1

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

**STRUCTURE-BINDING RELATIONSHIP
OF STEROIDAL COMPOUNDS TO
CYTOCHROME P-448**

by

ZENITH CHAN, B.Sc. (U.of O.)

A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the requirements for the degree of
Master of Science
Department of Chemistry

Carleton University
Ottawa, Ontario
December 1990

© copyright
1990, Zenith Chan

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-68930-7

Canada

The undersigned recommend to the Faculty of Graduate Studies
and Research acceptance of the thesis

STRUCTURE-BINDING RELATIONSHIP
OF STEROIDAL COMPOUNDS TO
CYTOCHROME P-448

submitted by Zenith Chan, B.Sc.
in partial fulfilment of the requirements for
the degree of Master of Science

Thesis Supervisor

Chair, Department of Chemistry

Carleton University
1990

ABSTRACT

Cytochrome P-448, an isozyme of the Poly-Substrate Mono-Oxidase cytochrome P-450, is known to be solely responsible for the metabolic activation of the environmental carcinogens, polycyclic aromatic hydrocarbons (PAH's). Apart from PAH's, the physiological substrates for this enzyme have not been firmly identified. Since steroids have an analogous multicyclic structure, they have been proposed as the one of the natural substrates for cytochrome P-448. This study tested and supported the hypothesis by demonstrating the inhibition capability of steroidal compounds in a standard cytochrome P-448 activity assay. It also showed that planar and highly hydrophobic molecules displaying a better binding affinity to cytochrome P-448 than those which are corrugated and polar. Therefore PAH's coincidentally bind more readily to cytochrome P-448 than other physiological substrates.

ACKNOWLEDGEMENTS

I wish to thank my supervisor, Dr. Bryan R. Hollebone for his patience and support during the course of my graduate work. His insights and suggestions helped the development of my own creative thinking.

I wish to extend my gratitude to Lorna Brownlee and Faliana Chan for their help in dosing and caretaking of the test animals. I would also like to thank Chantale Ouimet for proof-reading this thesis for me and the members of Dr. Hollebone's laboratory, Jeff Warner, Pam Wolff and Cathy Shewchuk, who all helped me one way or another.

Lastly, I would like to dedicate this work to my parents, Peter and Rachel Chan, and all my good friends for their support through my lifetime.

TABLE OF CONTENTS

Acceptance Sheet	ii
Abstract	iii
Acknowledgement	iv
Tables of Contents	v
List of Tables	viii
List of Figures	ix
List of Abbreviations	x
A. Introduction	1
A-1. Poly-Substrate Mono-Oxidase System and its function	1
A-2. Differences between cytochromes P-450 and P-448	5
A-3. Physiological function of cytochrome P-448	7
A-4. Thermodynamics and enzymatic control	9
B. Hypothesis	12
C. Experimental Design	13
C-1. Inhibitor parameter	13
C-1a. Planarity	13
C-1b. Corrugation: determination of Total Torsional Angle	14
C-2. Enzyme behaviour	15
C-3. Control reaction: ethoxyresorufin deethylation	19
D. Methods and Materials	23
D-1. Chemicals	23
D-2. Reagents, buffers and supporting chemicals	24
D-3. Animals and pretreatment	25
D-4. Preparation of gel columns	27
D-5. Isolation of microsomes	28

D-6. Determination of protein concentration	30
D-7. Fluorometric assay of ethoxyresorufin deethylation	31
E.Results	34
E-1. Independent parameter	34
E-2. Dependent parameter	45
E-2a.Cytochrome P-448 activity: ethoxyresorufin deethylation control	45
E-2b.Determination of % inhibition by steroidal test compounds	45
E-2c.Relationship between steroidal concentration and % inhibition	46
F.Discussion	60
F-1. Evaluation of inhibition capacity	60
F-2. Correlation of molecular planarity and inhibitor binding	63
F-3. Effect of molecular hydrophobicity on inhibitor binding	65
F-4. Concerted effect of planarity and hydrophobicity	70
F-5. Conclusions	74
G.References	76
H.Appendices	80
A.General calculations	80
B.Ethoxyresorufin deethylation activity data	81
C.% inhibition data	91
D.Linear relationship between independent (P) and	

dependent (pI') parameters	95
E. Polynomial equations for the fitted sigmoid curves and the calculation of pI'	97
E. Calculation of hydrophobicity	100
F. Correlation of HxP to pI'	105

LIST OF TABLES

Table I	Endocyclic torsional angles of AND	36
Table II	Endocyclic torsional angles of CDE	37
Table III	Endocyclic torsional angles of DHT	38
Table IV	Endocyclic torsional angles of 146AND	39
Table V	Endocyclic torsional angles of ES	40
Table VI	Endocyclic torsional angles of DHE	41
Table VII	Endocyclic torsional angles of EQ4	42
Table VIII	Endocyclic torsional angles of EQ5	43
Table IX	Summary of the Total Torsional Angle and planarity parameters for the test steroidal inhibitors	44
Table X	Relationship of p[CDE] and % inhibition	48
Table XI	Relationship of p[DHT] and % inhibition	48
Table XII	Relationship of p[146AND] and % inhibition	49
Table XIII	Relationship of p[ES] and % inhibition	49
Table XIV	Relationship of p[DHE] and % inhibition	50
Table XV	Relationship of p[EQ4] and % inhibition	50
Table XVI	Relationship of p[EQ5] and % inhibition	51
Table XVII	Relationship of p[Chry] and % inhibition	51
Table XVIII	Comparison of molecular planarity and the binding strength of the test steroidal inhibitors	62
Table XIX	Comparison of planarity and hydrophobicity parameters of the test steroidal inhibitors	67

LIST OF FIGURES

Figure 1	Model reaction mechanism for cytochrome P-450	2
Figure 2	Metabolism of benzo(a)pyrene to arene oxide, dihydrodiol and to the bay-region epoxide, benzo(a)pyrene-7,8-diol-9,10-epoxide	4
Figure 3	Minimum area of the binding site of cytochrome P-448	6
Figure 4	Structures of chrysene and 5 α -androstande	8
Figure 5	Deethylation of 7-ethoxyresorufin to resorufin by cytochrome P-448	20
Figure 6	Structures of the steroidal inhibitors and 7-ethoxyresorufin	32
Figure 7	Numbering of carbon atoms on the 4-ring steroidal system	33
Figure 8	Relationship between p[CDE] and % inhibition	52
Figure 9	Relationship between p[DHT] and % inhibition	53
Figure 10	Relationship between p[146AND] and % inhibition	54
Figure 11	Relationship between p[ES] and % inhibition	55
Figure 12	Relationship between p[DHE] and % inhibition	56
Figure 13	Relationship between p[EQ4] and % inhibition	57
Figure 14	Relationship between p[EQ5] and % inhibition	58
Figure 15	Relationship between p[Chry] and % inhibition	59
Figure 16	Relationship between planarity (P) and binding affinity (pI')	64
Figure 17	Relationship between hydrophobicity (H) and binding affinity (pI')	68
Figure 18	Correlation between the concerted property of hydrophobicity and planarity (HxP) and the binding affinity (pI')	69

