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Synthesis, physico-chemical and biological properties of 5- and 6-fluoro-1,2,3,4-tetrahydro-7,12-dimethylbenz(a)anthracene

Abood, Norman Anthony, Ph.D.

The Ohio State University, 1987
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OF 5- AND 6-FLUORO-1,2,3,4-TETRAHYDRO-
7,12-DIMETHYLBENZ(a)ANTHRACENE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Norman Anthony Abood, B.S., M.S., R.Ph.

*****

The Ohio State University
1987

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DEDICATION

To Mom, Dad and Karen
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PUBLICATIONS


FIELDS OF STUDY

Major Field: Organic Medicinal Chemistry
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<tr>
<td>1. Benz(a)anthracene</td>
<td>BA</td>
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<tr>
<td>2. Benzo(a)pyrene</td>
<td>B(α)P</td>
</tr>
<tr>
<td>3. 7,12-Dimethylbenz(a)anthracene</td>
<td>DMBA</td>
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<td>5. High performance liquid chromatography</td>
<td>HPLC</td>
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<td>6. Human neonatal foreskin fibroblast</td>
<td>HNF</td>
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<td>7. Lithium aluminum hydride</td>
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Polynuclear aromatic hydrocarbons (PAH) are ubiquitous environmental pollutants formed by the incomplete combustion of fossil fuels and other carbonaceous materials.\textsuperscript{1,2} Thousands of these compounds are carcinogenic\textsuperscript{3-5} and the past 50 years has seen an intense effort to characterize these materials and delineate the mechanisms by which they induce carcinogenesis. A vast majority of these investigations support the proposal that PAH are metabolized to reactive electrophilic intermediates which initiate a carcinogenic response following covalent bonding to DNA.\textsuperscript{6-10}

The two PAH that have received the most attention are benzo(a)-pyrene (B(a)P,\textsuperscript{1}) and 7,12-dimethylbenz(a)anthracene (DMBA\textsuperscript{2}). B(a)P was the first identified highly carcinogenic PAH isolated from combustion products\textsuperscript{11} and DMBA was found to be the most potent PAH skin carcinogen in rodents.\textsuperscript{12}

Considerable evidence suggests that for B(a)P, the ultimate carcinogen is the B(a)P-7,8-dihydrodiol-9,10-epoxide(\textsuperscript{5}).\textsuperscript{13-17} B(a)P is converted to \textsuperscript{5} in three steps (Scheme 1). The monooxygenase enzyme cytochrome P-450 catalyzes the conversion of B(a)P to the 7,8-epoxide. Hydratase mediated epoxide ring opening by a \textit{trans} addition of water to the nonbenzylic position provides \textit{trans}-dihydrodiol \textsuperscript{4}.\textsuperscript{5}
Scheme 1: Structure and metabolism of \( \text{B(\(a\))P} \) and DMBA leading to carcinogenic diol-epoxides.
Subsequent cytochrome P-450 metabolism provides dihydrodiol-epoxide via epoxidation of the 9,10-double bond. The enzymes behave in a highly enantioselective manner to give predominantly the (+)7R,8S-diol-9S,10R-epoxide (absolute configuration shown in Scheme 1). Interestingly, of the four stereochemical possibilities, this enantiomer is the most potent skin tumor initiator and lung adenoma inducer in mice. Similarly, DMBA-3,4-dihydrodiol-1,2-epoxide (8) has been implicated as the ultimate carcinogen. However, DMBA appears to undergo nonenantioselective metabolism to give the two racemic syn and anti diastereoisomers of the 3,4-dihydrodiol-1,2-epoxide (syn and anti refer to the relative orientation of the 1,2-epoxide to the benzylic hydroxyl moiety).

Cytochrome P-450 is an NADPH dependent hemoprotein oxidase enzyme. Cytochrome P-450 activates molecular oxygen to the highly reactive, electron deficient, valence isomeric iron species (Scheme 2). The catalytic sequence involves 1) binding of the substrate, 2) NADPH mediated reduction of NADPH cytochrome P-450 reductase, 3) transfer of one of the electrons made available to the heme-iron(III) complex, 4) binding of molecular oxygen to give a cytochrome P-450-dioxygen complex, 5) transfer of the second electron from cytochrome P-450 reductase, 6) cleavage of the oxygen-oxygen bond with concurrent incorporation of the distal oxygen atom into a water molecule, and 7) transfer of the second oxygen atom to the substrate and dissociation of the product.
Scheme 2: Catalytic cycle for oxygen activation and oxidative metabolism by cytochrome P-450. Brackets denote the prosthetic heme group.

The iron(III) species oxidizes aliphatic and aromatic hydrocarbons (Scheme 3). A nonconcerted mechanism has been proposed as the predominant pathway for aliphatic and allylic hydroxylation based on evidence supporting isotopic scrambling, racemization and allylic rearrangements of hydrocarbon substrates (reviewed in Ref. 29). However, 7α- and 11α-hydroxylation of steroids and benzylic
hydroxylation of ethyl benzene\textsuperscript{32} occur with high retention of stereochemistry and may represent instances where the receptor site maintains the conformation of the substrate. Thus, abstraction of hydrogen radical (Scheme 3, path a) generates a free radical and a ferric bound hydroxyl radical, 18, which undergoes radical combination to form the hydroxylated product 19. Aromatic hydroxylation seems to occur by two mechanisms: 1) epoxidation (path b or c) followed by chemical ring opening, hydride migration and keto-enol tautomerization (NIH shift)\textsuperscript{33} (path d), or 2) direct insertion of oxene oxygen into the aryl hydrogen bond (path e).\textsuperscript{34}

The NIH shift is supported by experiments with 1-[\textsuperscript{2}H]- and 2-[\textsuperscript{2}H]-naphthalene whereby hydroxylation at the site of deuterium substitution occurs with partial retention of deuterium fixed at the adjacent position as governed by a kinetic deuterium isotope effect.\textsuperscript{33} Based upon kinetic experiments using 3,5-di-[\textsuperscript{2}H]- and 1,4,6-tri-[\textsuperscript{2}H]-monosubstituted benzenes, Hanzlik, et al.\textsuperscript{33} were able to calculate the fractional rate of hydroxylation occurring by direct insertion or NIH shift of the epoxide and found that direct insertion competes efficiently depending upon the nature of the substituent and the site of hydroxylation. The mechanism for the epoxidation of aromatic molecules (i.e., concerted electrophilic addition of oxygen (path c) or stepwise oxidation via a cation radical intermediate (path b)) is uncertain, however kinetic data obtained from the P-450 mediated metabolism of the monohalobenzene series (Ar-X, X=F,Cl,Br,I) suggests the latter.\textsuperscript{35}
Scheme 3: Proposed pathways for the hydroxylation of aliphatic and aromatic hydrocarbons.

In 1976, Jerina proposed the bay-region hypothesis to predict which PAH would be carcinogenic and which regioisomeric metabolites would be principally responsible for initiation of the carcinogenic event. According to this hypothesis, "...the diol epoxides of angular benzo-rings in which the epoxide group formed part of a bay-region of the carcinogenic hydrocarbons would be prime candidates as ultimate carcinogens if metabolically formed." B(α)P and DMBA,
as well as several other carcinogenic PAH, fit the prediction of the bay-region hypothesis. Additionally, enantioselective metabolism studies of various sites in the B(α)P nucleus lead to the proposed steric model for the catalytic binding site of cytochrome P-450c (Figure 1). For oxidation of PAH, rat liver cytochrome P-450c is the most active (50-200 fold) of three P-450 isozymes.

![Steric model for the catalytic binding site of P-450c.](image)

Figure 1. Steric model for the catalytic binding site of P-450c. Heavy lines represent boundaries derived from the enantioselective epoxidation of B(α)P; shaded areas are regions of forbidden binding; dashed rings are areas of allowed binding for other PAH (taken from Ref. 19, p. 257).

This model has been successful in predicting the enantioselective epoxidation of a number of PAH including B- and D-ring metabolites of DMBA. However, it fails to explain the apparent loss of enantioselective metabolism in the A-ring. Notably, Jerina observed that the 3,4-epoxide precursor to the bay-region dihydrodiol-epoxide of benz(a)anthracene (BA), unlike the B- and D-ring monoepoxides and
the various monoepoxides of B(α)P, racemizes via an oxepine intermediate (Scheme 4). Additionally, A-ring metabolism of DMBA by cytochrome P-450c does not fit within the minimal boundaries proposed in the steric model; thus, these boundaries have been expanded (dotted lines, Figure 1).

Scheme 4: Racemization of benz(a)anthracene-3,4-epoxide.

Jerina also points out that conformational and electronic parameters influence the tumorigenic activity of diol epoxide metabolites. Significant tumorigenicity was confined only to those diol epoxides having preferred pseudodiequatorial hydroxyl group conformations and 1) a sterically hindered bay region or 2) a fairly high reactivity. High reactivity refers to the propensity of these diol epoxides to form stabilized arylmethyl carbocations via solvolysis of the epoxide. Interestingly, bay-region arylmethyl cations have higher $E_{\text{deloc}}/\beta$ (a quantum chemical measure of cation stability) than nonbay-region cations. Invariably, bay-region methyl cations are substituted at $\alpha$-positions on the remaining aromatic nucleus. Methyl cations at $\beta$-positions (nonbay-region) are stabilized to a lesser degree by the aromatic nucleus; these correspond to a lower $E_{\text{deloc}}/\beta$ (Figure 2).
Figure 2. Stabilization of arylmethyl cations at bay-region and nonbay-region sites in BA and B(α)P (Taken from ref. 37).
These steric and electronic effects will be illustrated later as part of a discussion on SAR of methylated and fluorinated analogues of B(α)P, BA and DMBA.

A second hypothesis on the carcinogenesis of PAH that has received lesser attention is the cation radical hypothesis advanced by Cavalleri and Rogan.\(^{38}\) This hypothesis attempts to explain trends in the carcinogenicity of various PAH systems based upon the ability of these compounds to undergo one-electron oxidation to form radical cations. PAH with relatively low ionization potentials (<7.35 eV)\(^{39}\) form radical cations in vitro in the presence of horseradish peroxidase or prostaglandin H synthetase. In order for a PAH radical cation to elicit a carcinogenic response, a high degree of localization of the positive charge is necessary thus providing for sufficient reactivity with cellular nucleophiles. B(α)P has been shown to undergo metabolism in vitro with horseradish peroxidase and subsequently to covalently bond to DNA via nucleophilic attack at C-6 (Scheme 5), the site of highest charge localization.\(^{38,40}\) 6-Methyl-B(α)P [MB(α)P] also covalently bonds with DNA nucleophiles through the C-6 methyl group (Scheme 6) owing to localization of charge density on carbon adjacent to the methyl group.\(^{41}\) It remains to be determined whether these hydrocarbon adducts are responsible for initiating carcinogenesis.
Scheme 5. Mechanism of trapping of B(\(\alpha\))P radical cation by a nucleophile (Nu).

Scheme 6. Stepwise one-electron oxidation of 6-methylB(\(\alpha\))P and subsequent trapping by a nucleophile (Nu) (taken from Ref. 43, p. 291).
Interestingly, in nucleophilic trapping experiments, cation radicals of DMBA (in an I\textsubscript{2}/pyridine system\textsuperscript{42}) formed pyridinium adducts at C-5, 7-CH\textsubscript{3} and 12-CH\textsubscript{3}. In addition, evidence accumulated on the differential metabolism of selected PAH on mouse skin vs rat mammary gland suggest that those compounds which induce carcinogenesis in the latter do so via a peroxidase mediated pathway. Cavalieri reasoned that the monooxygenase system, required for epoxide formation, is not operative in this tissue\textsuperscript{43} since it was reported\textsuperscript{14} that B(\alpha)P-7,8-dihydrodiol is not a potent carcinogen in rat mammary gland. However, later, Marnett observed prostaglandin H synthetase mediated epoxidation of B(\alpha)P-dihydrodiol \textit{in vitro} and this led to covalent bonding of the PAH.\textsuperscript{44-46}

Intercalative binding of hydrocarbon metabolites to DNA also appears to be an important event for potent carcinogenic PAH. Ultimate PAH dihydrodiol-epoxide metabolites have been shown to undergo noncovalent intercalation prior to covalent bonding.\textsuperscript{47-49} Proximate carcinogens (e.g., B(\alpha)P-7,8-dihydrodiol) also bond to DNA via intercalation.\textsuperscript{50} This bonding has been proposed as a mechanism for \textit{in vivo} intranuclear pooling of dihydrodiol metabolites.\textsuperscript{50} Once localized, this PAH may be subject to further metabolism by nuclear membrane bound cytochrome P-450.\textsuperscript{51,52} Interestingly, 90% of the DNA intercalated B(\alpha)P-7,8-dihydrodiol-9,10-epoxide is converted to tetrols while 10% or less form covalent DNA adducts.\textsuperscript{49}

Surprisingly, alkyl and alicyclic groups on the aromatic nucleus appear to enhance the intercalative bonding of PAH.\textsuperscript{48} Owing to electronic contributions, these groups destabilize the highest
occupied orbitals, thus lowering the ionization potential of the aromatic nucleus. This seems to be more important than steric contributions of small alkyl groups which would serve to inhibit intercalation. Presumably, lowering of the ionization potential favors PAH-nucleotide base stacking in DNA because of a favored "charge transfer" complex between the orbitals of the overlapping aromatic rings.48

9,10-Dimethylanthracene (20) and 1,2,3,4-tetrahydro-7,12-dimethylbenz(a)anthracene(TH-DMBA; 21), which are metabolism models for the anthracene ring system produced as a result of A-ring metabolism in DMBA, respectively, intercalate 6.7 and 3.5 times better than anthracene.48 Additionally, TH-DMBA binds 10.8 times better than 5,6-dihydrobenz(a)anthracene, the K-region metabolism model for the very weak carcinogen DMBA-5,6-epoxide.48 Similar results were observed when metabolites and metabolism models of B(α)P were employed in intercalative studies.47

![Chemical structures](attachment:chemical_structures.png)

A popular approach employed to elucidate possible sites of metabolic activation has involved use of monosubstituted PAH derivatives. For example, methyl substitution at the site of metabolism might be expected to block carcinogenicity as a result of decreased metabolism,53-55 but methyl group oxidation may complicate the
situation. Fluorine substitution for hydrogen also has been used to probe critical sites of metabolic activation. Advantages include:

1) apparent C-F bond stability and 2) fluorine size (respective van der Waals radii for F and H are 1.35Å and 1.2Å). Thus, fluorine has been proposed to block metabolism at the site of substitution with minimal steric effects at sites proximal to fluorine in the molecule.

