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IN VITRO INHIBITION OF 3-METHYLCHOLANTHRENE-INDUCED RAT HEPATIC ARYL HYDROCARBON HYDROXYLASE BY 8-ACYL-7-HYDROXYCOUMARINS

STRUCTURE–ACTIVITY RELATIONSHIPS AND METABOLITE PROFILES

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Abstract—A series of 4,5,6-substituted-8-acyl-7-hydroxycoumains was synthesized, and their inhibitory potencies towards 3-methylcholanthrene-induced rat hepatic microsomal aryl hydrocarbon hydroxylase activity were studied, both qualitatively and quantitatively. Using the Hansch approach to structure–activity relationships and computer analysis of the data, we have shown that the inhibitory potency could be related to the lipophilicity and molecular size of the compounds. ^{13}C NMR spectroscopy was used to determine electron density at particular carbons of the candidate inhibitors. The electron density was used as an additional parameter in the fitting of potency curves and was found to be of significance. Thus, the potency of a candidate inhibitor could be related to its lipophilicity, molecular size, and electron distribution. The existence of a dipole at the enzyme active site is postulated. The distribution of benzo[*a*]pyrene metabolites, both in the presence and absence of several derivatives, was studied with high pressure liquid chromatography. There was a significant decrease of the 7,8- and 9,10-dihydrodiols in the presence of these inhibitors. The comparative decrease in each of the dihydrodiols was not related to the potency of the inhibitor. The possibility of epoxide hydrolase inhibition by the 4,5,6-substituted-8-acyl-7-hydroxycoumarins was also examined; no epoxide hydrolase inhibition or stimulation was detected at concentrations 10–100 times greater than those which inhibited aryl hydrocarbon hydroxylase by 50%. The decreased formation of the 7,8- and 9,10-arene oxides was therefore postulated to be responsible for the decreased 7,8- and 9,10-dihydrodiol formations in the presence of inhibitor. Inhibition is postulated to have occurred at the enzyme active site, probably by means of a perturbation of the electron cloud density and the active site such that oxygenation at the 7,8- and 9,10-positions was unfavourable.

Cytochrome P-450, the terminal oxidase of an NADPH-dependent electron transport chain, participates in the biotransformation of a wide range of structurally different compounds including steroids, fatty acids, chemical carcinogens, insecticides and hydrocarbons [1]. The heterogeneous nature of the P-450 family has been established through the effects of various inducers such as MC† [2–4]. MC induction is closely associated with AHH induction which is responsible for the conversion of a number of environmental carcinogens to their ultimate carcinogenic form in mammalian cells [5, 6]. Enzyme inhibitors have been used in mechanistic studies of metabolic activation, but very little work has been reported on the inhibition of polycyclic-aromatic-induced P-450.

In a series of compounds that were evaluated with respect to AHH inhibition [7, 8], 7,8-benzoflavone and 9-chloro-7*H*-dibenzo-[*a*, *g*]-carbazole were shown to inhibit MC-induced, but not phenobarbital-induced or control AHH activity, whereas 6-aminochrysene affected both the MC-induced and control enzyme similarly [8]. Further work [9] on an extended benzoflavone series did not clearly indicate whether steric or electronic (or both) influences were operative, but the data suggested the possibility of exploring the nature of the enzyme active sites responsible for BP hydroxylation by selectively modified inhibitory compounds.

8-Acetyl-7-hydroxy-4-methylcoumarin has been shown [10] to be an inhibitor of phenobarbital-induced P-450-mediated aniline hydroxylase activity. Intramolecular bonding between the 7-hydroxy and 8-acyl function had been established [10], and its consequent pseudo polycyclic ring structure suggested that it might also be an inhibitor of MC-induced microsome-mediated AHH activity.

With the modification of xenobiotic metabolism, one can expect a parallel modification of the mutagenicity and carcinogenicity of compounds such as polycyclic aromatic hydrocarbons in the presence of

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† Abbreviations: MC, 3-methylcholanthrene; AHH, aryl hydrocarbon hydroxylase; Hepes, sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate; I_{50} values, concentration of inhibitor which inhibits the reaction by 50%; BP, benzo[*a*]pyrene; HPLC, high pressure liquid chromatography; BP-9,10-dihydrodiol, *trans*-9,10-dihydroxy-9,10-dihydrobenzo[*a*]pyrene; BP-4,5-dihydrodiol, *trans*-4,5-dihydroxy-4,5-dihydrobenzo[*a*]pyrene; BP-7,8-dihydrodiol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene.

such compounds. The ellipticines, for example, have been shown to be potent inhibitors of BP metabolism, decreasing the covalent binding of BP metabolites to DNA and their mutagenicity in the *Salmonella*/microsome test system [11].

In this paper, we study a series of 8-acyl-7-hydroxycoumarins as potential AHH inhibitors, their structure–activity relationships, and BP metabolite profiles produced in the presence of several of the more interesting inhibitors.