LIST OF ABBREVIATIONS

146AND	1,4,6-androstatrien-3,17-dione
AND	Androstane
BNF	β -naphthoflavone
BP	Benzo(a)pyrene
CDE	3,5-cholestadiene
Chry	Chrysene
DHE	6-dehydroestrone
DHT	1-dehydrotestosterone
[E]	Enzyme Concentration
EH	Epoxide Hydrolase
EQ4	Equilin
EQ5	d-Equilenin
ES	Estrone
[I]	Inhibitor Concentration
PSMO	Poly-Substrate Mono-Oxidase
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, Reduced Form
PAH's	Polycyclic Aromatic Hydrocarbons
pI	-logarithmic value of inhibitor concentration
[S]	Substrate Concentration

A. Introduction

A-1. Poly-Substrate Mono-Oxidase System and its function

Polycyclic aromatic hydrocarbons (PAH's) have long been implicated as the leading cause in chemical carcinogenesis¹. PAH's are activated to ultimate carcinogens, which are very reactive towards DNA due to their electrophilicity, by the Poly-Substrate Mono-Oxidase (PSMO) enzyme system^{2 3 4 5}. This is an ubiquitous family of enzymes⁶, also known as monooxygenases, that catalyse dioxygen activation using two electrons and two protons, with the insertion of one oxygen atom from O₂ into an organic substrate and the formation of water (equation 1 & figure 1⁷).

EQUATION 1

Many of these monooxygenases contain a heme protein called cytochrome P-450 which is the site of dioxygen activation. The absorption maximum seen near 450 nm for the Fe²⁺-CO complex is characteristic of the axial thiolate ligation provided by a protein cysteinyl residue, hence the

name cytochrome P-450 (EC 1.14.14.1). These cytochrome P-450-dependent monooxygenases are involved in many steps of the biosynthesis and biodegradation of endogenous compounds^{8 9 10} such as steroids¹¹, fatty acids,¹² prostaglandins¹³ and leukotrienes¹⁴. More importantly, they play a key role in the oxidative metabolism of exogenous compounds such as drugs and other environmental products allowing their elimination from the living organisms¹⁵. The apparent hydroxylation of lipophilic xenobiotics decreases their hydrophobicity and enables them to be coupled or conjugated with an endogenous substrate (e.g. UDP-glucuronic acid, acetyl-Co-A, glutathione, glycine and phosphoadenosyl phosphosulphate) to yield polar molecules. This is the intended reaction of cytochromes P-450 which essentially provides a protective mechanism against the accumulation and adverse effects of xenobiotic chemicals by increasing their polarity for subsequent excretion¹⁶.

However, cytochromes P-450 can also catalyse the activation of chemicals to reactive electrophilic intermediates which interact with vital cellular macromolecules. Such accidental reactions could give rise to toxicity and carcinogenicity¹⁷, since many carcinogens, such as PAH's, aromatic amines and aflatoxins express their toxicity only after oxidative metabolism. It was soon realised that more than one form of cytochromes P-450 exists, since an additional form of cytochromes P-450 could be

demonstrated spectrophotometrically after treatment with PAH's¹⁸. This sub-type of cytochromes P-450 can be called cytochrome P-448, although it is also known under other more specific isozyme terminologies (P-450c, P-450IA, P-450LM₄, etc.) depending on the type of animals used (rats, mice, rabbits or hamsters) and methods of determination (spectroscopic, immunological or genetic)¹⁹. Cytochrome P-448 is known to metabolically activate PAH's almost exclusively²⁰. As a result, PAH's such as benzo(a)pyrene¹²¹ (a procarcinogen) can be converted to a so-called bay region epoxide (figure 2)^{22 23}. This type of arene oxide is rather stable and sterically resistant to epoxide hydrolase (EH) enzyme action².

Figure 2 Metabolism of benzo(a)pyrene to arene oxide, dihydrodiol, and the bay-region epoxide, benzo(a)pyrene-7,8-diol-9,10-epoxide

Eventually this bay region epoxide (an ultimate carcinogen) can interact with cellular DNA and cause carcinogenic mutation. The balance between activating and

detoxifying enzymes is thus often the determining factor in expression of toxicity^{24 25}. This may be altered by selective induction of cytochrome P-450 isozymes following exposure to chemicals and by factors such as age, nutrition and diet²⁶. It is anomalous and most curious that the body has devised an enzyme system solely to achieve carcinogenesis^{26 27}. That brings one to question the physiological purpose for the presence of P-448.

A-2. Differences between cytochromes P-450 and P-448

P-450 and P-448 are notional names denoting the absorption maximum of the CO ligand spectra of the cytochromes with reduced iron (Fe^{2+}). Cytochrome P-450 is found at low levels in most tissues but it is principally distributed in the liver where the cytochrome is readily inducible²⁷. Cytochrome P-450 is less readily inducible in other tissues. Cytochrome P-450 is never detected nor induced in the placenta²⁸ and is present in neonates at low levels which increase with age²⁹. Cytochrome P-448 is distributed at low levels in most tissues including the placenta²⁸. It is highly inducible in liver and placenta, and is readily induced in lung, kidney and gut²⁸. Both cytochromes P-448 and P-450 are slightly inducible in the adrenals, with the former almost twice as inducible as that of the latter³⁰. Cytochrome P-448 has a predominate presence in neonates and its level decreases

with age²⁹. Both cytochromes P-450 and P-448 possess more than one isoform of enzymes¹⁹.

Work distinguishing between cytochromes P-450 and P-448 in terms of their inducibility³¹ and protein structure³² has been well documented. Cytochrome P-450 possesses a substrate-binding site of broad specificity which can accommodate a diversity of molecular structures²⁰ with a small area/depth ratio. These structures are usually very flexible that can adopt a more or less globular shape²⁰. P-448 is known to have a narrower substrate specificity to essentially planar and rigid molecules with large area/depth ratio²⁰ and can carry out highly

stereoselective oxygenation^{33 34}.

Based on investigations into the absolute configurations of several benzo(a)pyrene metabolites, a model has been proposed to define the minimum area of the cytochrome P-448 binding site²² (figure 3).

Figure 3 Minimum area of the binding site of cytochrome P-448

PAH's, therefore, appear to be the ideal substrates for cytochrome P-448.