All possible monomethylated B(α)P have been synthesized and their ability to exhibit two-stage skin tumor-initiating activity in female SENCAR mice has been reported (Table 1). For the first time, these data allowed for a reliable comparison of relative potencies in the same assay system. Consistent with the bay-region hypothesis, methyl substitution at positions 7, 8, 9 and 10 in B(α)P abolishes tumor-initiating activity at 200 nmol doses. However, these data contradict findings that 7-MB(α)P and B(α)P exhibit similar sarcomagenic activities in male Long Evans rats when injected subcutaneously (2.5 mg and 1.0 mg, respectively). Methyl substitution at positions 2, 5 and 6 results in PAH having weak tumor-initiating activity, whereas 3- and 4-MB(α)P have potencies similar to those observed for B(α)P. Interestingly, 11-MB(α)P is three fold more potent than B(α)P. This may, in part, be explained by increased bay-region steric strain caused by substitution at the 11 position.
Table 1: Skin Tumor-Initiating Activity of Various Monomethylated Derivatives of B(\(\alpha\))P after TPA Promotion (Taken from Ref. 60).

B(\(\alpha\))P and its monomethylated derivatives were applied topically at a dose of 200 nmol, followed 1 week later by twice-weekly applications of 2\(\mu\)g of TPA. Results were determined at 15 weeks.

<table>
<thead>
<tr>
<th>Initiator</th>
<th>No. of mice</th>
<th>Papillomas/mouse</th>
<th>% of mice with tumor</th>
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<tbody>
<tr>
<td>Control (TPA promotion only)</td>
<td>29</td>
<td>0.1</td>
<td>6</td>
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<tr>
<td>B((\alpha))P</td>
<td>30</td>
<td>2.2</td>
<td>67</td>
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<tr>
<td>1-Methyl-B((\alpha))P</td>
<td>29</td>
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<tr>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8-Methyl-B((\alpha))P</td>
<td>28</td>
<td>0.03</td>
<td>3</td>
</tr>
<tr>
<td>9-Methyl-B((\alpha))P</td>
<td>29</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10-Methyl-B((\alpha))P</td>
<td>29</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>11-Methyl-B((\alpha))P</td>
<td>30</td>
<td>5.6</td>
<td>90</td>
</tr>
<tr>
<td>12-Methyl-B((\alpha))P</td>
<td>29</td>
<td>1.9</td>
<td>69</td>
</tr>
<tr>
<td>7,8-Diol-9-Methyl-B((\alpha))P</td>
<td>28</td>
<td>0.07</td>
<td>7</td>
</tr>
<tr>
<td>7,8-Diol-B((\alpha))P</td>
<td>30</td>
<td>2.53</td>
<td>77</td>
</tr>
</tbody>
</table>

Unfortunately, a detailed explanation of these results cannot be devised, since no definitive metabolism studies have been conducted. Attempts to address why the bay-region blocked 7-MB(\(\alpha\))P PAH is carcinogenic through metabolism studies\(^{64,65}\) revealed that a 7,8-dihydrodiol does form, but to a relatively small extent. However, 7-MB-(\(\alpha\))P-7,8-dihydrodiol is not mutagenic in the Ames assay (with microsomal activation), but in 10T1/2 cells 7-MB(\(\alpha\))P was covalently bonded to DNA at 1/8 the level of B(\(\alpha\))P.\(^{65}\) This bonding was observed in the absence of 7,8-dihydrodiol formation. Thus, the site of metabolism...
important to carcinogenesis for 7-MB(α)P remains unknown. Furthermore, no correlation was found between bacterial mutagenicity and tumor initiating activity in the methylated B(α)P series.\(^6\) Nonetheless, correlations were observed between calculated bay-region carbonium ion stability and carcinogenicity.\(^6\) Such calculations predict that the 11-methyl isomer is most carcinogenic.

In contrast to hydroxymethyl-B(α)P analogues, various halomethyl-B(α)P PAH were found to be among the most potent direct acting mutagens in the Ames assay.\(^6\) These compounds are representative of possible reactive arylmethyl carbonium ion intermediates formed either by one-electron oxidation,\(^3\) or through solvation of the esterified hydroxymethyl metabolite.\(^6\) The first direct evidence for the latter mechanism resulting in a mutagenic product was described by Watabe et al.\(^7\) for the 6-hydroxymethyl sulfate ester of DMBA. 7-Hydroxymethyl-DMBA (7-HM-DMBA), while being a potent carcinogen, is not mutagenic in the Ames assay in the absence of microsomal enzymes. When 7-HM-DMBA was incubated with Wistar rat liver cytosolic sulfotransferase, the product was more mutagenic than DMBA or 7-HM-DMBA in the presence of hepatic microsomal monooxygenase. The mutagenicity of the purified product was lost upon treatment with sulfatase or NaOH.

6-MB(α)P is a potent carcinogen upon injection into fetal Swiss mice,\(^7\) but was a weak carcinogen when painted on mouse skin.\(^6\) Formation of 6-MB(α)P-7,8-dihydrodiol is 1/6 that of B(α)P-7,8-dihydrodiol in rat liver microsomes.\(^7\) For the corresponding epoxides, formation of 6-MB(α)P-7,8-dihydrodiol-9,10-epoxide is 1/2 that of B(α)P-7,8-dihydrodiol-9,10-epoxide.\(^7\) This metabolic behavior was
attributed to a peri steric effect of the 6-methyl group inhibiting bay-region metabolism to the proximate 7,8-dihydriodiol carcinogen and to a peri steric effect altering the conformation of this dihydriodiol thus retarding epoxidation at the 9,10- position. Furthermore, the 6-peri methyl group forces the resulting trans-7,8-dihydriodiol to adopt a pseudodiaxial orientation. The dramatic decrease in carcinogenic potency of several peri methylated PAH was attributed to such a steric effect on dihydriodiol conformation (Figure 3). 

![Figure 3: 6-Peri effect on the conformation of bay-region B(a)P-7,8-dihydriodiol.](image)

Only the 6-, 7-, 8-, 9- and 10-fluoro-B(a)P [FB(a)P] analogues have been synthesized and evaluated. In addition to the size difference (F is 40% larger than H), the aryl-fluorine bond dissociation energy (116 kcal/mol for fluorobenzene) is greater than the bond dissociation energy for the aryl-hydrogen bond (103 kcal/mol) in benzene. Thus, fluorine has been proposed to block or reduce metabolism at the formal double bond to which it is attached as well as at adjacent peri positions. Direct insertion of oxygen between the C-F vs C-H bond should be inhibited owing to the greater bond strength of the former. Strongly electronegative fluorine
withdraws electron density from the formal double bond to which it is linked thus retarding epoxidation at the site of substitution.\textsuperscript{78,79}

However, if epoxidation were to occur, the product likely would undergo internal rearrangements in preference to reaction with nucleophiles. Examples of $\alpha$-fluoroepoxides are unknown, however, $\alpha$-chlorostyrene oxide (22) rearranges violently affording chloroacetophenone (23) and chlorophenylacetaldehyde (24). Consequently, if fluoroarene oxide 25 were to have been formed it would be expected to rearrange affording $\alpha$-fluorophenol (26). Alternatively, epoxide hydrolase might hydrate the epoxide and the resulting fluorinated dihydrodiol 27 would be expected to spontaneously decompose to catechol 28 which might further oxidize to quinone 29.\textsuperscript{79} However, neither of these pathways have been substantiated.

Scheme 7: Rearrangements of Haloepoxides.
As predicted by the bay-region hypothesis, the 7-, 8-, 9- and 10-FB(α)P analogues (at 400 nmol) were all significantly less tumorigenic than B(α)P (at 200 nmol) upon topical administration to female SENCAR mice (Table 2). Furthermore, hepatic metabolism of 7- and 8-FB(α)P did not produce the 7,8-dihydriodiol nor did the 9- and 10-FB(α)P produce the 9,10-dihydriodiol.

Table 2: Skin Tumor-Initiating Activity of B(α)P and 5 of its Isomeric Monofluorinated Derivatives. (Taken from Ref. 79)

Thirty female SENCAR mice received a single topical application of compound in 200μl of acetone. One week later, the mice received twice-weekly topical applications of TPA (3.2 nmol) in 200μl of acetone. From 28 to 30 mice in each experimental group were alive at the end of 16 weeks of tumor promotion.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose</th>
<th>% of mice with papillomas</th>
<th>Papillomas/mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>B(α)P</td>
<td>200</td>
<td>67</td>
<td>2.90 ± 0.52a</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>90</td>
<td>5.70 ± 0.58</td>
</tr>
<tr>
<td>6-FB(α)P</td>
<td>200</td>
<td>43</td>
<td>0.53 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>50</td>
<td>0.83 ± 0.21</td>
</tr>
<tr>
<td>7-FB(α)P</td>
<td>200</td>
<td>13</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>10</td>
<td>0.13 ± 0.08</td>
</tr>
<tr>
<td>8-FB(α)P</td>
<td>200</td>
<td>10</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>7</td>
<td>0.07 ± 0.05</td>
</tr>
<tr>
<td>9-FB(α)P</td>
<td>200</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10-FB(α)P</td>
<td>200</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>7</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>B(α)P only</td>
<td>400</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TPA only</td>
<td>3.2</td>
<td>10</td>
<td>0.10 ± 0.05</td>
</tr>
</tbody>
</table>

6-FluoroB(α)P possesses approximately 30-50% of the skin tumor-initiating activity of B(α)P. The decrease in carcinogenicity was attributed to a 6-fluoro peri effect, but the mechanism of this effect had not been previously elucidated. In fact, the 6-fluoro
group caused a 1-2 fold enhancement in 7,8-dihydrodiol formation relative to B(α)P. Additionally, the rat liver enzyme metabolites, 1,6- and 3,6-B(α)P-quinones, were formed with concomitant loss of fluorine. Formation of the 6-fluoro bay-region dihydrodiol-epoxide was decreased by 50% relative to the epoxide formed from B(α)P. Thus, overall, the amounts of 6-FB(α)P-7,8-dihydrodiol-9,10-epoxide and B(α)P-7,8-dihydrodiol-9,10-epoxide were approximately equal. Conformational analysis revealed that electrostatic repulsion of the peri fluorine by non-bonding electrons on oxygen caused the 7,8-dihydrodiol to adopt a pseudoaxial orientation; this metabolite did not display strong mutagenic activity toward Chinese hamster V79 cells. Furthermore, dihydrodiol-epoxides of several PAH and substituted PAH, in which hydroxyl groups occupy a pseudodiagonal conformation, were found to possess low carcinogenic and mutagenic activity while those dihydrodiol-epoxides, in which the hydroxyl groups occupy a pseudoequatorial conformation, are highly tumorogenic when derived from carcinogenic PAH. These differences in carcinogenicity may be due to differences in conformation (i.e., alter intercalative behavior) or differences in the solvolytic properties of the epoxide as a result of changes in conformation (e.g., pH-independent solvolysis of the weak carcinogen, (+)-anti-6-fluoro-B(α)P-dihydrodiol-epoxide to tetrols is enhanced compared to the corresponding unflorinated compound). Benz(a)anthracene (BA) is considered a weak carcinogen and the formation of all possible dihydrodiols and dihydrodiol-epoxides have
been observed.\textsuperscript{83,84} For BA, it appears that both the 3,4-dihydrodiol-1,2-epoxide and the 8,9-dihydrodiol-10,11-epoxide form covalent adducts with DNA. Structure-activity relationships on the 12 mono-methyl regioisomers of BA (MBA) by several investigators indicate that 12-MBA is more potent than 1-, 2-, 3-, 4-, 5-, 9-, 10- and 11-MBA.\textsuperscript{85} This is consistent with the hypothesis that increasing steric strain in the bay-region enhances carcinogenic potency.\textsuperscript{74} However, 12-MBA is less tumorigenic than 7-MBA, but of equal potency to 6- and 8-MBA. The reasons for these observations remain unclear. An exemplary study appears in Table 3. These investigations are the first to reveal the tumor-initiating activity of a complete series in this single tumor model.\textsuperscript{85}

Table 3: Skin Tumor-Initiating Activity of the 12 MBA's (Taken from Ref. 85).

