The Mechanism of Adduct Formation between Reduced Flavins and Arene Epoxides[1]

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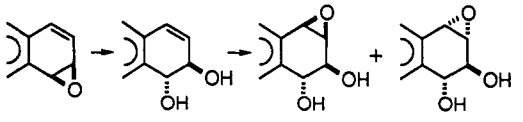
The mechanism of nucleophilic epoxide opening by reduced flavins, a potentially relevant transformation to the carcinogenesis of polycyclic aromatic hydrocarbons, was investigated. In the spontaneous epoxide hydrolysis pH region, the reaction pathway between the epoxide and the dihydroflavin is bimolecular epoxide opening. Chromatographic analysis of the reaction of (±)-1a,2,3,7b-tetrahydro-(1aα,2α,3β,7bα)-naphth[1,2-*b*]oxirene-2,3-diol (**1**) with 5,10-dihydro-7,8,10-trimethylbenzo[*g*]pteridine-2,4(1*H*,3*H*)-dione (LFH_2, dihydrolumiflavin) in 9/1 (v/v) aqueous pH 7.86 Tris buffer–dioxane yielded the flavin N(5) adduct **8** as the only major product. Under the same conditions, (±)-1a,2,3,7b-tetrahydro-(1aα,2β,3α,7bα)-naphth[1,2-*b*]oxirene-2,3-diol (**2**) gave the N(5) adduct **10**, N(3) adducts (the stereoisomers **11a** and **12a**), and an unknown adduct (possibly the C(4a) adduct), each in similar yields. The tetrahydronaphthalene oxide, (±)-1a,2,3,7b-tetrahydro-(1aα,7bα)-naphth[1,2-*b*]oxirene (**3**), gave the N(5) adduct **14** and the C(4a) adduct **16**, in approximately a 2/1 ratio. The C(4a) adduct was not stable, however, and was transformed to a secondary adduct. A comparison of the absorption spectra indicated that the outcome of **1** and **3** with $FMNH_2$ was similar to that for LFH_2. The reaction of **2** with $FMNH_2$ gave an adduct, assigned to that of a C(4a) adduct, in addition to the N(5) and N(3) adducts. While the reactions of **1** (pH 5.1) and **2** (pH 4.3) with LFH_2 under acidic conditions gave only the N(5) adducts, that of **3** at pH 6.6 gave the same adducts as observed at pH 7.86. Most of these adducts have been isolated. All of the adducts (except **12a**) exhibited *trans* stereochemistry with respect to epoxide opening. Rate constants for these reactions were determined by chromatographic monitoring of the epoxide disappearance and for these epoxides in the spontaneous region (pH approximately 7.5) are in the range of 0.1–0.9 M^{-1} s^{-1}. The magnitude of these rate constants indicates that the rate constant for nucleophilic, bimolecular attack by the dihydroflavin on these epoxides is comparable to that of the thiolate anion (rate constants for the 2-thioethanol thiolate opening of **1** and **2** are 0.27 and 0.85 M^{-1} s^{-1}, respectively, under conditions comparable to this study: Becker, A. R.; Janusz, J. M.; Bruice, T. C. *J. Am. Chem. Soc.* **1979**, *101*, 5679–5687). The behavior of the epoxides with the dihydroflavins provides one of the few quantitative estimates of the nucleophilic capacity of the dihydroflavin and confirms the perception of the dihydroflavin as an exceptionally reactive nucleophile.

From epidemiological and experimental studies over the past 60 years, polycyclic aromatic hydrocarbons are proven as causative agents in several human cancers.[2,3] It is now known that the basis for this phenomenon is initial metabolism of the arenes to arene epoxides, by the cytochrome P-450 monooxygenases,[4] followed by hydrolysis of these arene oxides to the *trans*-diols and further oxygenation at the adjacent double bond. The epoxide of these arene oxides can possess exceptional electrophilic reactivity.[5,6] Depending on arene ring structure and diol epoxide stereochemistry, these diol epoxides may express this electrophilic reactivity by initiating the carcinogenic process through DNA alkylation.[3,6] The paradigm for this process is the metabolism of benzo[*a*]pyrene to the illustrated diol epoxide substructures.



Our interest in the electrophilic properties of these epoxides was piqued by the extraordinary observation by

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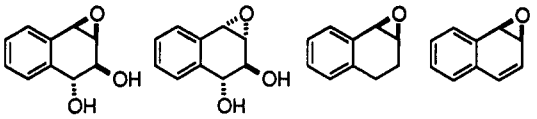
Yang and Gelboin[7a,b] that the highly reactive benzo[a]-pyrene diol epoxides effected *nonenzymatic* oxidation of the coenzyme NADPH, generating significant quantities of the product of epoxide reductive cleavage, the 7,8,9-triol.[7c] While the ability of dihydronicotinamides to undergo oxidation by carbocations is well established;[8] in this instance[7a,b] it is remarkable that epoxide reduction is competitive with epoxide hydrolysis. This outcome demands a broader mechanistic view, beyond that of discriminate nucleophile capture, of the electrophilic behavior of arene diol epoxides. With the intention of evaluating the capability of arene oxides as *oxidants*, we elected to examine the (much less reactive) tetrahydronaphthalene diol epoxides, in the presence of a kinetically much more facile (relative to dihydronicotinamides) reductant, the dihydroflavin. At the start of this study we were aware that the reaction could follow either of two distinct pathways (the flavin acting as a *reductant* or as a *nucleophile*[9] toward the epoxide). It was with these possibilities in mind that an examination of the reaction between epoxides 1–4 and the dihydroflavin was undertaken.

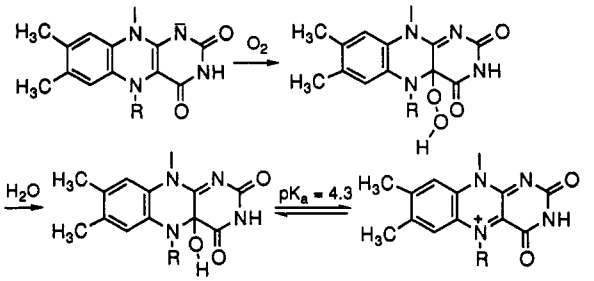


(±)-1 (±)-2 (±)-3 (±)-4

Results

Detection and Characterization of Adducts. The behavior of the four epoxides in the presence of two separate dihydroflavins (FlH_2), dihydrolumiflavin (LFH_2) and dihydroriboflavin 5′-phosphate ($FMNH_2$), was observed. Each reaction was evaluated at two different pHs (one in the hydronium ion catalyzed and the other in the pH-independent hydrolysis region[10] of the epoxide) in a 9:1 aqueous buffer–dioxane solvent. These reactions used dihydroflavin, generated by catalytic reduction, and were conducted under anaerobic conditions to preclude dihydroflavin oxidation. *The dominant reaction pathway for epoxides 1–3 in the spontaneous pH region was the formation of covalent flavin–epoxide adducts.* In the faster acid catalyzed region, covalent adduct formation was still observed but did not always represent the dominant pathway. Epoxide 4 converted to 1-naphthalenol[11] without dihydroflavin trapping in both pH regions. Evidence for adduct formation between the epoxides and FlH_2 was found, firstly, in the HPLC analysis of reaction mixtures. The hydrolysis of epoxides in the spontaneous pH region, in the absence of dihydroflavin, was evaluated by HPLC analysis and found to occur much more slowly than the dihydroflavin reaction.[12]

Detection and Isolation of the Adducts from Reaction with LFH_2. Direct evidence of adduct formation was found by HPLC analysis of the reaction progress of epoxides 1, 2, and 3 with 1,5-dihydrolumiflavin (LFH_2). The chromatograms for the early reaction times of epoxide 1 with LFH_2 at pH 7.86 (Figure 1, Ia) show the disappearance of epoxide without the appearance of any distinct adduct peaks.[13] One new peak (retention time t_R 4.8 min) was seen at 10 h reaction time, identified as the *reduction* product, (±)-(1α,2β,3β)-1,2,3,4-tetrahydro-1,2,3-naphthalentriol.[14] The yield of this triol (35%) accounts, however, for only one-third of the starting epoxide. A basis for this discrepancy is provided by chromatograms of an *oxidized* reaction mixture. When a reaction portion was removed and air oxidized, and then left in the dark for 3 h, the HPLC chromatogram (Figure 1, Ib) exhibited a new peak (t_R 8.5 min). This new material derives from oxidative conversion of the major product of the reaction. It is known that N(5) adducts air oxidize, via the flavin radicals, to 4a-peroxy derivatives,[15] which exchange in water (via the flavinium cation) to provide the 5-alkyl-4a-hydroxy pseudobases.[9,16]



The transformed adduct, isolated by preparative HPLC, has an absorption spectrum[17] with λ_{max} at 343, 305, and 283 nm. Dihydroflavin N(5) adducts have a single absorption band centered around 335–350 nm, while N(5) alkyl pseudobases[9,18] possess the three absorption bands

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(12) Becker, A. R.; Janusz, J. M.; Rogers, D. Z.; Bruice, T. C. *J. Am. Chem. Soc.* **1978**, *100*, 3244–3246. The nucleophilic reaction of the dihydroflavin with the epoxide, in the spontaneous hydrolysis pH region for the epoxide, was much faster than hydrolysis. The nucleophilic reaction was competitive with hydrolysis in the hydronium ion-catalyzed hydrolysis pH region. A more complete discussion on the hydrolysis of these epoxides under the conditions used in this study is given elsewhere: Lee, Y. T. *Bull. Kor. Chem. Soc.*, submitted.