A-3. Physiological function of cytochrome P-448

A primary function of the placenta is steroid biogenesis²⁷. It provides a physical barrier to protect the foetus and to regulate the amount of steroids that are essential to foetal development. As the highly inducible P-448 is the only significant cytochrome P-450 isozyme present in the placenta, it is highly probable that steroids are the physiological substrates of cytochrome P-448. Neither foetus nor placenta possesses all the essential enzymes in the metabolic pathway of steroids (e.g. oestrogen and progesterone). The two tissues act in concert (the foeto-placental unit) and complement each other to complete the biosynthesis of steroids³⁵. This arrangement provides protection for the foetus from high levels of biologically active steroid hormones. This can also explain why cytochrome P-448 predominates in neonates since steroid hormones are important in regulating their proper growth and development. As maturation and development of the animal are completed, steroid hormones play a lesser role in bodily function thus lowering the required presence of cytochrome P-448.

The narrow specificity of P-448 makes it a good candidate to be linked to steroid metabolism, which requires precise enzymatic catalysis to achieve optimal biological activity. The ability of cytochrome P-448 to oxygenate at sterically

hindered positions in molecules can be compared to the enzymatic process in steroid metabolism, which also requires oxygenation at conformationally hindered positions³. The basic steroidal carbon skeleton of 4 fused aliphatic rings certainly resembles to the fused aromatic rings of PAH's. The binding site of cytochrome P-450 allows diverse structures with globular shapes to fit and is not stereospecific in its oxidation reactions. In contrast, with its large area/depth binding site and stereoselectivity²⁰, cytochrome P-448 is suitable to carry out sterically hindered reactions on the large molecules of fused rings, such as oxidation and aromatisation³⁶. Therefore, it is likely that cytochrome P-448 is biologically designated for the regulatory steroid metabolism²⁷.

A-4. Thermodynamics and enzymatic control

Figure 4 Structures of chrysene (left) and 5α-androstane (right)

The first step in exploring the hypothesis that steroid molecules are the natural substrate for cytochrome P-448 is to

study the substrate-enzyme binding affinities of selected steroids. Chrysene (figure 4), an aromatic and essentially planar analogue of the steroidal skeleton with a 2-fold rotation axis of symmetry, is predominately epoxidised at the 1,2- and 3,4- positions by cytochrome P-448³⁷. By analogy, if 5 α -androsterane (figure 4), an aliphatic and highly corrugated molecule, is fitted into the binding site of cytochrome P-448, it would be oxygenated preferentially on rings A and D. Cytochrome P-448 should oxidise on the α -face of the 5 α -androsterane molecule, which is on the flip side of the exocyclic methyl groups on the β -face. This is not unlike the natural metabolism of steroids, where most chemical modifications occur at rings A and D. Therefore, 5 α -androsterane and chrysene should define the extremities of the structural requirement for binding of multiple-ring molecules to cytochrome P-448.

The rate of oxidation at any site on the substrate is dependent on concentrations of both substrate and cofactors:

$$\text{Rate} = k_1 [\text{substrate}] [\text{cofactors}]$$

EQUATION 2

The observed rate of reaction is dependent on the free energy barrier according to the equation expressed by van't Hoff and Arrhenius³⁸. For any such second order reaction,

$$\ln k_1 = \ln P - \frac{\Delta G^*}{RT}$$

EQUATION 3

where P is the probability of reaction at transition state.
 Since $P = 1$, thus $\ln P = 0$.

$$\Delta G^* = \Delta H^* - T\Delta S^*$$

EQUATION 4

I>Enthalpy ΔH^* = strength of bond(s) to be broken

II>Entropy ΔS^* = ease of fitting substrate into active site (i.e. the frequency factor)

When designing the experimental methodology, it is necessary to separate the influences of enthalpy and entropy by keeping one constant while varying the other.

The enzymatic reaction of cytochromes P-450 depends exclusively on enthalpy since the weakest C-H bond of the molecule is preferentially oxygenated³⁹. This is understandable since P-450 can bind with various molecules with no positional preference and the rapid rotation of molecules allows the enzymatic active site to "pick out" and attack the weakest bond. The rate of reaction is inversely

proportional to the bond strength of weakest C-H bond³⁹. Cytochrome P-448 behaves more like a conventional enzyme than cytochrome P-450, since it displays a narrow specificity for substrates²⁰. This specificity is characteristic for most enzymes because they usually perform their function by orienting well defined molecules to specific reactions in the metabolic pathways of substrates and cofactors. This suggests that entropic control plays a dominant role in most forms of enzymatic catalysis. Thus, the reaction rate at the active site of cytochrome P-448 likewise should be dominated by the ease of binding of substrate and the $T\Delta S^*$ term is the dominant component in governing the free energy, ΔG^* , of the reaction (equation 4).

B. Hypothesis

The literature suggests that the large but shallow^{20 22} binding site of cytochrome P-448 is biologically designed for physiological steroids. However, it appears that the planarity of PAH's enables them to fit more avidly than the steroids. Therefore, by introducing successive double bondings into rings A and B in the steroidal structure, causing flattening of the rings, it should be possible to increase the competitive binding of these structures on cytochrome P-448 against the naturally saturated ring system. This should be measurable as concentration-dependent decreases in the maximal velocity of metabolism of a standard cytochrome P-448 substrate. The more competitive the steroidal compound, the more effectively it would inhibit the test reaction.

C. Experimental Design

C-1. *Inhibitor parameter*

C-1a. Planarity

To allow an investigation of the proposed relationship between structure and the effectiveness of binding for steroid-like molecules at the cytochrome P-448 active site, the exact nature of the structure parameter of the inhibitor molecules must be defined. It would be desirable to know the molecular dimensions of all these inhibitor molecules from available crystal structure data so that the molecular planarity could be deduced directly. In the absence of such data, there are quantum mechanical programs such as the Modified Intermediate Neglect of Differential Overlap (MINDO/3) which can provide predictions of molecular structures from molecular orbital calculations⁴⁰. The planarity or "flatness" is not uniform throughout any molecule: aliphatic rings of the steroid molecules are thicker than aromatic regions. A very simple parameter such as an average molecular depth is not discriminatory enough because the same depth could be achieved with a small or large number of aliphatic C-C bonds in specific positions. A more discriminating parameter involves defining the frequency of

deviation from planarity, that is the "bumpiness" or corrugation of the molecule. Corrugation is highly dependent on the conformational relationship among the carbon atoms on the steroidal backbone. The endocyclic torsional angles centred about the C-C bonds conveniently define the conformation of the steroidal rings ⁴¹ ⁴². The torsional angle of C-C bonds in aromatic rings is essentially 0° due to their planar structures. Conversely, the angles in aliphatic rings are of considerable size because of the boat, chair or twist conformation associated with them. This parameter is probably the most directly related to the binding to protein surfaces because it is also related to the number of carbon atoms which can come into contact with the binding site. The more corrugated the molecule, the fewer carbon atoms are available to contact directly with the protein surface.