Groups of 30 seven-week old female mice were initiated with the indicated dose of hydrocarbon in 200\textmu l of acetone and treated with 10\mu g of TPA in 200\textmu l of acetone twice weekly for the duration of the experiment. aThe incidence of papillomas was tabulated at 21 weeks of promotion.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose(nmol)</th>
<th>% Mice with papillomas\textsuperscript{a}</th>
<th>No. of papillomas per mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>--</td>
<td>3</td>
<td>0.03</td>
</tr>
<tr>
<td>BA</td>
<td>400</td>
<td>23</td>
<td>0.3</td>
</tr>
<tr>
<td>1-MBA</td>
<td>400</td>
<td>27</td>
<td>0.3</td>
</tr>
<tr>
<td>2-MBA</td>
<td>400</td>
<td>7</td>
<td>0.07</td>
</tr>
<tr>
<td>3-MBA</td>
<td>400</td>
<td>13</td>
<td>0.2</td>
</tr>
<tr>
<td>4-MBA</td>
<td>400</td>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td>5-MBA</td>
<td>400</td>
<td>17</td>
<td>0.23</td>
</tr>
<tr>
<td>6-MBA</td>
<td>30</td>
<td>27</td>
<td>0.3</td>
</tr>
<tr>
<td>100</td>
<td>10</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>33</td>
<td>0.6</td>
<td></td>
</tr>
</tbody>
</table>
Table 3 (continued)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose(nmol)</th>
<th>% of Mice with papillomas</th>
<th>No. of papillomas per mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-MBA</td>
<td>30</td>
<td>28</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>70</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>77</td>
<td>4.9</td>
</tr>
<tr>
<td>8-MBA</td>
<td>30</td>
<td>17</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>23</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>40</td>
<td>1.03</td>
</tr>
<tr>
<td>9-MBA</td>
<td>400</td>
<td>28</td>
<td>0.55</td>
</tr>
<tr>
<td>10-MBA</td>
<td>400</td>
<td>13</td>
<td>0.23</td>
</tr>
<tr>
<td>11-MBA</td>
<td>400</td>
<td>10</td>
<td>0.1</td>
</tr>
<tr>
<td>12-MBA</td>
<td>30</td>
<td>23</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>17</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>52</td>
<td>1.03</td>
</tr>
<tr>
<td>DMBA</td>
<td>9</td>
<td>97</td>
<td>8.14</td>
</tr>
</tbody>
</table>

As with BA, the 3,4- and 8,9-dihydrodiols of 7-MBA were the major metabolic products resulting from treatment with rat-liver microsomes. These metabolites were also found in cultured mouse skin. However, only the 3,4-dihydrodiol formed covalent DNA adducts through the dihydrodiol-epoxide intermediate. Structure-activity relationships for the monofluoro analogues of 7-MBA also point to the importance of bay-region metabolism leading to carcinogenesis. Interestingly, 5-F-7-MBA has approximately 1/6 the tumor-initiating activity of 7-MBA. 12-F-7-MBA is more than three times as active as 7-MBA. These two compounds, respectively, illustrate a 5-F peri effect on decreasing bay-region carcinogenicity and a 12-F peri effect on increasing bay-region steric strain. In contrast, 2-F-7-MBA and 3-F-7-MBA possess potent sarcomagenic activity in Long Evans rats. Unlike 6-M and 6-F-B(α)P, 5-F-7-MBA does not form any detectable bay-region 3,4-dihydrodiol metabolites.
upon treatment with rat liver microsomes, suggesting that for this compound the peri effect blocks metabolism. Unfortunately, metabolism studies with 5,7-dimethylbenz(a)anthracene have not been carried out even though its tumor initiating activity is approximately equal to that of 5-F-7-MBA. Notably, however, the peri effect of fluorine in 7-F-BA does not block the formation of 5,6- and 8,9-dihydrodiols. Conformational analysis revealed that these diols assume a pseudodiplaxial relationship owing to electrostatic repulsive forces.

If the effect of fluorine at the 12 position is to increase steric strain, then replacement with a methyl group should further increase steric strain and enhance carcinogenic potency. Indeed, 7,12-dimethylbenz(a)anthracene (DMBA) is approximately 40 fold more potent as a skin tumor-initiator than is 12-F-7-MBA. Furthermore, DMBA is known to be the most carcinogenic PAH in several tumor models. DMBA (10 nmol) was also determined to be approximately 20 fold more potent than B(α)P, causing 9.2 papillomas/mouse in 100% of the mice, whereas B(α)P (100 nmol) causes 2.4 papillomas/mouse in 64% of the mice. Using both rat-liver microsomes and cultured mouse skin, 3,4-, 5,6-, 8,9- and 10,11-dihydrodiol metabolites are formed. Of these, the 8,9-dihydrodiol is followed by the 3,4-dihydrodiol as the major metabolites. Incubation of DMBA with mouse embryo cells generates covalent DNA adducts which exhibit fluorescence spectra consistent with an anthracene chromophore. These findings suggest involvement of a bay-region dihydrodiol-epoxide.
Inhibition of DNA bonding without inhibition of overall metabolism by treating cultured mouse embryo cells with 1,1,1-tri-chloropropen-2,3-oxide (an epoxide hydrolase inhibitor) further substantiate the intermediacy of a dihydriodiol-epoxide metabolite. In support of the 3,4-dihydriodiol-1,2-epoxide hypothesis, DMBA-3,4-dihydriodiol and 7-hydroxymethyl-12-MBA-3,4-dihydriodiol are more mutagenic than DMBA in the Ames assay. DMBA-3,4-dihydriodiol is also more active than DMBA as a mouse skin tumor-initiator. Double labeling experiments with [3H]-DMBA and [14C]-adenine or [14C]-guanine, followed by separation of the DNA adducts using HPLC phenylborate column chromatography provided evidence for the presence of bay-region syn- and anti-dihydriodiol-epoxide deoxyadenosine and anti-dihydriodiol-epoxide deoxyguanosine adducts.

The total binding of the bay-region dihydriodiol-epoxide metabolites of DMBA to adenine is significantly greater than the total binding of analogous B(α)P metabolites to adenine. Since DMBA is a more potent carcinogen than B(α)P, Dipple et al. have suggested that the total binding of carcinogenic PAH to adenine correlates most closely with the tumorigenic activity of DMBA. Structures of these adducts are unproven. Biological evaluation of the recently synthesized syn- and anti-7,12-dimethylbenz(a)anthracene-3,4-dihydriodiol-1,2-epoxide is of considerable interest in this regard.

Much of the earlier evidence concerning structure-activity relationships of various monofluoro analogues of DMBA in mouse skin tumor-initiation models supports the bay-region hypothesis. Thus, in this system, 1-, 2-, 4- and 5-F-DMBA (200 nmol) have more than an
85% decrease in tumorigenic potency compared to DMBA. Full activity is maintained for the 9- and 11-fluoro isomers and 10-F-DMBA is 2 fold more potent than DMBA as a tumor initiator. These results parallel the sarcomagenic activity of these compounds as complete carcinogens after intramuscular injection (9.1 μmol, 2.5 mg) into Long Evans rats. However, 4-F-DMBA maintains approximately 50% of the sarcomagenic activity compared to DMBA. In contrast, 4-F-DMBA (0.1 μmol/mouse/wk) remain inactive after 30 weeks as a complete carcinogen when painted on mouse skin. These discrepancies suggest the possibility for altered routes of metabolism, especially in subcutaneous tissue. However, these routes have not been rigorously explored.

In keeping with the bay-region hypothesis, DMBA and 11-F-DMBA are equally potent in their ability to induce mutations eliminating ouabain resistance in Chinese hamster V79 cells. Such induction only takes place in the presence of secondary hamster embryo cells as metabolic activators. One- and 5-F-DMBA are three orders of magnitude less active than DMBA, and 2-F-DMBA is inactive as a mutagen in these cells.

Fluorine substitution in the A-ring or 5 position blocks rat liver microsomal metabolism to 3,4-dihydrodiols and these compounds do not form covalent, bay-region DNA adducts in Syrian hamster embryo cells. DMBA and 11-F-DMBA do form 3,4-dihydrodiols and had nearly identical DNA binding profiles as detected by HPLC. Furthermore, modulation of the amount of 8,9- and 10,11-dihydrodiol produced vs the position of fluorine in the A-ring and K-region suggest that a
back-resonance effect of the nonbonding electrons of fluorine to increase electron density at α-positions (sites of preferred electrophilic aromatic substitution) in the fused-ring aromatic structure is operant. This effect has been invoked to explain the 2-fold increase in carcinogenic potency of 10-F-DMBA since back-resonance shows an increase in electron density at position 4, presumably favoring 3,4-epoxidation and subsequent dihydrodiol formation (Figure 5).

![Figure 4: Back-resonance Effect of Fluorine to Increase Electron Density at Position 4 in DMBA.](image)

It is unfortunate that careful tumor-initiation and metabolism experiments with 6- and 8-F-DMBA have not been conducted, since these compounds also exhibit the same back-resonance effect. More recently, 9- and 10-F-DMBA were studied in mouse skin. In these studies, a positive correlation was found between the level of a specific covalent DNA adduct (tentatively assigned anti-dihydrodiol-epoxide deoxyadenosine 30) and tumor-initiating activity. This assignment was based upon HPLC techniques (described earlier) used to identify the nature of the covalent DNA adducts derived by reaction with syn- and anti-dihydrodiol-epoxides of DMBA.
The synthesis and mutagenicity of the A-ring reduced PAH 1,2,3,4-tetrahydro-7,12-dimethylbenz(a)anthracene (TH-DMBA, 21) was reported by Witlak et al. in 1979. Unexpectedly, TH-DMBA showed a dose dependent amutagenic response in three strains of Salmonella typhimurium (Ames assay) with or without hepatic S-9 microsomal activation, but DMBA was mutagenic only in the presence of the S-9 mix. In contrast, TH-DMBA had 1/10 the mutagenic activity of DMBA in Chinese hamster embryo cells only in the presence of secondary hamster embryo cells as metabolic activators. Perhaps in S. typhimurium, TH-DMBA is either an automutagen or this PAH undergoes bacterial metabolism to mutagenic products; in Chinese hamster embryo cells TH-DMBA requires metabolic activation prior to eliciting the mutagenic response. TH-DMBA has potent tumor-initiating activity in Swiss and SENCAR mice. In the latter, TH-DMBA (100 nmol) is approximately 1/10 as potent a tumor-initiator as DMBA and is approximately three fold more potent than B(α)P.
In the methylated anthracene series, 2,9,10-trimethylanthracene (31) exhibits a greater dose dependent mutagenic response than 9,10-dimethylanthracene (20) in two strains of S. typhimurium, but only in the presence of the hepatic S-9 mix.\textsuperscript{110} 2,9,10-Trimethylanthracene (1 \mu g) was three fold more potent a tumor-initiator in CD-1 mice compared to 9,10-dimethylanthracene, but is approximately 1/6 as potent as B(\alpha)P.\textsuperscript{110} Metabolism of methylated anthracenes by rat liver microsomes provides evidence for formation of epoxides peri to the methyl group since the corresponding dihydrodiols are formed.

\[
\text{31}
\]

TH-DMBA (0.5 \mu g/ml) transforms human neonatal foreskin fibroblasts (HNF) cells at 1/6 the dose of B(\alpha)P as measured by anchorage independent growth in soft agar.\textsuperscript{111} These transformed cells are invasive on chick embryo skin organ culture and form a fibrosarcoma.\textsuperscript{111} DMBA (1 \mu g/ml) is inactive. Furthermore, [\textsuperscript{3}H]-TH-DMBA and [\textsuperscript{3}H]-B(\alpha)P localize in the nucleus (1.4x10^7 molecules/nuclear residue and 1.6x10^9 molecules/nuclear residue, respectively) in HNF cells whereas DMBA is randomly dispersed throughout the cytoplasm with no detectable amounts in the nucleus.\textsuperscript{112} B(\alpha)P binds to a cytoplasmic lipoprotein complex prior to transport across the nuclear membrane. TH-DMBA and B(\alpha)P do not appear to undergo cytoplasmic
metabolism to oxygenated products. Under conditions for cell transformation, 95% of the radiolabelled TH-DMBA is recovered unmetabolized. In light of the dramatic differences in biological profiles between DMBA and TH-DMBA, it seems unlikely that TH-DMBA undergoes metabolic aromatization of the A-ring in order to elicit a mutagenic or carcinogenic response. The potent carcinogenic activity of the 5-membered A-ring analogue 2,3-dihydro-5,11-dimethyl-1H-cyclopenta(a)anthracene (32) (incapable of metabolic A-ring aromatization) further supports this hypothesis.
Statement of the Objective

TH-DMBA has been proposed to undergo either low level nuclear metabolism and highly specific covalent DNA bonding or interaction with DNA in an as yet unknown, nonclassical sense (e.g., physical bonding or cation radicals). It was recently shown that TH-DMBA, but not DMBA, forms two covalent adducts with cellular DNA in HNF cells as detected by a very sensitive \(^{32}\text{P}\) postlabelling technique. To investigate possible metabolic requirements leading to TH-DMBA-induced carcinogenesis and for structure-toxicity relationships (STR) studies, we desired the 6 possible aryl monofluoro analogues of this PAH. As previously illustrated for DMBA and 7-MBA, substitution of fluorine is anticipated to block or reduce carcinogenicity if inserted into a site critical for metabolism. For example, if in TH-DMBA, D-ring metabolism leads to carcinogenesis, then fluorine substitution at position 9 or 10 is anticipated to block the event. Also, fluorine substitution at positions 5 and 6 may modulate carcinogenic potency owing to electronic influences on the production of D-ring metabolites. Loss of carcinogenic potency by the 5- and 6-monofluoro analogues would point to the importance of a B-ring epoxide as a possible carcinogenic precursor, but loss of carcinogenicity by only one of these fluoro analogues might reflect inhibition of metabolites at a position near the fluoro substitution site.
The carcinogenic potency of these aryl fluoro analogues will be assessed for their ability to transform HNF cells in culture. The level and number of covalent adducts will be determined by chromatography following a $[^{32}P]$ postlabeling technique.$^{114,115}$

In this dissertation, we describe the regioselective syntheses for the 5- and 6-fluoro-TH-DMBA analogues (F-TH-DMBA, 33, 34) and compare their physico-chemical and biological properties to the four D-ring aryl-F regioisomers.