(13) The unstable adduct is argued below to derive from epoxide alkylation of the flavin; since dihydroflavins are acidic they are not expected to give discernable reversed-phase peaks in the absence of a phase modifier.

(14) As discussed below this triol derives from the flavin–epoxide N(5) adduct. Its yield was estimated by use of the extinction coefficients of the epoxide and the triol. A complete discussion on the mechanism of its formation will be presented elsewhere.

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(16) (a) Kemal, C.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 995–999. Kemal, C.; Bruice, T. C. *J. Am. Chem. Soc.* **1976**, *98*, 3955–3964. (b) Merenyi, G.; Lind, J. *J. Am. Chem. Soc.* **1991**, *113*, 3146–3153.

(17) A figure showing the spectral changes accompanying this reaction is provided in the supplementary material.

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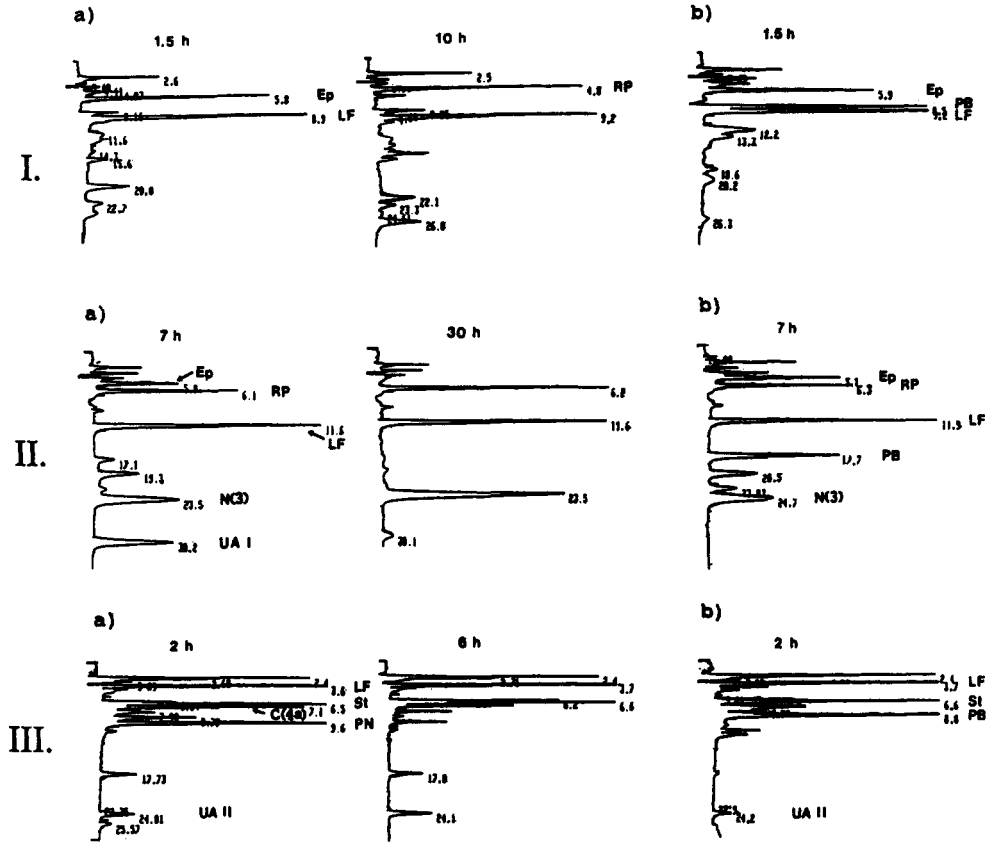
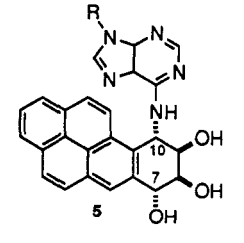


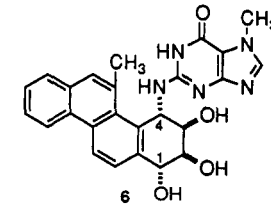
Figure 1. Time course by HPLC monitoring of the reaction of the epoxides with lumiflavin. I: epoxide 1 (1 mM) with LFH_2 (1 mM) at 25 °C in pH 7.86 90:10 aqueous Tris buffer–dioxane. Portions were eluted (a) without delay and (b) after 3 h following air oxidation. Samples were eluted on a C-18 column using a mobile phase of 44:1:55 CH_3OH–dioxane–H_2O at a flow rate of 0.8 mL min^{-1}, with detection at 210 nm. Ep: epoxide. RP: reduction product. PB: pseudobase. II: epoxide 2 (1 mM) with LFH_2 (1 mM) at 25 °C in pH 7.86 90:10 aqueous Tris buffer–dioxane. Portions of the reaction were eluted (a) without delay and (b) after 2 h following air oxidation. The HPLC conditions were identical to those of I. Ep: epoxide. RP: reduction product. PB: pseudobase. N(3): N(3) adduct. UA I: unknown adduct I. III: time course by HPLC monitoring of the reaction of epoxide 3 (1 mM) with LFH_2 (1 mM) at 25 °C in pH 7.86 90:10 aqueous Tris buffer–dioxane. Portions of the reaction were eluted (a) without delay and (b) after 6 h following air oxidation. The HPLC mobile phase was 44:1:55 CH_3CN–dioxane–H_2O. C(4a): C(4a) adduct. PN: peroxy N(5) adduct. UA II: unknown adduct II. PB: pseudobase. St: internal standard (2-hydroxyindan).

observed at these wavelengths. These spectral data suggest oxidative formation of a pseudobase product, a conclusion confirmed by its FAB mass and ^1H NMR spectra. As would be anticipated,[16a,17a] dissolution of this product in MeOH yields a methanol adduct, by pseudobase exchange; its absorption spectrum is similar to that of the starting pseudobase.

A structural assignment to the unstable N(5) adduct follows from the following observations. The triol reduction product appears well after epoxide has disappeared, and hence after flavin–epoxide adduct formation. It is isolated in sufficient quantities so as to demand that it derive from the major epoxide–flavin adduct, the N(5) adduct. The triol ^1H NMR spectrum exhibits a benzylic methylene, indicating flavin N(5) attachment to the epoxide C(4′). Among the four pairs of stereoisomers allowed by the two stereogenic centers of the pseudobase (at the flavin C(4a) and the C(4″) of the triol), only one pair was detected. In the ^1H NMR spectrum of the isolated, transformed adduct are doublets at δ 4.33 (J = 9.6 Hz) and 4.51 (J = 3.4 Hz) which can be collectively assigned to the C(1′) and C(4′) protons. The vicinal coupling between the hydrogens of C(2′) and C(3′) is 2.2 Hz. Although the tetrahydronaphthalene ring system is conformationally mobile (with the consequence that its coupling constants may not be diagnostic of relative stereochemistry),[19] in this instance the bulky flavin clearly selects for itself a C-4′ pseudoaxial placement, analogous to what is seen[19a] for the nucleoside adducts of the larger arene systems (for example, 5 and 6).[20] Thus, in the arene



$J_{7,8}$= 9 Hz; $J_{9,10}$= Small (unresolved)
Cis-adduct: $J_{7,8}$= 3 Hz; $J_{9,10}$ = 4 Hz



$J_{1,2}$= 8.3 Hz; $J_{2,3}$ = 2.3 Hz;
$J_{3,4}$ = 3.8 Hz

of this pseudobase adduct, the C(1)′ and C(2′) protons are pseudoaxial and the C(3′) and C(4′) hydrogens pseudoequatorial.[21] As the available data do not distinguish

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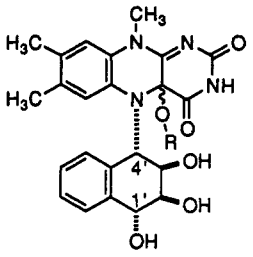
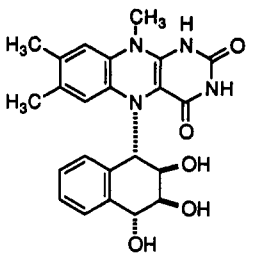
the relative stereochemistry of the flavin C(4a) substituent, the best formulation for the pseudobase structure (**7a**) leaves this assignment uncertain. By inference, therefore, the structure of the initial N(5) adduct is **8**.