C-1b. Corrugation: Determination of Total Torsional Angle

Corrugation will be defined as the Total Torsional Angle. All the endocyclic torsional angles of each steroidal compound are simply added together to give a Total Torsional Angle. An endocyclic torsional angle is centred about a given bond and may be graphically displayed in a Newman projection, i.e. a view of the molecule looking down the bond in question. The torsional angle involving four bonded atoms A-B-C-D is positive if, when a projection is made looking from B to C,

the A-B bond can eclipse the C-D bond when rotated in a clockwise direction by less than 180° ⁴³. The angle has the same sign when viewed looking from atom C to B as from atom B to C. However, since molecular corrugation depends on the magnitude of these torsional angles only, the signs of the angles are omitted and the absolute value of each torsional angle is taken. This definition for corrugation is only valid for steroidal compounds with a 5α -skeleton. If the 5β -steroidal compounds are used, the formula must be adjusted to account for the ring fusion angle between rings A and B.

The Total Torsional Angle defines the degree of molecular corrugation measured as the deviation away from a planar, aromatic structure. The larger the Total Torsional Angle, the less planar will be the steroidal structure since planarity is inversely proportional to corrugation. This defines molecular planarity as inversely proportional to the Total Torsional Angle. To obtain an arbitrary planarity parameter each molecule in this study can be normalised by subtracting the Total Torsional Angle of the molecule from a general reference compound.

C-2. Enzyme behaviour

A general scheme for enzymatic reaction is shown in equation 5. Since O_2 is usually in abundance and NADPH can be

added in excess, cytochrome P-448 reaction is assumed to follow this typical enzymatic mechanism scheme⁴⁴ where k_1 , k_{-1} and k_2 are rate constants. The substrate concentration is adjusted so that pseudo-1st order rate conditions can be applied to the *in vitro* assay of enzyme behaviour.

EQUATION 5

Traditionally, it would be desirable to examine the binding affinity of steroidal structures on cytochrome P-448 employing these Michaelis-Menten kinetics in an *in vitro* system. Accordingly, test animals are dosed with a chemical that selectively induces cytochrome P-448⁴⁵ in a short term study. The enzyme is then isolated from the livers which have been first removed from the animals. The pseudo-1st order *in vitro* system would contain the solubilised microsomal protein (i.e. cytochrome P-448) saturated with O_2 and NADPH, leaving the substrate concentration as the only variable. Since this eliminates all cofactors as determinants of the rate of reaction, the reaction rates can be determined by directly monitoring the rate of disappearance of test substrates⁴⁶ or indirectly through coupled cofactors such as NADPH as is done with cytochrome P-450⁴⁷.

If the reaction rate is inhibited by the binding of any competitive steroidal substrates, then the observed rate of reaction will be slower than that of the control reaction. Then the hypothesis predicts that androstane and chrysene should respectively define the upper and lower extremities of the reduced reaction rate, with the various steroidal alkenes falling in between the two. The full rate equation for a competitively inhibited reaction is⁴⁸:

$$\text{Rate} = \frac{V_{\max} [S]}{[S] + K_m (1 + [I]/K_i)}$$

EQUATION 6

Here [S] is substrate concentration, [I] is the inhibitor concentration, K_i is the association constant between inhibitor and enzyme, $V_{\max}$ is the maximum reaction rate and K_m is the Michaelis constant. The Michaelis constant has the units of concentration and is a combination of constants k_1 , k_{-1} and k_2 depending whether a steady-state or equilibrium condition is assumed⁴⁴. It represents the concentration of substrate necessary to ensure reaction control rather than diffusion control of the reaction. The smaller this value, the more effectively the substrate binds to the active site, thus $1/K_m$ is often used to define the binding affinity of a specific substrate. From equation 6, K_m is modified by the term describing the inhibitor effect. In this term, the ratio $[I]/K_i$ represents the apparent increase in K_m of the substrate

due to competitive action of the inhibitor. This increase in K_m corresponds to an apparent reduction in the binding affinity of the original substrate, due to the concentration of inhibitor [I] and its binding efficiency $1/K_i$.

Since V_{max} is assumed constant because the same standard substrate is always used, this assay becomes a test for the entropic control of the reaction. In general, the apparent K_m and V_{max} can be obtained as from the slope and the y-intercept of a double-reciprocal plot ($1/\text{rate}$ vs $1/[S]$) according to the Lineweaver-Burke equation (equation 7), which is basically the reciprocal of the Michaelis-Menten equation (equation 6). The K_i value, which defines binding affinity of the inhibitor to the enzyme, can be readily obtained from the Lineweaver-Burke plot.

$$\frac{1}{\text{rate}} = \frac{K_m(1+[I]/K_i)}{V_{max}[S]} + \frac{1}{V_{max}}$$

EQUATION 7

The Michaelis-Menten method can define the K_m value in a heterogeneous enzyme system provided that the enzyme is pure enough. However, that is not necessarily true in this study and multiple values of K_m can result. PSMO is such a heterogeneous system which cannot be purified without denaturation. It must be assayed in its microsomal lipid

matrix which also includes the supporting electron transfer enzymes. Even an indirect Michaelis-Menten assay using the disappearance of NADPH can only define the reaction rate for comparing the K_m constants of different substrates if the reaction remains fully coupled. For any heterogeneous system, there are numerous cellular metabolic reactions that involve either consumption or formation of NADPH. If these are not removed by tissue preparation or controlled in the measurement procedure, they confound the experiment. Even if a control experiment is done, the high background of irrelevant NADPH activity is expected to lower the significance of the P-448 reaction rate data. A further problem is that chrysene, like most PAH's, is itself fluorescent and it interferes at the wavelength region where NADPH disappearance is observed. Thus, if a different method is used for chrysene, comparison of alicyclic and aromatic hydrocarbons in cytochrome P-448 reactions will not be meaningful.

C-3. Control Reaction: Ethoxyresorufin Deethylation

For the present hypothesis, however, the complete Michaelis-Menten experiment is not necessary. Since the binding of molecules at the active site is of interest, only the relative entropic information contained within the apparent value of K_m is required. The enthalpic information which is essentially within V_{max} can be held constant as part

of the experimental control. This is done by using a single isoform of the enzyme which can be induced and tested *in vitro* under pseudo-1st order conditions with a single standard control substrate. Against this control, the relative binding capabilities of the steroidal molecules can be assessed by direct comparison of observed reaction rates. For convenience, induction can be achieved with any of the standard inducing agents for cytochrome P-448 including 3-methylcholanthrene (3MC), β -naphthoflavone (BNF), isosafrole (ISO) and pregnenolone 16 α -carbonitrile (PCN)⁴⁹, all of which have multicyclic structures.

