations executed in aqueous deuterated borate buffer at pD = 10.4 and $T = 295$ K. Solutions of the reduced flavin derivatives **1b** and **3b** were prepared under argon with $Na_2S_2O_4$ ($c = 0.2$ mol L^{-1}). All titrations were at rapid host–guest complexation–decomplexation kinetics. Either the concentration of the host or guest was constant. All titrations were executed below the cac of $\sim 4.5 \times 10^{-3}$ mol L^{-1} of the oxidized flavinophane **1a**. For reasons of low solubility of the formed complexes, titrations with reduced trianionic host **1b** were limited to concentration ranges below 4.0×10^{-3} mol L^{-1}. For all runs where binding occurred, the plots of observed changes in chemical shift versus concentration could be evaluated by a nonlinear least-squares curve-fitting procedure for the formation of stoichiometric 1:1 complexes. Indications for the formation

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of complexes with higher stoichiometries were not obtained. The maximum changes in chemical shift, $\Delta\delta_{max}$, observed for resonances of the binding partner chosen at constant concentration, varied between 40 and 75% of the changes in chemical shift calculated for saturation binding, $\Delta\delta_{sat}$. The association constants and the free energies of formation for the various complexes analyzed are shown in Table II. The K_a values obtained for complexes of the reduced flavinophane **1b** represent true association constants, not perturbed by additional self-association equilibria in the solutions. On the other hand, the K_a values calculated for complexes of the oxidized flavin derivatives **1a** and **3a** represent *apparent* association constants since host–guest complexation occurs in competition with self-association.[21c] The true association constants have larger values than the apparent constants given in Table II. Control studies showed no interactions between the diphenylmethane unit **10** and the guests in the concentration ranges below 4.5×10^{-3} mol L^{-1}.

Hydrophobic Stacking Complexation by the Oxidized Flavin Derivatives 1a and 3a

In ^1H NMR titrations with [**3a**] = 4.0×10^{-4} to 4.0×10^{-3} mol L^{-1} and [naphthalene guest] = 5.0×10^{-4} mol L^{-1}, all guest resonances move considerably upfield, generally by 0.1–0.4 ppm.[37] Similarly, if the concentrations of host and guest are reversed, all isoalloxazine resonances appear at increasingly higher field. As an example, at [**14**] = 5.0×10^{-4} mol L^{-1} and [**3a**] = 2.0×10^{-3} mol L^{-1}, the following upfield shifts are observed for the guest proton resonances: $\Delta\delta$ 0.34 ppm (1-H; calculated $\Delta\delta_{sat}$ 0.69), 0.26 (3-H), 0.28 (4-H), 0.33 (5-H; $\Delta\delta_{sat}$ 0.67), 0.22 (7-H), 0.31 (8-H; $\Delta\delta_{sat}$ 0.64). Similarly, at [**3a**] = 1.0×10^{-3} mol L^{-1} and [**14**] = 1.5×10^{-3} mol L^{-1}, the isoalloxazine resonances are shifted upfield by 0.26 ppm (9-H), 0.08 (7-H), 0.14 (6-H), >0.26 ($N(1)$-CH_2),[38] and 0.40 (CH_2CH_3). The protons of the butanoic acid side chain in proximity to the tricyclic unit also encounter upfield shifts, whereas the signal of the methylene group α to the carboxylate is virtually unaffected ($\Delta\delta < 0.01$).

The upfield shifts of the isoalloxazine resonances in the presence of the naphthalene guests resemble those seen in the self-association of **3a**. Together with the upfield shifts of the guest resonances, they are indicative of a strong hydrophobic π–π stacking complexation between the isoalloxazine and the substituted naphthalene. Such hydrophobic π–π stacking interactions of isoalloxazines with various aromatic molecules had been previously observed,[39,40] and calculations had been performed on their geometry.[41] However, quantitative experimental evaluations of the energetics involved were not provided. The *apparent* association constants of the 1:1 complexes of **3a** and the corresponding free binding energies between 3.0 and 4.0 kcal mol^{-1} demonstrate that π–π stacking interactions represent an important binding mode in flavin chemistry. In aqueous solution, the entropically and enthalpically favorable[42] desolvation of the complementary hydrophobic surfaces of the binding partners presumably represents a major driving force for complexation. Correspondingly, the strength of both self-association and host–guest interactions decreases upon addition of organic cosolvents, e.g. of methanol-d_4 or dimethyl-d_6 sulfoxide.[32,43] In addition, AM1 calculations[44,45]

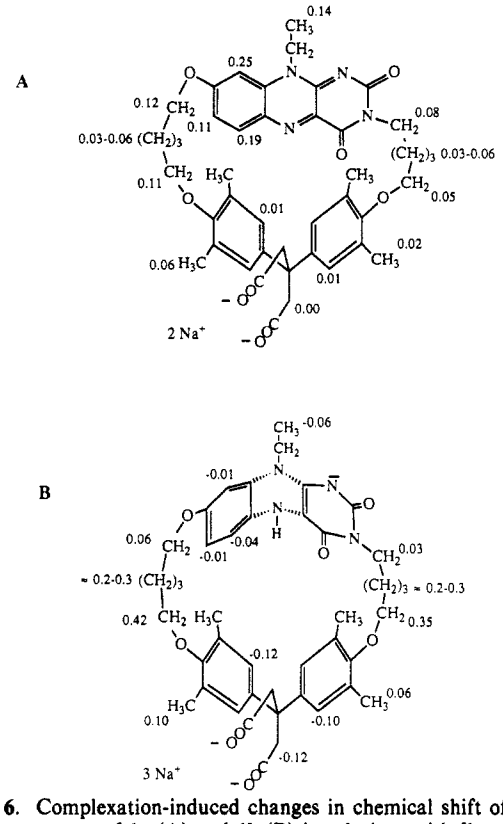

Figure 6. Complexation-induced changes in chemical shift of the ^1H NMR resonances of **1a** (A) and **1b** (B) in solutions with [host] = 5×10^{-4} mol L^{-1} and [2-naphthol] = 1.0×10^{-2} mol L^{-1} in deuterated borate buffer, pD = 10.4, T = 295 K; – = downfield shift.

