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A Mechanistic Study of the Dihydroflavin Reductive Cleavage of the Dihydroflavin–Tetrahydronaphthalene Epoxide Adducts[1]

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Dihydroflavins are facile reducing agents and potent nucleophiles. The dihydroflavin nucleophilic reactivity, as measured by the rate of covalent flavin adduct formation with tetrahydronaphthalene epoxides, is comparable to that of the thiolate anion (Y. T. Lee and J. F. Fisher (1993) *J. Org. Chem.* **58**, 3712). In these reactions there appears subsequent to the nucleophilic cleavage of the epoxide by the dihydroflavin the product corresponding to formal hydride reduction product (at the benzylic carbon) of these epoxides. Thus the reaction of (±)-1a,2,3,7b-tetrahydro-(1aα,2α,3β,7bα)-naphth[1,2-*b*]oxirene-2,3-diol (**1**), (±)-1a,2,3,7b-tetrahydro-(1aα,2β,3α,7bα)-naphth[1,2-*b*]oxirene-2,3-diol (**2**), and (±)-1a,2,3,7b-tetrahydro-(1aα,7bα)-naphth[1,2-*b*]oxirene (**3**) in 9:1 (v/v) aqueous Tris buffer–dioxane, at both acidic and neutral pH, with $FMNH_2$ and 1,5-dihydrolumiflavin (LFH_2) gave (following covalent flavin–epoxide adduct formation) the products having a methylene group at the benzylic position. The reduction product yield was proportional to the yield of the N(5) flavin–epoxide adduct intermediate, and the rate of the reaction was proportional to the dihydroflavin concentration. These observations are consistent with these reduction products resulting from bimolecular reaction between the dihydroflavin–epoxide adduct and a second molecule of dihydroflavin.

INTRODUCTION

A portion of the metabolism of the polycyclic aromatic hydrocarbons (PAHs) corresponds to sequential epoxidation (catalyzed by the cytochrome P-450 monooxygenases), epoxide hydrolysis (catalyzed by epoxide hydrolase), and reepoxidation (*1*). Some of the resulting diol epoxides express potent mutagenic activity via electrophilic modification of DNA (*1,2*). The nucleophilic interception of these (and other) xenobiotic-derived electrophiles is an important metabolic detoxication process. An evaluation of the reaction between three tetrahydronaphthalene epoxides (models for the much more reactive and mutagenic PAHs) with dihydroflavins, undertaken to evaluate

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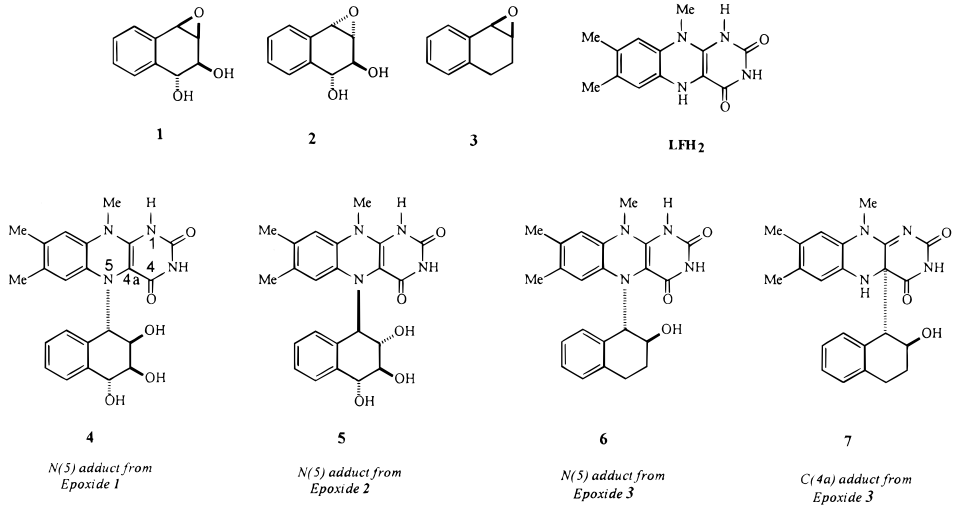
the relative capability of the dihydroflavin (FlH_2) as a reductant or as a nucleophile toward arene epoxides, established that the dominant pathway at early reaction times was nucleophilic epoxide opening by the FlH_2 to give various (depending on the identity of the nucleophilic atom of the flavins) covalent flavin–epoxide adducts (*3*). The nucleophilic capability of the dihydroflavin is known (*4*).