EXPERIMENTAL

All solvents were distilled before use, and reagents used in enzyme preparations were analytical reagent grade. Biochemicals were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Boehringer-Mannheim (West Germany). Male, white Wistar-derived rats (100–150 g) were obtained from the University of Sydney Animal House (Castle Hill, NSW, Australia). [^{3}H]BP and [^{3}H]styrene oxide were purchased from Amersham Australia Pty. Ltd. BP metabolite reference standards were provided by P. J. Little, NIH, Research Triangle Park, NC. Microanalyses were performed by the Australian Mineral Development Laboratories, Fisherman's Bend and Port Melbourne (Victoria, Australia).

Preparation of microsomes. The livers of male Wistar-derived rats (pretreated for 2 days with 25 mg/kg MC in corn oil prior to killing) were homogenized in potassium phosphate buffer (pH 7.4, 0.1 M) to make a 12.5% (w/v) suspension, and microsomes were prepared by differential centrifugation. Microsomal pellets were resuspended in Hepes (pH 7.4, 0.01 M) and stored for a maximum of 7 days at −20° or for a maximum of 3 months at −70° until assayed. The microsome preparation was shown to be stable over this time with respect to its AHH activity. Protein was determined colorimetrically according to the biuret method [12].

AHH. An assay, using radiolabeled substrate, based on that of De Pierre *et al.* [13] and Van Cantford *et al.* [14] was used. The incubation mixture consisted of 0.5 mM $NADP^+$, 3 mM isocitrate, 0.22 units of isocitric dehydrogenase, 5 mM $MgCl_2$, 0.2 mg microsomal protein, and 80 mM [^{3}H]BP (added in 50 μl acetone; 12.5 μCi/μmole) in 1 ml buffer (pH 7.4, 0.01 M Hepes). All test compounds studied for inhibitory properties were added in dimethyl sulfoxide.

The incubation was carried out at 37° for 15 min in a water bath with constant shaking. The reaction was stopped and treated as described by Van Cantfort *et al.* [14] except that the aqueous phase was extracted three times with hexane. The aqueous phase (0.5 ml) was then counted in aqueous counting solution (A.C.S. Amersham; 25 ± 2% counting efficiency). The reaction was shown to be linear with respect to protein concentration up to 0.2 mg/ml. Eighty nanomoles of BP per milliliter represents at least ten times the K_m under these conditions [15, 16]. The inhibitor was added in 0.05 ml of dimethyl sulfoxide. This concentration of dimethyl sulfoxide decreased the specific activity of AHH by only 10% when compared to a dimethyl sulfoxide free incubation. The test compounds were not metabolized in the above system (HPLC analysis, unpublished results).

Concentrations inhibiting 50% of MC-induced AHH activity (I_{50} values) were determined by a range of eight inhibitor concentrations in duplicate.

Metabolite analysis by HPLC. Incubations with hepatic microsomes obtained from MC-induced animals were carried out with the assay medium and incubation method described above, except that the incubation time was 10 min. The reaction was terminated by vortexing for 60 sec after the addition of 3 ml of ethyl acetate. The extraction procedure was repeated three times. More than 99% of the radioactivity was found in the organic phase.

Extracts from three identical incubations were pooled and evaporated in a stream of nitrogen, while being protected from light. The residue was dissolved in 0.05 ml acetone. BP and its metabolites were separated by HPLC using an Altex model 330 liquid chromatography system with a Browlee RP-8 10 μm analytical column. The column was eluted in a series of steps; a gradient of 40–55% methanol in water (1.5% change/min) was followed by a 55% methanol in water isocratic phase for 65 min; then, a rapid change to 100% methanol over 2 min was followed by a 100% methanol isocratic phase. The flow-rate was 1.5 ml/min for the initial gradient and isocratic stages and then increased to 2 ml/min. The column was operated at 40° and the eluent was monitored at 254 nm. Fractions were collected at 60-sec intervals, and the radioactivity was determined. Retention times of reference standards were also obtained in this system.

Epoxide hydrolase activity. Epoxide hydrolase activity was measured by the incubation of 2 mM [^{3}H]styrene oxide (added in 0.02 ml acetonitrile, 127 μCi/μmole) and 0.5 mg microsomal protein in 0.4 ml buffer (pH 9, 0.125 M Tris). The mixture was incubated at 37° for 5 min in a shaking water bath. The reaction was stopped by rapid cooling of the incubation tubes in ice. The incubation mixture was then extracted three times with 4 ml vol. of hexane. The radioactivity of a 0.1 ml aliquot of the aqueous phase was determined. The reaction was shown to be linear to 0.5 mg microsomal protein and to 8 min under the above conditions. A 2 μM concentration of styrene oxide represents a saturating concentration of substrate.

Inhibitory studies were carried out by the addition of the test compound to the incubation mixture of dimethyl sulfoxide (0.02 ml). To determine whether metabolites of the test compound formed by microsomal oxidation might be inhibitors of epoxide hydrolase, we preincubated the test compound with 0.5 mM $NADP^+$, 3 μM isocitrate, 0.1 units of isocitrate dehydrogenase, 5 mM $MgCl_2$ and 0.25 mg microsomal protein in 0.4 ml buffer. After a 30-min incubation at 37°, the pH was adjusted to 9.0 with 0.125 M Tris buffer in a total Volume of 0.8 ml, and the epoxide hydrolase activity was assayed as described previously.