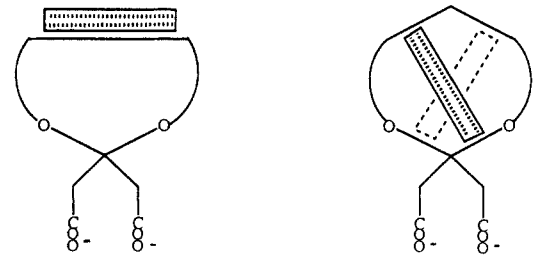

Figure 7. Different modes of complexation of naphthalene derivatives by the oxidized and the reduced flavinophane. Two favorable substrate orientations in the cavity of **1b** are shown.[48]

of the Mulliken charges for the individual atoms of **3a** and the naphthalene derivatives **11**–**13** in Table II indicate the potential for a strong dipolar contribution to the π–π stacking interaction as has been proposed for other systems.[46] The origin of the enhanced stability of the stacking complexes formed by the naphthalenecarboxylate **14** is not known.

For the oxidized flavinophane **1a**, CPK model examinations suggested that a cavity inclusion of aromatic guests would be possible sterically only if the guest is oriented cofacially to the isoalloxazine moiety. In ^1H NMR titrations, all protons of the four naphthalene guests ($c \approx 5 \times 10^{-4}$ mol L^{-1}) move upfield by ~0.2 to ~0.5 ppm upon addition of **1a** ($c \approx (3\text{–}4) \times 10^{-3}$ mol L^{-1}). Correspondingly, the isoalloxazine resonances in **1a** move upfield by ~0.1–0.25 ppm upon addition of a large excess of guest (Figure 6A). In the absence of suitable control runs with **3a**, these moderate complexation-induced shifts were at first taken as evidence for an incorporation of the naphthalene guests in the

(37) Obviously, "host" and "guest" are less well defined in the studies with **3a** and **3b** than in the studies with **1a** and **1b**. For consistency, the flavin derivatives are considered as the hosts in all binding studies.

(38) This resonance, in the spectrum of free **3a**, is masked by the HDO peak, and an accurate determination of the complexation-induced shift is not possible.

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cavity cofacially to the isoalloxazine unit.[6] However, the similarity between the *apparent* association constants calculated for the complexes of **3a** and **1a** (Table II) strongly suggests that the oxidized flavinophane, like the nonmacrocyclic derivative, prefers to bind aromatic guests externally in a hydrophobic $\pi-\pi$ stacking mode (Figure 7).[21b] Possibly, the macrocyclic cavity in **1a** is not sufficiently preorganized for binding or, more probably, it is too small for an energetically favorable guest incorporation.

The comparison of the upfield shifts of host and guest resonances observed upon complexation by **1a** and **3a** also suggests that cavity binding is not a relevant complexation mode of the oxidized flavinophane. The upfield shifts calculated at saturation binding for the isoalloxazine and guest resonances in the complexes of both flavin derivatives are of similar magnitude. Furthermore, as shown in Figure 6A, the resonances of the alkyl bridges around the cavity and the diphenylmethane unit in **1a** are only weakly or almost not affected by the interaction with the naphthalene guest. This is in contrast to what would be expected if the naphthalenes would bind in the cavity of **1a**. The observed weak shifts of some of the resonances of the bridges and the diphenylmethane unit may well be explained by conformational changes of **1a** upon external association with the guest.

The free energies of formation of host–guest stacking complexes by **1a** and **3a** are in the same range as the free energies calculated for self associating dimers of these flavin derivatives. The considerable magnitude of both association phenomena suggests an important role of $\pi-\pi$ stacking interactions in the binding of aromatic residues in proximity to the isoalloxazine unit of FAD or FMN at flavoenzyme active sites.

Cavity-Inclusion Complexation by the Reduced Flavinophane 1b

In sharp contrast to the oxidized isoalloxazine **3a**, the reduced compound **3b** does not form stable complexes with naphthalene derivatives in concentration ranges below 5×10^{-3} mol L^{-1} in the aqueous borate buffer. For all naphthalene guests ($c = 5 \times 10^{-4}$ mol L^{-1}) in the presence of a 10-fold excess of **3b**, no or only very weak ($\Delta\delta \ll 0.05$ ppm) upfield shifts of the proton resonances are observed. We explain these findings, together with the absence of significant self association by the reduced **3b**, with the presence of the negative charge at N-1. In addition, we believe that the absence of any stable association with **3b** provides evidence for the nonplanarity of the dihydroisoalloxazine unit in aqueous solution. More recently, an almost planar structure had been proposed for dihydroisoalloxazines in polar, e.g. aqueous solutions.[11a,47] For a planar reduced tricyclic structure, a considerable tendency for hydrophobic $\pi-\pi$ stacking can be expected. The absence of significant self association and stacking complexation by **3b** could indicate that isoalloxazines, in aqueous solution, take a similar bent shape as is observed by X-ray analysis for the crystalline state.[12]