For example, the reaction of epoxide **1** with dihydrolumiflavin (LFH_2) in 9:1 (v/v) aqueous Tris–dioxane pH 7.86 buffer gave the flavin N(5) adduct (±)-**4** as the only major product *at early time points*. Under the identical conditions epoxide **2** gave the N(5) adduct (±)-**5**, two (±)-N(3) adducts (*cis* and *trans* substituted), and an adduct of uncertain structure (speculated to be the C(4a) or the N(1) adduct). All were formed in similar yields. Epoxide **3** gave the N(5) adduct (±)-**6** and the C(4a) adduct (±)-**7**, in approximately a 2:1 ratio. The C(4a) adduct was not stable, however, and was transformed to a stable secondary adduct.



SCHEMES 1–7

The outcome of the reaction of both **1** and **3** with $FMNH_2$ was similar to that obtained for LFH_2. In the instance of the reaction of **2** with $FMNH_2$, three initial products are formed (assigned to adducts derived from C(4a), N(5), and N(3) attack of the dihydroflavin). 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 seen at pH 7.86. All of the adducts (except for a *cis* substituted N(3) adduct from epoxide **2**) exhibited *trans* stereochemistry with respect to epoxide opening. An unusual transformation in the product distribution, as evidenced by HPLC analysis, occurred at prolonged reaction times. At these late times the product of formal hydride reductive epoxide opening, at the benzylic carbon of the arene epoxide, appeared for each epoxide.

Yang and Gelboin (*5*) have reported the nonenzymatic reduction of the highly reactive benzo[*a*]pyrene diol epoxides by the coenzyme NADPH. In this instance the

mechanism appears to be NADPH reduction of the nascent arene carbocation formed as a solvolytic intermediate. Although the capability of reduced flavins (either under enzymatic or nonenzymatic conditions) to behave likewise may be presumed (particularly in light of the mechanistic versatility of the flavin structure (*6*)), a different mechanism is required. For these naphthalene epoxides, the alcohol reduction product appears *after* nucleophilic opening of the epoxide by the dihydroflavin. Hence the formation of the alcohol is not a result of direct hydride transfer (or other electron transfer process) between the dihydroflavin and epoxide, but is a reaction of the covalent flavin–epoxide adducts. Our observations on this transformation are presented.

MATERIALS AND METHODS

Instrumentation

Melting points are uncorrected. ^{1}H NMR coupling constants are uncorrected for non-first-order behavior. Analytical HPLC used isocratic elution on a 5 μm C-18 column (4.6 mm $\times$ 25 cm) at a flow rate of 0.8 mL min^{-1}. Preparative liquid chromatography used a 10-μm C-18 column (2.1 $\times$ 25 cm), with sample injection size of 1.7 mL and a flow rate of 4.5 mL min^{-1}. Detection was at 210 nm. The mobile phases are given in the experimentals.

Materials

Pt/asbestos catalyst was obtained from Baker. FMN, riboflavin, NADPH (type III), and isocitrate dehydrogenase (9.5 mg protein mL^{-1}, 3.1 units (mg protein)$^{-1}$) were obtained from Sigma. The epoxides (*7*) and lumiflavin (*3,8*) were prepared by published procedures. Spinach ferredoxin–NADP$^+$ reductase was purified by the method of Zanetti and Curti (*9*). The final enzyme solution contained 59 μM enzyme, 160 mM NaCl, 0.2 mM EDTA, and 0.2 mM dithiothreitol in 63.3 mM Tris pH 7.4 buffer.