Figure 5 Deethylation of 7-ethoxyresorufin to resorufin by cytochrome P-448

For the assay, 7-ethoxyresorufin can be used as the specific substrate for cytochrome P-448^{50 51}. Its deethylation to form resorufin has long been used to distinguish and measure the induction of cytochrome P-448

(figure 5)⁴⁹. It is also considered to be the specific reaction to determine the P-448 activity in biological tissues and much more sensitive and reproducible than assaying the activity of either aryl hydrocarbon hydroxylase and biphenyl 2-hydroxylase⁵². The reaction product, resorufin, conveniently emits fluorescence at a wavelength which is free of the interferences by PAH's or NADPH. In fact, the determination of 7-ethoxyresorufin O-deethylation activity in human tissue has been suggested as a potential marker to identify carcinogenic exposure⁵³. Although alkoxyresorufins are structurally different from most toxins, fluorescence studies using different alkoxyresorufins as substrates are common in determining the induction of P-448 and cytochromes P-450 in general by various chemicals^{54 55 56}.

Therefore, with this standard cytochrome P-448 assay, one can determine the sequence of binding affinities of various steroidal structures by setting up an inhibition study. If steroids are indeed physiological substrates of P-448, they could act as competitive inhibitors to the deethylation of ethoxyresorufin⁵⁷. This is because the steroidal structures will compete with ethoxyresorufin for the same binding site on cytochrome P-448. A similar study has been performed to determine the affinity of drugs for cytochrome P-450 by inhibition of p-nitrophenotole O-deethylation⁵⁸. Another study demonstrated that aryl hydrocarbon hydroxylase is

inhibited by steroids⁵⁹. However the proposed inhibition study on ethoxyresorufin deethylation should provide a more definite demonstration that cytochrome P-448 is involved.

The progress of the deethylation reaction is not linear with time due to the back-inhibition by resorufin⁶⁰ and usually only the initial reaction rate is noted. To avoid such problems, low concentrations of microsomal protein are used, together with relatively high ethoxyresorufin concentrations to prevent substrate depletion⁶⁰.

Steroidal compounds are added to the reaction mixture before the reaction is initiated⁵⁹ by NADPH instead of being added after the reaction commences⁶⁰. This is because adding the steroid inhibitor during the reaction will require additional time for the fluorometric cell to re-equilibrate and causes a break in the initial data.

D. Methods and materials

D-1. Chemicals

β -naphthoflavone (BNF), chrysene, resorufin were purchased from Aldrich Chemical Co. (Milwaukee Wis). 7-ethoxyresorufin, reduced form nicotinamide adenine dinucleotide phosphate (NADPH), androstane, 1-dehydrotestosterone, estrone, 6-dehydroestrone, equilin and d-equilenin were purchased from Sigma Chemical Co. (St. Louis MO). 3,5-cholestadiene and 1,4,6-androstatrien-3,17-dione were purchased from Steraloids, Inc. (Wilton NH). All purchased chemicals had 95% purity except 6-dehydroestrone with 90%.

D-2. Reagents, buffers and supporting chemicals

Bovine serum albumin, Folin-Ciocalteu's reagent (2 N), monobasic and dibasic potassium phosphates (99%) were purchased from Sigma Chemical Co. (St. Louis MO). Tris(hydroxymethyl)aminomethane was purchased from Aldrich Chemical Co. (Milwaukee Wis.). Sepharose CL-2B gel was purchased from Pharmacia (Uppsalla, Sweden).

Potassium phosphate buffer was prepared as 0.1 M solution (K_2HPO_4 13.519 g/L, KH_2PO_4 3.046 g/L) adjusted to pH 7.4 with 20% NaOH and was subsequently degassed. Tris buffer was prepared as 0.1 M solution (12.114g/L) adjusted to pH 7.8 by concentrated HCl. Both buffers were kept refrigerated until use. All buffers and reagents were made up with Millipore deionised water.

D-3. Animals and pretreatments

This animal work was approved by the Carleton University Animal Care Committee and carried out in facilities inspected regularly by Ontario Ministry of Agriculture and Food. The animal housing room was thoroughly washed and cleaned with detergent and disinfectant before the animals arrived.

Male Sprague-Dawley rats (Charles River, Montreal) weighing 200-230g, were ordered in 2 sets of four. Sprague-Dawley rats were used to provide continuity with previous work both in these laboratories and in other studies. This strain is very docile and easy to handle. At this age and weight, the rats were nearing maturity, the liver weight to animal weight ratio was constant as organs and body were still growing. Male rats were used since the PSMO system is affected by the cycles of the female endocrine system. They were housed in metal hanging cages, previously cleaned with hot water and disinfectant, and were provided with free access to food (Purina rat chow) and water. The animal room was equipped with fluorescent lighting set to 12-hour day-night cycle. Ventilation of the room was provided by high efficiency particulate filter capable of excluding 99.9% of all particles exceeding the size of 0.3 μm . Twice daily, the rats were taken out of their cages and handled individually to minimise stress from handling during the dosing process later.

After accommodation for 3 days, the rats were then starved for 16 to 24 hours. They were dosed once by gavage with BNF (1 mmol or 82 mg per kg) as a suspension in 1 mL corn oil (Mazola). For each dose a 10 mL glass syringe with a steel oral dosing tube (7-8 cm) attached was filled to 1 mL with the required dose excluding air bubbles. The rat was held vertically by the nape of the neck and the back, and the dosing tube was inserted carefully down the oesophagus into the stomach. The dose was then administered, the tube removed, and the rat returned to its cage. This dosage was roughly 1/5 of that of the LD₅₀ for rats⁶¹. Concurrent control treatments with 1 mL of corn oil were also carried out.

After a 72-hour rest period, animals were suffocated with CO₂ in a sealed polyethylene bag. Once suffocation was ascertained by feeling the absence of a heartbeat, the rat was removed from the bag and placed on its back. The skin over the abdomen was pulled away from the body and the abdomen opened with scissors. The diaphragm and the rib cage were cut to expose the liver. It was then removed for enzyme isolation.

D-4. Preparation of gel columns⁶²

Sepharose CL-2B gel was washed on a Bucher funnel with three volumes of acetone followed by 0.15 M KCl until no acetone odour was detected. Then, the gel was suspended and equilibrated with ice cold 0.1 M potassium phosphate buffer, pH 7.4, to a slurry. Both the gel slurry and the buffer were degassed. Eight clean 10 mm X 30 cm glass columns, two for each rat liver sample, fitted with filter and stopcock were rinsed first with acetone, then with phosphate buffer. 16 ml. of potassium phosphate buffer were then put into each column and the level was marked. With the stopcock open, the gel slurry was gently poured down the column and packed to a volume of 16 mL. The level of buffer was never allowed to drop below that of the gel bed. Excess gel was removed by disturbing the gel bed and withdrawing with a Pasteur pipette. The packed columns were kept cool in the refrigerator until use. During separation, sample and eluent fluids were allowed to flow freely through the columns, no pumps or fraction collectors were required.