A strikingly different complexation behavior is observed with the reduced flavinophane **1b**. CPK model examinations suggest that a suitably sized cavity, similar in shape to the binding sites in bis(diphenylmethane) hosts[29], forms if the dihydroisoalloxazine unit in **1b** bends outwards. All titration results, indeed, support that naphthalene derivatives are encapsulated in an axial-like orientation in the cavity of **1b** as in Figure 7.[48] In support of such a geometry, which resembles the geometry of the arene complexes of bis(diphenylmethane) hosts, the resonances of the methylene bridges of **1b** move considerably upfield upon complexation (Figure 6B). Correspondingly, all aromatic host resonances and the signals for the acetic acid residues in **1b** move downfield in the complex. Besides this characteristic upfield and downfield shift pattern of the host resonances, the specific large upfield shifts of some of the guest resonances further support the cavity-inclusion geometry schematically shown in Figure 7. As an example, the resonances of 6-hydroxy-2-naphthonitrile (**11**, $c = 5 \times 10^{-4}$ mol L^{-1}) in the presence of **1b** ($c = 4.0 \times 10^{-3}$ mol L^{-1}, degree of saturation ~55%) encounter the following upfield shifts: $\Delta\delta$ 1.31 (1-H), 0.60 (3-H), 0.49 (4-H), 0.14 (5-H), 0.02 (7-H), 0.43 (8-H). The complexation-induced shifts show that the ring with the electron-withdrawing cyano group is preferentially incorporated in the cavity. An incorporation of the hydroxy-substituted ring of **11**, deprotonated at pD 10.4, is less favorable since larger desolvation energies are required and repulsive charge–charge interactions with the negative charge at N-1 of **1b** could occur. The large, selective complexation-induced shifts expecially of the guest resonances 1-H ($\Delta\delta_{sat}$ = 2.74 ppm), 3-H, and 4-H are incompatible with a $\pi-\pi$-stacking complex geometry and strongly support the cavity inclusion geometry shown in Figure 7.

Conclusions

The host–guest binding interactions and the self-association of the novel flavinophane **1** and the nonmacrocyclic isoalloxazine **3** have been investigated in an aqueous borate buffer at pH 10.0 in concentration ranges below 5×10^{-3} mol L^{-1}. Whereas the oxidized forms **1a** and **3a** show very similar binding and association properties, striking differences in binding ability are observed between the reduced cyclophane **1b** and the nonmacrocyclic model **3b**. The two oxidized derivatives **1a** and **3a** undergo a very similar strong self-association, and the experimental data are best explained by the formation of dimers, stabilized by hydrophobic $\pi-\pi$-stacking interactions. The free energies of formation of the dimers are between 3 and 4 kcal mol^{-1}. A significant self-association is not observed with the reduced forms **1b** and **3b**. The two oxidized flavin derivatives **1a** and **3a** also show very similar capabilities in host–guest binding. They both form hydrophobic $\pi-\pi$ stacking complexes with naphthalene derivatives stabilized by free energies of 3–4 kcal mol^{-1}. The experimental data indicate that the flavinophane **1a** does not bind substrates to a significant extent inside the cavity, and that the $\pi-\pi$ stacking between guest and isoalloxazine moiety occurs externally. On the basis of the findings in our association and complexation studies with the oxidized **1a** and **3a**, a considerable role of $\pi-\pi$ stacking interactions must be assumed in the binding of aromatic substrates in proximity to the isoalloxazine unit of FAD or FMN at flavoenzyme active sites.

Whereas no complexation ability is observed for the dihydroisoalloxazine **3b**, the reduced flavinophane **1b** forms cavity inclusion complexes with naphthalene derivatives. In these complexes, of similar stability as the external stacking complexes formed by the oxidized host, the guests take an axial-like orientation and undergo $\pi-\pi$ stacking and edge-to-face dipolar interactions with the aromatic rings of the two spacers.

The initial target of developing a redox-switchable host has been partially achieved. The flavinophane **1** clearly can be switched from cavity binding to noncavity binding in a redox process. However, the hydrophobic $\pi-\pi$ stacking association, which occurs externally with **1a**, is of the same strength as the cavity binding with **1b** and was underestimated in the initial design. The present study shows that flavinophanes resembling **1** but with an externally bridged and, hence, shielded isoalloxazine should exhibit the desired redox-dependent complexation behavior. The bridging of the isoalloxazine will prevent external $\pi-\pi$ stacking, and binding will only occur in the larger cavity of the reduced flavin-host.

Experimental Section

General. ^{1}H NMR spectra were performed on Bruker AM 500 or AF 200 spectrometers. All association studies were carried out at 500 MHz

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(48) The schematic inclusion geometry shown in Figure 7B with two possible, rapidly equilibrating orientations of the guest is supported for bis(diphenylmethane) hosts by both X-ray crystallography and computer modelling studies.[29] In each of the two favorable inclusion geometries shown, the naphthalene guest undergoes $\pi-\pi$ stacking interactions with one aromatic ring of the diphenylmethane unit and the benzene or pyrimidinedione ring of the dihydroisoalloxazine. In addition, the complexes are stabilized by edge-to-face dipolar interactions between the aromatic hydrogens of the guest and the two other π-systems of the spacers. The ^{1}H NMR spectra measured at fast exchange reflect a time-averaged complex geometry with the guest located in the plane passing through the central carbon atom of the diphenylmethane unit and the two central nitrogens N-5 and N-10 of the dihydroisoalloxazine unit.

at 295 K. The δ values for the analytical spectra are in ppm relative to Me_4Si. Mass spectra were recorded on a AEI MS902 high-resolution mass spectrometer. EI mass spectra were carried out at 70 eV, and FAB mass spectra were carried out in a *m*-nitrobenzyl alcohol matrix. IR spectra were recorded on a Perkin-Elmer PE 580 instrument. Electronic absorption spectra were recorded with a Varian Cary 2300 instrument. Emission spectra were measured on a Spex 212 fluorolog. Melting points were obtained on an Electrothermal heated-stage apparatus and are uncorrected. Elemental analyses were performed by Spang Microanalytical Laboratory, Eagle Harbor, MI, and Galbraith Laboratories Inc., Knoxville, TN. Analytical thin-layer chromatography was conducted with E. Merck silica gel 60 F-254 precoated plates. Column chromatography was performed on E. Merck silica gel (Kieselgel 60, 70–230 mesh). Reagents were purchased from Aldrich and used without further purification unless specified otherwise. *N,N*-Dimethylformamide (DMF) was purified by drying over calcium hydride followed by fractional distillation and stored over E. Merck alumina, activity grade I.