Hansch analysis. The Hansch analysis was carried out with the use of a multiparameter equation-fitting FUNFIT interactive time-sharing program for general curve-fitting. This program computes the coefficients for the chosen form of the equation and

generates statistical data on the quality of the resultant fit.

Synthesis. All compounds prepared in this work gave proton NMR and mass spectra in agreement with their assigned structures and were homogeneous by TLC. The newly synthesized compounds had elemental analysis within ±0.4% of the calculated values.

4-Methyl-7-hydroxycoumarin [17], 7-hydroxycoumarin [18], 4-phenyl-7-hydroxycoumarin [19], 5-methyl-7-hydroxycoumarin [20], 4-methyl-6-ethyl-7-hydroxycoumarin [21], 4-methyl-6-chloro-7-hydroxycoumarin [22], and 4-propyl-7-hydroxycoumarin [23] were prepared by the cited literature methods. The melting points were in agreement with published values. Mass spectra and proton NMR data were in agreement with the assigned structures. The coumarin was then esterified with four times the molar equivalent of the appropriate acid chloride or anhydride. The esters were then used in the Fries rearrangement with an equimolar amount of aluminium chloride to give the desired product. When necessary, the compounds were purified by column chromatography on silica (100–200 mesh or type 60 plate grade) or by preparative TLC. All compounds were recrystallized from ethanol.

p-Ethyl and *p*-propyl benzoic acid were prepared by the Grignard reaction of *p*-ethyl [24] and *p*-propyl bromobenzene [24] with $CO_{2(s)}$ [25] respectively.

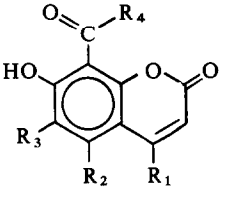
^{13}C NMR data. ^{13}C Spectra were run in 15% dimethyl sulfoxide/deuterated chloroform with trimethylsilane as an internal standard on a Joel CFT-20 spectrophotometer operating at 25 MH_z, in the fourier transform mode. A digital resolution of ±0.5 ppm was achieved by use of a 4500 H_z spectal width and 4K data points.

RESULTS AND DISCUSSION

Physical organic chemical characterization of the test compounds. The properties of compounds 1–25 are summarized in Table 1. Chemical shift assignments (Table 2) are based on multiplicity in the single frequency off resonance decoupled spectrum, the use of established substituent parameters, and internal consistency.

Analysis of AHH inhibitors. The common structural feature of compounds I-1 to I-25 is the 8-acyl-7-hydroxy function which may be considered as being an additional fused ring [9]. The most apparent feature of the inhibition data (Table 3) was the significant enchancement of activity in those compounds which possess an 8-benzoyl group such as: I-4 compared with I-1; I-10 compared with I-9; I-12

Table 1. 4,5,6-Substituted-7-hydroxy-8-acylcoumarins



Compound No.	R_1	R_2	R_3	R_4	m.p. (°)	Lit. m.p. (°)	Ref.
1	CH_3	—	—	CH_3	162–163	162–163	17
2	CH_3	—	—	n-C_3H_7	146–148	147–148	26
3	CH_3	—	—	$CH{=}CHCH_3$	136–138		
4	CH_3	—	—	C_6H_5	210	210	27
5	CH_3	—	—	$pCH_3C_6H_5$	258–260	260	28
6	CH_3	—	—	$mCH_3C_6H_4$	250		
7	CH_3	—	—	$oCH_3C_6H_4$	242–245	245	26
8	CH_3	—	—	$pC_2H_5C_6H_4$	242–245		
9	—	—	—	CH_3	164–166	167	29
10	—	—	—	C_6H_5	198–200		
11	C_6H_5	—	—	CH_3	162–164	165	30
12	C_6H_5	—	—	C_6H_5	221–223	225	30
13	C_6H_5	—	—	$pCH_3C_6H_4$	200–202		
14	—	CH_3	—	CH_3	190–192		
15	—	CH_3	—	C_2H_5	195–196		
16	—	CH_3	—	C_6H_5	223–226		
17	—	CH_3	—	$pCH_3C_6H_4$	218–220		
18	—	CH_3	—	$pC_2H_5C_6H_4$	200–202		
19	—	CH_3	—	$p(n$-$C_3H_7)C_6H_4$	188–190		
20	CH_3	—	Cl	C_6H_5	200–202	202	31
21	CH_3	—	Cl	$pCH_3C_6H_4$	191–193		
22	CH_3	—	C_2H_5	CH_3	136–138	140	32
23	CH_3	—	C_2H_5	C_6H_5	160	160–161	33
24	n-C_3H_7	—	—	C_6H_5	197–200		
25	n-C_3H_7	—	—	$pCH_3C_6H_4$	198–200		