![Chemical structures](image)
**Results and Discussion**

**Synthesis:**

Construction of each F-TH-DMBA constitutes a regioselective total synthesis. Synthesis of 5-F-TH-DMBA was envisioned to proceed through keto acid 35 (Scheme 8). Reaction of the Grignard reagent derived from 1-bromo-4-fluoro-5,6,7,8-tetrahydronapthalene (36) with phthalic anhydride would provide the desired keto acid 35. Conversion of similar keto acids to substituted 7,12-dimethylbenz(a)anthracene analogues has been reported. 116-118

![Scheme 8: Retrosynthetic of 5-F-TH-DMBA.](image)

Compound 36 was synthesized from commercially available 1-amino-5,6,7,8-tetrahydronaphthalene (37). Acetylation and bromination afforded haloamide 38 and hydrolysis of the amide provided the amine.
hydrochloride 39 in good overall yield.\textsuperscript{119} Diazotization of the amine and treatment with 48% tetrafluoroboric acid provided the unstable diazonium tetrafluoroborate salt 40, which was thermolyzed in

\[
\begin{align*}
39 & \xrightarrow{1) \text{NaNO}_2, 6\text{N HCl}} 40 \\
36 & \xrightarrow{2) 170^\circ \text{C}} \quad R = \text{F}
\end{align*}
\]

in the absence of solvent providing intermediate 36 (63% yield from 37). The Grignard reagent of 36 was prepared by the method of Newman and Tuncay.\textsuperscript{120} Reaction of this Grignard reagent with phthalic anhydride provided keto acid 41 which was resistant to ring closure in conc. \(\text{H}_2\text{SO}_4\). Zinc dust/potassium hydroxide reduction of 41 provided carboxylic acid 42 which then underwent ring closure in conc. \(\text{H}_2\text{SO}_4\) to anthrone 43 in 68% yield (55.5% from 36). Dichromate
oxidation of the anthrone produced anthraquinone \(44\) in 72\% yield. Treatment of \(44\) with excess methyl magnesium bromide provided a diastereomeric mixture of diols \(45\) (82\%) and this diol mixture was

deoxygenated with low valent titanium\(^{121}\) in a 60\% yield to afford the desired 5-F-TH-DMBA \(33\) (12\% overall yield from \(37\)). \(\text{SnCl}_2/\text{HCl}\) reductive dehydration\(^ {118}\) or HI/MeOH-\(\text{SnCl}_2/\text{HCl}\) treatment\(^ {122}\) converted \(45\) to \(33\) in 26\% and 27\% yields, respectively.
Scheme 9: Retrosynthesis of 6-F-TH-DMBA.

Synthesis of 6-F-TH-DMBA \(34\) was again envisioned to proceed through a keto acid intermediate \(46\) (Scheme 9). Electrophilic addition of phthalic anhydride to 2-fluoro-5,6,7,8-tetrahydronaphthalene \(47\) was expected to occur predominantly at the remaining \(\beta\) position; Freidel-Crafts acylation of tetrailin takes place at the \(\beta\) position\(^{123,124}\) and Freidel-Crafts phthaloylation of 2-methyl-5,6,7,8-tetrahydronaphthalene \(48\) also takes place at the remaining \(\beta\) position\(^{125}\).

Fluorotetrailin \(47\) was prepared according to a modification of Sy et al.\(^{126}\) Catalytic hydrogenation of the keto acid \(49\), rather than Clemensen reduction\(^{126}\), increased the yield of the corresponding phenylbutyric acid \(50\) from 70% to 91%. Sublimation of the crude polyphosphoric acid (PPA) cyclized product \(51\)\(^{126,127}\) increased the yield from 62% to 90%.
Clemenssen reduction of fluorotetralone \(51\) afforded fluorotetralin \(47\) in 77% yield (63% yield from \(49\), compared with 26% yield from \(49\)).

Friedel-Crafts phthaloylation of \(47\) afforded keto acid \(46\) (69%) the structure of which was confirmed by \(^1H\) and \(^19F\) NMR spectroscopy. A characteristic doublet \((J_{H5-F}=11.5\) Hz) for ortho coupling in the \(^1H\) NMR spectrum and doublet of doublets \((J_{H5-F}=11.3\) Hz and \(J_{H8-F}=7.6\) Hz) observed for ortho and meta proton coupling to the fluorine resonance signal substantiated the structural assignment. Formation of keto acid \(46\) was accompanied by a mixture of less than 10% other regioisomers as determined by \(^1H\) and \(^19F\) NMR. These \(1\)-substituted tetrailins were easily removed by a single
recrystallization from benzene. Reduction (Zn/KOH) afforded acid 52 (94%), ring closure (conc. H₂SO₄) produced anthrone 53 (96%) and dichromate oxidation yielded quinone 54 (73%). Treatment of 54 with excess methyl magnesium bromide afforded a diastereomeric mixture of diols 55 (cis/trans; 3.4:1.0; 85%).

\[ \text{Conversion of 55 to target 34 was best carried out (65%) by deoxygenation using low-valent titanium}^{121} \text{ (21% overall yield from 49). With a modification of the SnCl₂/HCl reductive dehydration procedure,}^{118} \text{ 34 was obtained in a maximum 23% yield. This modification employed excess SnCl₂ rather than excess HCl. Excess HCl}^{118} \text{ or tandem HI/MeOH-SnCl₂/HCl}^{122} \text{ transformed 55 to 34 in 5-10% yields. Under these highly acidic conditions, the product was always contaminated with significant amounts of the exo methylene tautomers 56 and 57. Careful chromatography (silica gel, hexane) of contaminated 34} \]
allowed for the separation of 56, whereas 57 co-chromatographed with 34. The complete separation of 57 and 34 was successfully accomplished utilizing a charge-transfer (10% 2,4,7-trinitrofluorenone/silica gel, hexane) chromatographic technique.  

The structure of the exo methylene tautomer 56 was confirmed by synthesis and a proton NOE difference study. Reaction of the intermediate keto acid 46 with methyl magnesium bromide afforded phthalide 58 (88%) and Zn/KOH reduction yielded free acid 59 (54%). Ring closure with concentrated H₂SO₄ provided anthrone 60 (77%). Without isolation of intermediates, the keto acid could be converted to the anthrone in approximately 61% overall yield. Refluxing 60 with methyl Grignard for 3 h produced tertiary alcohol 61 which was
dehydrated by acidification of the reaction mixture with 4N HCl (reflux 4 h). The exo methylene tautomer 56 (52%) was separated from the intermediate alcohol 61 (8.5%) by column chromatography on silica gel/methylene chloride. Noteworthy, under these conditions there was no appreciable isomerization of 56 to 34.

The structure of 56 was confirmed by 270 MHz proton NOE difference experiments (Table 4). The alternating positive and negative NOE effect for the respective two and three spin systems involving H-ll, H-A, H-B and H-I confirmed the assignment of the exo methylene function to position 12 and established assignments for the two nonequivalent vinyl proton resonance signals. The most compelling evidence for the structural assignment was derived from the NOE effect obtained upon irradiation of H-B wherein the area of resonance signal for H-1 and H-A was increased by 10% and 32%, respectively, and for H-ll decreased by 4%. Thus, the exo methylene function must be neighboring the benzyl methylene protons on C-1. Additionally, the methyl proton resonance signal for 56 was observed as the expected doublet (δ 1.34, J=7 Hz) and the neighboring tertiary proton as a quartet (δ 4.38, J=7 Hz). Compound 57 must also be an exo methylene tautomer owing to vinyl proton resonance signals at δ 5.8-5.9 (multiplet), a methyl proton resonance signal as an expected doublet at δ 1.29 (J=7 Hz), and a tertiary proton resonance signal at δ 4.20 (quartet, J=7 Hz). Since NOE experiments established the structure for 56, 57 must be the 7-methylene regioisomer.
Table 4: NOE Enhancements for Four Protons of 6-Fluoro-1,2,3,4,7,12-hexahydro-7-methyl-12-methylenebenz(a)anthracene (56)
Measured on a Degassed CDCl₃ Solution (0.22 M)

<table>
<thead>
<tr>
<th>Irradiated Proton</th>
<th>Observed Proton</th>
<th>Irradiated Proton ppm, a mult</th>
<th>Observed Proton ppm, a mult</th>
<th>% Area Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-11</td>
<td>H-11</td>
<td>7.5, m</td>
<td>5.48, s</td>
<td>-1</td>
</tr>
<tr>
<td>H-11</td>
<td>H-A</td>
<td>7.5, m</td>
<td>5.82, s</td>
<td>7</td>
</tr>
<tr>
<td>H-A</td>
<td>H-11</td>
<td>5.82, s</td>
<td>7.5, m</td>
<td>14</td>
</tr>
<tr>
<td>H-A</td>
<td>H-B</td>
<td>5.82, s</td>
<td>5.48, d</td>
<td>30</td>
</tr>
<tr>
<td>H-B</td>
<td>H-11</td>
<td>5.48, d</td>
<td>2.8, m</td>
<td>-1</td>
</tr>
<tr>
<td>H-B</td>
<td>H-A</td>
<td>5.48, d</td>
<td>5.82, s</td>
<td>32</td>
</tr>
<tr>
<td>H-B</td>
<td>H-1</td>
<td>5.48, d</td>
<td>2.8, m</td>
<td>10</td>
</tr>
</tbody>
</table>

aMe₄Si as internal standard

Comparative Acid-Catalyzed Isomerization:

The observation that significant amounts of exo methylene tautomers were formed when the classical methods (high acid concentration) were used to convert 55 to 34 prompted us to study the comparative acid catalyzed isomerization of 34 and 56 (Table 5). In refluxing benzene containing TsOH, 34 reached thermodynamic equilibrium with methylene tautomers 56 and 57 after 1 h (34/56/57=1.0:0.6:2.8). In the absence of acid catalysis, no isomerization was observed after 3 h. When 56 was treated with TsOH in refluxing benzene, the thermodynamic isomeric product ratio was not obtained after 1 or 5 h. After 72 h, the product ratio was similar to the ratio observed when
34 was subjected to acid-catalyzed isomerization for 1 h. These data are markedly different from results observed for acid-catalyzed isomerization of TH-DMBA (21) and 5F-TH-DMBA (33) which after 72 h only afforded fully aromatic to 7-methylene isomer ratios of 13:1 and 8:1, respectively.

Table 5: Comparative Isomerization of 6-F-1,2,3,4-tetrahydro 7,12-dimethylbenz(a)anthracene (34) and its 12-Methylene Tautomer (56) in Refluxing Benzene in the Presence or Absence of Acid Catalysis.

<table>
<thead>
<tr>
<th>treated</th>
<th>conditions</th>
<th>34</th>
<th>56</th>
<th>57</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>reflux, d,e 1 h</td>
<td>1.0±0.1</td>
<td>0.3±0.1</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>34</td>
<td>reflux, d,e 5 h</td>
<td>1.0</td>
<td>0.4±0.2</td>
<td>3.1±0.1</td>
</tr>
<tr>
<td>34</td>
<td>reflux, g,h 3 h</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>56</td>
<td>reflux, d,e 1 h</td>
<td>1.0</td>
<td>27.0±1.0</td>
<td>2.0±0.9</td>
</tr>
<tr>
<td>56</td>
<td>reflux, d,e 5 h</td>
<td>1.0</td>
<td>4.6±0.8</td>
<td>3.2±0.4</td>
</tr>
<tr>
<td>56</td>
<td>reflux, d,e 72 h</td>
<td>1.0</td>
<td>0.6±0.1</td>
<td>2.8±0.4</td>
</tr>
<tr>
<td>56</td>
<td>reflux, g,h 5 h</td>
<td>0.0</td>
<td>1.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

a10 mg of PAH. bDetermined by integration of $^1$H NMR spectra in CDCl$_3$ of the vinyl proton resonance signals for 56 and 57 and H-8, H-11 multiplet for 34. cThe 7-methylene tautomer of 34. dP-Toluene-sulfonic acid (2.5 mg) in 5 mL of benzene. The reaction mixture was passed through a disposable Pasteur pipet containing a 7-cm column of basic alumina (Woelm) and eluted with 6-8 mL of benzene. The eluate was concentrated under reduced pressure and traces of benzene were removed by repeated evaporation from CC$_1$$_4$ under reduced pressure. The residue was dissolved in CDCl$_3$ for NMR studies. fProduct ratios are reported relative to 34. gBenzene (5 mL); no acid added. hSame workup as in e, but no column chromatography was necessary.

The product ratios were determined by integration of protons unique to each isomer in the NMR spectrum of the product mixture. Figure 6 is the $^1$H NMR spectrum of 56. The two nonequivalent vinyl proton resonance signals for the 12-methylene group appear at 5.8 and 5.5 $\delta$. For the 7-methylene tautomer 57 (Figure 7), the two vinyl
protons appear as a multiplet at 5.85 δ. The aromatic protons, H8 and H11 on 6-F-TH-DMBA, 34 (Figure 8) appeared as an AA'BB' pattern at 8.1-8.4 δ. Thus, for example, when the 12-exo methylene tautomer 56 was heated with TsOH in refluxing benzene for 5 h (Figure 9), a product ratio of 56/34/57=4.0:1.0:2.8 was obtained. This product ratio was determined by the integration of the two aromatic protons for 34 and one vinyl proton (5.5 δ) for 56. Subtraction of the latter integration from the total integration at 5.8 δ provided the integration for the two vinyl protons for 57.

The rate of conversion of the fully aromatic PAH 34 to 7-exo methylene isomer 57 was considerably faster than the isomerization of the 12-exo methylene isomer 56 to 57. Thus, conversion of 56 to 34 was the slow step. Possibly this reflects resonance stabilization by the 6-F group of the intermediate cation 62 derived from protonation of 34.

\[
\begin{array}{c}
\text{H}_{3}C\text{H}_{3}F \\
C_{3}\text{H}_{3}(CH)\text{F} \\
\text{CH}_{3}\text{F}
\end{array}
\]

Protonation of 56 would yield cation 63 which cannot be resonance stabilized by fluorine. Additionally, unfavorable steric interactions between the 7-\text{CH}_{3} group and the 6-F substituent and between the 12-\text{CH}_{3} and 1-\text{CH}_{2} groups became more severe as the
compound passed from puckered $56$ to planar $34$ (Scheme 10). Tautomerization of $34$ to $57$ was more rapid owing to relief of steric strain. Although $5$-F-TH-DMBA $33$ would have an inverse effect on stabilization of the respective cations, for these compounds, no such $\text{CH}_3$-F peri interaction was present and little isomerization to exo methylene tautomers was possible; the fully aromatic system is thermodynamically most stable.