Synthesis. 10-Ethyl-8-methoxybenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione (5). A mixture of 9.3 g (60 mmol) of 6-(*N*-ethylamino)uracil[18a] and 23.0 g (170 mmol) of *p*-nitrosoanisole[18b] in 20 mL of acetic anhydride/glacial acetic acid (3:1) was heated under reflux for 30 min. The dark green color of the nitrosoanisole changed to dark orange, and a precipitate formed. After the mixture was allowed to cool to room temperature, the orange microcrystalline solid was collected by filtration, washed with methanol followed by CH_2Cl_2, and dried in vacuo to yield 9.8 g (60%) of **5**: mp >320° C; IR (KBr) ν (N—H) 3500, (C=O) 1710, 1665 cm^{-1}; ^1H NMR (500 MHz, DMF-d_7) δ 1.42 (t, J = 7.1 Hz, 3 H, NCH_2CH_3), 4.14 (s, 3 H, OCH_3), 4.79 (q, J = 7.1 Hz, 2 H, NCH_2CH_3), 7.08 (d, J = 2.5 Hz, 1 H, 9-H), 7.33 (dd, J = 9.1 and 2.5 Hz, 1 H, 7-H), 8.09 (d, J = 9.1 Hz, 1 H, 6-H); MS (EI), m/z (relative intensity) 272 (M^+, 32) 244 ($M^+ - C_2H_4$, 100). Anal. Calcd for $C_{13}H_{12}N_4O_3$ (272.3): C, 57.35; H, 4.41; N, 20.59. Found: C, 57.64; H, 4.17; N, 20.40.

10-Ethyl-8-hydroxybenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione (6). To a suspension of 10.4 g (38 mmol) of **5** in 250 mL of dry 1,2-dichloroethane under N_2 was added a total of 84.9 g (32.4 mL, 0.34 mol) of BBr_3 via syringe. The dark orange mixture was heated under reflux for 12 h. The reaction mixture was then cooled in an ice–water bath and methanol added dropwise until foaming ceased. The solvent and volatile materials were removed in vacuo to yield 9.8 g (99%) of crude **6**. This material was used in the following reaction without further purification: mp >300 °C; IR (KBr) ν (C=O) 1700, 1650 cm^{-1}; ^1H NMR (500 MHz, Me_2SO-d_6) δ 1.31 (t, J = 7.2 Hz, 3 H, NCH_2CH_3), 4.55 (q, J = 7.2 Hz, 2 H, NCH_2CH_3), 7.05 (d, J = 2.2 Hz, 1 H, 9-H), 7.12 (dd, J = 8.9 and 2.2 Hz, 1 H, 7-H), 7.95 (d, J = 8.9 Hz, 1 H, 6-H); MS (EI), m/z (relative intensity) 258 (M^+, 7), 230 ($M^+ - C_2H_4$, 100); HRMS m/z (M^+, $C_{12}H_{10}N_4O_3$) calcd 258.0754, obsd 258.0759.

3-(5-Chloropentyl)-8-[(5-chloropentyl)oxy]-10-ethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione (7). A mixture of 3.6 g (14 mmol) of crude phenol **6**, 27.2 g (84 mmol) of Cs_2CO_3, and 51.0 g (360 mmol) of 1,5-dichloropentane in dry DMF was heated to 90 °C for 12 h under N_2. The warm reaction mixture was filtered through a Celite pad, and the residue was washed with DMF. The solvent and excess of 1,5-dichloropentane were removed by distillation at reduced pressure. The dark oily residue was taken up in the minimum amount of CH_2Cl_2 and deposited on top of a silica gel column. The residual 1,5-dichloropentane was eluted with CH_2Cl_2. The product was eluted with a gradient of ethylacetate (1–10%) in CH_2Cl_2 to give the pure solid dichloride **7**: 4.5 g (69%); mp 142–143 °C (toluene/hexane); IR (KBr) ν(C=O) 1700, 1660 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 1.51 (t, J = 7.1 Hz, 3 H, NCH_2CH_3), 1.5–2.0 (m, 12 H, CH_2), 3.55 (t, J = 6.7 Hz, 2 H, CH_2Cl), 3.61 (t, J = 6.7 Hz, 2 H, CH_2Cl), 4.11 (t, J = 7.4 Hz, 2 H, NCH_2CH_2), 4.21 (t, J = 6.3 Hz, 2 H, OCH_2), 4.74 (q, J = 7.1 Hz, 2 H, NCH_2CH_3), 6.95 (d, J = 2.5 Hz, 1 H, 9-H), 7.21 (dd, J = 9.1 and 2.5 Hz, 1 H, 7-H), 8.20 (d, J = 9.1 Hz, 1 H, 6-H); MS (EI), m/z (relative intensity) 468 ($M^+ + 2$, 68), 466 (M^+, 100). Anal. Calcd for $C_{22}H_{28}N_4O_3Cl_2$ (467.4): C, 56.54; H, 5.99; N, 11.99. Found: C, 56.62; H, 6.03; N, 11.74.