Table 2. ^{13}C NMR shift assignments* (ppm)

Coumarin substituents	C-2	C-3	C-4	C-4a	C-5	C-6	C-7	C-8	C-8a
(a) 8-Benzoyl-7-hydroxy δ(CO) 193.00 ppm; $\delta(C_6H_5)$ 128.66 (2C),129.44 (2C), 133.73 and 137.02 ppm	160.12	112.28	143.84	111.51	129.98	113.39	158.79	115.51	152.56
(b) 8-Benzoyl-7-hydroxy-4-methyl δ(CO) 193.05 ppm; $\delta(C_6H_5)$ 128.59 (2C),129.37 (2C), 133.66 and 137.06 ppm; $\delta(CH_3)$ 18.73 ppm	158.51	111.20	151.92	112.41	126.51	112.99	158.51	114.98	152.81
(c) 8-Benzoyl-7-hydroxy-5-methyl δ(CO) 195.66 ppm; $\delta(C_6H_5)$ 129.56 (2C), 129.33 (2C), 133.56 and 136.61 ppm; $\delta(CH_3)$ 18.47 ppm	160.13	111.41	140.85	110.31	138.78	114.40	158.25	112.31	152.81
(d) 8-Benzoyl-7-hydroxy-4-methyl-6-ethyl δ(CO) 194.31 ppm; $\delta(C_6H_5)$ 128.50 (2C), 129.17 (2C), 133.42 and 137.70 ppm; $\delta(CH_2CH_3)$ 13.94 and 22.81 ppm; $\delta(CH_3)$ 18.64 ppm	159.71	111.25	150.31	112.31	126.17	128.84	156.69	114.41	152.86
(e) 8-Benzoyl-7-hydroxy-4-methyl-6-chloro δ(CO) 191.46 ppm; $\delta(C_6H_5)$ 128.78 (2C), 129.28 (2C), 133.96 and 136.59 ppm; $\delta(CH_3)$ 18.47 ppm	159.13	112.40	151.99	113.50	126.12	118.20	153.09	117.07	150.01
(f) 8-Benzoyl-7-hydroxy-4-phenyl δ(CO) 193.21 ppm; $\delta 8(C_6H_5)$ 128.64 (2C), 129.32 (2C), 133.84 and 137.08 ppm; $\delta 4$(CH) 127.6 (2C), 128.9 (2C), 130.6 and 141.6 ppm	159.12	111.15	155.90	112.52	128.88	113.79	158.68	115.43	152.63
(g) 8-Benzoyl-7-hydroxy-4-propyl δ(CO) 193.09 ppm; $\delta(C_6H_5)$ 128.62 (2C), 129.42 (2C), 133.62 and 137.08 ppm; $\delta(CH_2CH_2Ch_3)$ 14.0, 25.2 and 38.5 ppm	160.22	112.15	156.81	112.41	129.98	113.39	158.62	114.62	152.93

* ^{13}C Spectra were run in 15% dimethyl sulfoxide/deuterated chloroform using trimethylsilane as an internal standard on a Joel CFT-20 spectrophotometer operating at 25 MH_z in the Fourier transform mode. All resonances represent one carbon unless otherwise stated.

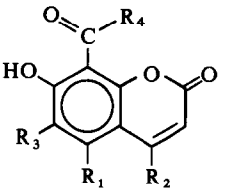
compared with I-11; and I-16 compared with I-14. This is not unexpected if one considers that these compounds would possess a four-ring planar system similar to other polycyclic aromatic hydrocarbons which are metabolized by this system or their inhibitors such as 7,8-benzoflavone or 6-aminochrysene [9]. The effect of modifying the 4, 5 and 6 positions of the coumarin nucleus is shown by the comparison of the activities of I-4, I-10, I-12, I-16, I-23, and I-24. The nonsubstituted coumarins, such as I-10, were very poor inhibitors; activity was increased markedly by all but one of the substitution patterns examined, i.e. 6-chloro-7-hydroxy-4-methyl.

A hydrophobic binding site close to the orientation of the 4-substituent of the coumarin ring is expected, if one compares the potency of those compounds with a 4-alkyl substituent and those without ring substituents. The results for the 4-propyl substituent compared with a 4-methyl substituent suggest, however, that this area is small, accommodating only one or two carbon atoms. A 4-phenyl substituent is a bulky hydrophobic functional group, which could be expected to interact sterically and electronically with the cytochrome enzyme-active site.

I-11 was a better inhibitor than the corresponding 4-methyl coumarin I-1. However, the inhibitory potencies of compounds I-12 and I-13, and of the corresponding 4-methyl inhibitors I-4 and I-5, were not significantly different. The effects of steric hindrance, increased lipophilicity, and electronic interaction due to the 4-phenyl substituent are thus not clearly separated. Compounds in the group of those with a 5-methyl substituent were, however, the most active of those tested. The lipophilicities and molar refractivities of compounds with a 4-methyl or 5-methyl substituent are identical. Therefore, one must postulate either the existence of a hydrophobic binding site near the orientation of the 5-substituent or an electronic interaction caused by the presence of a 5-methyl group. Evidence for the latter was provided by the analysis of the quantitative structure–activity relationship (*vide infra*).