Scheme 10: Tautomerization of 6-F-TH-DMBA

TH-DMBA PAH $21$ and $33$ each have one peri interaction, but 6-F derivative $34$ has an additional $\text{CH}_3$-F peri interaction serving to drive acid catalyzed isomerization to the thermodynamically more stable 7-exo methylene compound $57$ which is the sterically least crowded of the two ($56$ and $57$) exo methylene regioisomers formed. These data are in agreement with isomerization studies using polymethylated anthracenes. $^{130,131}$ At least two peri $\text{CH}_3$-$\text{CH}_3$ interactions were required for the complete isomerization to thermodynamically more stable, sterically less crowded exo methylene tautomers, as determined by $^1$H NMR spectroscopy. Thus, compound $34$ may comprise
the minimum steric requirements \((\text{CH}_3-\text{CH}_2\) and \(\text{CH}_3-F\) steric interaction\) for the partial isomerization of methylated anthracenes.

These data may be compared to recent observations derived from acid catalyzed equilibration studies using the four D-ring analogues.\(^{132,133}\) The 8- and 11-F regioisomers (Table 6) undergo isomerization to the thermodynamically more stable 7-exo methylene tautomer owing to a fluoro \textit{peri} effect. As with TH-DMBA and 5F-TH-DMBA, 9- and 10-F regioisomers (lacking a \textit{peri} fluorine interaction) do not isomerize to any appreciable extent.

### Table 6: Thermodynamic Product Ratios for the Acid-Catalyzed Isomerization of TH-DMBA and Its 6 Monofluoro Analogues.\(^{a}\)

<table>
<thead>
<tr>
<th>TH-DMBA Analogues</th>
<th>Product Ratio(^b)</th>
<th>(\Delta G_{\pm SD}(B \Rightarrow A))</th>
<th>(\Delta G_{\pm SD}(B \Rightarrow C))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH-DMBA</td>
<td>0</td>
<td>13.0 ± 2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>5-F</td>
<td>0</td>
<td>8.5 ± 0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>6-F</td>
<td>0.6 ± 0.1</td>
<td>1.0</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>8-F</td>
<td>0.3 ± 0.1</td>
<td>1.0</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>9-F</td>
<td>0</td>
<td>8.0</td>
<td>1.0</td>
</tr>
<tr>
<td>10-F</td>
<td>0</td>
<td>10.5 ± 2.5</td>
<td>1.0</td>
</tr>
<tr>
<td>11-F</td>
<td>0</td>
<td>1.0</td>
<td>10.5 ± 1.2, &gt; 3.3(^e)</td>
</tr>
</tbody>
</table>

\(^a\)Conditions and workup for the isomerization reaction were as described in Table 5.\(^b\)Determined by integration of unique protons in \(^1\)H NMR spectrum. \(^c\)Values reported in kcal/mol. \(^d\)A, B, and C are the 12-exo methylene tautomer, fully aromatic PAH and 7-exo methylene tautomer, respectively. \(^e\)Limited by the sensitivity of \(^1\)H NMR integration.

The \textit{peri} effect of fluorine on thermodynamic product stability is most dramatic for the 11-F regioisomer.\(^{63}\) The net steric effect of the 11-F and 1-CH\(_2\) moieties on the C\(_{12}\)-CH\(_3\) is to force the C\(_{12}\)-CH\(_3\) out of the anthracene plain ring (a type of sandwiching effect). To
further relieve steric strain, protonation and rehybridization of $C_{12}$ yields the 7-exo methylene tautomer. The steric effect of fluorine at positions 6 and 8 may create a slight twist in the anthracene nucleus. Protonation and rehybridization of $C_{12}$ is favored over $C_7$ since the steric repulsive force of the 1-CH$_2$ moiety is greater than the steric repulsive force of fluorine at position 6 or 8 (van der Waals radii for CH$_2$ and F are 2.0Å and 1.35Å, respectively). Thus, isomerization to 7-exo methylene tautomers is preferred. Further work would be necessary to elucidate the small difference in peri effects observed for the 6-F vs. 8-F isomer.
Figure 5. 90 MHz $^1$H-NMR Spectrum of 12-exo methylene tautomer 56.
Figure 6. 90 MHz $^1$H-NMR Spectrum of 7-exo methylene tautomer 57.
Figure 7. 90 MHz $^1$H-NMR Spectrum of 34.
Figure 8. 90 MHz $^1$H-NMR Spectrum of the product mixture obtained after refluxing 56 in benzene with TsOH for 5 h.
Biological Results:

HNF cell transformation and covalent DNA binding studies were conducted by Dr. H. Lalitha Kumari. The preliminary results of these experiments are summarized in Table 7. Clearly, TH-DMBA, 6-, 10- and 11-F-TH-DMBA transformed HNF cells in culture as evidenced by anchorage independent growth in soft agar. TH-DMBA formed two covalent adducts with DNA, as does 10- and 11-F-TH-DMBA. 6-F-TH-DMBA formed 3-4 adducts. At the same dose, 5-F-TH-DMBA did not transform HNF cells and did not form covalent adducts with DNA. These results reveal a correlation between covalent bonding and carcinogenicity for this PAH series as evidenced by formation of the transformed phenotype. Fluorine substitution at position 6, 10 and 11 did not interfere with the induction of carcinogenesis. However, such substitution does appear to markedly alter the nature and degree of covalent adducts formed. Also, there appeared to be a significant modulation in the transforming frequency of these PAH depending upon the site of fluorine substitution. Back-resonance effects of fluorine may be responsible for this modulation, however a more complete conclusion is pending biological evaluation of 8- and 9-F-TH-DMBA.
Table 7: Cellular DNA Adducts and Transformation Efficiency of TH-DMBA and Fluoro Analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Adduct #</th>
<th>RAL $a \times 10^7$</th>
<th>Colonies/50,000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH-DMBA</td>
<td>1</td>
<td>0.2-0.62</td>
<td>72 ± 7.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.3-1.38</td>
<td></td>
</tr>
<tr>
<td>5F-TH-DMBA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6F-TH-DMBA</td>
<td>1</td>
<td>0.12-0.32</td>
<td>131 ± 12.7</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.11-0.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.15-0.56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.08-0.28</td>
<td></td>
</tr>
<tr>
<td>10F-TH-DMBA</td>
<td>1</td>
<td>0.12</td>
<td>80 ± 12.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>11F-TH-DMBA</td>
<td>1</td>
<td>0.08</td>
<td>58.5 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>Untreated Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$RAL = relative adduct labelling at the 1 μg/ml level

D-ring dihydrodiol-epoxides were anticipated not to be carcinogenic since the 7- and 12-methyl groups would force any dihydrodiol precursors to adopt pseudodi-axial orientations. Our preliminary results with 10- and 11-F-TH-DMBA support this possibility. Furthermore, our results do not support the hypothesis that simple epoxides peri to methyl substituents are likely carcinogenic precursors.110

Thus, TH-DMBA and its fluorinated analogues continue to offer a unique opportunity to study altered routes of metabolism leading to carcinogenesis in HNF cells in the absence of conventional carcinogenic epoxide and dihydrodiol-epoxide metabolites. Based on our preliminary observation that 5-F-TH-DMBA was the only non-carcinogen in this series, two possibilities exist for the
carcinogenesis of TH-DMBA. These are: 1) metabolism at position 5 resulting in covalent binding to DNA, via a cation radical intermediate (c.f. Scheme 5) or 2) metabolic oxidation at the benzylic 1 or 4 positions, possibly via either a reactive benzylic cation intermediate (c.f. Scheme 6) or benzylic alcohol. For example, 6- and 10-F-TH-DMBA were more carcinogenic than TH-DMBA while 11-F-TH-DMBA was less carcinogenic. The most dramatic modulation in carcinogenic potency was seen for 5- and 6-F-TH-DMBA, in which fluorine was substituted in the ring directly adjacent to the C-1 carbocation. Fluoro substitution in the remote D-ring resulted in a more subtle modulation in carcinogenic potency.

In the DMBA series, fluorine substitution at position 10 resulted in a two fold increase in tumor-initiating activity.\textsuperscript{104} It was hypothesized that such substitution might enhance formation of the 3,4-dihydrodiol through a resonance effect to increase electron density at position 4. An alternate possibility may be that such substitution stabilizes the C-1 carbocation derived from the bay-region dihydrodiol-epoxide metabolite. Both effects may in fact be operating for this compound.

However, for 10-F-TH-DMBA, no such resonance effect to enhance electron density at position 4 can occur. Thus, the F-TH-DMBA series may provide us with an opportunity to selectively study the fluorine resonance effect on C-1 carbocation reactivity as it relates to carcinogenic potency. On the other hand, 5-F-DMBA was shown to be a weak carcinogen and this was attributed to fluorines ability to block metabolic epoxidation of the 3,4-double bond.\textsuperscript{91,105} Loss of
carcinogenic activity with 5-F-TH-DMBA may also result from an inability to metabolically activate the adjacent 4 position.

Clearly further work is needed to characterize the covalent nucleotide adducts of TH-DMBA in order to confirm these hypotheses. One approach may be to synthesize molecules that represent the putative electrophilic metabolites and react them with DNA or homonucleotide polymers. This would provide PAH-nucleotide markers of known structure for comparison with actual TH-DMBA-nucleotide adducts. Benzylic bromides such as 1- and 4-bromo-TH-DMBA (Figure 9) may be promising synthetic targets for the evaluation of TH-DMBA nucleotide adducts.

Figure 9: Structure of 1- and 4-bromo-TH-DMBA.
Experimental

Melting points were determined in open capillaries with a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Mass spectra were recorded with a Kratos MS-30 operating at 70 eV. Infra-red spectra were recorded on Beckman Model No. IR-4210 or 4230 spectrophotometers. $^1$H-NMR spectra were determined on a Brucker HX-90E or an IBM, NR-80 spectrometer. NOE experiments were carried out on an IBM NR/270 FT-NMR spectrometer. Me$_4$Si was used as an internal standard and chemical shifts are reported on the $\delta$ scale with peak multiplicities: d, doublet; dd, double of doublets; m, multiplet; s, singlet; and t, triplet. Purification of compounds was carried out by column chromatography over silica gel 60 mesh (E. Merck) and 100-200 mesh (Fisher Scientific Co.) and also by preparative thin layer chromatography (TLC) over precoated silica gel GF plates (E. Merck). THF, after distilling over CaH$_2$, was heated at reflux over Na and benzophenone and distilled under N$_2$ prior to distillation. Synthesis and purification of the target PAH were always carried out under yellow light. Indication of purity was by HPLC (Varian 5000) using a Zorbax ODS 6.2mm x 25 cm column with UV monitor (254nm) and gradient elution 60% MeOH to 100% MeOH over 30 min. By this procedure no 7- or 12 oxidation products were detected and in all samples submitted for biological investigations which were carried out under yellow light. Elemental analyses were performed by Galbraith Laboratories, Inc., Knoxville, TN.
1-Bromo-4-Fluoro-5,6,7,8-tetrahydronaphthalene (36). An aqueous solution of NaNO₂ (4.30 g/10 mL, 61 mmol) was added dropwise to an ice cooled, stirred mixture of 39 (16.0 g, 61 mmol), H₂O (40 mL) and conc. HCl (20 mL). The yellow solution was filtered and 48% HBF₄ (30 mL) was added to the ice cooled filtrate. After stirring for 15 min, the diazonium tetrafluoroborate 40 [19.20 g (97%; mp 123-124°C dec)] was filtered, washed with H₂O, MeOH/Et₂O (1:1) and Et₂O successively, and dried in a vacuum desiccator (rt, 1 torr) overnight. IR (KBR) 2280 (⁻N≡N) cm⁻¹; ¹H NMR (d₆-DMSO) δ 1.7-1.9 (m, 4H, H-6, H-7), 2.7-2.9 (m, 2H, H-5), 3.0-3.2 (m, 2H, H-8), 8.12 (d, 1H, J=8.9 Hz, H-3), 8.44 (d, 1H, J=8.6 Hz, H-2).

In a round bottom flask fitted with a reflux condenser, 40 (18.9 g, 58 mmol) was thermolyzed in a 170°C oil bath for 20 min. The dark oil was taken up in hexane, filtered, and the solvent removed under reduced pressure. Distillation afforded 36 (11.22 g, 82% based on 39) as a colorless liquid; bp 88-89°C (1.2 torr.). ¹H NMR (d₆-DMSO) δ 1.6-1.9 (m, 4H, H-6, H-7), 2.5-2.8 (m, 4H, H-5, H-8), 6.95 (dd, 1H, Jₜₜ=J₂₃=9 Hz, H-3), 7.42 (dd, 1H, Jₜₜ=8.9 Hz, Jₜ₂=5.4 Hz, H-2). Anal. Calcd for C₁₀H₁0BrF: C, 52.43; H, 4.40; F, 8.29. Found: C, 52.52; H, 4.44; F, 8.31.

2-[(4-Fluoro-5,6,7,8-tetrahydro-1-naphthalenyl)carbonyl]benzoic Acid (41). A solution of 36 (10.00 g, 44 mmol) and ethylene dibromide (0.36 mL) in 105 mL of dry THF/benzene (2:1) was added dropwise over 1 h to a stirred mixture of sublimed Mg (2.91 g, 121 mmol) in THF (25 mL) under N₂, and refluxed for 45 min. The Grignard
was injected into a warmed solution of phthalic anhydride (6.98 g, 47 mmol) in benzene (180 mL) and refluxed under N₂ for 5 h. The yellow reaction mixture was cooled in an ice bath and carefully quenched with 10% HCl solution (60 mL). The organic layer was separated and the aqueous layer extracted (Et₂O). The organic fractions were combined, dried (MgSO₄) and concentrated under reduced pressure. Recrystallization (benzene) of the light yellow product gave 41 (11.10 g, 85%): mp 208-210°C; IR (KBr) 2400-3200 (CO₂H) 1670 (C=O), 1690 (C=O) cm⁻¹; ¹H NMR (CDCl₃) δ 1.6-1.9 (m, 4H, H-6', H-7'), 2.6-2.9 (m, 2H, H-8'), 3.0-3.3 (m, 2H, H-5'), 6.71 (dd, 1H, J₆-F = J₇-F = 8.6 Hz, H-3'), 6.99 (dd, J₉-H = 8.6 Hz, J₈-H = 6.0 Hz, H-2'), 7.3-7.8 (m, 3H, H-3, H-4, H-5), 7.9-8.1 (m, 1H, H-6). Anal. Calcd for C₁₈H₁₅F₀₃: C, 72.46; H, 5.07, F, 6.36. Found: C, 72.84, H, 5.03, F, 6.22.