D-5. Isolation of microsomes⁶²

Once the livers were removed from the rats, they were quickly placed in an ice cold 0.15 M KCl solution containing 0.1 mM Na₂EDTA. All subsequent operations were carried out at 0-4°C using cold solutions. The adhering tissues were cut free and the livers were rinsed several times in KCl-EDTA to remove all visible blood. The livers were then blotted dry by filter paper and put into 0.1 M potassium phosphate buffer (4 mL buffer/g liver). They were chopped by scissors into small pieces and crushed by placing under a pre-cooled, motor-driven Kinematica GmbH microtome. One fifteen second pulse at a setting of six was required. The homogenate from each liver was transferred to chilled centrifuge tubes and was centrifuged at 12,000 X g (10,000 r.p.m.) for 10 minutes in a Beckman Model L (type 30A rotor) ultracentrifuge refrigerated at 4°C to remove cell debris, nuclei and mitochondria.

After centrifugation the supernatant was quickly and carefully decanted and collected in cold beaker with the floating fat removed by a Pasteur pipette to yield the postmitochondrial supernatant fraction. 4 mL of this supernatant was layered on the rinsed and drained gel column and allowed to absorb into the gel. The outflowing eluent was discarded. The walls of the column were then washed with about 1 mL of eluent buffer (0.1 M potassium phosphate

buffer), and after its absorption into the gel 10 mL of the eluent buffer was applied to the column. The microsomes were collected in 7 mL of eluate from each column and all microsomal fractions from the same sample were pooled together in a chilled beaker. Each column was washed with 20 ml eluent buffer before reusing. The columns were used up to 4 times before the gel was washed and columns repacked. Sample aliquots of microsomes in polypropylene cryotubes were frozen in liquid N₂ and stored at -65 to -70°C in a Kelvinator series 500 Ultracold Freezer until ready for enzyme analyses.

D-6. Determination of protein concentration

A revised version of the Lowry method⁶³ was used for the estimation of protein content in microsomal preparation. A protein standard of 500 µg/mL was prepared with bovine serum albumin in 0.5 M NaOH. The sample was prepared in 0.5 M NaOH to a dilution between 250 and 1000 µg/mL. A triplicate of the reagent blank was set up with three 1 mL aliquots of 0.5 M NaOH in glass tubes. Duplicate samples were set up by pipetting aliquots of 0.1 to 0.3 mL diluted test samples and making the volumes up to 1 mL with 0.5 M NaOH. Duplicate BSA standards between 50 to 200 µg, in 25 µg increments, were set up by pipetting appropriate volumes of aliquots of the stock standard making up to 1 mL with NaOH. 5 mL of freshly prepared Lowry C reagent (1 % CuSO₄ : 2 % KNa tartarate: 2 % Na₂CO₃ = 1:1:100) was added to all tubes and mixed well. 0.5 mL of freshly diluted 1 N Folin-Ciocalteu's phenol reagent was added to all tubes after at least 10 minutes and mixed well. The contents of the tube were mixed again after 30 minutes and their absorbance values were subsequently measured at 720 nm on a Beckman DU-7 UV-Visible spectrophotometer. After subtracting the mean value of the triplicate blanks from the mean values of the standard and the samples, a programmable calculator (Casio) with linear regression analysis was used to interpolate and convert absorbance values to µg of protein (Appendix A).

D-7. Fluorometric assay of ethoxyresorufin deethylation

Reactions were carried out in fluorometer cuvettes at 37°C using a Varian SF-330 spectrofluorometer. The reaction system, containing 40 µg solubilised microsomal protein, 1 µM ethoxyresorufin substrate (200 µL of a 0.01 mM solution in Tris-HCl buffer), 10 µL acetone (with or without appropriately dissolved inhibitor) and 0.1 M Tris-HCl buffer pH 7.8, was equilibrated for 4 minutes at 37°C. The reaction was then initiated by the addition of 250 µM NADPH (10 µL of a 50 mM solution in Tris-HCl buffer). The ensuing reaction should sustain in linear rates for at least 3 minutes. The final reaction volume was 2 mL. The reaction rate was measured directly by the increasing fluorescence of the reaction mixture as displayed on a chart recorder (Appendix A). The fluorometer settings were: excitation slit 10 nm, emission slit 20 nm; excitation and emission wavelengths, 530 nm and 586 nm, respectively. The fluorometer was calibrated with 10 µL of resorufin (0.01 mM in ethanol). Six different concentrations and a control for each steroidal inhibitor were performed in triplicate and were repeated twice with different microsomal samples. The steroidal inhibitors used were androstane (AND), 3,5-cholestadiene (CDE), 1-dehydrotestosterone (DHT), 1,4,6-androstatrien-3,17-dione (146AND), estrone (ES), 6-dehydroestrone (DHE), equilin (EQ4), equilenin (EQ5) and chrysene (Chry).

Figure 6 Structures of the steroidal inhibitors and 7-ethoxyresorufin

Figure 7 Numbering of carbon atoms on the 4-ring steroidal system

E. Results

E-1. Independent parameter

The chemical structures of the nine selected steroidal compounds and the standard substrate, 7-ethoxyresorufin, are depicted in figure 6. The numbering of carbon atoms on a steroidal backbone is shown in figure 7.

The endocyclic torsional angles of all eight steroids structures were obtained from the Atlas of Steroid Structures⁶⁴ and are displayed in Tables I-VIII. The Total Torsional Angle of each steroid is summed up at the bottom of the tables. No significant torsional angle data could be gathered for chrysene in the literature because of its essentially flat aromatic structure. It is known that carbon atoms 2, 3 and 4 on chrysene do deviate 0.0025 Å from the mean plane⁶⁵ and this value is considered to be quite significant in terms of crystallographic studies. However, it is of little importance here since the deviation of the carbon atoms of the rest of steroidal compounds are only known to the nearest 0.01 Å. Therefore, the Total Torsional Angle for chrysene will be taken to be zero.

The highly corrugated androstane molecule and the

essentially flat chrysene molecule are used to define the lowest and highest extremities of planarity, respectively. To obtain an arbitrary planarity parameter each molecule in this study can be normalised by subtracting the Total Torsional Angle of the molecule from that of androstane. The Total Torsional Angle of the steroidal inhibitors and the value of the planarity parameters obtained by this normalisation are summarised in table IX.