Activity of the candidate inhibitors was abolished with the introduction of a 6-chloro group into the aromatic portion of the coumarin nucleus. This was shown to be due to an effect on electron density rather than steric repulsion (*vide infra*). With the introduction of a 6-ethyl group into the aromatic ring of the coumarin nucleus, I-22 did not show a significant increase in potency when compared with I-1, but I-23 was approximately two to three times more active than the corresponding inhibitor I-4. This suggests a favourable electron density interaction in the region of the 6-substituent, thus placing

Table 3. Inhibition of AHH in MC-induced rat liver microsomes



Compound No. (I)	R_1	R_2	R_3	R_4	$I_{50} \times 10^{-5}$ M*
1	CH_3	—	—	CH_3	85
2	CH_3	—	—	n-C_3H_7	70
3	CH_3	—	—	$CH{=}CHCH_3$	200
4	CH_3	—	—	C_6H_5	25
5	CH_3	—	—	$pCH_3C_6H_4$	8
6	CH_3	—	—	$mCH_3C_6H_4$	12
7	CH_3	—	—	$oCH_3C_6H_4$	9.5
8	CH_3	—	—	$pC_2H_5C_6H_4$	0.8
9	—	—	—	CH_3	370
10	—	—	—	C_6H_5	172
11	C_6H_5	—	—	CH_3	36
12	C_6H_5	—	—	C_6H_5	18.5
13	C_6H_5	—	—	$pCH_3C_6H_4$	7
14	—	CH_3	—	CH_3	58
15	—	CH_3	—	C_2H_5	48
16	—	CH_3	—	C_6H_5	14.5
17	—	CH_3	—	$pCH_3C_6H_4$	0.8
18	—	CH_3	—	$pC_2H_5C_6H_4$	0.83
19	—	CH_3	—	$p(n$-$C_3H_7)C_6H_4$	0.4
20	CH_3	—	Cl	C_6H_5	N
21	CH_3	—	Cl	$pCH_3C_6H_4$	N
22	CH_3	—	C_2H_5	Ch_3	93
23	CH_3	—	C_2H_5	C_6H_5	9.3
23	n-C_3H_7	—	—	C_6H_5	27
25	n-C_3H_7	—	—	$pCH_3C_6H_4$	6

* Control value ± S.E. (number of observations) was 2.8 ± 0.5 nmoles BP oxidized·(mgmicrosomal protein)$^{-1}$·min^{-1} (12). N = no inhibition observed at 100×10^{-5} M test compound. All I_{50} values represent the average of three determinations, which agreed within ±10%.

the three pseudo polycyclic aromatic rings in a more favourable position for interaction with the enzyme active site.

Regression analysis of the inhibition data. The relative rates of oxidative metabolism of drugs have been shown by many workers [34–36] to be related to the relative lipophilic character of the molecule. The apparent relationship between potency and hydrophobicity and the possible involvement of other physico-chemical parameters led to multiple regression analyses of the data of Table 3 to identify the factors which influence the potency of these microsomal oxidase inhibitors (Table 4).

The data were fitted to various forms of the equation:

$$pI_{50} = a \log P + b(\log P)^2 + CMR + dE + f$$

where pI_{50} is the negative log of the molar I_{50} value, P is the octanol/water partition coefficient, MR is the molecular refractivity, and E a parameter relating to the electron densities at sites within the molecule. The log P of coumarin used was 1.39 [37]. This value was corrected for hydrogen bonding between the 7-hydroxy and 8-carbonyl functions by +0.63 which is the average difference in log P between *ortho*- and *para*-substituted compounds in which hydrogen bonding would be expected to occur. The molar refractivity (MR) was used as an index of "bulkiness" of the compounds. The MR of each compound was calculated as the sum of individual substituent values. The MR of coumarin was arbitrarily set at 0. The additive approach to determining MR has been justified previously [38]. 0 and MR values for substituents were obtained from Refs. 39 and 40.

A correlation between lipophilicity and inhibitory activity was found for I-1, I-2, I-4 to I-19, and I-22 to I-25 (Table 4). A first-order analysis of log P explained 57% of the variance of the regression. A second-order (parabolic) analysis was not statistically justified (P = 0.05). The regression was only slightly improved by the inclusion of MR (Table 4).