2-[(4-Fluoro-5,6,7,8-tetrahydro-1-naphthalenyl)methyl]benzoic Acid (42). A mixture of 41 (5.70 g, 19 mmol), Zn dust (42.0 g, activated by washing with dilute aq.HCl), CuSO₄ (82 mg) and pyridine (5 mL) in 10% KOH solution (70 mL) was refluxed for 26 h. The reaction mixture was filtered while hot and the filter cake was washed with H₂O. The filtrate was poured onto ice/conc. HCl solution and the white precipitate was filtered and dried affording 42 (5.24 g, 96%); mp 183-184°C. The analytical sample melted at 183-184.5°C (benzene/petroleum ether): IR (KBr) 2400-3200 (CO₂H), 1690 (C=O) cm⁻¹; ¹H NMR (CDCl₃) δ 1.6-1.9 (m, 4H, H-6', H-7'), 2.5-2.9 (m, 4H, H-5', H-8'), 4.34 (s, 1H, Ar-CH₂-Ar), 6.6-6.8 (m, 2H, H-2', H-3'), 6.9-7.1 (m, 1H, H-3), 7.2-7.6 (m, 2H, H-4, H-5), 8.0-8.2
5-Fluoro-2,3,4,12-tetrahydrobenz[a]anthracene-7(1H)-one (43). A mixture of 42 (5.20 g, 18 mmol) in conc. H$_2$SO$_4$ (100 mL) was stirred at rt for 1 h. The red solution was poured onto ice and extracted (CH$_2$Cl$_2$). The organic fractions were combined, dried (MgSO$_4$) and concentrated under reduced pressure. The residue was triturated with MeOH, filtered and dried affording 3.75 g of crude 43; mp 178-183°C. Recrystallization (benzene/EtOH) afforded pure 43 (3.30 g, 68%); mp 184-189°C; IR (KBr) 1650 (C=O) cm$^{-1}$; $^1$H NMR (CDCl$_3$) $\delta$ 1.7-2.0 (m, 4H, H-2, H-3), 2.6-2.9 (m, 4H, H-1, H-4), 3.98 (s, 2H, H-12), 7.3-7.7 (m, 3H, H-9, H-10, H-11), 7.80 (d, 1H, $J_{H-F}$=10.2 Hz, H-6), 8.2-8.4 (m, 1H, H-8). Anal. Calcd for C$_{18}$H$_{15}$FO: C, 81.17; H, 5.68; F, 4.68. Found: C, 81.23; H, 5.72; F, 4.74.

5-Fluoro-1,2,3,4-tetrahydrobenz[a]anthracene-7,12-dione (44). To a refluxing mixture of K$_2$Cr$_2$O$_7$ (3.42 g, 12 mmol) in HOAc (60 mL), 43 (2.96 g, 11 mmol) was added in a single portion. After 10 min of refluxing, the hot solution was poured onto ice, filtered and dried. Column chromatography (silica gel/CH$_2$Cl$_2$) afforded 44 (2.25 g, 72%) after recrystallization from benzene/EtOH; mp 166-167°C; IR (KBr) 1670 (C=O) cm$^{-1}$; $^1$H NMR (CDCl$_3$) $\delta$ 1.7-2.0 (m, 4H, H-2, H-3), 2.7-3.0 (m, 2H, H-4), 3.3-3.5 (m, 2H, H-1), 7.6-7.9 (m, 3H, H-6, H-9, H-10), 8.1-8.3 (m, 2H, H-8, H-11). Anal. Calcd for C$_{18}$H$_{13}$FO$_2$: C, 77.12; H, 4.68; F, 6.78. Found: C, 77.30; H, 4.74; F, 6.76.
cis/trans-5-Fluoro-1,2,3,4,7,12-hexahydro-7,12-dimethylbenz[a]-anthracene-7,12-diol (45). An ether solution of 2.8 M MeMgBr (Aldrich, 15 mL, 42 mmol) was injected into a stirred solution of 44 (1.00 g, 3.6 mmol) in dry benzene (30 mL) under N₂ at rt. The yellow solution was refluxed for 7 h, cooled to rt and slowly poured onto ice. The mixture was extracted (EtOAc), dried (MgSO₄) and concentrated under reduced pressure. The residue was recrystallized from benzene/petroleum ether affording 45 (0.93 g, 81%) as a diastereomeric mixture of diols: IR (KBr) 3150-3600 (OH) cm⁻¹. Anal. Calcd for C₂₀H₂₁F₀₂: C, 76.88; H, 6.78; F, 6.08. Found: C, 77.04; H, 6.95; F, 6.02.

5-Fluoro-1,2,3,4-tetrahydro-7,12-dimethylbenz(a)anthracene (33). Low-valent titanium was prepared by slowly adding LiAlH₄ (24 mg, 0.64 mmol) to a stirred suspension of TiCl₃ (197 mg, 1.28 mmol) in dry THF (5 mL) under a stream of N₂ at 0°C. The mixture was stirred at 0°C for 10 min and refluxed for 1 h. To this black suspension was added 45 (100 mg, 0.32 mmol) at 0°C under a stream of N₂. The mixture was refluxed for 3 h, cooled to 0°C, quenched with 2N HCl (2 mL), diluted with H₂O and extracted (CH₂Cl₂). The organic fractions were combined, washed (H₂O), dried (Na₂SO₄) and concentrated under reduced pressure. Column chromatography (silica gel-hexane) of the yellow, fluorescent residue afforded 53 mg (60%) of 33 after recrystallization from CH₂Cl₂/MeOH: mp 116-116.5°C; >99% pure by HPLC analysis; ¹H NMR (CDCl₃) δ 1.5-2.2 (m, 4H, H-2, H-3), 2.8-3.0 (m, 2H, H-4); 2.94 (s, 3H, CH₃), 3.12 (s, 3H, CH₃), 3.2-3.4 (m, 2H, H-1), 7.3-7.6 (m, 2H, H-9, H-10), 7.63 (d, 1H, J_H-F=12.7 Hz, H-6), 8.1-8.3
(m, 2H, H-8, H-11). Anal. Calcd for C$_{20}$H$_{19}$F: C, 86.30; H, 6.88; F, 6.82. Found: C, 86.29; H, 6.87; F, 6.83.

2-[(3-Fluoro-5,6,7,8-tetrahydro-2-naphthalenyl)carbonyl]benzoic Acid (46) and its Methyl Ester. To a stirred mixture of 47$_{126}$ (5.25 g, 35 mmol) and phthalic anhydride (4.71 g, 32 mmol) in α-dichlorobenzene (ODCB, 50 mL), was added AlCl$_3$ (8.46 g, 64 mmol) in three portions over 15 min. The reaction mixture was stirred at 95°C for 5 h. The deep red solution was poured onto ice/conc. HCl and extracted (EtOAc). The organic fractions were combined, dried (MgSO$_4$), filtered and evaporated under reduced pressure removing only the EtOAc. Petroleum ether (150 mL) was added to the ODCB fraction. Cooling yielded 8.20 g of pale yellow product, mp 174-182°C. Recrystallization from benzene gave 46 (6.55 g, 69%) as pale yellow crystals (mp 179.5-184.5°C) of sufficient purity for subsequent reactions. Fisher esterification provided an analytical sample of methyl ester; mp 107.5-109°C (MeOH); IR (KBr) 1725 (CO$_2$Me), 1670 (ArOOAr), 1280 (C-O) cm$^{-1}$; $^1$H NMR (CDCl$_3$) ð 1.6-1.9 (m, 4H, 2CH$_2$), 2.6-2.9 (m, 4H, 2CH$_2$), 3.72 (s, 3H, OCH$_3$), 6.74 (d, 1H, J$_{H-F}$=11.8 Hz, H-4'), 7.3-7.4 (m, 1H, H-1), 7.4-7.7 (m, 3H, H-3, H-4, H-5), 7.9-8.0 (m, 1H, H-6). Anal. Calcd for C$_{19}$H$_{17}$O$_3$F: C, 73.05; H, 5.49; F, 6.09. Found: C, 73.05; H, 5.61; F, 6.06.

Alkyline hydrolysis of methyl ester of 46 afforded analytically pure 46; mp 184.5-184°C (benzene/hexane); IR (KBr) 1670-1720 (C=O's), 2400-3300 (CO$_2$H) cm$^{-1}$; $^1$H NMR (CDCl$_3$) ð 1.7-2.0 (m, 4H, 2CH$_2$), 2.6-2.9 (br s, 4H, 2CH$_2$), 6.72 (d, 1H, J$_{H-F}$=11.5 Hz, H-4'), 6.6-7.2 (br s, 1H, CO$_2$H, exchangeable), 7.3-7.4 (m, 1H, H-1'), 7.4-7.7 (m,
3H, H-3, H-4, H-5), 8.0-8.1 (m, 1H, H-6). Anal. Calcd for C_{18}H_{15}O_{3}F: C, 72.46; H, 5.07; F, 6.37. Found: C, 72.69; H, 5.15; F, 6.06.

2-[(3-Fluoro-5,6,7,8-tetrahydro-2-naphthalenyl)methyl]benzoic Acid (52). A solution of 46 (3.91 g, 13 mmol), CuSO₄ (60 mg), Zn dust (30 g; activated by washing with 10% HCl), pyridine (4 mL) in 10% KOH solution was refluxed for 21 h. The hot mixture was filtered and the filter cake washed with H₂O. The filtrate was poured onto ice/conc. HCl and the white precipitate was filtered, washed with H₂O and dried affording 52 (3.51 g, 94%); mp 151-152°C. The analytical sample melted at 157-158°C (benzene/hexane): IR (KBr) 1690 (CO₂H), 2400-3200 (CO₂H) cm⁻¹; ¹H NMR (CDCl₃) δ 1.6-1.9 (m, 4H, 2CH₂), 2.5-2.8 (m, 4H, 2CH₂), 4.41 (s, 3H, ArO⁻Ar), 4.5-6.0 (br s, 1H, C₀₂H), 6.73 (d, 2H, J₁°=9.9 Hz, H-1'), H-1, H-4'), 7.1-7.6 (m, 3H, H-3, H-4, H-5), 8.01 (dd, 1H, Jortho=8.0 Hz, Jmeta=1.5 Hz, H-6). Anal. Calcd for C_{18}H_{17}O₂F: C, 76.02; H, 6.03; F, 6.69. Found: C, 75.79; H, 6.07; F, 6.47.

6-Fluoro-2,3,4,7-tetrahydrobenz[a]anthracene-12(1H)-one (53). A mixture of 52 (4.00 g, 14 mmol) in conc. H₂SO₄ (100 mL) was stirred at rt for 1 h. The red solution was poured onto ice and the yellow precipitate was filtered, washed with H₂O, and dried affording 53 (3.61 g, 96%); mp 138.5-141.5°C. The analytical sample melted at 144-144.5°C (benzene/hexane): IR (KBr) 1655 (C=O) cm⁻¹; ¹H NMR (CDCl₃) δ 1.7-2.0 (m, 4H, 2CH₂), 2.7-3.0 (m, 2H, H-4), 3.2-3.5 (m, 2H, H-1), 4.22 (s, 2H, H-7), 7.03 (d, 1H, J₁°=9.9 Hz, H-5), 7.3-7.7 (m, 3H, H-8, H-9, H-10), 8.1-8.3 (m, 1H, H-11). Anal. Calcd for
6-Fluoro-1,2,3,4-tetrahydrobenz[a]anthracene-7,12-dione (54). A mixture of 53 (3.60 g, 13 mmol) and K₂Cr₂O₇ (5.95 g, 20 mmol) in glacial HOAc (60 mL) was refluxed for 20 min. The reaction mixture was poured onto ice. The yellow precipitate was filtered, washed thoroughly with H₂O, dried and chromatographed on a silica gel column using CH₂Cl₂ as eluent to give 54 (2.62 g, 73%) as yellow needles from benzene/EtOH: mp 190-191°C; IR (KBr) 1670 (CO) cm⁻¹; ¹H NMR (CDCl₃) δ 1.7-2.0 (m, 4H, 2CH₂), 2.8-3.0 (m, 2H, H-4), 3.2-3.4 (m, 2H, H-1), 7.18 (d, 1H, J₉-F=12.1 Hz, H-5), 7.6-7.8 (m, 2H, H-9, H-10), 8.1-8.3 (m, 2H, H-8, H-11). Anal. Calcd for C₁₈H₁₃O₂F: C, 77.12; H, 4.68; F, 6.78. Found: C, 77.08; H, 4.70; F, 6.62.

cis-6-Fluoro-1,2,3,4,7,12-hexahydro-7,12-dimethylben[a]-anthracene-7,12-diol (55a) and trans-6-Fluoro-1,2,3,4,7,12-hexahydro-7,12-dimethylben[a]anthracene-7,12-diol (55b). A solution of 2.8 M MeMgBr in Et₂O (Aldrich) was added to a stirred solution of 54 (1.00 g, 3.6 mmol) in dry benzene (30 mL) at rt under N₂ and refluxed for 3 h. The ice-cooled solution was quenched by the dropwise addition of H₂O (20 mL). The benzene layer was decanted and the aqueous layer was extracted (EtOAc). The organic fractions were combined, washed (sat. NaCl solution) and dried (MgSO₄). The solvent was evaporated and the residue was crystallized from benzene/petroleum ether to give 0.63 g of white crystalline cis-diol, 55a. The analytical sample melted at 190.5-191.5°C (benzene/petroleum ether). Column chromatography of the residue from the concentrated mother liquor (silica
gel-\(\text{CH}_2\text{Cl}_2\) gave 0.21 g of trans-diol, 55b after recrystallization from benzene/petroleum ether; mp 158-160°C. An additional 0.09 g of cis-diol 55a was eluted providing a combined yield of 0.93 g (84%) of diols 55a and 55b (3.4:1.0).