(±)-**7a**, R = H
(±)-**7b**, R = CH_3



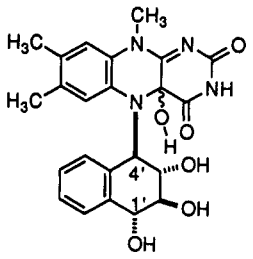
(±)-**8**

Although the HPLC chromatograms (Figure 1, I) show **8** to be the predominant product, other products appeared in small yield. These must be adducts also, since they were not observed with either LFH_2 or epoxide alone. Theoretically, they may be primary adducts from attack by N(1), O(2α), N(3), O(4α) or they may arise from further transformations of the primary adducts. Given this complexity, and the impossibility of ascertaining respective extinction coefficients at the detection wavelength (210 nm), the yield for the formation of **8** can only be estimated. Since the extinction coefficient for the K-band of aromatic compounds is fairly constant (substituent independent)[22] the chromatogram peak areas are roughly proportional to the respective yields. Thus, assuming quantitative conversion of the N(5) adduct to the pseudobase, the yield of **8** at 1.5 h was 56%. A yield may also be estimated from the progressive increase in LF concentration following air oxidation of the reaction. If this increase resulted entirely from oxidation of the N(5) adduct, a yield of 68% is estimated. The true yield is likely between these two values.

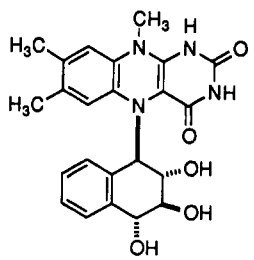
Like the pH 7.86 reaction, the reaction of LFH_2 and epoxide **1** at pH 5.1 failed to provide distinct product peaks upon direct HPLC analysis. However, the oxidized reaction mixture showed the formation of one peak, which coeluted with the pseudobase from the pH 7.86 reaction in small yield (approximately 20%). This indicates that while the *trans* N(5) adduct **8** was formed, the nucleophilic reaction of the reduced flavin is only partially competitive with acid-catalyzed epoxide solvolysis.

The behavior of epoxide **2** with LFH_2 is more complicated. Under the identical reaction, workup, and HPLC conditions as for epoxide **1** at pH 7.86, two major adducts (t_R 23.5 and 30.2 min) were seen at early reaction times (Figure 1, IIa). The triol reduction product (t_R 6.2 min) appeared also in significant yield (approximately 30%). Upon air oxidation of the reaction, a new peak (t_R 17.7 min) appeared (Figure 1, IIb). As was seen for epoxide **1**, this reflects an oxidative transformation of an initial N(5) adduct to the pseudobase (identified as **9** by the absorption, FAB MS, and ^1H NMR analysis of the isolated material).

This regiochemistry for this structural assignment follows from the structure of the reduction product (HPLC yield of 63%), which again arises via the N(5) adduct (and possibly also by the t_R 30.2 min adduct). The benzylic methylene of the reduction product indicates prior N(5) attachment to the arene C(4′). The ^1H NMR of **9** has resonances at δ 4.21 (d, J = 8.7 Hz) and 4.33 (d, J approximately 8 Hz) which are collectively assigned as the C(1′) and C(4′) hydrogens. Although the two doublets cannot be assigned with certainty, the coupling constant magnitude for both is too large for a *cis* C(3′),C(4′) relationship.[19a] This indicates an all-pseudoaxial orientation for the cyclohexenyl hydrogens.[21] By inference from the pseudobase structure **9**, the initial adduct from dihydroflavin attack on the epoxide is **10**.

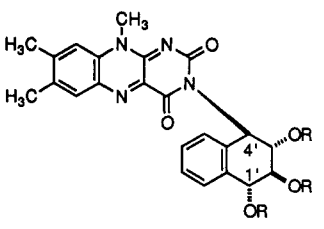


(±)-**9**

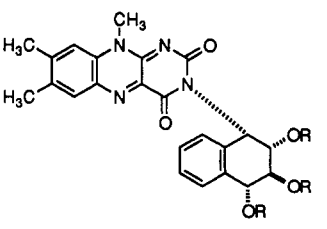


(±)-**10**

The isolated material from the t_R 23.5 min peak was yellow and by ^1H NMR consisted of two compounds in approximately a 10:1 ratio. Following peracetylation and FAB MS analysis of the separated components, the two were identified as isomers. The observed $(M + H)^+$ of 435 corresponds to an *oxidized* flavin (confirmed by the absorption spectra) LF–epoxide **2** adduct. The ^1H NMR spectrum of the more abundant adduct has doublets at δ 5.06 (J = 8.7 Hz) and 7.26 (J = 10.2 Hz) corresponding to the C(4′) and C(1′) hydrogens, respectively. The large difference in chemical shift indicates flavin attachment to C(4′), with the large coupling constant (J = 8.7 Hz) between the C(3′) and C(4′) protons indicating a *trans* substitution. Of the flavin nitrogens, only attachment to N(3) permits a stable oxidized flavin–arene oxide adduct with an unperturbed oxidized flavin absorption spectrum. Accordingly, this adduct is assigned as the N(3)–C(4′) structure **11** (**11a**, initial adduct; **11b**, isolated triacetylated derivative). Further characterization of the minor isomer was precluded by a lack of material, but as the available physical data indicate an isomeric relationship, it reasonably may be formulated as the stereoisomer **12b**.



(±)-**11a**, R = H
(±)-**11b**, R = Ac



(±)-**12a**, R = H
(±)-**12b**, R = Ac

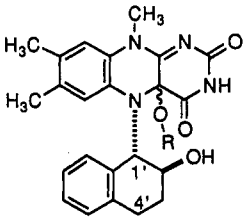
The material from the t_R 30.2 min peak was too unstable for characterization (unknown adduct I). A possible structure is discussed later. Thus, at 7 h, three major adducts are found. The respective yields (estimated from the peak areas) are N(5), 29%, N(3), 30% and unknown

(21) (a) Abraham, R. J.; Gottschalck, H.; Paulsen, H.; Thomas, W. A. *J. Chem. Soc.* **1965**, 6268–6277. (b) Sayer, J. M.; Yagi, H.; Silverton, J. V.; Friedman, S. L.; Whalen, D. L.; Jerina, D. M. *J. Am. Chem. Soc.* **1982**, *104*, 1972–1978. (c) Sayer, J. M.; Whalen, D. L.; Friedman, S. L.; Paik, A.; Yagi, H.; Vyas, K. P.; Jerina, D. M. *J. Am. Chem. Soc.* **1984**, *106*, 226–233.

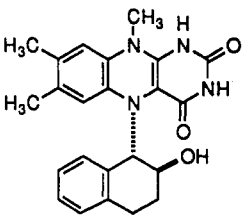
(22) Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectrometric Identification of Organic Compounds*, 4th ed.; Wiley: New York, 1981; p 322.

adduct I, 26%. As with epoxide 1, the acid reaction of epoxide 2 (pH 4.3) gave only the N(5) adduct in poor (10%) yield.

The HPLC chromatogram (Figure 1, III) after 2 h reaction at pH 7.86 of epoxide 3 with LFH_2 showed two major adducts (t_R of 7.1 and 9.7 min) and the product of reductive epoxide cleavage, (±)-1,2,3,4-tetrahydro-2-naphthalenol. Upon oxidation of the reaction mixture, the two adducts disappeared and were replaced by a single peak (t_R 8.8 min). This newly formed compound is a pseudobase, both by its spectral data and conversion in methanol to the 4a-methoxy pseudobase. As will be shown later, this pseudobase was formed from the (t_R 9.7 min) 4a-peroxy derivative of the N(5) adduct. The other primary adduct (t_R 7.1 min) derives from flavin C(4a) opening of the epoxide and transforms to a secondary product (unknown adduct II) which precipitates from the reaction mixture (see below). Therefore, the t_R 9.7 min adduct is a precursor to the (±)-1,2,3,4-tetrahydro-2-naphthalenol. Accordingly, the juncture carbon of the arene in the dihydroflavin–epoxide adduct is again the benzylic carbon. The large vicinal coupling constant (9.3 Hz) between the C(1′) and C(2′) hydrogens indicates a pseudobase structure having the trans relative stereochemistry of **13a**. Again, by inference, the initial N(5) adduct is **14**. The t_R 9.7 min adduct was not stable to isolation and converted to the C-4a pseudobase at both pH 5.7 and 7.7.