At this stage it became apparent that the electronic characteristics of the substituents needed to be evalu-

Table 4. Regression analysis of the inhibition of AHH in MC-induced rat liver microsomes: compounds (1), (2), (4–19), (22–25)*

	r	r^2%	s	$F_{(x,y)}$	Eq.
$pI_{50} = 0.53 (\pm 0.10)$ lot $P + 2.16 (\pm 0.32)$	0.75	57.2	0.26	$29.4_{(1,21)}$	1
$pI_{50} = 0.68 (\pm 0.54)$ log $P - 0.03 (\pm 0.09)$ log $p^2 + 1.97 (\pm 0.74)$	0.75	57.1	0.30	$12.7_{(2,20)}$	2
Compare equations 3 and 2					
$F = 0.022$					
$F_{(1,19)0.5} = 4.41$					
$pI_{50} = 1.81 (\pm 0.69)$ log $P - 0.11 (\pm 0.06)$ *MR* $+ 2.60 (\pm 0.39)$	0.79	63.4	0.23	$18.2_{(2,20)}$	3
Compare equations 4 and 2					
$F = 3.27$					
$F_{(1,19)0.5} = 4.41$					
$pI_{50} = 0.54 (\pm 0.09)$ log $P + 0.02 (\pm 0.01)$ σ^5 $- 0.03 (\pm 0.01)$ $\sigma^6 - 7.98 (\pm 0.95)$	0.82	68.5	0.21	$14.5_{(2,20)}$	4
Comparing equations 5 and 2					
$F = 6.815$					
$F_{(1,19)0.5} = 4.41$					
$pI_{50} = 1.19 (\pm 0.71)$ log $P - 0.10 (\pm 0.05)$ *MR* $+ 0.03 (\pm 0.01)$ $\sigma^5 - 0.03 (\pm 0.01)$ σ^6 $+ 1.21 (\pm 1.12)$	0.87	75.2	0.18	$10.9_{(3,19)}$	5
Comparing equations 6 and 2					
$F = 13.06$					
$F_{(2,18)0.5} = 3.55$					

* Abbreviations: pI_{50} = the negative log 10 at I_{50} (molar) value; P = octanol/water partition coefficient; *MR* = molecular refractivity; σ^5, σ^6 = ^{13}C NMR shift for 5 and 6 carbons, respectively; r = correlation coefficient; s = standard deviation from the regression; F and F_i are the values calculated from the F ratio with the number of degrees of freedom (y, x) at the indicated probability level.

ated. The simplest method of evaluating the electronic contribution of substituents is to use Hammett substituent constants σ [39, 40]. Other approaches include those of separating the field and resonance components [41] which may also be weighted [42] for *ortho*, *meta*, and *para* substitution. However, very little attention has focused on the applicability of the existing constants for heterocyclic systems such as coumarins, although the thiophene [43], benzimidazole, benzotriazole, indole and indazole [44] systems have been examined.

The best approach depends on the linear relationship between ^{13}C NMR shifts and net carbon charges [45]. A correlation between chemical shifts and Hammett σ constants has also been noted [46, 47]. In the case of coumarin, a satisfactory fit between Hammett constants and ^{13}C shifts has also been found for the "side chain" positions, i.e. 2, 3, 4, 4a, and 8a [48]. The chemical shifts for all positions of the 8-benzoyl-7-hydroxycoumarins were assigned, and it was found that, as expected, the chemical shifts for all positions except 4, 5, and 6 were relatively constant. The chemical shifts for the 4, 5, and 6 positions for which ^{13}C NMRs were run (I-4, I-10. I-12, I-16, I-20, I-23, and I-24) were expected to be constant for that coumarin ring substitution pattern when different 8-acyl functions were present. The 4, 5 and 6 position chemical shifts were used as an additional parameter for regression analysis.

Using chemical shifts at carbons 5 and 6 (σ^5 and σ^6 respectively) as well as log P and *MR*, a good correlation was achieved for I-1, I-2, I-4 to I-19, and I-22 to I-25. The squared correlation matrix for equation 5 is shown in Table 5. From equations 4 and 5, to optimise activity carbon 5 of the coumarin should be deshielded and carbon 6 shielded. From this, one can postulate the existence of a dipole on the receptor surface. The interaction between the cytochrome and potent inhibitors or good substrates would be such that electrostatic repulsion is minimized. Thus, electrostatic repulsion explains the absence of inhibitory activity of the 6-choro-7-hydroxy-4-methyl-coumarins where the 6 position would be relatively deshielded.

Table 5. Squared correlation matrix*

	Log P	*MR*	σ^5	σ^6
Log P	1.0			
MR	0.66	1.00		
σ^5	0.00	0.01	1.00	
σ^6	0.03	0.00	0.86	1.00

* Abbreviations are the same as used in Table 4.

Multiple regression analysis of the AHH activities in MC-induced microsomes revealed that activity was associated with three factors: (1) the hydrophobicity of the compound, (2) the electron distribution, and (3) its molecular refractivity. These results show that the fit of the inhibitors is not an "all-or-none" situation, but rather a binding or partial insertion of groups of moderate size on or into a macromolecular "pouch" in a continuous linear process. But, because the free energy change does not closely parallel only partioning or molecular volume or electronic factors, one must assume that there is not a great deal of flexibility in the macromolecular receptor site.