For cis-55a: IR (KBr) 3150-3600 (OH) cm\(^{-1}\); \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.61 (d, 3H, \(J=0.7\) Hz, 12-\(\text{CH}_3\)), 1.70 (d, 3H, \(J=0.95\) Hz, 7-\(\text{CH}_3\)), 1.4-2.0 (m, 4H, H-2, H-3), 2.49 (d, 1H, \(J=0.95\) Hz, 12-OH), 2.7-2.9 (m, 2H, H-4), 3.1-3.3 (m, 2H, H-1), 3.46 (d, 1H, \(J_{\text{HF}}=14.3\) Hz, 7-OH, exchangeable), 6.80 (d, 1H, \(J_{\text{HF}}=13.7\) Hz, H-5), 7.2-7.4 (m, 2H, H-9, H-10), 7.6-7.7 (m, 2H, H-8, H-11). Anal. Calcd for C\(_{20}\)H\(_{21}\)O\(_2\)F: C, 76.88; H, 6.78; F, 6.08. Found: C, 76.68; H, 6.90; F, 5.91.

For trans-55b: \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.78 (s, 3H, 12-\(\text{CH}_3\)), 1.87 (d, 3H, \(J_{\text{HF}}=1.6\) Hz, 7-\(\text{CH}_3\)), 1.7-2.1 (m, 4H, H-2, H-3), 2.22 (s, 1H, 12-OH, exchangeable), 2.7-2.9 (m, 2H, H-4), 3.12 (d, 1H, \(J_{\text{HF}}=9.9\) Hz, 7-OH, exchangeable), 3.2-3.4 (m, 2H, H-1), 6.87 (d, 1H, \(J_{\text{HF}}=13.3\) Hz, H-5), 7.3-7.5 (m, 2H, H-9, H-10), 7.7-7.8 (m, 2H, H-8, H-11).

6-Fluoro-1,2,3,4-tetrahydro-7,12-dimethylben[al]anthracene (34). The synthesis of 34 from 55a (100 mg, 0.32 mmol) was carried out according to procedure for the preparation of 33. The reaction mixture was refluxed for 3 h, cooled to 0°C, diluted with H\(_2\)O (20 mL) and worked up accordingly. Column chromatography (silica gel-hexane) afforded 58 mg (65%) of 34 after recrystallization from benzene/MeOH; mp 69.5-70°C; >99% pure by HPLC analysis. \(^1\)H NMR (CDCl\(_3\)) \(\delta\) 1.5-2.1 (m, 4H, H-2, H-3), 2.8-3.0 (m, 2H, H-4), 3.13 (s, 3H, 12-\(\text{CH}_3\)), 3.23 (d, 3H, \(J_{\text{HF}}=5.4\) Hz, 7-\(\text{CH}_3\)), 3.0-3.3 (m, 2H, H-1), 6.73 (d, 1H, \(J_{\text{HF}}=15.3\) Hz, H-5), 7.4-7.6 (m, 2H, H-9, H-10), 8.2-8.6 (m, 2H, H-8,
Anal. Calcd for C_{20}H_{19}F: C, 86.30; H, 6.88; F, 6.82. Found:
C, 86.10; H, 6.89; F, 6.82.

6-Fluoro-1,2,3,4-tetrahydro-7,12-dimethylbenz[a]anthracene (34).
Seven drops of conc. HCl (0.18 g, 2 mmol) were added to a stirred
suspension of SnCl\textsubscript{2}2H\textsubscript{2}O (3.00 g, 13 mmol) in Et\textsubscript{2}O (20 mL). The
supernatant (~15 mL) was decanted after 10 min of stirring. Under
yellow light, 55\textsubscript{a} (0.6 mmol) was added to the supernatant solution in
two portions, stirred at rt for 1 h, quenched with H\textsubscript{2}O (10 mL) and
stirred for 10 min. The yellow reaction mixture was diluted with H\textsubscript{2}O
and extracted (benzene). The organic fractions were combined, washed
(sat. NaCl solution), dried (MgSO\textsubscript{4}), and evaporated under reduced
pressure. Chromatography of the residue (silica gel-hexane) gave 34
(0.41 g, 23%) as yellow needles after recrystallization (benzene/
MeOH); mp 65-66°C.

3-(3-Fluoro-5,6,7,8-tetrahydro-2-naphthalenyl)-3-methyl-1(3H)-
isobenzofuranone (58). A solution of 2.8 M MeMgBr (Aldrich, 7.6 mL,
21 mmol) was slowly added to a rapidly stirred solution of 46
(2.00 g, 7 mmol) in dry Et\textsubscript{2}O (64 mL) and dry benzene (16 mL) at rt
under N\textsubscript{2}. The dark mixture was refluxed for 2.5 h, cooled in an ice
bath, and quenched with dilute HCl solution and warmed for 1 h. The
mixture was extracted (Et\textsubscript{2}O) and the organic layers were combined,
washed (5% NaHCO\textsubscript{3} solution), dried (MgSO\textsubscript{4}) and concentrated under
reduced pressure. The residue was crystallized from benzene-
petroleum ether affording 58 (1.75 g, 88%); mp 89-90°C: IR (KBr)
1765 cm\textsuperscript{-1}; \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \delta 1.6-1.8 (m, 4H, 2CH\textsubscript{2}), 2.05 (d, 3H,
J\textsubscript{H-F}=1.6 Hz, CH\textsubscript{3}), 2.6-2.8 (m, 4H, 2CH\textsubscript{2}), 6.77 (d, 2H, J\textsubscript{H-F}=12.7 Hz,
H-4'), 7.19 (d, 1H, J_{H-F}=8.2 Hz, H-1'), 7.3-7.7 (m, 3H, H-4, H-5, H-6), 7.8-8.0 (m, 1H, H-7). Anal. Calcd for C_{19}H_{17}O_2F: C, 76.99; H, 5.79; F, 6.41. Found: C, 76.99; H, 5.90; F, 6.13.

2-[1-(3-Fluoro-5,6,7,8-tetrahydro-2-naphthalenyl)ethyl]benzoic Acid (59). A mixture of 58 (1.00 g, 3.4 mmol), CuSO_4 (12 mg), Zn dust (6.0 g, activated by treatment with dilute acid), pyridine (8 drops) in 10% KOH (10 mL) was refluxed for 20 h. The hot mixture was filtered and the filter cake washed with H_2O. The filtrate was poured onto ice/conc HCl and the white precipitate was filtered, washed with H_2O and dried affording 59 (0.54 g, 54%) as off white crystals after recrystallization from benzene-petroleum ether; mp 132-134°C; IR (KBr) 3200-2500 (OH), 1680 (C=O) cm^{-1}; ^1H NMR (CDCl_3) δ 1.61 (d, 3H, J=7.3 Hz, CH_3), 1.7-1.9 (m, 4H, 2CH_2), 2.6-2.8 (m, 4H, 2OH_2), 5.42 (q, 1H, J=7.1 Hz, OHCH_3), 6.65 (d, 1H, J_{H-F}=11.1 Hz, H-4'), 6.90 (d, 1H, J_{H-F}=7.9 Hz, H-1'), 7.1-7.6 (m, 3H, H-3, H-4, H-5), 8.0-8.2 (m, 1H, H-6).

6-Fluoro-2,3,4,7-tetrahydro-7-methylbenz[a]anthracen-12(1H)-one (60). A mixture of 59 (0.53 g, 1.8 mmol) in conc. H_2SO_4 (7 mL) was stirred at rt for 1 h. The deep red solution was poured onto ice and extracted (EtOAc). The organic fractions were combined, washed (sat. NaHCO_3), dried (MgSO_4) and evaporated under reduced pressure. Column chromatography of the residue on silica gel with CH_2Cl_2 as eluent afforded 60 (0.38 g, 77%) as off white crystals after recrystallization from petroleum ether; mp 104-105°C; IR (KBr) 1665 (C=O) cm^{-1}; ^1H NMR (CDCl_3) δ 1.49 (d, 3H, J=7.3 Hz, 7-CH_3), 1.6-2.1 (m, 4H, H-2,
6-Fluoro-1,2,3,4,7,12-hexahydro-7,12-dimethylbenz[a]anthracene-12-ol (61) and 6-Fluoro-1,2,3,4,7,12-hexahydro-7-methyl-12-methylene-benz[a]anthracene (56). A solution of 2.8 M MeMgBr (10 mL, Aldrich) was added to a solution of 60 (1.10 g, 3.9 mmol) in anhydrous Et₂O (30 mL). The purple solution was refluxed for 3 h and quenched by the dropwise addition of 4N HCl (30 mL). Benzene (30 mL) was added and the mixture was refluxed for 4 h. The organic layer was separated and the aqueous layer was extracted (Et₂O). The organic fractions were combined, dried (MgSO₄) and evaporated under reduced pressure. The oily residue was chromatographed on a silica gel column with hexane-CH₂Cl₂ (1:1) as eluent to give 56 (0.56 g, 52%) as white crystals from EtOH; mp 107.5-108.5°C. Compound 61 was eluted from the column using CH₂Cl₂ as eluent affording 0.10 g of white crystals upon trituration with petroleum ether; mp 150.5-152°C.

For 61: IR (KBr) 3540 (OH) cm⁻¹; ¹H NMR (CDCl₃) δ 1.44 (d, 3H, J=7.3 Hz, 7-CH₃), 1.79 (s, 3H, 12-CH₃), 1.6-2.0 (m, 4H, H-2, H-3), 2.05 (s, 1H, 12-OH, exchangeable), 2.7-2.9 (m, 2H, H-4), 3.1-3.3 (m, 2H, H-1), 4.38 (q, 1H, J=7.3 Hz, H-7), 6.77 (d, 1H, J=10.2 Hz, H-5), 7.2-7.4 (m, 3H, H-8, H-9, H-10), 7.7-7.9 (m, 1H, H-11). Anal. Calcd for C₁₉H₁₇OF: C, 81.39; H, 6.12; F, 6.78. Found: C, 81.02; H, 6.25; F, 6.53.
For 56: \textsuperscript{1}H NMR (CDCl\textsubscript{3}) \( \delta \) 1.34 (d, 3H, J=7.0 Hz, 7-CH\textsubscript{3}), 1.5-2.2 (m, 4H, H-2, H-3), 2.2-2.4 (m, 2H, H-4), 2.4-2.8 (m, 2H, H-1), 4.38 (q, 1H, J=7.0 Hz, H-7), 5.49 (d, 1H, J=0.9 Hz, bay region vinyl H), 5.82 (s, 1H, vinyl H), 6.75 (d, 1H, J\textsubscript{H-F}=9.8 Hz, H-5), 7.2-7.4 (m, 3H, H-8, H-9, H-10), 7.4-7.6 (m, 1H, H-11). MS, \textit{m/e} calcd 278.1471. Found: 278.1481. Anal. Calcd for C\textsubscript{20}H\textsubscript{19}F: C, 86.30; H, 6.88; F, 6.82. Found: C, 86.16; H, 6.89; F, 6.52.
APPENDIX A

HNF CELL TRANSFORMATION AND COVALENT DNA BINDING STUDIES
These biological studies were conducted by Dr. H. Lalitha Kumari in the laboratory of Dr. George E. Milo at the O.S.U. Comprehensive Cancer Center.

The ability of TH-DMBA and some of its fluoro analogues to transform HNF cells and form covalent DNA adducts has been investigated. Cultured HNF cells at population doubling (PDL) five were synchronized by seeding into nutritionally deficient medium wherein the cells were arrested in the $G_1$ phase (Scheme II). Twenty-four hours later, the cells were released by feeding with complete medium containing 10% fetal bovine serum (FBS) and 0.5 U/ml insulin as a growth factor. Ten hours later, when the cells were in the $G_1/S$ phase, they were treated with carcinogen (1 $\mu$g/mL) for 12 h. At the end of this treatment period (path a), the cells were washed twice with minimum essential medium (MEM) and split at a 1:2 ratio into selection medium (8 x amino acid medium). At 75% confluency, the cells were serially passaged at 1:10 split ratio into selection medium. After 16-20 population doublings, the cells were seeded into soft agar. Three weeks later, the plates were scored for colony formation.
To investigate carcinogen-DNA adduct formation, the cells were cultured and synchronized as previously described (Scheme 11). Ten hours after cells were released from synchrony, they were treated with carcinogen. Ten hours later, the cells were scraped and washed with MEM by centrifugation and the washed cells were lysed by suspending in 1% sodium dodecyl sulfate containing 1mM EDTA. The
lysate was incubated at 38°C for 30 min with 500 μg/mL of proteinase K and extracted with phenol and phenol/chloroform mixture (path b). The DNA was precipitated by addition of 1 volume of ethanol at -19°C in the presence of 0.5 M NaCl. The precipitate was washed with 70% ethanol and treated with a mixture of RNase T₁ (50 U/mL) and RNase A (100 μg/mL). Following a second phenol extraction, the purified DNA dissolved in 0.1 M sodium chloride/0.01 M sodium citrate buffer (SSC) and used for adduct analysis.