10-Ethyl-3-[3-[(ethyloxy)carbonyl]propyl]-8-methoxybenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione (8). A mixture of 0.50 g (1.8 mmol) of **5**, 0.39 g (2.0 mmol) of ethyl 4-bromobutanoate, and 0.51 g (3.7 mmol) of K_2CO_3 in 15 mL of dry DMF was heated to 60 °C under N_2 for 12 h. The dark mixture was allowed to cool to room temperature and filtered. The solid was washed with a small amount of DMF. The solvent of the filtrate was removed by distillation under reduced pressure to yield 0.52 g (74%) of a yellow solid which was recrystallized from ethanol: mp 199–201 °C; IR (KBr) ν (C=O) 1720, 1705, 1690 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3/CD_3OD$, 95:5) δ 1.25 (t, J = 7.1 Hz, 3 H, OCH_2CH_3), 1.50 (t, J = 7.2 Hz, 3 H, NCH_2CH_3), 2.09 (qn, J = 7.1 Hz, 2 H, $CH_2CH_2CH_2$), 2.43 (t, J = 7.6 Hz, 2 H, CH_2COOH), 4.05 (s, 3 H, OCH_3), 4.15–4.2 (m, 4 H, OCH_2CH_3, NCH_2CH_2), 4.75 (q, J = 7.2 Hz, 2 H, NCH_2CH_3), 6.95 (d, J = 2.4 Hz, 1 H, 9-H), 7.23 (dd, J = 9.2 and 2.4 Hz, 1 H, 7-H), 8.24 (d, J = 9.2 Hz, 1 H, 6-H); MS (EI), m/z (relative intensity) 386 (M^+, 100). Anal. Calcd for $C_{19}H_{22}N_4O_5$ (386.4): C, 59.06; H, 5.74; N, 14.50. Found: C, 59.33; H, 5.94; N, 14.50.

3-(3-Carboxypropyl)-10-ethyl-8-methoxybenzo[*g*]pteridine-2,4-(3*H*,10*H*)-dione (3). A total of 0.42 g (1.1 mmol) of **8** in 10 mL of 88% formic acid and 0.25 mL of methanesulfonic acid was heated to reflux for 4 h under N_2. Most of the acid was removed by distillation under reduced pressure. Upon dilution of the resultant yellow oil with water, a bright yellow solid formed. The solid was collected by filtration, washed with cold water, and dried in vacuo over P_2O_5 to yield 0.35 g (89%) of **3**: mp 263–264 °C (water); IR (KBr) ν (C=O) 1705 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3/CD_3OD$, 95:5) δ 1.51 (t, J = 7.2 Hz, 3 H, NCH_2CH_3), 2.10 (m, 2 H, $CH_2CH_2CH_2$), 2.44 (t, J = 7.5 Hz, 2 H, CH_2COOH), 4.06 (s, 3 H, OCH_3), 4.18 (t, J = 7.0, 2 H, NCH_2CH_2), 4.75 (q, J = 7.2 Hz, 2 H, NCH_2CH_3), 6.98 (d, J = 2.2 Hz, 1 H, H-9), 7.22 (dd, J = 9.2 and 2.2 Hz, 1 H, H-7), 8.23 (d, J = 9.2 Hz, 1 H, H-6); MS (EI), m/z (relative intensity) 358 (M^+, 100). Anal. Calcd for $C_{17}H_{18}N_4O_5 \cdot H_2O$ (376.4): C, 54.24; H, 5.32; N, 14.89. Found: C, 54.41; H, 5.50; N, 14.88.

3,3-Bis(4-hydroxy-3,5-dimethylphenyl)pentanedioic Acid Diethyl Ester (9). A mixture of 49.0 g (0.40 mol) of 2,6-dimethylphenol and 36.0 g (0.18 mol) of diethyl 1,3-acetonedicarboxylate was chilled to 0 °C in an ice bath with mechanical stirring. A total of 72 mL (1.4 mol) of concentrated H_2SO_4 was added such that the temperature remained below 5 °C. After the addition was complete, the mixture was allowed to warm to room temperature. A light pink solid formed upon standing for 24 h. This solid was collected by filtration, washed thoroughly with water, and dried in vacuo over P_2O_5. Recrystallization from nitromethane gave 68.8 g (75%) of **9** as diamond-shaped crystals: mp 177–178 °C; IR (KBr) ν (C=O) 1750 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.98 (t, J = 7.2 Hz, 6 H, CH_2CH_3), 2.14 (s, 12 H, $ArCH_3$), 3.42 (s, 4 H, CH_2COOEt), 3.87 (q, J = 7.2 Hz, 4 H, CH_2CH_3), 4.64 (br s, 2 H, OH), 6.71 (s, 4 H, ArH); MS (EI), m/z (relative intensity) 428 (M^+, 54), 341 (M^+ – CH_2COOEt, 100). Anal. Calcd for $C_{25}H_{32}O_6$ (428.5): C, 70.09; H, 7.47. Found: C, 69.95; H, 7.64.

3,3-Bis(4-methoxy-3,5-dimethylphenyl)pentanedioic Acid (10). A total of 4.28 g (10 mmol) of **9** was treated with 113 mmol (7.0 mL, 16.0 g) of iodomethane and 5.52 g (40 mmol) of K_2CO_3 in 100 mL of dry acetone. The mixture was heated to reflux under N_2 for 12 h. The solvent was removed in vacuo to give a solid residue. This residue was taken up in 50 mL of 5% KOH in methoxyethanol and heated under reflux for 3 h. Upon acidification with concentrated HCl, while cooling in an ice bath, a white precipitate formed. The solid was collected by vacuum filtration, washed thoroughly with water, and dried in vacuo to give 1.5 g (37%) of **10**: mp 202–204 °C; IR (KBr) ν (C=O) 1720 cm^{-1}; ^1H NMR (200 MHz, $CDCl_3$) δ 2.18 (s, 12 H, $ArCH_3$), 3.50 (s, 4 H, CH_2COOH), 3.67 (s, 6 H, OCH_3), 6.71 (s, 4 H, ArH); MS (EI) m/z (relative intensity) 400 (M^+, 0.6), 382 ($M^+ - H_2O$, 27), 296 (100); HRMS m/z ($M^+ - H_2O$, $C_{23}H_{28}O_6$) calcd 382.1781, obsd 382.1763.