The overall poor regression correlation may be explained in terms of an unidentified physico-chem-

ical parameter. But, as a consequence of the multiplicity of cytochromes present in MC-induced preparations [49] which may have varying susceptibilities to inhibition, it may not be possible to obtain completely satisfactory regression analyses unless an enzymically homogeneous preparation is used.

BP metabolite profiles. The assay procedure used is able to separate and quantitate a number of BP metabolite fractions. Although the reactive arene oxide intermediates are not detected, if it is assumed that the dihydrodiols originate from arene oxide precursors [50], then the detection of these dihydrodiols provides a measure of the amount of epoxide formed.

The metabolite profiles could potentially indicate the stage at which metabolite formation is inhibited. From this the inhibition mechanisms may be postulated. Whereas the assignment of a particular profile area to only one metabolite is not possible, the retention times of reference metabolite standards may be used to indicate the possible metabolites to which the peaks may correlate. Good separation of individual quinones or phenols was not possible on the program used. Typical chromatographic profiles of BP metabolites are shown in Fig. 1.

Table 6 shows the relative distribution of BP metabolites in the absence of inhibitor. Table 7 shows the effect of three inhibitors of BP metabolite formation. In the presence of 7-hydroxy-4-methyl-8-(*p*-toluolyl)coumarin, the ratios of the uninhibited to inhibited dihydrodiol, quinone, and phenol metabolites were approximately 3, 1.1, and 1.7 respectively. More significantly, the ratios of the uninhibited to inhibited 9,10-dihydrodiol, 4,5-dihydrodiol, and 7,8-dihydrodiol metabolites were approximately 7, 1.15, and 3 respectively.

The data indicate that metabolism of BP has been inhibited almost entirely in the 7, 8, 9, 10 ring of the BP molecule. In the presence of 7-hydroxy-4-phenyl-8-(*p*-toluoyl)coumarin and 8-(*p*-*n*-propylbenzoyl)-7-hydroxy-5-methylcoumarin, similar data were obtained. Although metabolism was more generally inhibited in the case of the latter two inhibitors, the 7, 8, 9, and 10 positions were again those at which oxygenation was most inhibited.

Stereochemical factors in the metabolism of compounds by monooxygenases, such as the more extensive metabolism of *S*-(+)-amphetamine compared with the *R*-(−) isomer [51], suggest the need for "steering groups" to enable orientation near the heme and "activated oxygen". Polycyclic aromatic hydrocarbons, however, do not have functional groups that may serve in this capacity; consequently, a wide variety of possible sites of interaction are expected. A fairly indiscriminant resonant interaction with the prophyrin ring of the heme may occur, dictated only by differences in electron density of either the polycyclic aromatic hydrocarbons itself or of the enzyme active site.

The different cytochrome P-450 isozymes exhibit both position and stereo selectivity [52]. This selectivity is expected if one considers that the amino acid sequence and secondary structure would determine the enzyme active site, i.e. the heme environment and, consequently, the electron density around the porphyrin ring. The HPLC data, which indicate that metabolism in the 7,8- and 9,10-positions is most inhibited, suggest that inhibitor binding is occurring

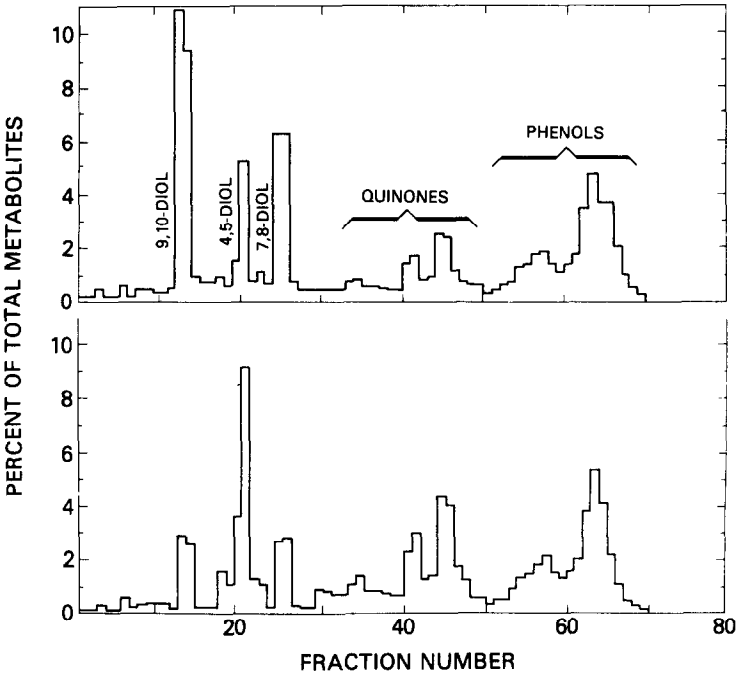


Fig. 1. Upper profile: Chromatographic profile of BP metabolites obtained in the absence of inhibitor. Specific AHH activity: 3.75 nmoles·min^{-1}·(mg protein)$^{-1}$. Lower profile: Chromatographic profile of BP metabolites obtained in the presence of 7-hydroxy-4-methyl-8-(*p*-toluoyl)coumarin (80 μM). Specific AHH activity: 1.87 nmoles·min^{-1}·(mg protein)$^{-1}$.