*(±)-1,2,3,4-Tetrahydro-(1α,2β,3β)-naphthalene-1,2,3-triol (**8a**).* The authentic material for comparison to the triol product from the FlH_2-epoxide **1** adduct was made by $NaBH_3CN$–$BF_3{\cdot}Et_2O$ reduction (*10*) of epoxide **1**, mp 138–139°C (MeOH–benzene) (Found: C, 66.34; H, 6.81. $C_{10}H_{12}O_3$ requires C, 66.65; H, 6.71%); TLC R_f (silica gel) = 0.39 (10:1 EtOAc/abs EtOH); δ_H (300 MHz; CD_3CN) (*J* values in Hz) 7.43–7.07 (m, 4 H, aryl), 4.62 (d, 1 H, 1-H, $J_{1,2}$ 6.4), 4.15 (m, 1 H, 3-H), 3.74 (dd, 1 H, 2-H, $J_{2,3}$ 2.1), 3.00 (dd, 1 H, 4-H *cis* to 3-H, $J_{3,4(cis)}$ 4.7, $J_{4,4'}$ 17.0), 2.85 (dd, 1 H, 4-H *trans* to 3-H, $J_{3,4(trans)}$ 6.1); δ_C (300 MHz, CD_3OD) C: 137.69, 135.47; CH: 130.18, 129.81, 128.57, 127.28, 75.54, 73.03, 68.67; CH_2: 35.08; *m/z* (EI, 70 eV) 162 (M^+-H_2O, 100%), 149 (33), 144 (M^+-2H_2O, 52), 120 (52), 119 (50); *m/z* (FAB, glycerol matrix) 457 ([MH^+ + (3 $\times$ matrix)], 8%), 365 ([MH^+ + (2 $\times$ matrix)], 26), 273 ([MH^+ + (1 $\times$ matrix)], 51), 255 ([MH^+ − H_2O + matrix], 20), 179 ([M − H]$^+$, 6), 163 ([M − H_2O]$^+$, 100), 145 ([M − 2H_2O]$^+$, 74), 117 (78).

*(±)-(1,2,3)-Tri-O-acetyl-1,2,3,4-tetrahydro-(1α,2β,3β)-naphthalene-1,2,3-triol (**8b**).* Triol **8a** was acetylated (Ac_2O–pyridine) and the product recrystallized from benzene–hexane, mp 104–105°C; δ_H (300 MHz, CD_3CN) 7.33–7.14 (m, 4 H, aryl), 6.23 (d, 1 H, 1-H, $J_{1,2}$ 6.1), 5.53 (ddd, 1 H, 3-H), 5.36 (dd, 1 H, 2-H, $J_{2,3}$ 2.1), 3.83 (dd, 1 H, 4-H *trans* to 3-H, $J_{3,4(trans)}$ 7.0), 3.22 (dd, 1 H, 4-H *cis* to 3-H, $J_{3,4(cis)}$ 4.8,

$J_{4,4'}$ 17.1), 2.13 (s, 3 H), 2.06 (s, 6 H); *m/z* (EI, 20 eV, high resolution) (*11*) 246 (M^+ − AcOH, 0.8%, +1.0 mmu), 204 (M^+ − AcOH − CH_2CO, 3, +0.6 mmu), 186 (M^+ − 2AcOH, 16, −0.9 mmu), 161 (21), 144 (M^+ − 2AcOH − CH_2CO, 100, +1.0 mmu).