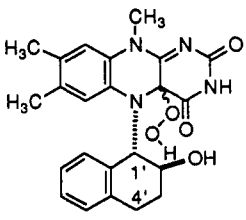


(±)-**13a**, R = H
(±)-**13b**, R = CH_3

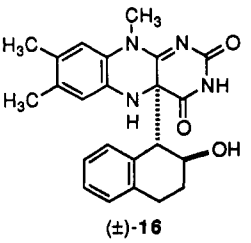


(±)-**14**

This observation, and its spectral data (λ_{max} approximately 370 nm),[15,16a] suggest it to be the 4a-peroxy N(5) adduct **15**. The t_R 7.1 min adduct was not stable to isolation. The absorption spectrum of the HPLC eluent containing this adduct resembled closely that[18] of 3-methyl-4a-benzyl-4a,5-dihydrolumiflavin, a flavin C(4a) adduct. This similarity[9] is the basis for an assignment of structure. As was mentioned, this adduct transformed to a secondary product (unknown adduct II) at later stages of the reaction. The NMR spectrum of this secondary adduct shows the C(4a) of the LF moiety attached to the C(1′) of the arene with a *trans* configuration. Accordingly, a tentative assignment of structure to that of the C(4a) adduct **16** is made.

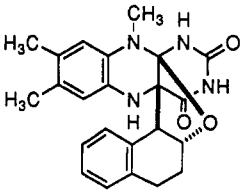


(±)-**15**

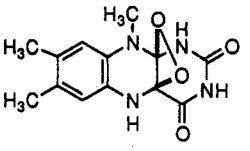


(±)-**16**

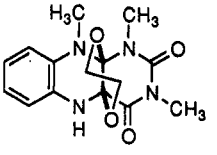
Epoxide 3 is unique among the three epoxides in that it gave an adduct of uncertain structure (unknown adduct II, t_R 24 min in Figure 1, III) as a precipitate at late reaction times. Its molecular mass (M_r 404, deduced from EI and FAB MS) indicates it to be isomeric with a (primary) dihydroflavin–epoxide adduct. It was observed that in the reaction of the epoxide with photochemically generated LFH_2,[23] **15** depleted much more slowly, but the rate of formation of this precipitate was similar. This excludes **15**, the N-5 adduct, as its source. The only other possibility is the C(4a) adduct. The lack of an absorption peak above 309 nm requires a C(10a)–N(1) single bond. The absence of an hydroxyl band in the IR, and the adduct's inability to form an acetyl derivative, suggest alkylation of the arene 2-hydroxyl. In the ^1H NMR spectrum of the isolated adduct, the δ 4.00 doublet collapses with irradiation of the δ 4.72 multiplet. This vicinal pair must correspond to the C(1′) and C(2′) hydrogens, respectively, in a *trans*-diaxial (J = 8.9 Hz) relationship. By the previous reasoning, the arene connects to the LF at the C(1′) position. The δ 7.74, 6.45, and 3.42 singlets in $CDCl_3$ were less shielded in both pyridine and DMSO, identifying three exchangeable protons (assigned as the hydrogens of N(3), N(1), and N(5), respectively). With regard to interpretation of the ^{13}C NMR spectra, the data for 4a-benzyl-3-methyl-4a,5-dihydrolumiflavin[24] and *trans*-1-methyl-2-hydroxytetralin[25] are useful. The most telling signals are the δ 62.9 and 97.8 resonances. It is difficult to assign these peaks to anything other than tetrahedral 4a and 10a carbons, respectively. This unknown adduct decomposed to LF in acidic aqueous solution (pH 1.0) or upon standing on silica gel. A plausible formulation with respect to all these data is structure **17**. Mager,[26] in proposing the 4a,10a-dioxetane



(±)-**17**



18



19

derivative **18** as an intermediate in flavin-mediated hydroxylations, prepared the 4a,10a-ethylenedioxy adduct **19**.[27] However, an adduct with a free unprotected N(1) H group has never been prepared. Mager's proposal would be more credible should structure **17** be verified (an example of a similar intramolecular trapping is presented by Zaugg *et al.*[28]). Lastly, the yields of the adducts at 2 h (estimated by HPLC integration and assuming quantitative conversion of the N(5) adduct to the peroxy

(23) Massey, V.; Stankovich, M.; Hemmerich, P. *Biochemistry* **1978**, *17*, 1–8. Lumiflavin photoreduction was carried out at 30 °C in pH 6.9 Tris–dioxane (9/1) containing 10 mM EDTA. Final reaction concentrations were 0.5 mM epoxide and 0.3 mM LFH_2.

(24) (a) Ghisla, S.; Hastings, J. W.; Favaudon, V.; Lhoste, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1978**, *75*, 5860–5863. (b) Kim, J. M.; Cho, I. S.; Mariano, P. S. *J. Org. Chem.* **1991**, *56*, 4943–4955.

(25) Schneider, H. J.; Agrawal, P. K. *Org. Magn. Res.* **1984**, *22*, 180–186.

(26) Mager, H. I. X. *Eur. J. Biochem.* **1984**, *142*, 199–202.

(27) Mager, H. I. X. *Tetrahedron* **1977**, *33*, 981–989.

(28) Zaugg, H. E.; Papendick, V.; Michaels, R. J. *J. Am. Chem. Soc.* **1964**, *86*, 1399–1401.

derivative) are 47% of the N(5) adduct and 27% of the C(4a) adduct (including unknown adduct II).

The reaction of epoxide 3 at pH 6.6 yielded HPLC chromatograms similar to those obtained at pH 7.86. The C(4a) and 4a-peroxy N(5) adducts appeared on the chromatogram early, and the unknown adduct II precipitated from the reaction solution at later reaction times. The identical pseudobase compound appears after oxidation of the reaction mixture. The relative yields of the adducts are also similar.

Epoxide 4 quantitatively converted[11] to 1-naphthol without bimolecular reaction with the flavin, at both acidic and neutral pH.

Detection of Adducts by HPLC Analysis: Reactions with $FMNH_2$. The reactions of epoxides 1–3 with $FMNH_2$ were followed by withdrawing reaction portions at appropriate times for HPLC analysis, using procedures similar to those described in Figure 1 for the LFH_2 reactions. The chromatographic profiles were evaluated both by the disappearance of the epoxide peak and the increase of the FMN peak. At acidic pH's, in addition to this change, the epoxide hydrolysis products appeared. The increase of FMN peak area as the reaction proceeded is attributed to the overlap of the adducts, having greater extinction coefficients than FMN at the 210 nm detection wavelength.

Spectrophotometric Detection of Adduct Formation. Evidence for adduct formation was provided by the spectral changes accompanying the reactions of epoxides 1–3 with FlH_2 ($FMNH_2$ at acidic and basic pH; LFH_2 only at basic pH, as it is too insoluble at the acid pH). Thus, the spectra[17] for the reaction of epoxide 1 with LFH_2 at pH 7.9 showed a decrease in absorbance throughout the whole spectrum, without new band formation. This would appear to contradict the HPLC analysis, which provided evidence for an N(5) adduct in high yield. N(5) adducts typically have a distinct band between 335 and 350 nm in organic solvents. Whereas an N(5) adduct is in an unionized state with respect to N(1)H group in these solvents, it is ionized in a pH 7.9 aqueous solution (pK_a = 6.5). A possibility is that this ionization results in a more shoulder-like band near 345 nm. Supporting this inference is the λ_{max} value of 3-methyl-5-benzyl-4a,5-dihydrolumiflavin in 2 M NH_3 (345, 290 nm), the interval of which is much shorter than that in CH_3OH (340, 250 nm).[29] The reaction of epoxide 1 with $FMNH_2$ at pH 7.8 exhibited a similar spectral change,[17] showing the N(5) alkylation was also the major pathway. The spectral change for the reaction of epoxide 1 with $FMNH_2$ at pH 5.1 did not aid in the mechanistic characterization; the shoulder at 390 nm became more featureless as the reaction proceeded.

The reaction of epoxide 2 with LFH_2 at pH 7.9 showed a quite pronounced spectral change.[17] The shoulder near 340 nm changed to a band with λ_{max} at 350 nm. Since both the N(5) and N(3) adducts detected by HPLC analysis are not expected to have a distinct band at this wavelength, this band likely results from the formation of unknown adduct I. Accordingly, a characterization of unknown adduct I may be attempted based on this λ_{max}. A 350-nm band is quite unusual for a C(4a) adduct (λ_{max} more typically near 360 nm);[9,29] nonetheless, well-characterized C(4a) adducts with λ_{max} as short as 327 nm are known.[30] An O(2α) adduct (λ_{max} 480 nm)[31] is ruled out. An O(4α) adduct is also unlikely since substitution at the O(4α) position has been reported to occur (with the 5-acetyl derivative) only after the O(2α) position is substituted.[32] Dialkyl adducts such as the N(5),C(4a)–, N(5),N(1)–, N(5),O(2α)–, and N(5),O(4α)– are not possibilities since unknown adduct I is a primary adduct (as is the N(3) adduct; appearance of unknown adduct I does not lag behind that of the N(3) adduct). A final possibility is an adduct at N(1). In contrast to numerous examples of N(5) and C(4a) adducts, only a few examples of N(1) adducts are known. A wide range of λ_{max} values for the adduct have been reported (340 nm[33] and 370 nm[31]), probably due to their instability. As far as the λ_{max} is concerned, formation of an N(1) adduct is not inconsistent with the spectral change. Thus, the possibilities are N(1) adduct or C(4a) adduct. Although these possibilities cannot be distinguished with certainty, given the established nucleophilic character of C(4a), even including this study, this is the more probable assignment. In support of this conclusion, the spectral changes for the reaction of epoxide 2 with $FMNH_2$ at pH 7.8 were similar but showed distinctly the new band at 357 nm. This corresponds to C(4a) adduct formation. The basis for the rather different λ_{max} values for the two C(4a) adducts is not at all obvious. The reaction of epoxide 2 with $FMNH_2$ at pH 4.3 showed a spectral change too trivial for adduct characterization.