Table 6. Relative distribution of BP metabolites*

Expt. No.	Specific activity [nmoles·min^{-1}·(mg protein)$^{-1}$]					
	Dihydrodiols					
	9,10-	4,5-	7,8-	Quinones	Phenols	Total
1	0.86	0.43	0.58	0.66	1.22	3.75
2	0.94	0.46	0.53	0.71	1.18	3.82
3	0.83	0.41	0.59	0.65	1.26	3.74

* Variation among these experiments was ±12%. Incubation conditions: 80 μM BP, 0.2 mg (MC-induced) microsomal protein, 10-min incubation period.

near the enzyme active site. The binding apparently alters the electron density around the porphyrin ring such that metabolism in the 7,8- and 9,10-positions is more unfavourable.

HPLC has been used [53, 54] to study the mechanism of 7,8-benoflavone inhibition. It was found [53] that 7,8-benzoflavone acts on the oxidase to inhibit oxygenation at all sites of the BP molecule, or on a prior component of the microsomal electron transport chain. However, 15,16-dihydro-11-methyl-cyclo-penta[*a*]phenanthren-17-one metabolism [54] was shown to be differentially inhibited by 7,8-benzoflavone.

There does not appear to be a correlation between the inhibitory potency and the degree of inhibition at the 7,8- and 9,10-positions. For example, 8-(*p*-*n*-propylbenzoyl)-7-hydroxy-5-methylcoumarin was the most potent inhibitor tested, but 7-hydroxy-4-methyl-8-(*p*-toluoyl)coumarin was the most potent inhibitor of metabolism at the 7,8- and 9,10-positions of BP.

Absence of effect on epoxide hydrolase. A decrease in dihydrodiol formation may occur with the inhibition of epoxide hydrolase which hydrates reactive primary expoxides produced by AHH to form dihydrodiols [50]. The dihydrodiols, in turn, may be converted into highly electrophilic diol-epoxides [55]. The possibility of epoxide hydrolase inhibition by the candidate inhibitors was examined, but no detectable epoxide hydrolase inhibtion or stimulation was observed up to 10–100 times those required to inhibit AHH by 50%. 7-Hydroxy-4-methyl-8-(*p*-toluoyl)coumarin was preincubated as described to determine whether metabolites of the inhibitor, formed by microsomal oxidation, might be epoxide hydrolase inhibitors. No inhibition of epoxide hydrolase was detected.

Epoxide hydrolase inhibitors described to date [56–58] are of a limited chemical type; hence, it is not surprising that the tested compounds were not expoxide hydrolase inhibitors.

The decreased formation of the 7,8- and 9,10-arene oxides must, therefore, be postulated as being responsible for the decreased 7,8- and 9,10-dihydrodiol formation in the presence of inhibitor. We postulate that inhibition occurs at the enzyme active site, probably through a perturbation of the electron cloud density (of the site) such that oxygenation at the 7,8- and 9,10-positions is unfavourable.

A two-stage metabolic activation involving the formation of the 7,8-dihydrodiol, which is further metabolized by the oxidation of the adjacent 9,10-olefinic bond, has been proposed [55]. The 7,8-dihydrodiol but not the 9,10-dihydrodiol has been shown to be converted by cells into an intermediate that reacts with DNA [59], and more recent studies have established the 7,8-diol-9,10-epoxides as the proximate carcinogens [60]. The inhibitors described

Table 7. Effect of inhibitors on BP metabolite formation*

Inhibitor (I)	Concn*†	Exp. No.	Ratio: Control specific activity / Specific activity in the presence of I					
				Diols				
			1	2	3	Total	Quinones	Phenols
7-Hydroxy-4-methyl-8-(*p*-toluoyl)coumarin	80	1	7.17	1.13	4.46	2.96	1.20	1.77
		2	6.69	1.22	4.75	3.08	1.15	1.65
		3	7.67	1.15	4.35	3.09	1.08	1.69
7-Hydroxy-4-phenyl-8-(*p*-toluoyl)coumarin	70	1	2.96	1.23	2.90	2.22	1.43	2.10
		2	3.11	1.22	2.85	2.23	1.38	2.11
		3	3.07	1.29	2.71	2.20	1.45	2.06
8-(*p*-*n*-Propylbenzoyl)-7-hydroxy-5-methylcoumarin	4	1	2.60	1.26	3.05	2.17	1.17	2.25
		2	2.90	1.34	3.17	2.35	1.17	2.19
		3	2.88	1.45	3.59	2.41	1.20	2.30

* Incubation conditions: 80 μM BP, 0.2 mg (MC-induced) microsomal protein, 10-min incubation period.

† The concentration of inhibitor used was the concentration which had been shown previously to inhibit BP metabolism by 50%.

may be helpful in defining the role of the cytochrome P-450 mediated monooxygenases in polycyclic hydrocarbon induced toxicity and carcinogenicity.

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