*(±)-1,2,3,4-Tetrahydro-(1α,2β,3α)-naphthalene-1,2,3-triol (**9a**).* The reduction of **2** with $NaBH_3CN/BF_3{\cdot}Et_2O$ to prepare for authentic material for comparison to the triol product from the FlH_2–epoxide **2** reaction was unsuccessful; hence an alternative procedure was used. Epoxide **2** (25 mg, 0.14 mmol) in Et_2O (15 mL) was added to a solution of $LiAlH_4$ (100 mg, 2.6 mmol) in Et_2O (4 mL). After 2 h, the reaction mixture was cooled and quenched by the successive addition of water (0.1 mL), 15% aq NaOH (0.1 mL), and a second water portion (0.3 mL). The solid was removed by filtration. The filtrate was dried (K_2CO_3) and the solvent evaporated. The product was purified by preparative silica TLC (R_f 0.25 in 3:1 Et_2O/EtOAc) to give **9a** (6.6 mg, 26%), mp 150–151°C (MeOH–benzene); TLC R_f (silica gel) = 0.43 (10:1 EtOAc/abs EtOH); δ_H (300 MHz, CD_3CN) 7.47–7.07 (m, 4 H, aryl), 4.44 (d, 1 H, 1-H, $J_{1,2}$ 8.1), 3.75 (ddd, 1 H, 3-H), 3.44 (dd, 1 H, 2-H, $J_{2,3}$ 9.4), 3.08 (dd, 1 H, 4-H *cis* to 3-H, $J_{3,4(cis)}$ 5.7, $J_{4,4'}$ 16.3), 2.74 (dd, 1 H, 4-H *trans* to 3-H, $J_{3,4(trans)}$ 10.1); δ_C (300 MHz, CD_3OD) C: 138.93, 134.26; CH: 129.55, 128.58, 128.40, 127.51, 79.54, 75.11 70.79; CH_2: 38.08; *m/z* (EI, 20 eV) 180 (M^+, 10%), 162 ($M^+ - H_2O$, 24), 144 ($M^+ - 2H_2O$, 100), 120 (58), 119 (48), 116 (96); *m/z* (FAB, glycerol matrix) 457 ([MH^+ + (3 × matrix)], 11%), 365 ([MH^+ + (2 × matrix)], 33), 273 ([MH^+ + (1 × matrix)], 71), 255 ([$MH^+ - H_2O$ + matrix], 20), 179 ([M − H]$^+$, 7), 163 ([$M-H_2O$]$^+$, 100), 145 ([$M-2H_2O$]$^+$, 77), 117 (82).

*(±)-(1,2,3)-Tri-O-acetyl-1,2,3,4-tetrahydro-(1α,2β,3α)-naphthalene-1,2,3-triol (**9b**).* Acetylation (Ac_2O/pyridine) of the triol **9a** gave a triacetate; δ_H (300 MHz, $CDCl_3$) 7.34–7.12 (m, 4 H, aryl), 6.17 (d, 1 H, 1-H, $J_{1,2}$ 7.0), 5.42 (dd, 1 H, 2-H, $J_{2,3}$ 9.2), 5.22 (ddd, 1 H, 3-H), 3.26 (dd, 1 H, 4-H *cis* to 3-H, $J_{3,4(cis)}$ 5.4, $J_{4,4'}$ 15.9), 3.01 (dd, 1 H, 4-H *trans* to 3-H, $J_{3,4(trans)}$ 9.5), 2.17 (s, 6 H), 2.13 (s, 3 H); *m/z* (EI, 30 eV, high resolution) 204 (M–AcOH–CH_2O, 2%, +1.3 mmu), 186 (M–2AcOH, 14, +0.1 mmu), 161 (23), 145 (92), 144 (M–2AcOH–CH_2O, 100, +1.7 mmu), 43 (80).