A 5 μg sample (Scheme 12) of the purified DNA was digested with 5.0 μg each of micrococcal endonuclease and spleen phosphodiesterase. For adduct identification, an aliquot of this digest was extracted with n-butanol in the presence of a transferring agent (tetrabutylammonium chloride). The butanol layer was evaporated and the residue dissolved in water and labelled with [γ-³²P]-ATP in the presence of T₄-polynucleotide kinase affording 3',5'-deoxyribonucleotide bis-(phosphate). This was subjected to four directional chromatography and exposed to screen enhanced autoradiography to give a fingerprint of the nucleotide adducts. In order to quantitate the number of nucleotides, an aliquot of the digest was treated with the kinase in the absence of butanol extraction and subjected to two directional chromatography followed by screen enhanced radiography. From a knowledge of the total number of nucleotides labelled, the relative number of adducts formed was calculated.
Purified DNA from Carcinogen Treated Cells

- micrococcal endonuclease;
- spleen phosphodiesterase

Normal Nucleotides + Nucleotide-Carcinogen Adducts
(Ap + Tp + Cp + Gp) + Xp + Xp + ...

dilute; γ-32P-ATP +
T₄-polynucleotide kinase

extract with butanol
Xp + Yp + ...

Finger Prints

*Ap + *Tp + *Cp + *Gp + *Xp...

2-D chromatography;
autoradiography

γ-32P-ATP + T₄-polynucleotide kinase

*XP + *YP

4-D chromatography;
autoradiography

Finger Prints

Scheme 12: Analysis of Carcinogen-DNA Adducts by ³²P-Postlabelling Method.

The four directional chromatography (Scheme 13) employed 1.1 M LiCl (D₁) and 2.5 M NH₄CHO₂ pH 3.5 (D₂) to remove phosphate and normal nucleotides, 3.0 M LiCHO₂ containing 8.5 M urea at pH 3.5 (D₃) and 0.8 M LiCl containing 0.5 M tris HCl and 8.5 M urea at pH 8 (D₄) to mobilize the nucleotide-PAH adducts. Chromatograms were further developed in the D₄ direction using 0.35 M MgCl₂ to remove traces of background.
Scheme 13: Schematic diagram of four directional (D) chromatography on a PEI cellulose plate for resolution of $[^{32}\text{P}]$ labelled carcinogen nucleotide adducts.
APPENDIX B

BIOLOGICAL EXPERIMENTAL METHODS
Cell Synchronization, Carcinogen Treatment and Transformation.

HNF cells confluent for no more than 12-16 h at PDL 3-5 were blocked at the G1 phase of the cell cycle by seeding the cells in Dulbecco's modified Eagle's minimum essential medium, deficient in arginine and glutamine and supplemented with 1.0 mM sodium pyruvate, 50 μg/ml gentocin and 10% dialyzed FBS as described previously.135-137 Twenty-four hours later, the cells were released from the block by feeding with CM supplemented with 0.5 units/ml insulin. Freshly prepared solutions of carcinogens were added 10 h after release when cells were entering early S phase. At the end of the 12 h treatment period, control and treated cell cultures were rinsed 3 times with MEM and subpassaged at a 1:2 split ratio into CM containing 0.8 mM nonessential amino acids, 25 vitamins and 20% FBS (selection medium). When the cell populations were 75% confluent, they were serially passaged at a 1:10 split ratio into the selection medium til PDL 16-20. Approximately 50,000 cells were seeded in soft agar according to the method previously described.135-137

Anchorage Independent Growth. Carcinogen treated and control cell populations were trypsinized and seeded at 50,000 cell/25 cm² well in 2.0 ml of 0.33% soft agar in Dulbecco's LoCal medium supplemented with 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2.0 mM glutamine, 50 g/ml gentamycin, 0.2% sodium bicarbonate, 1 x essential vitamins, 1 x essential amino acids and 20% FBS. The cells were layered over 5.0 ml of a 2.0% agar base prepared in RPMI 1629 medium or McCoy's medium supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2.0 mM glutamine, 50 μg/ml
gentamycin, 2.0% sodium bicarbonate, 1 x essential vitamins, 1 x essential amino acids and 20% FBS. The soft agar cultures were incubated at 37°C in a 4% CO₂ enriched air atmosphere. The cells were fed once in 8-10 days with supplemented 1 x LoCal medium. Three weeks later, plates were scored for the colonies containing 50-100 cells. TH-DMBA (21) was included as a positive control in all experiments.

**Induction of Carcinogenic Event.** At the onset of scheduled DNA synthesis (i.e., 10 h after release from block), a freshly prepared solution of each compound was added to the cells. When acetone served as a solvent, its concentration did not exceed 0.1% and cells treated with the same concentration of acetone served as control. Treated and untreated cells were incubated for 10-12 h. At the end of the treatment period, the cell monolayers were rinsed twice with MEM and scraped. Cells were washed twice with MEM by centrifugation at 1500 rpm for 5.0 min at 4°C. Cell pellets were quickly frozen and stored at -70°C until used.

**Isolation of DNA.** To isolate nuclear DNA, at the end of the treatment period, the cells were washed twice with cold HBSS with Ca^{++} and Mg^{++}. The cells are then treated for 1-2 min with 0.5% Triton X-100 in Hank's balanced salt solution (HBSS) to release cytoplasm and attach the nuclei to the plate. The attached nuclei were then washed twice with cold HBSS lacking Ca^{++} and Mg^{++} and incubated at 37°C with 2.0 ml of 100 mM NaCl: 50 mM tris-HCl pH 7.4:10 mM EDTA (NTE) containing 0.5% sarkosyl to lyse the nuclei. The lysate was then transferred to the tube and incubated with
proteinase-K (250 μg/ml) for 2.0 h at 50°C. The lysate was extracted successively with 1 volume each of phenol (5.0 min), 1:1 mixture of phenol/chloroform (chloroform/isoamylalcohol 24:1) (3.0 min) and chloroform (3.0 min.). The phases were separated by centrifugation at 3000 rpm for 10 min in a IEC centrifuge. After addition of 0.1 volume of 5 M NaCl, the DNA was precipitated by addition of 1 volume of cold ethanol. The precipitated DNA was washed three times with cold 70% ethanol to remove salts. Traces of ethanol were removed with a pasteur pipette and the DNA was dissolved in 2.0 ml of 0.01 X SSC containing 1.0 mM EDTA. The RNA was destroyed by incubating at 38°C for 30 min with a mixture of RNase T<sub>1</sub> (50 μg/ml) and RNase A (100 μg/ml) in 50 mM Tris-HCl pH 7.4. After extraction of this solution with phenol, the DNA was precipitated as described above. The DNA was dissolved in 0.1XSSC containing 1.0 mM EDTA and its concentration estimated spectrophotometrically assuming 20 A<sub>260</sub> units/mg DNA. The solution was stored at -70°C after quick freezing in dry ice/acetone.

**Detection of Carcinogen-DNA Adducts and Normal Nucleotides.** Carcinogen DNA adducts were analyzed by using [³²P] postlabelling procedures described by Gupta, et al.<sup>114,115</sup>

1. **Synthesis of [γ⁻³²P]·ATP.** The synthesis of labelled ATP was carried out using a Promega Biotec Gamma Prep-A system (Promega Biotec, Madison, WI). Carrier free [³²P] (5.0 mCi in 50 μl water) was added to the reaction mixture supplied by promega Biotec. The mixture was vortexed gently and incubated at room temperature for 50 min. The reaction was stopped by incubating the contents for
5.0 min at 90°C and then cooled on ice. This system synthesized approximately 5 mCi of [γ-32P]-ATP in 100 μl total volume. To check the purity, a small aliquot was diluted 50 fold with water (approximately 1 μCi of the sample in 1 μl) and was applied 2.0 cm from the bottom of a 5x20 cm polyethyleneimine (PEI) cellulose sheet. The sheet was developed in 1.5 M LiCl to 15 cm. Spots were located by autoradiography at room temperature for 10 min. Greater than 97% of the radioactivity was routinely localized in the ATP spot. The preparation was used immediately or stored at -80°C for up to 14 days.

2. Digestion of DNA. Control or carcinogen treated DNA was hydrolyzed to deoxyribonucleoside 3' monophosphate by incubating 5 μg of DNA with 5.0 μg each of micrococcal nuclease and spleen phosphodiesterase in 12.5 μl of 10.0 mM sodium succinate (pH 6.5) and 5.0 mM CaCl2 at 38°C for 4.0 h. The digest was diluted to 50 μl (0.1 μg/μl DNA) with water.

3. Isolation of DNA. Ten μl of the diluted digest was mixed with 5.0 μl each of 100 mM ammonium formate (pH 3.5), 10 mM tetrabutyl ammonium chloride and 30 μl of water. The mixture was extracted twice with 1.0 volume of double distilled n-butanol in 1.5 ml Eppendorf tubes by mixing for 30 sec on a vortex mixer. Phases were separated by centrifugation for 1.0 min in a microfuge. The combined organic phases from the two extractions were back extracted twice with 90 μl of water each time to remove trace contaminant of normal nucleotides. The butanol extract was neutralized by adding 1.0 μl of 200 mM Tris-HCl pH 9.5 and evaporated to dryness (speed vac concentration; or by passing N2 or by
4. [\(^{32}\)P] Labelling of Isolated Adducts. The adduct residue from 1.0 g of DNA was dissolved in 10.0 \(\mu\)l of water. To this solution is added a 5.0 \(\mu\)l aliquot of a radioactive mix containing 2.25 \(\mu\)l of buffer mix (300 mM Tris-HCl pH 9.5, 100 mM MgCl\(_2\), 100 mM dithiothreitol, 10 mM spermidine), 4.0 \(\mu\)l of carrier free \([\gamma-^{32}\)P\]-ATP [200 Ci], 0.75 \(\mu\)l of polynucleotide kinase (10 U/\(\mu\)l) and 0.5 \(\mu\)l of water. The remainder of the radioactive mix was used for labelling normal nucleotides. After agitating the reaction mixture by pipetting, it was incubated at 38\(^{\circ}\)C for 1.0 h. The labelling conditions were essentially the same irrespective of the DNA quantity used for adduct isolation except that the amount of \([\gamma-^{32}\)P\]-ATP used vary as follows: 100-150 Ci (<5.0 \(\mu\)g DNA), 150-200 Ci (6-10 \(\mu\)g DNA).

5. [\(^{32}\)P] Labelling of Total Nucleotides. Five microliters of the DNA digest was further diluted to 500 \(\mu\)l with water. A 5.0 \(\mu\)l aliquot was mixed with 2.5 \(\mu\)l of the radioactive mix used for adduct labelling. After incubation at 38\(^{\circ}\)C for 1.0 h, 1.5 \(\mu\)l of a solution containing 2.0 \(\mu\)g/\(\mu\)l amount each of carrier dpAp, dpCp, dpGp, dpTp and 1.0 \(\mu\)l of potato apyrase (15 milliunits/\(\mu\)l) was added, and the incubation was continued for another 30 min. The labelled digest was then diluted to 250 \(\mu\)l (0.02 ng DNA/\(\mu\)l) with 10 mM Tris-HCl (pH 9.5 containing 5.0 mM EDTA.

6. Separation by TLC of [\(^{32}\)P] Labelled Adduct DNA from Unmodified Nucleotides. To remove residual labelled normal nucleotides, unused \([\gamma-^{32}\)P\]-ATP, \([^{32}\)P\]1 and other radioactive contaminants and to resolve [\(^{32}\)P] adducts, a four directional PEI-cellulose TLC
system was used (Scheme 13). The reaction mixture (13 μl) was spotted on a PEI cellulose plate and developed overnight (18 h) with 1.1 M LiCl. A 12 cm long Whatman #1 wick was attached to the top of the sheet by stapling. The wet sheet was cut 15 cm from the bottom. The upper portion of the plate along with the wick which contains the bulk of the radioactivity is discarded. The lower portion of the chromatogram is twice soaked without drying in 500 ml of deionized water in a flat tray for 10 min and 5.0 min and dried thoroughly before developing in the D2 direction with 2.5 M ammonium formate pH 3.5. After developing the D2 direction, the plate was cut 10 cm from the bottom of the plate and washing and drying processes were repeated. The development in the D3 direction was with 3.0 M lithium formate containing 8.5 M urea pH 3.5. After repeating the washing and drying steps, the chromatogram was cut in two at the mid point and developed in the D4 direction with 0.8 M lithium chloride, 0.5 M Tris, 8 M urea pH 8.0. After washing and drying, the plate was developed in the D4 direction with 0.35 M MgCl2 to remove traces of contaminants. The plates were washed once, dried thoroughly and then transferred to the cassette with enhanced screen for autoradiography. The x-ray film (XAR-Eastman Kodak Company, Rochester, NY) was exposed at -80°C for 18-96 h (as needed), developed and the film was realigned with the TLC plate, over a light-box. The radioactive areas associated with adducts were marked, cut out and quantitated by the Cerenkov assay method. Appropriate blank areas of the chromatogram were also assayed and their counts were subtracted from the sample counts.
7. **TLC of $^{32}$P-Labelled Normal DNA Nucleotides.** For 1-D separation of $^{32}$P labelled digest, 5.0 μl of the diluted labelled solution (~0.1 ng) was applied at 2.0 cm from the bottom edge of the PEI-cellulose TLC plate. The chromatogram was developed with 40 mM ammonium sulphate to about 16-18 cm above the origin. For 2-D separations, 5.0 μl of the diluted digest was applied at 2.0 cm each from the left hand and bottom edge of the PEI cellulose TLC plates (10 x 10 cm) which had been pre-equilibrated by soaking in 100 ml of 0.1 M ammonium formate pH 3.5 for 15 min and dried in a current of warm air. Development in the D₁ direction was with 1.5 M ammonium formate pH 3.5 to 8.0 cm above the origin. The layer was dried in warm air, soaked in 100 ml of 10 mM Tris base in water for 10 min and soaked in 200 μl of water for 5.0 min and dried. Development in the D₂ direction was with 0.2 M ammonium sulphate. After drying, the spots were located by screen enhanced autoradiography at room temperature for 10-20 min. Radioactivity of the individual spots was evaluated by the Cerenkov assay as described earlier. The experiments were run in duplicate and the experiments were repeated at least twice. The label of individual adducts were determined by averaging the values from these analyses.
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