10-Ethyl-4,10-dihydro-19,23,27,29-tetramethyl-2,4-dioxo-2*H*-8,3-(epoxypentanoxy[1,4]benzenemethano[1,4]benzeneoxypentano)benzo[*g*]pteridine-24,24-diacetic Acid Diethyl Ester (2). A mixture of 6.00 g (12.8 mmol) of **7**, 5.50 g (12.8 mmol) of **9**, and 16.6 g (51.0 mmol) of Cs_2CO_3 in 2.0 L of dry DMF was heated to 70 °C for 3 days under N_2. The reaction mixture was filtered through a Celite pad, and the pad was washed with additional DMF. The DMF was removed by distillation at reduced pressure to give a brown foamy solid. This crude material was dissolved in the minimum amount of CH_2Cl_2 and deposited on a silica gel column (4 × 55 cm). The product was eluted with a solvent gradient of ethyl acetate (0–20%) in CH_2Cl_2 to yield 1.3 g (12%) of the macrocyclic diester: mp 252–253 °C; IR (KBr) ν (C=O, ester) 1720, (C=O, amide) 1750, 1660 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 0.92 (t, J = 7.2 Hz, 6 H, OCH_2CH_3), 1.41 (t, J = 7.1 Hz, 3 H, NCH_2CH_3), 1.45–1.5 (m, 2 H, CH_2), 1.6–1.65 (m, 2 H, CH_2), 1.7–1.75 (m, 2 H, CH_2), 1.75–1.95 (m, 6 H, CH_2), 1.98 (s, 6 H, CH_3), 1.99 (s, 6 H, CH_3), 3.41 (s, 4 H, CH_2COOEt), 3.57 and 3.59 (2 t, J = 6.7 Hz, 4 H, OCH_2CH_2), 3.84 (q, J = 7.2 Hz, 4 H, OCH_2CH_3), 4.26 (t, J = 7.2 Hz, 2 H, NCH_2CH_2), 4.41 (t, J = 5.9 Hz, 2 H, OCH_2CH_2), 4.65 (q br, J = 7.1 Hz, 2 H, NCH_2CH_3), 6.60 (s, 2 H, ArH), 6.62 (s, 2 H, ArH), 6.91 (d, J = 2.3 Hz, 1 H, 9-H), 7.18 (dd, J = 2.3 and 9.2 Hz, 1 H, 7-H), 8.16 (d, J = 9.2 Hz, 1 H, 6-H); MS (EI, 16 eV) m/z (relative intensity) 822 (M^+, 100). Anal. Calcd for $C_{47}H_{58}O_9N_4$ (823.0): C, 68.61; H, 7.05; N, 6.81. Found: C, 68.57; H, 7.16; N, 6.66.

10-Ethyl-4,10-dihydro-19,23,27,29-tetramethyl-2,4-dioxo-2*H*-8,3-(epoxypentanoxy[1,4]benzenemethano[1,4]benzeneoxypentano)benzo[*g*]pteridine-24,24-diacetic Acid (1). A solution of 0.70 g (0.85 mmol) of **2** in 15 mL of 88% formic acid and 0.5 mL of methanesulfonic acid was heated under reflux for 12 h under N_2. The cooled yellow solution was diluted with 15 mL of water, and a yellow precipitate formed. This material was collected by filtration, washed thoroughly with water, air-

dried, and finally washed with chloroform. After drying in vacuo, 0.59 g (90%) of **1** was obtained as a bright yellow powder: mp 293–294 °C (methanol); IR (KBr) ν (C=O) 1720, 1705, 1645 cm^{-1}; ^{1}H NMR (500 MHz, Me_2SO-d_6) δ 1.17 (t, J = 7.2 Hz, 3 H, CH_3), 1.3–1.35 (m, 2 H, CH_2), 1.5–1.55 (m, 2 H, CH_2), 1.6–1.65 (m, 2 H, CH_2), 1.65–1.7 (m, 2 H, CH_2), 1.75–1.8 (m, 4 H, CH_2), 1.90 (s, 6 H, CH_3), 1.98 (s, 6 H, CH_3), 3.36 (s, 4 H, CH_2CO), 3.51 (t, J = 6.7 Hz, 4 H, OCH_2), 4.02 (t, J = 6.4 Hz, 2 H, NCH_2CH_2), 4.49 (t, J = 6.5 Hz, 2 H, OCH_2), 4.60 (br q, J = 7.2 Hz, 2 H, NCH_2CH_3), 6.65 (s, 2 H, ArH), 6.71 (s, 2 H, ArH), 7.25 (d, J = 2.4 Hz, 1 H, 9-H), 7.31 (dd, J = 9.1 and 2.4 Hz, 1 H, 7-H), 8.02 (d, J = 9.1 Hz, 1 H, 6-H); MS (FAB) m/z (relative intensity) 767 (M^+ + 1, 100). Anal. Calcd for $C_{43}H_{50}O_9N_4$ (766.9): C, 67.36; H, 6.53; N, 7.31. Found: C, 67.14; H, 6.53; N, 7.16.

^{1}H NMR Self-Association and Complexation Studies. Sample Preparation. The ^{1}H NMR binding titrations were carried out by preparing a series of seven to nine samples containing varying concentrations of **1** or **3** (c = 5 × 10^{-4} – 4.5 × 10^{-3} mol L^{-1}) using a stock solution of the guest (c = 5.0 × 10^{-4} mol L^{-1}) in deuterated borate buffer. In the case of 2-naphthol as guest, titrations with either constant amounts of guest or host were run. For the latter, a stock solution of the host (c = 5.0 × 10^{-4} mol L^{-1}) was used to prepare solutions of varying concentration of 2-naphthol (c = 1 × 10^{-3}–1 × 10^{-2} mol L^{-1}). The stock solutions were prepared with deuterated borate buffer (c = 0.2 mol L^{-1}) in D_2O (99.9 atom % D) adjusted to pD = 10.4 (pH = 10) with 40% NaOD in D_2O. The D_3BO_3 was prepared by three recrystallizations of H_3BO_3 from D_2O (99.9 atom % D). The pH was measured with an Orion Research ionalyzer 901, the pH-electrode Orion Research 81-01-00, and the silver–silver chloride reference electrode Fisher Scientific 13-620-53. The HDO peak was taken as internal standard.