*(±)-1,2,3,4-Tetrahydro-2-naphthalenol (**10**).* Authentic material for comparison to the alcohol product from the FlH_2–epoxide **3** adduct. Tetralone (0.5 g, 3.42 mmol) in Et_2O (5 mL) was added dropwise to a solution of $LiAlH_4$ (0.26 g, 6.84 mmol) in Et_2O (10 mL). After stirring for 30 min, the reaction mixture was cooled, and water (0.3 mL) and 2 N aq H_2SO_4 (2 mL) were added successively. The mixture was filtered. The filtrate was dried (K_2CO_3) and then evaporated to leave a colorless liquid. The crystals, obtained from petroleum ether, melted in the process of isolation. δ_H (300 MHz, $CDCl_3$) 7.24–7.07 (m, 4 H, aryl), 4.16 (m, 1 H, 2-H), 3.08 (dd, 1 H, 1-H *cis* to 2-H, $J_{1,1'}$ 16.2, $J_{1,2(cis)}$ 4.7), 2.83 (ddd, 1 H, 4-H, $J_{3,4}$ 5.8, 5.8, 6.2, and 9.0, $J_{4,4'}$ 17.0), 2.76 (dd, 1 H, 1-H *trans* to 2-H, $J_{1,2(trans)}$ 7.5), 2.05 (m, 1 H, 3-H), 1.82 (m, 1 H, 3-H), *m/z* (EI, 70 eV) 148 (12, M^+), 130 (100, M^+-H_2O), 104 (82, $M^+-H_2O-C_2H_4$).

Reaction of the Epoxides with Catalytically Generated FlH_2

The reactions of the epoxides (1 mM) with $FMNH_2$ (2 mM) or LFH_2 (1 mM at neutral pH, much less than 1 mM at acidic pH) were run in the two pH regions for

epoxide hydrolysis (hydronium-ion-catalyzed and the pH-independent) (*12*) using a 9:1 (v/v) 20 mM buffer–dioxane solvent at 25°C (*3*).

Quantification of Reduction Products

Yields were determined from the HPLC peak areas of the products relative to an internal standard. The raw peak integration was corrected for the respective $\lambda_{210\ nm}$ differences by determination of the relative peak intensities for standard solutions of the epoxides and reduction products. These ratios were 1:8a = 1.49 and 3:10 = 1.41. The 2:9a ratio was taken to be identical to that for **1 : 8a**. The internal standards were *cis*-1,2-indanediol (*13*) (for **1** and **2**) and 2-indanol (for the reaction of **3**).

Preparative HPLC Separation of the Reduction Products

The reduction products were isolated using mobile phases of 20:80 CH_3CN/H_2O for **8a** and **9a**, and 40:60 CH_3CN/H_2O for **10**.

Reaction of the Epoxides with Enzymatically Generated $FMNH_2$ and 1,5-Dihydroriboflavin

Final concentrations are given parenthetically. Sodium isocitrate (3.5 mg, 10 mM) and $MnCl_2 \cdot 4H_2O$ (1.3 mg, 5 mM) were placed in a Schlenk tube under N_2. Deoxygenated solutions (by repetitive N_2 evacuation and equilibration) of 1.0 mL 95:5 20 mM Tris pH 7.9 buffer–dioxane, 0.1 mL of 1.4 mM NADPH (0.1 mM), and 0.1 mL of 1.4 mM FMN or riboflavin (0.1 mM) were added by syringe. After further N_2 deoxygenation, the catalysts were added (50 μL of isocitrate dehydrogenase, 90 units/mL, and 10 μL of ferredoxin-$NADP^+$ reductase, final concentration of 4 μM). The reaction was initiated with addition of 30 μL of 10^{-2} M epoxide (0.2 mM) in dioxane. After 2 days, 0.2 mL of the reaction mixture was withdrawn and evaporated to dryness. The residue was dissolved in MeOH (0.3 mL) and filtered for HPLC analysis. Control reactions omitted the flavin.

Chemical Competence of Adducts to Produce the Reduction Products

The N(5) adducts were not stable to isolation. Thus, an attempt was made to prepare them, by catalytic hydrogenation, from the pseudobase. A dioxane solution of the pseudobase, obtained from air oxidation of the reaction of **3** with LFH_2, was added to a deoxygenated solution of 9:1 20 mM Tris pH 7.8 buffer–dioxane containing Pt/asbestos catalyst. Hydrogen gas was bubbled through the solution for a few minutes, and the solution was then set aside for 10 h. HPLC analysis showed that the pseudobase was unreactive. Repetition of this hydrogenation reaction, at pH 4.9, also showed no evidence of N(5) adduct formation.