The reaction of epoxide 3 with LFH_2 at pH 7.9 gave a pronounced spectral change.[17] A new band appeared at 363 nm, as the absorbance throughout the spectrum decreased. The new band must again be that of the C(4a) adduct. The irreversible absorbance decrease at all wavelengths is accounted for by the loss in material resulting from precipitation of unknown adduct II. The reaction of epoxide 3 with $FMNH_2$ at pH 7.8 showed a similar change, save for a lesser absorbance decrease across the spectrum. This observation shows that the same type of adducts as above were formed, but the relative yield of the C(4a) adduct compared to that of the N(5) adduct was smaller. Since the highest λ_{max} of unknown adduct II (which forms from the C(4a) adduct) is 309 nm, the less the C(4a) adduct forms, the less the spectral change. The reaction of epoxide 3 with $FMNH_2$ at pH 6.6 showed a spectral change similar to those at neutral pH, indicating a similar reaction course.

Detection of Adducts by the Spectra of Oxidized Reaction Mixtures. Evidence of adduct formation was also found in the spectral change of the oxidized reaction mixture. The spectrum[17] observed in the reaction of epoxide 1 with LFH_2 at pH 7.86 showed the progressive conversion of the oxidized flavin spectrum at time 0 to an adduct spectrum (λ_{max} near 355 nm). Other reactions showed similar changes. Although these spectra provide convincing evidence of adduct formation, they hardly distinguish among the structural possibilities.

Kinetics of Adduct Formation. The rate constants for adduct formation between the three epoxides and FlH_2

(29) Walker, W. H.; Hemmerich, P.; Massey, V. *Helv. Chim. Acta.* **1967**, *50*, 2269–2279.

(30) Chan, T. W.; Bruice, T. C. *Biochemistry* **1978**, *17*, 4784–4793.

(31) Dudley, K. H.; Ehrenberg, A.; Hemmerich, P.; Müller, F. *Helv. Chim. Acta* **1964**, *47*, 1354–1383.

(32) Dudley, K. H.; Hemmerich, P. *Helv. Chim. Acta* **1967**, *50*, 355–363.

(33) Ghisla, S.; Massey, V.; Lhoste, J. M.; Mayhew, S. G. *Biochemistry* **1974**, *13*, 589–597. Müller, F.; Grande, H. J.; Jarbandhan, T. in *Flavins and Flavoproteins*; Singer, T. P., Ed.; Elsevier: Amsterdam, 1976; pp 38–50.

Table I. Rate Constants for Adduct Formation between the Epoxides and FlH_2[a]

epoxide	FlH_2	pH	k_2 (M^{-1} s^{-1}) by HPLC	k_2 (M^{-1} s^{-1}) by spectrophotometry[b]
Spontaneous Epoxide Hydrolysis pH Region				
I	$FMNH_2$	7.70	3.4×10^{-1}	2.8×10^{-1} (332 nm), 5.7×10^{-1} (420 nm)
	LFH_2	7.86	4.2×10^{-1}	3.8×10^{-1} (332 nm), 6.3×10^{-1} (420 nm)
II	$FMNH_2$	7.70	1.2×10^{-1}	2.5×10^{-1} (336 nm)
	LFH_2	7.86	1.6×10^{-1}	1.8×10^{-1} (336 nm) 3.1×10^{-1} (392 nm)
III	$FMNH_2$	7.70	5.2×10^{-1}	2.5×10^{-1} (336 nm), 5.9×10^{-1} (380 nm)
	LFH_2	7.86	8.4×10^{-1}	3.4×10^{-1} (336 nm), 9.8×10^{-1} (380 nm)
Hydronium Ion-Catalyzed Epoxide Hydrolysis pH Region				
I	$FMNH_2$	5.16	1×10^{-2}	
II	$FMNH_2$	4.35	2.5×10^{-2}	
III	$FMNH_2$	6.50	1.4×10^{-1}	

[a] Reactions were carried out at 25 °C in 90:10 buffer–dioxane using 1 or 2 mM epoxide and 0.5 or 1 mM FlH_2. [b] Two sets of rate constants were obtained from the two indicated wavelengths.

were determined by both HPLC analysis and spectrophotometry (Table I). The kinetic data were fitted well by bimolecular rate equations (see Experimental Section; for these equations the reactant concentrations were determined from HPLC peak area or from the spectral absorbance change), implicating a bimolecular (S_N2) mechanism. The rate constants for the reactions with LFH_2 at acidic pH could not be obtained, due to the limited solubility of LFH_2. The rate constants for $FMNH_2$ at acidic pH with the epoxides were obtained solely by HPLC analysis, due to insignificant absorbance changes.

The spectral changes of the reactions showed different rates at different wavelengths. For example, in the reaction of epoxide 1 with both $FMNH_2$ and LFH_2, the rate of absorbance decrease between 350 and 300 nm was substantially slower than between 380 and 460 nm. Therefore, spectrophotometry does not give unique rate constants. However, it was possible to confirm the reliability of the rate constants determined by HPLC analysis by evaluating the absorbance changes at two different wavelengths, and determining two rate constants (assuming monophasic kinetics). For most of the reactions at neutral pH, the rate constants determined by the HPLC analysis are between the two rate constants determined from the absorbance changes. The dual absorbance changes appear to result from the transformation of initially formed adducts to secondary adducts, and also to Fl_{ox} and the reduction products of the epoxides. In the case of epoxide 1, the rate constants determined by HPLC analysis are closer to the ones determined by spectrophotometry at 332 nm. Dual absorbance changes would result, for example, when the secondary adducts have extinction coefficients similar to those of initial adducts at 332 nm, but higher at 420 nm. Although not detected by HPLC analysis, it is possible that the N(5) adduct undergoes a slow transformation to a secondary adduct, causing biphasic kinetics as in the reaction of epoxide 3. The reaction of epoxide 2 with $FMNH_2$ at pH 7.8 shows a large absorbance change only at 336 nm. Since the absorbance change at later reaction times is considerably faster than expected from the early absorbance decrease, the true rate constant for this reaction is believed to be lower than that (2.5×10^{-1} M^{-1} s^{-1}) determined assuming monophasic kinetics. Thus, the HPLC analysis rate constant (1.2×10^{-1} M^{-1} s^{-1}) is the more correct. In the reactions of epoxide 3, the biphasic kinetics must be related to the behavior of unknown adduct II.

Discussion

The facile bimolecular cleavage of these three epoxides by dihydroflavins in the pH region of spontaneous epoxide hydrolysis is the outstanding observation of this study. It may be commented upon in terms of mechanism with respect to the epoxide, in terms of the plethora of products, and in terms of mechanism with respect to the flavin.

Mechanism of Epoxide Cleavage. The pH-independent "spontaneous" epoxide hydrolysis which occurs at neutral pH corresponds to rate-determining bond heterolysis to the zwitterion (or where water acts as a general acid, the carbocation).[10,34] Hence, all reactions of the epoxides involving the zwitterion intermediate must proceed at an identical (or slower) rate compared to the rate of hydrolysis.[5d] The simple observation that dihydroflavin attack occurs faster than this process eliminates this intermediate from further consideration. Given the observation of bimolecular kinetics it is certain that adduct formation at neutral pH occurs exclusively by an S_N2 mechanism. All of the adducts (except for the trace product 12 of epoxide 2) exhibit trans stereochemistry, consistent with the S_N2 mechanism. The behavior of the dihydroflavin is thus little different from the bimolecular opening of these same epoxides by the thiolate anion.[12,34,35] Indeed, a comparison between the thiolate and the dihydroflavin is most instructive. In their exhaustive analysis of the hydrolysis and thiolate cleavage reactions of epoxides 1 and 2, Becker *et al.*[12] observed that in aqueous solution the effect of intramolecular hydrogen bonding, of which 2 is capable but 1 is not, is lost. In its absence, the rate constant for thiolate opening of the epoxide for these two epoxides is quite similar (1, 0.51 M^{-1} s^{-1}, and 2, 0.64 M^{-1} s^{-1}, at 30 °C).[12] The magnitude of these rate constants may be compared with that of dihydroflavin under similar circumstances (1, 0.34–0.42 M^{-1} s^{-1}, and 2, 0.12–0.16 M^{-1} s^{-1}, Table I). The rate constants are nearly identical. Hence, the dihydroflavin is a polarizable nucleophile (as is demanded for facile epoxide opening)[5a] of near-identical ability as the classic thiolate anion.