Table I *Sum of endocyclic torsional angles of AND*

	Ring	Location	Torsional angle (°)
=====			
Androstane (AND)	A	C ₁₀ -C ₁ -C ₂ -C ₃	58
		C ₁ -C ₂ -C ₃ -C ₄	53
		C ₂ -C ₃ -C ₄ -C ₅	53
		C ₃ -C ₄ -C ₅ -C ₁₀	57
		C ₄ -C ₅ -C ₁₀ -C ₁	58
		C ₅ -C ₁₀ -C ₁ -C ₂	58

B	C ₁₀ -C ₅ -C ₆ -C ₇	65	
	C ₅ -C ₆ -C ₇ -C ₈	59	
	C ₆ -C ₇ -C ₈ -C ₉	54	
	C ₇ -C ₈ -C ₉ -C ₁₀	55	
	C ₈ -C ₉ -C ₁₀ -C ₅	57	
	C ₉ -C ₁₀ -C ₅ -C ₆	61	
-----			351
C	C ₁₄ -C ₈ -C ₉ -C ₁₁	56	
	C ₈ -C ₉ -C ₁₁ -C ₁₂	55	
	C ₉ -C ₁₁ -C ₁₂ -C ₁₃	52	
	C ₁₁ -C ₁₂ -C ₁₃ -C ₁₄	51	
	C ₁₂ -C ₁₃ -C ₁₄ -C ₈	56	
	C ₁₃ -C ₁₄ -C ₈ -C ₉	59	
-----			329
D	C ₁₇ -C ₁₃ -C ₁₄ -C ₁₅	46	
	C ₁₃ -C ₁₄ -C ₁₅ -C ₁₆	32	
	C ₁₄ -C ₁₅ -C ₁₆ -C ₁₇	6	
	C ₁₅ -C ₁₆ -C ₁₇ -C ₁₃	23	
	C ₁₆ -C ₁₇ -C ₁₃ -C ₁₄	42	
-----			149
Total (A+B+C+D)			1166

Table II Sum of endocyclic torsional angles of CDE

	Ring	Location	Torsional angle (°)
=====			
3,5-cholesta- diene (CDE)	A	C ₁₀ -C ₁ -C ₂ -C ₃	56
		C ₁ -C ₂ -C ₃ -C ₄	27
		C ₂ -C ₃ -C ₄ -C ₅	3
		C ₃ -C ₄ -C ₅ -C ₁₀	7
		C ₄ -C ₅ -C ₁₀ -C ₁	47
		C ₅ -C ₁₀ -C ₁ -C ₂	50

			190
	B	C ₁₀ -C ₅ -C ₆ -C ₇	4
		C ₅ -C ₆ -C ₇ -C ₈	16
		C ₆ -C ₇ -C ₈ -C ₉	49
		C ₇ -C ₈ -C ₉ -C ₁₀	65
		C ₈ -C ₉ -C ₁₀ -C ₅	45
		C ₉ -C ₁₀ -C ₅ -C ₆	13

			192
	C	C ₁₄ -C ₈ -C ₉ -C ₁₁	48
		C ₈ -C ₉ -C ₁₁ -C ₁₂	50
		C ₉ -C ₁₁ -C ₁₂ -C ₁₃	56
		C ₁₁ -C ₁₂ -C ₁₃ -C ₁₄	60
		C ₁₂ -C ₁₃ -C ₁₄ -C ₈	61
		C ₁₃ -C ₁₄ -C ₈ -C ₉	55

			330
	D	C ₁₇ -C ₁₃ -C ₁₄ -C ₁₅	45
		C ₁₃ -C ₁₄ -C ₁₅ -C ₁₆	34
		C ₁₄ -C ₁₅ -C ₁₆ -C ₁₇	9
		C ₁₅ -C ₁₆ -C ₁₇ -C ₁₃	19
		C ₁₆ -C ₁₇ -C ₁₃ -C ₁₄	39

			146
Total (A+B+C+D)			858

Table III Sum of endocyclic torsional angles of DHT

	Ring	Location	Torsional angle (°)
=====			
1-dehydro- testosterone (DHT)	A	C ₁₀ -C ₁ -C ₂ -C ₃	2
		C ₁ -C ₂ -C ₃ -C ₄	6
		C ₂ -C ₃ -C ₄ -C ₅	5
		C ₃ -C ₄ -C ₅ -C ₁₀	1
		C ₄ -C ₅ -C ₁₀ -C ₁	3
		C ₅ -C ₁₀ -C ₁ -C ₂	2

			19
	B	C ₁₀ -C ₅ -C ₆ -C ₇	61
		C ₅ -C ₆ -C ₇ -C ₈	58
		C ₆ -C ₇ -C ₈ -C ₉	55
		C ₇ -C ₈ -C ₉ -C ₁₀	52
		C ₈ -C ₉ -C ₁₀ -C ₅	50
		C ₉ -C ₁₀ -C ₅ -C ₆	57

			333
	C	C ₁₄ -C ₈ -C ₉ -C ₁₁	59
		C ₈ -C ₉ -C ₁₁ -C ₁₂	59
		C ₉ -C ₁₁ -C ₁₂ -C ₁₃	58
		C ₁₁ -C ₁₂ -C ₁₃ -C ₁₄	57
		C ₁₂ -C ₁₃ -C ₁₄ -C ₈	61
		C ₁₃ -C ₁₄ -C ₈ -C ₉	62

			356
	D	C ₁₇ -C ₁₃ -C ₁₄ -C ₁₅	46
		C ₁₃ -C ₁₄ -C ₁₅ -C ₁₆	32
		C ₁₄ -C ₁₅ -C ₁₆ -C ₁₇	6
		C ₁₅ -C ₁₆ -C ₁₇ -C ₁₃	23
		C ₁₆ -C ₁₇ -C ₁₃ -C ₁₄	42

			149
Total (A+B+C+D)			857

Table IV *Sum of endocyclic torsional angles of 146AND*

	Ring	Location	Torsional angle (°)
=====			
1,4,6-andro- statrien-3,17- dione (146AND)	A	C ₁₀ -C ₁ -C ₂ -C ₃	2
		C ₁ -C ₂ -C ₃ -C ₄	0
		C ₂ -C ₃ -C ₄ -C ₅	4
		C ₃ -C ₄ -C ₅ -C ₁₀	11
		C ₄ -C ₅ -C ₁₀ -C ₁	11
		C ₅ -C ₁₀ -C ₁ -C ₂	7

			35
	B	C ₁₀ -C ₅ -C ₆ -C ₇	21
		C ₅ -C ₆ -C ₇ -C ₈	5
		C ₆ -C ₇ -C ₈ -C ₉	18
		C ₇ -C ₈ -C ₉ -C ₁₀	46
		C ₈ -C ₉ -C ₁₀ -C ₅	60
		C ₉ -C ₁₀ -C ₅ -C ₆	47

			197
	C	C ₁₄ -C ₈ -C ₉ -C ₁₁	60
		C ₈ -C ₉ -C ₁₁ -C ₁₂	57
		C ₉ -C ₁₁ -C ₁₂ -C ₁₃	54
		C ₁₁ -C ₁₂ -C ₁₃ -C ₁₄	54
		C ₁₂ -C ₁₃ -C ₁₄ -C ₈	61
		C ₁₃ -C ₁₄ -C ₈ -C ₉	63