Likewise the C(4a) adduct was unstable to isolation. The HPLC eluent (60:40 MeOH/0.01% aq Na_2HPO_4) containing this adduct was combined with Tris buffer to give 9:1 (v/v) Tris pH 8 buffer–MeOH solution, and this solution was set aside overnight. HPLC analysis showed the formation of LF and of the hydrolysis products (*cis* and *trans* diols) of the epoxide, in addition to some unknown products. No reduction product was detected.

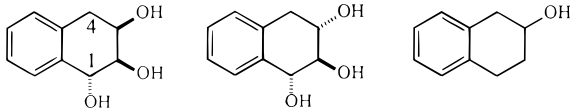
Rate Dependence of the Reduction on FlH_2 Concentration

This reaction used the same procedures as described above. To minimize the pH change resulting from different concentrations of $FMNH_2$, the pH of the FMN solution was adjusted with NaOH to pH 7.9 prior to reduction. The concentrations of the reactants were 1 and 2 mM (epoxide) and 1, 2, and 4 mM ($FMNH_2$). Ionic strength was held constant at 0.1 M with KCl. The reaction mixtures were analyzed at two time points: first, at the time when the disappearance of epoxide was nearly complete and, second, at the time when formation of the reduction product was approximately half of the final yield. Each reaction was done in duplicate.

RESULTS

Reaction of the Epoxides with Nonenzymatically Generated FlH_2

HPLC analysis (direct injection of reaction aliquots) of the reaction of **1** with LFH_2 at pH 7.86 demonstrated the disappearance of epoxide (approximately 75% within 1.5 h) (Fig. 1, Set Ia). No distinct adduct peak was seen. The new peak at t_R 4.8 min appearing at 10 h reaction time (Fig. 1, Set Ib) was identified as (±)-1,2,3,4-tetrahydro-(1α,2β,3β)-naphthalene-1,2,3-triol (±)-**8a** on the following basis. The product of $BF_3{\cdot}Et_2O$ catalyzed $NaBH_3CN$ reduction of **1** was identical to the product of the dihydroflavin–epoxide reaction. Its ^{1}H NMR spectrum exhibited diastereotopic methylene hydrogen resonances at δ 3.00 and δ 2.85, forming the AB parts of an ABX pattern ($J_{A,B} = -17$ Hz and $J_{A,X} = 6.1$, $J_{B,X} = 4.7$ Hz). The ^{13}C NMR spectrum, mass (EI and FAB) spectra, and elemental analysis data confirm the triol structure. Peracetylation (Ac_2O/pyridine) gave a triacetyl derivative **8b**, having in its ^{1}H NMR a 6 H singlet at δ 2.06 and a 3 H singlet at δ 2.13 and the unambiguous benzylic methylene ($J_{4,4'} = -17.1$ Hz, $J_{3,4}$ and $J_{3,4'} = 7.0$ and 4.8 Hz). The fragmentation pattern of the EI mass spectrum was that of a triacetyl derivative.



8a, R=H
8b, R=Ac

9a, R=H
9b, R=Ac

10

SCHEMES 8–10

At 1.5 h reaction time, despite 75% loss of epoxide, little if any **8a** was seen. At 10 h reaction time, despite nearly complete loss of the epoxide, [**8a**] was only one-third that of the starting epoxide. A mechanistic pathway explaining the discrepancy between the epoxide disappearance and the triol appearance was revealed by an HPLC analysis of an *oxidized* reaction mixture. When a reaction aliquot (from the reaction of **1** and dihydrolumiflavin) was removed, O_2 oxidized, and left to stand in the dark for 3 h prior to HPLC analysis, the resulting chromatogram (Fig. 1, Set II) showed a new peak (t_R 8.5 min). This peak was identified (*3*) as the 5-alkyl-4a-hydroxy pseudo base (*4,14*) derived from the flavin–epoxide N(5) adduct. *Hence the triol*