Rather little can be said concerning the mechanism of flavin cleavage of the epoxides in the acid-catalyzed region; clearly, there is the possibility of new pathways. Since the pH in the reactions of epoxide 1 (pH 5.1) and 2 (pH 4.3) with $FMNH_2$ are lower than the pK_a of $FMNH_2$ (6.7), these reactions may occur between the neutral $FMNH_2$ and the epoxide. Since the neutral dihydroflavin is less reactive than the anionic dihydroflavin (FlH^-) in electrophilic alkylations,[36] these reactions may require the hydronium catalyst. Regardless, although epoxide hydrolysis now competes with the bimolecular dihydroflavin adduct formation, the reaction outcome still reflects a potent nucleophilic character of the dihydroflavin.

The Dihydroflavin Nucleophile. The versatility of the flavin coenzyme is unparalleled.[37] This versatility

(34) Bruice, T. C.; Bruice, P. Y. *Acc. Chem. Res.* **1980**, 378–384.
(35) Yagi, H.; Hernandez, O.; Jerina, D. M. *J. Am. Chem. Soc.* **1976**, *97*, 6881–6883.
(36) Blankenhorn, G.; Ghisla, S.; Hemmerich, P. Z. *Naturforsch.* **1972**, *27b*, 1038–1040.
(37) (a) Bruice, T. C. *Acc. Chem. Res.* **1980**, *13*, 256–262. Ghisla, S., Massey, V. *Eur. J. Biochem.* **1989**, *181*, 1–17.

derives from an eclectic reaction manifold, where the numerous competitive processes (characterized by small activation barrier differences) that exist *in vitro*[37a] are individually optimized by each unique flavoenzyme.[37b] In this study with epoxides, therefore, it is not surprising that the dominant pathways involve competition for nucleophilic attack by the flavin N(5) and C(4a) atoms. These same two atoms are seen to trace individual reaction pathways *in vitro* and *in vivo*. In the instance of *in vitro* dihydroflavin reduction of carbonyls, covalent flavin adducts are not involved, yet there is exceptionally fast nucleophilic interception (in a nonproductive equilibrium) of the carbonyl by the N(5) of the dihydroflavin.[38] To date, these studies by Bruice and colleagues represent the best evidence in favor of the enhanced nucleophilic character of the dihydroflavin. In contrast, nitroalkane anion addition to oxidized flavin occurs to C(4a),[29] and the thiol/disulfide redox equilibrium mediated by the flavin also involves C(4a) adduct intermediacy.[39] The inactivation of flavoproteins by modified substrates may, in part, represent nucleophilic attack of the dihydroflavin.[40] Further, there are circumstances where C(4a),N(5) substituent migration is observed.[41] All in all, the nucleophilic tendency of both the N(5) and C(4a) atoms has been recognized previously, and it is not surprising that both participate in epoxide opening.

The appearance of N(3) adducts from epoxide **2** is, however, nothing short of remarkable. No obvious explanation for this occurrence may be provided. One may speculate that this epoxide, and this epoxide alone, forms a flavin pyrimidinediol epoxide hydrogen-bonded complex which facilitates the N(3) attack. With the larger arene diol epoxides, stacking complexes are formed between the diol epoxide and nucleotides[42] and between oxidized flavins (FMN) and other species,[43] as evidenced by facilitated general acid catalysis for epoxide hydrolysis.

Implications with Respect to the Carcinogenesis of Polycyclic Aromatic Hydrocarbons. This study raises the possibility that these arene epoxides may undergo adduct-forming reactions *in vivo* with reduced flavins, possibly leading to inactivation of select flavoenzymes. Flavoenzymes are intimately involved in the processes of xenobiotic metabolism.[44] It is possible that the sensitivity of the flavoenzyme cytochrome P-450 reductase to the benzo[*a*]pyrene diol epoxides[45] may derive from direct dihydroflavin alkylation. On the other hand, adduct formation between epoxides and reduced flavins may represent a detoxification reaction, by prevention of arene oxide alkylation of DNA. There is promise to the continuing study of the flavin molecule as a nucleophile, both with respect to flavoenzyme mechanism and to the interception of ultimate carcinogens.

Experimental Section

Instrumentation. Fast atom bombardment (FAB) mass spectra were recorded either at the University of Minnesota or at the Midwest Center for Mass Spectrometry, University of Nebraska. The coupling constants (Hz) in the ^{1}H NMR are reported as observed (uncorrected for non-first-order behavior).

Chromatography. Analytical HPLC samples were eluted isocratically on a 5-μm C-18 column (4.6 mm × 25 cm) at a flow rate of 0.8 mL min^{-1}. Detection was done primarily at 210 nm and occasionally at 350 nm or 445 nm. Mobile phases are given in the individual experimentals. Preparative liquid chromatography used a 10-μm C-18 column (2.1 × 25 cm). The sample injection of 1.7 mL was eluted at a flow rate of 4.5 mL min^{-1}.

Materials. Pt on asbestos was obtained from Baker. FMN was purchased from Sigma and was used as received. All buffers were made with distilled water passed through an ion-exchange purification system. Dioxane was purified by distillation from sodium. Deoxygenated nitrogen was prepared by passage through heated BASF catalyst. The epoxides[46] and lumiflavin[47] were prepared by published procedures. Lumiflavin was separated from an acidic impurity by neutral alumina chromatography (Brockman activity I) using a 10:1 $CHCl_3$–MeOH mobile phase.

Reaction of the Epoxides with FlH_2. The reactions of the epoxides (1 or 2 mM) with $FMNH_2$ (1 or 2 mM) or LFH_2 (1 mM, ≪1 mM for reactions at acidic pH) were run in 90:10 (v/v) 20 mM buffer–dioxane at 25 °C. The pH determinations of the neutral buffer solutions containing FlH_2 were made under anaerobic conditions. The pH of the acidic buffer–dioxane solutions containing LFH_2 were considered to be unchanged by LF reduction, since the solubility of LFH_2 at acidic pH is low. Catalytic hydrogenation with Pt/asbestos catalyst, in a modified Thunberg vessel[48] to allow for filtration of the catalyst, was used for flavin reduction.

HPLC Analysis of Adduct Formation. Portions from the reactions were removed for HPLC analysis two or three times during the reaction, starting when adduct formation was nearly complete and ending 1 day later. For epoxides **1** and **2**, portions (0.2 mL) of the reaction mixture with withdrawn and added to 0.8 mL of 90:10 H_2O–CH_3OH, and a 0.2 mL portion of the diluted solution was combined with 0.1 mL of MeOH. A 0.1-mL portion of this was used for the HPLC analysis, using a mobile phase of 44:1:55 MeOH–dioxane–H_2O. The dioxane is used as a mobile-phase component in order to eliminate the impurity peaks from the dioxane cosolvent of the reactions. The reaction of epoxide **3** was similarly followed by HPLC using a 44:1:55 CH_3CN–dioxane–H_2O mobile phase.

Spectrophotometric Analysis of Anaerobic Reactions. For these studies, a 5- or 10-mm pathlength Thunberg cell having an upper adaptor with two side arms was used.[48] The flavin solution (0.5 mM) in 90:10 buffer–dioxane and the Pt catalyst were placed in the upper tube, and 30 μL of 5 × 10^{-2} M epoxide in dioxane (to give a final concentration of 1 mM) was placed in one of the side arms. After flavin reduction, a 1.3-mL portion of the FlH_2 solution was drained into the Thunberg cell and the reaction started by addition of the epoxide solution.

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Detection of Adducts by Spectrophotometric Evaluation of the Oxidized Reactions. A portion of the reaction was withdrawn at appropriate times, diluted with pH 7.9 90:10 Tris–dioxane, and oxidized by air bubbling in the dark. After 10 min the spectrum was recorded.

HPLC Determination of the Kinetics of Adduct Formation. Reactants and product concentrations were evaluated by HPLC analysis of portions removed at early reaction times (within 1 h). Since adduct formation occurs via a bimolecular kinetics the following equation[50] for second-order kinetics was used to determine the rate constants at neutral pH

$$[1/(a-b)]\ln[b(a-x)/a(b-x)] = k_2t$$

where a and b are the initial concentrations of reactants and x is the concentration of reactants that have been consumed. All unknowns can be determined from the decrease in the epoxide concentration. In the case of equal concentrations of reactants the equation[50] $x/[a(a-x)] = k_2t$ was used. Under acidic conditions, hydrolysis of the epoxide is competitive with adduct formation. The equation correlating k_1, k_2, epoxide, and FlH_2 concentration, and time, is not experimentally accessible.[50] However, since k_1 can be determined, and the concentration of free FlH_2 can be estimated from the concentrations of epoxide and hydrolysis products, the equation[50] $a = a_i - (k_1/k_2)\ln[b_i/b - (b_i - b)]$ (where a_i and a are the concentration of epoxide at the initial time and later time, respectively, and b_i and b are the corresponding FlH_2 concentrations) was used to obtain k_2.