The 12 samples for the ^{1}H NMR self-association studies were prepared from a stock solution of **1** or **3** (c = 4.5 × 10^{-3} mol L^{-1}) in the deuterated borate buffer. Aliquots of the stock solutions were diluted with additional buffer solution with use of calibrated micropipets to give solutions ranging in concentration from 3 × 10^{-4} to 4.5 × 10^{-3} mol L^{-1}.

The solutions of the reduced **1b** and **3b** were prepared by the addition of solid sodium dithionite to the deoxygenated samples in the NMR tubes under argon to give solutions with [$Na_2S_2O_4$] = 0.2 mol L^{-1}. Identical titration results were obtained when each sample was prepared with an amount of sodium dithionite equal to twice the concentration of the flavin derivative. The samples were deoxygenated by bubbling argon through the solutions in the NMR tubes with a long hypodermic needle for about 10 min. The completeness of reduction was checked by monitoring the disappearance of the fluorescence of the oxidized flavin derivative with the long-wavelength (366 nm) excitation of a UV handlamp.

Evaluation of Self-Assocation Constants. Self-association constants for **1a** and **3a** were calculated by three different methods using the concentration-dependent chemical shifts of the isoalloxazine proton 6-H observed over the concentration range of 3.0 × 10^{-4}–3.5 × 10^{-3} mol L^{-1}. The methods of (i) Jentschura and Lippert[36] and (ii) Menger and Whitesell[35] are linear graphical methods, while the method of (iii) Bangerter and Chan[32b] is based on a nonlinear least-squares calculation. The equation for each method is given below. In all cases, the chemical shift of the monomer, δ_1, was obtained by linear extrapolation from the low concentration end of a plot of δ_{obs} versus concentration. For **1a**, δ_1 was estimated to be 8.002 ppm, while the δ_{obs} at 3.0 × 10^{-4} mol L^{-1} was 7.961 ppm. The estimated δ_1 for **3a** was 8.032 ppm, and δ_{obs} at 3.0 × 10^{-4} mol L^{-1} was 7.997 ppm. For methods i and ii, the chemical shift of the pure dimer, δ_2, was estimated by linear extrapolation from the high concentration end of a plot of δ_{obs} versus the reciprocal square root of the total isoalloxazine concentration. This technique gave a δ_2 for **1a** of 7.739 ppm (δ_{obs} = 7.868 at 3.5 × 10^{-3} mol L^{-1}) and a δ_2 of 7.699 ppm for **3a** (δ_{obs} = 7.849 at 3.5 × 10^{-3} mol L^{-1}). In the case of method iii, the least-squares fit yielded a δ_2 of 7.684 for **1a** and 7.592 for **3a**. The equations for the three methods are given below:

$$2C_{tot}K_{dim} = \frac{1 - (\delta_{obs} - \delta_2)/(\delta_1 - \delta_2)}{(\delta_{obs} - \delta_2)^2/(\delta_1 - \delta_2)^2} \quad \text{(i)}$$

where C_{tot} is the total concentration, K_{dim} is the self-association constant, and δ_{obs} is the observed chemical shift.

$$\ln (C_{tot}\delta_{rel}) = n \ln [C_{tot}(\delta_{agg} - \delta_{rel})] + \ln K_{dim} + \ln (n) - (n - 1) \ln \delta_{agg} \quad \text{(ii)}$$

where $\delta_{rel} = \delta_{obs} - \delta_1$, $\delta_{agg} = \delta_{obs} - \delta_2$. A plot of ln ($C_{tot}\delta_{rel}$) versus ln [$C_{tot}(\delta_{agg} - \delta_{rel})$] gives n, the aggregation number, as the slope, and K_{dim} can then be calculated. For **1a** and **3a**, the aggregation numbers were calculated as 2.13 and 2.11, respectively.

$$\delta_{obs} - \delta_1 = (\delta_2 - \delta_1)\frac{(4K_{dim}C_{tot} + 1) - (1 + 8K_{dim}C_{tot})^{1/2}}{4K_{dim}C_{tot}} \quad \text{(iii)}$$

Here, the plot of the observed chemical shift change, δ_{obs}, as a function of concentration, C_{tot}, is fitted by using a nonlinear least-squares procedure.

Examples of ^{1}H NMR Binding Titrations. Complex **1b·11**: [host] = 4.0 × 10^{-4}–4.0 × 10^{-3} mol L^{-1}, [guest] = 5.0 × 10^{-4} mol L^{-1}; 1-H $\Delta\delta_{max\ obsd}$ 1.31, $\Delta\delta_{sat.\ calcd}$ 2.74, K_a = 247 L mol^{-1}; 4-H $\Delta\delta_{max\ obsd}$ 0.49, $\Delta\delta_{sat.\ calcd}$ 1.11, K_a = 209 L mol^{-1}.

Complex **1a·12**: [guest] = 8.0 × 10^{-4}–1.0 × 10^{-2} mol L^{-1}, [host] = 4.0 × 10^{-4} mol L^{-1}; 6-$H_{isoallox}$ $\Delta\delta_{max\ obsd}$ 0.19, $\Delta\delta_{sat.\ calcd}$ 0.30, K_a = 158 L mol^{-1}; $ArOCH_2$ $\Delta\delta_{max\ obsd}$ 0.12, $\Delta\delta_{sat.\ calcd}$ 0.20, K_a = 131 L mol^{-1}.

Complex **1b·13**: [host] = 2.0 × 10^{-4}–2.5 × 10^{-3} mol L^{-1}, [guest] = 5.0 × 10^{-4} mol L^{-1}; 5-H $\Delta\delta_{max\ obsd}$ 0.47, $\Delta\delta_{sat.\ calcd}$ 0.84, K_a = 365 L mol^{-1}; 8-H $\Delta\delta_{max\ obsd}$ 0.38, $\Delta\delta_{sat.\ calcd}$ 0.92, K_a = 290 L mol^{-1}.