Spectrophotometric Kinetic Evaluation of Adduct Formation. The rate constants for adduct formation at neutral pH were also determined by monitoring the epoxide and FlH_2 concentrations by absorbance, using the second-order kinetic equation above. The rate constants at the acidic pH values could not be obtained due to severe biphasic kinetics or to too small absorbance changes.

Kinetics of Epoxide Hydrolysis. The pseudo-first-order rate constants for the hydrolyses of epoxides 1–3 were obtained by essentially the same procedure (HPLC time course evaluation of epoxide and hydrolysis product concentrations) as for adduct formation.

Isolation of the Epoxide–LFH_2 Adducts. This was accomplished from larger scale reactions (typically 50 mL) of the epoxides (1.50–2 mM) with LFH_2 (1 mM) at pH 7.9. The flavin solution was reduced and filtered to remove the catalyst. The reaction was initiated by injection of a deoxygenated epoxide solution in dioxane. Adduct isolation was done by the following procedures.

Adducts of Epoxide 1. After 2 h, the reaction is oxidized by air and then left in the dark for 3 h. Concentration of the products, for preparative HPLC purification, was done by batch absorption on a C-18 silica column and elution with CH_3CN. The CH_3CN fraction containing the adducts and LF was concentrated to 10 mL and purified in portions by preparative C-18 HPLC, using a mobile phase of 30:70 CH_3CN–H_2O at a flow rate of 4.5 mL min^{-1}. Adduct **7** (t_R = 20 min) was obtained as a white solid upon evaporation.

5,10-Dihydro-4a-hydroxy-7,8,10-trimethyl-5-[(1α,2β,3β)-1,2,3-trihydroxy-1,2,3,4-tetrahydro-4β-naphthalenyl]-(±)-benzo[g]pteridine-2,4(3*H*,4a*H*)-dione (7a) $C_{23}H_{24}N_4O_6$: UV–vis (CH_3CN) λ_{max} nm (rel absorbance) 219 (100), 275 (shoulder, 20), 283 (22), 305 (19), 343 (26); MS (FAB, DTT/DTE matrix, positive ion) m/z (rel intensity) 85 (100), 177 (7), 257 (19, LF + H), 258 (11), 453 (6, M + H), 475 (1, M + Na); (negative ion) 241 (49), 255 (56), 256 (100, LF), 451 (4, M-H); ^1H NMR (300 MHz, CD_3CN): δ 8.35 (d, 1 H, H-5′), 7.46 (dd, 1 H, H-6′), 7.36 (dd, 1 H, H-7′), 7.32 (d, 1 H, H-8″), 7.14 (s, 1 H, H-9), 6.25 (s, 1 H, H-6), 4.51 (d, 1 H, H-4′), 4.33 (d, 1 H, H-1′), 3.80 (dd, 1 H, H-3′), 3.67 (dd, 1 H, H-2′), 3.62 (s, 3H, N-10 CH_3), 2.21 (s, 3 H, C-8 CH_3), 1.88 (s, 3 H, C-7 CH_3); $J_{1',2'}$ = 9.8, $J_{2',3'}$ = 2.2, $J_{3',4'}$ = 3.4, $J_{5',6'}$ = 7.8, $J_{7',8'}$ = 7.6 Hz.

5,10-Dihydro-4a-methoxy-7,8,10-trimethyl-5-[(1α,2β,3β)-1,2,3-trihydroxy-1,2,3,4-tetrahydro-4β-naphthalenyl]-(±)-benzo[g]pteridine-2,4(3*H*,4a*H*)-dione (7b) $C_{24}H_{26}N_4O_6$. The transformation of the hydroxy pseudobase **7a** to the 4a-methoxy pseudobase was accomplished by dissolution in MeOH, containing a trace amount of acetic acid, for 10 d in the dark. The solvent was evaporated, and the residue purified by preparative HPLC column (30:70 CH_3CN–H_2O mobile phase). The product (t_R = 22 min) was collected as a white solid by evaporation of the eluent: UV–vis (CH_3CN) λ_{max} nm (rel absorbance) 219 (100), 281 (24), 305 (21), 342 (30).

Adducts of Epoxide 2. The reaction mixture at 5 h was oxidized by air bubbling and the pseudobase **9** (t_R 23 min) isolated by preparative HPLC (as described above) using 22:78 CH_3CN–H_2O as the mobile phase.

5,10-Dihydro-4a-hydroxy-7,8,10-trimethyl-5-[(1β,2α,3β)-1,2,3-trihydroxy-1,2,3,4-tetrahydro-4α-naphthalenyl]-(±)-benzo[g]pteridine-2,4(3*H*,4a*H*)-dione (9) $C_{23}H_{24}N_4O_6$: UV–vis (CH_3CN) λ_{max} nm (rel absorbance) 218 (100), 272 (28), 281 (23), 305 (14), 340 (22); MS (FAB, DTT/DTE matrix, positive ion) m/z (rel intensity) 119 (100), 212 (57), 245 (82), 257 (72, LF + H), 258 (48), 261 (55), 279 (50), 453 (36, M + H), 475 (3, M + Na); (negative ion) 153 (100), 185 (27), 256 (25, LF), 297 (42), 311 (58), 451 (26, M-H); ^1H NMR (300 MHz, CD_3CN): δ 8.34 (d, 1 H, H-8′), 7.46 – 7.31 (H-5′, -6′ and -7′), 7.14 (s, 1 H, H-9), 6.09 (s, 1 H, H-6), 4.33 (d, 1 H, H-1′ or -4′), 4.21 (d, 1 H, H-1′ or -4′); $J_{1',2'}$ and $J_{3',4'}$ = 8.0 or 8.7 Hz. The δ 4.33 doublet overlapped with an impurity peak.

5,10-Dihydro-4a-hydroxy-7,8,10-trimethyl-3-[(1β,2α,3β)-1,2,3-triacetoxy-1,2,3,4-tetrahydro-4α-naphthalenyl]-(±)-benzo[g]pteridine-2,4(3*H*,4a*H*)-dione (11b) and 5,10-Dihydro-4a-hydroxy-7,8,10-trimethyl-3-[(1β,2α,3β)-1,2,3-triacetoxy-1,2,3,4-tetrahydro-4β-naphthalenyl]-(±)-benzo[g]pteridine-2,4(3*H*,4a*H*)-dione (12b). The yellow compound, isolated from the 24 h reaction by preparative HPLC (30:70 CH_3CN–H_2O mobile phase) was a mixture of two isomeric N(3) adducts and was contaminated with LF. The adducts were separated from LF by preparative silica TLC (10:1 $CHCl_3$–MeOH). The unresolved adducts were extracted from the silica (by repeated washing with EtOAc–MeOH) and the solvent evaporated to provide a yellow residue. This residue (2 mg) was dissolved in 0.3 mL of Ac_2O containing 50 μL of Et_3N and 5 mg of (dimethylamino)-pyridine. After 20 h at ambient temperature, the reaction was diluted with cold water and then extracted with $CHCl_3$. The $CHCl_3$ extract is washed with 5% NaOH and water and dried ($MgSO_4$) and the solvent evaporated. The residue was purified by preparative TLC (EtOAc). The relative yield of the major (**11b**, R_f 0.6) to minor product (**12b**, R_f 0.7) was approximately 10:1.

11b, $C_{29}H_{28}N_4O_8$: UV–vis (CH_3CN) λ_{max} nm (rel absorbance) 225 (100), 270 (88), 356 (20), 445 (29); MS (FAB, DTT/DTE matrix, positive ion) m/z (rel intensity) 383 (17), 399 (22), 441 (17, M – 2AcOH + H), 501 (16, M – AcOH + H), 561 [100, (M + H)$^+$], 562 [50, (M + 2H)$^+$], 583 [4, (M + Na)$^+$]; (negative ion) 153 (100), 381 (81), 560 (78, M); IR (FT, $CHCl_3$) 1749, 1686, 1556, 1227 cm^{-1}; ^1H NMR (300 MHz, CD_3CN): δ 8.27 (s, 1 H, H-6), 7.38 (s, 1 H, H-9), 7.26 (d, 1 H, H-1′), 7.23 (d, 1 H, H-8′), 7.11 (dd, 1 H, H-7′), 6.95 (dd, 1 H, H-6′), 6.41 (d, 1 H, H-5′), 6.30 (dd, 1 H, H-3′), 5.64 (dd, 1 H, H-2′), 5.06 (d, 1 H, H-4′), 4.04 (s, 3 H, N-10 CH_3), 2.64 and 2.57 (s, 3 H, C-7 and -8 CH_3), 2.24, 2.06, 1.88 (s, each 3 H, acetyl); $J_{1',2'}$ = 10.2, $J_{2',3'}$ = 10.4, $J_{3',4'}$ = 8.7, $J_{5',6'}$ = 7.8, $J_{6',7'}$ = 7.4, $J_{7',8'}$ = 7.4 Hz.

12b, $C_{29}H_{28}N_4O_8$: UV–vis (CH_3CN) λ_{max} nm (rel absorbance) 225 (100), 270 (88), 358 (20), 445 (29); MS (FAB, DTT/DTE matrix, positive ion) m/z (rel intensity) 383 (36), 399 (45), 441 [29, (M – 2AcOH + H)$^+$], 501 [18, (M – AcOH + H)$^+$], 561 (100, M + H), 562 [48, (M + 2H)$^+$], 583 [7, (M + Na)$^+$]; (negative ion) 153 (100), 381 (33), 560 (16, M); IR (FT,$CHCl_3$) 1748, 1684, 1558, 1227 cm^{-1}.

Unknown adduct I (t_R 30 min) was not stable to isolation.

Adducts of Epoxide 3. The reaction was air oxidized at 2 h. The pseudobase **13a** was isolated by preparative HPLC (t_R 32 min, 40:60 CH_3CN–H_2O) following the above procedure.

5,10-Dihydro-4a-hydroxy-7,8,10-trimethyl-5-(*trans*-2-hydroxy-1,2,3,4-tetrahydro-1-naphthalenyl)-(±+)-benzo[g]-pteridine-2,4(3*H*,4a*H*)-dione (13a) $C_{23}H_{24}N_4O_4$: UV–vis (CH_3CN) λ_{max} nm (rel absorbance 219 (100), 275 (sh, 17), 283 (23), 305 (18), 343 (28); MS (FAB, DTT/DTE matrix, positive ion) m/z (rel intensity) 129 (1.8), 147 (1.6), 186 (1.8), 230 (1.6), 243 (1.5), 257 [100, (LF + H)$^+$], 275 (0.7), 421 [0.4, (M + H)$^+$], 443 [0.2,

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(M + Na)$^+$]; (negative ion) 256 (100, LF), 403 [33, (M – OH)$^-$], 419 [32, (M – H)$^-$]; ^{1}H NMR (300 MHz, CD_3CN) δ 8.9 (s, br, 1 H, N-3H), 8.30 (d, 1 H, H-8′), 7.33 (dd, 1 H, H-7′), 7.24 (dd, 1 H, H-6′), 7.13 (s, 1 H, H-9), 7.11 (d, 1 H, H-5′), 6.14 (s, 1 H, H-6), 4.86 (s, br, 1 H, 4a-OH?), 4.21 (d, 1 H, H-1′), 3.60 (s, N-10 CH_3), 3.54 (m, 1 H, H-2′), 2.96 (d, 1 H, C-2 OH), 2.68 (m, 1 H, H-4′), 2.21 (s, 3 H, C-8 CH_3), 1.88 (s, 3 H, 7-OH_3); $J_{1',2'}$ = 9.3, $J_{5',6'}$ = 7.8, $J_{6',7'}$ = 7.2, $J_{7',8'}$ = 7.4 Hz.

5,10-Dihydro-4a-methoxy-7,8,10-trimethyl-5-(*trans*-2-hydroxy-1,2,3,4-tetrahydro-1-naphthalenyl)-(±)-benzo[*g*]pteridine-2,4(3*H*,4a*H*)-dione (13b) $C_{24}H_{26}N_4O_4$. The transformation of the pseudobase to this 4a-methoxy derivative used the same procedure as described for **7**, but with a reaction time of 3 d. The product was isolated by preparative HPLC (t_R 45 min, 65:35 MeOH–H_2O): UV–vis (CH_3CN) λ_{max} nm (rel absorbance) 217 (100), 248 (42, sh), 267 (28), 282 (23), 306 (16), 341 (27); MS (FAB, DTT/DTE matrix, positive ion) *m/z* (rel intensity) 119 (100) 155 (57), 193 (9), 257 (16), 261 (14), 279 (12), 309 (12), 435 [6, (M + H)$^+$].

5,10-Dihydro-4a-hydroperoxy-7,8,10-trimethyl-5-(*trans*-2-hydroxy-1,2,3,4-tetrahydro-1-naphthalenyl)-(±)-benzo[*g*]pteridine-2,4(3*H*,4a*H*)-dione (15) $C_{23}H_{24}N_4O_5$. The material was unstable to isolation; only an absorption spectrum was obtained of the HPLC eluent. A portion of the reaction mixture at 2 h was applied to the preparative HPLC column using a 40:60 CH_3CN–H_2O mobile phase. The t_R 37 min peak was collected and the absorption spectrum taken immediately: UV–vis λ_{max} nm (rel absorbance) 213 (100), 265 (37), 271 (36, sh), 279 (35), 362–375 (16).

5,10-Dihydro-7,8,10-trimethyl-4a-(*trans*-2-hydroxy-1,2,3,4-tetrahydro-1-naphthalenyl)-(±)-benzo[*g*]pteridine-2,4-(3*H*,4a*H*)-dione (16) $C_{23}H_{24}N_4O_3$. The material assigned as the C(4a) adduct **16** was not stable to isolation. An absorption spectrum was obtained from the HPLC eluent from the 2 h reaction using 60:40 MeOH–0.01% aqueous Na_2HPO_4 as the mobile phase: UV–vis λ_{max} nm (rel absorbance) 214 (100), 271 (35), 360 (15).

Unknown Adduct II (17), Assigned as (±)-(6aα,7aα,13aα,13bβ)-5,6,6a,8,13,13b-Hexahydro-8,10,11-trimethyl-7a,13a-(iminomethaniminomethano)naphtho[1′,2′:4,5]furo[2,3-*b*]quinoxaline-14,16-dione. The reaction of epoxide **3** yields a secondary adduct which precipitates from the reaction solution. Two days after initiation of the reaction, the precipitate was filtered and purified by recrystallization from MeOH. Further purification was achieved by neutral alumina (Brockman activity I) chromatography using 10:1 $CHCl_3$–MeOH as the mobile phase: UV–vis (CH_3CN) λ_{max} nm (rel absorbance) 216 (100), 251 (20), 309 (11); MS (EI, 20 eV) *m/z* (rel intensity) 104 (87), 129 (40), 130 (100), 146 (43), 256 (12, LF), 404 (10, M); MS (FAB, DTT matrix, positive ion) 103 (66), 119 (100), 135 (39), 155 (54), 404 (44, M), 405 (25, M + H); MS (FAB, NaCl matrix, positive ion) 103 (61), 119 (100), 135 (38), 155 (55), 177 (30), 309 (22), 404 [30, (M)$^+$], 405 (19, (M + H)$^+$], 427 [20, (M + Na)$^+$]; MS (FAB, DTT matrix) 404.1848, calcd for $C_{23}H_{24}N_4O_3$ 404.1848; IR (KBr) 3300, 2930, 1720, 1700, 1520, 1438, 1345, 1335, 1056, 1027, 990 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.74 (s, 1 H, N-3 H), 7.30–7.04 (m, 4 H, H-5′ to H-8′), 6.45 (s, 1 H, H of N-1), 6.41 and 6.22 (s, 1 H, H-6 and H-9), 4.72 (m, 1 H, H-2′), 4.00 (d, 1 H, H-1′), 3.42 (s, 1 H, NH-5 ?), 3.03 (s, 3 H, N-10 CH_3), 2.92 (m, H-4′), 2.49 and 2.15 (m, 1 H, H-3), 2.11 and 2.02 (s, 3 H, C-7 and C-8 CH_3) [The δ 4.00 doublet (J = 8.9 Hz) collapsed upon irradiation of the δ 4.72 multiplet. The δ 7.74, 6.45 and 3.42 singlets were considerably more shielded in pyridine-d_5 (δ 11.0, 8.55, 4.45) and DMSO-d_6 (δ 10.64, 9.13, 3.55). The δ 10.64 and 9.13 singlets (DMSO-d_6) disappeared by D_2O exchange; exchange of the δ 3.55 peak could not be observed due to the peak overlap with the H_2O resonance]; ^{13}C NMR (300 MHz, DMSO-d_6, (C, CH_2) 171.7 (C-4), 150.7 (C-2), 141.7 (C-9′), 131.0, 130.0, 128.7, 126.4 and 125.7 (C-5a,-7,-8,-9a,10′), 97.7 (C-10a), 62.9 (C-4a), 28.5 (C-3′), 25.5 (C-4′); (CH, CH_3): δ 129.9, 128.0, 127.2 and 126.1 (C-5′,-6′,7′,8′), 115.7 and 112.7 (C-6, -9), 74.7 (C-2′), 48.9 (C-1′), 30.5 (N-10 CH_3), 19.0 and 18.4 (C-7 and -8 CH_3). Despite exhaustive efforts crystals suitable for X-ray analysis were not obtained. This material transformed to LF upon standing on silica gel under light. It was incapable of acetylation (Ac_2O–pyridine).

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Supplementary Material Available: Six figures illustrating the spectral changes which accompany the reactions of the dihydroflavins and the epoxides (6 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.