Complex **1a·14**: [host] = 2.0 × 10^{-4}–3.5 × 10^{-3} mol L^{-1}, [guest] = 5.0 × 10^{-4} mol L^{-1}; 1-H $\Delta\delta_{max\ obsd}$ 0.25, $\Delta\delta_{sat.\ calcd}$ 0.39, K_a = 764 L mol^{-1}; 5-H $\Delta\delta_{max\ obsd}$ 0.39, $\Delta\delta_{sat.\ calcd}$ 0.57, K_a = 913 L mol^{-1}; 8-H $\Delta\delta_{max\ obsd}$ 0.267, $\Delta\delta_{sat.\ calcd}$ 0.40, K_a = 1124 L mol^{-1}.

Complex **3a·11**: [flavin] = 4.0 × 10^{-4}–5.0 × 10^{-3} mol L^{-1}, [naphthalene] = 5.0 × 10^{-4} mol L^{-1}; 1-H $\Delta\delta_{max\ obsd}$ 0.34, $\Delta\delta_{sat.\ calcd}$ 0.76, K_a = 155 L mol^{-1}; 4-H $\Delta\delta_{max\ obsd}$ 0.27, $\Delta\delta_{sat.\ calcd}$ 0.62, K_a = 157 L mol^{-1}; 5-H $\Delta\delta_{max\ obsd}$ 0.22, $\Delta\delta_{sat.\ calcd}$ 0.52, K_a = 148 L mol^{-1}.

Complex **3a·13**: [flavin] = 3.0 × 10^{-4}–3.0 × 10^{-3} mol L^{-1}, [naphthalene] = 5.0 × 10^{-4} mol L^{-1}; 1-H $\Delta\delta_{max\ obsd}$ 0.38, $\Delta\delta_{sat.\ calcd}$ 0.69, K_a = 457 L mol^{-1}; 4-H $\Delta\delta_{max\ obsd}$ 0.45, $\Delta\delta_{sat.\ calcd}$ 0.74, K_a = 549 L mol^{-1}; 8-H $\Delta\delta_{max\ obsd}$ 0.37, $\Delta\delta_{sat.\ calcd}$ 0.65, K_a = 491 L mol^{-1}.

Electrochemical Studies. Materials. Boric acid and NaOH were ACS grade and used without further purification. The borate buffer stock solution (c = 0.2 mol L^{-1}) was prepared as described above. All solutions were prepared with millipore water. Protective argon was purified by first passing through BTS catalyst (Fluka/BASF R3-11), reduced with hydrogen gas at 100 °C, then soda lime, and finally silica gel.

Cyclic Voltammetric Experiments. Cyclic voltammetric experiments were performed under argon in a conventional three-electrode cell with a BAS glassy carbon working electrode (MF-2012), a platinum wire counter electrode, and a BAS silver–silver chloride reference electrode (MF-2020) assembled in a Haber–Luggin capillary. The cell consisted of an outer water jacket which was connected to a thermostat. All experiments were performed at 25 °C. The working electrode was polished with 0.05-μm alumina (BAS CF-1050), washed with millipore water, and dried before each cyclic voltammetric experiment. The 10-mL solutions of **1** and **3** were prepared with the borate buffer stock solution, introduced into the cell with syringes, and purged with argon for 10 min. The BAS CV-27 voltammograph and the XY recorder MF-8050 were used for all CV studies.

Spectroelectrochemical Experiments. These experiments were carried out in a spectroelectrochemical cell with an optically transparent electrode, consisting of a platinum gauze electrode mounted on a Suprasil quartz window. A platinum wire was used as the auxiliary electrode, and a BAS silver–silver chloride electrode (MF-2020) was the reference electrode. Controlled-potential electrolysis was performed using the BAS CV-27 voltammograph while the UV/VIS spectra were recorded with a Varian Cary 2300 spectrophotometer. Solutions of **1** (7.05 × 10^{-4} mol L^{-1}) and **3** (1.05 × 10^{-3} mol L^{-1}) were prepared, purged with argon, and then transferred with gas syringes to the spectroelectrochemical cell under argon. Constant potentials of −730 (**1**) and −750 mV (**3**) were applied. The UV/vis spectra of the reduced compound were recorded after the absorbance of the peaks at λ ~430 nm had dropped to a very low, constant value, and the solutions had become colorless.

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Registry No. **1**, 116159-03-6; **1a·11**, 124991-85-1; **1a·12**, 124942-92-3; **1a·13**, 124991-84-0; **1a·14**, 124942-94-5; **1b**, 116191-48-1; **1b·11**, 124942-91-2; **1b·12**, 124942-97-8; **1b·13**, 124942-93-4; **1b·14**, 124942-98-9; **2**, 116191-47-0; **3**, 124942-87-6; **3a·11**, 124942-95-6; **3a·12**, 124942-99-0; **3a·13**, 124942-96-7; **3a·14**, 124943-00-6; **3b**, 124942-89-8; **5**, 116158-99-7; **6**, 116159-00-3; **7**, 116159-01-4; **8**, 124942-86-5; **9**, 116159-02-5; **10**, 124942-88-7; *p*-ONC_6H_4OMe, 1516-21-8; $ClCH_2(CH_2)_3CH_2Cl$, 628-76-2; $EtOCOCH_2CH_2CH_2Br$, 2969-81-5; 6-(*N*-ethylamino)uracil, 5770-53-6; 2,6-dimethylphenol, 576-26-1; diethyl 1,3-acetonedicarboxylate, 105-50-0.

Supplementary Material Available: Two tables including full cyclic voltammetry data for solutions of **1** and **3** (2 pages). Ordering information is given on any current masthead page.