			349
	D	C ₁₇ -C ₁₃ -C ₁₄ -C ₁₅	42
		C ₁₃ -C ₁₄ -C ₁₅ -C ₁₆	42
		C ₁₄ -C ₁₅ -C ₁₆ -C ₁₇	25
		C ₁₅ -C ₁₆ -C ₁₇ -C ₁₃	2
		C ₁₆ -C ₁₇ -C ₁₃ -C ₁₄	27

			138
Total (A+B+C+D)			719

Table V *Sum of endocyclic torsional angles of ES*

	Ring	Location	Torsional angle (°)
=====			
Estrone (ES)	A	C ₁₀ -C ₁ -C ₂ -C ₃	1
		C ₁ -C ₂ -C ₃ -C ₄	2
		C ₂ -C ₃ -C ₄ -C ₅	2
		C ₃ -C ₄ -C ₅ -C ₁₀	0
		C ₄ -C ₅ -C ₁₀ -C ₁	1
		C ₅ -C ₁₀ -C ₁ -C ₂	1

			7
	B	C ₁₀ -C ₅ -C ₆ -C ₇	15
		C ₅ -C ₆ -C ₇ -C ₈	48
		C ₆ -C ₇ -C ₈ -C ₉	68
		C ₇ -C ₈ -C ₉ -C ₁₀	57
		C ₈ -C ₉ -C ₁₀ -C ₅	20
		C ₉ -C ₁₀ -C ₅ -C ₆	1

			209
	C	C ₁₄ -C ₈ -C ₉ -C ₁₁	55
		C ₈ -C ₉ -C ₁₁ -C ₁₂	57
		C ₉ -C ₁₁ -C ₁₂ -C ₁₃	53
		C ₁₁ -C ₁₂ -C ₁₃ -C ₁₄	55
		C ₁₂ -C ₁₃ -C ₁₄ -C ₈	61
		C ₁₃ -C ₁₄ -C ₈ -C ₉	60

			341
	D	C ₁₇ -C ₁₃ -C ₁₄ -C ₁₅	42
		C ₁₃ -C ₁₄ -C ₁₅ -C ₁₆	42
		C ₁₄ -C ₁₅ -C ₁₆ -C ₁₇	25
		C ₁₅ -C ₁₆ -C ₁₇ -C ₁₃	2
		C ₁₆ -C ₁₇ -C ₁₃ -C ₁₄	27

			138
Total (A+B+C+D)			695

Table VI *Sum of endocyclic torsional angles of DHE*

	Ring	Location	Torsional angle (°)
=====			
6-dehydro- estrone (DHE)	A	C ₁₀ -C ₁ -C ₂ -C ₃	1
		C ₁ -C ₂ -C ₃ -C ₄	1
		C ₂ -C ₃ -C ₄ -C ₅	0
		C ₃ -C ₄ -C ₅ -C ₁₀	2
		C ₄ -C ₅ -C ₁₀ -C ₁	2
		C ₅ -C ₁₀ -C ₁ -C ₂	1

			7
	B	C ₁₀ -C ₅ -C ₆ -C ₇	16
		C ₅ -C ₆ -C ₇ -C ₈	4
		C ₆ -C ₇ -C ₈ -C ₉	46
		C ₇ -C ₈ -C ₉ -C ₁₀	60
		C ₈ -C ₉ -C ₁₀ -C ₅	33
		C ₉ -C ₁₀ -C ₅ -C ₆	0

			159
	C	C ₁₄ -C ₈ -C ₉ -C ₁₁	60
		C ₈ -C ₉ -C ₁₁ -C ₁₂	57
		C ₉ -C ₁₁ -C ₁₂ -C ₁₃	54
		C ₁₁ -C ₁₂ -C ₁₃ -C ₁₄	54
		C ₁₂ -C ₁₃ -C ₁₄ -C ₈	61
		C ₁₃ -C ₁₄ -C ₈ -C ₉	63

			349
	D	C ₁₇ -C ₁₃ -C ₁₄ -C ₁₅	42
		C ₁₃ -C ₁₄ -C ₁₅ -C ₁₆	42
		C ₁₄ -C ₁₅ -C ₁₆ -C ₁₇	25
		C ₁₅ -C ₁₆ -C ₁₇ -C ₁₃	2
		C ₁₆ -C ₁₇ -C ₁₃ -C ₁₄	27

			138
Total (A+B+C+D)			653

Table VII Sum of endocyclic torsional angles of EQ4

	Ring	Location	Torsional angle (°)
=====			
Equilin (EQ4)	A	C ₁₀ -C ₁ -C ₂ -C ₃	1
		C ₁ -C ₂ -C ₃ -C ₄	1
		C ₂ -C ₃ -C ₄ -C ₅	0
		C ₃ -C ₄ -C ₅ -C ₁₀	2
		C ₄ -C ₅ -C ₁₀ -C ₁	2
		C ₅ -C ₁₀ -C ₁ -C ₂	1

			7
	B	C ₁₀ -C ₅ -C ₆ -C ₇	21
		C ₅ -C ₆ -C ₇ -C ₈	48
		C ₆ -C ₇ -C ₈ -C ₉	2
		C ₇ -C ₈ -C ₉ -C ₁₀	25
		C ₈ -C ₉ -C ₁₀ -C ₅	26
		C ₉ -C ₁₀ -C ₅ -C ₆	1

			123
	C	C ₁₄ -C ₈ -C ₉ -C ₁₁	57
		C ₈ -C ₉ -C ₁₁ -C ₁₂	55
		C ₉ -C ₁₁ -C ₁₂ -C ₁₃	53
		C ₁₁ -C ₁₂ -C ₁₃ -C ₁₄	50
		C ₁₂ -C ₁₃ -C ₁₄ -C ₈	59
		C ₁₃ -C ₁₄ -C ₈ -C ₉	56

			330
	D	C ₁₇ -C ₁₃ -C ₁₄ -C ₁₅	42
		C ₁₃ -C ₁₄ -C ₁₅ -C ₁₆	42
		C ₁₄ -C ₁₅ -C ₁₆ -C ₁₇	25
		C ₁₅ -C ₁₆ -C ₁₇ -C ₁₃	2
		C ₁₆ -C ₁₇ -C ₁₃ -C ₁₄	27

			138
Total (A+B+C+D)			595

Table VIII *Sum of endocyclic torsional angles of EQ5*

	Ring	Location	Torsional angle (°)
=====			
d-Equilenin (EQ5)	A	C ₁₀ -C ₁ -C ₂ -C ₃	0
		C ₁ -C ₂ -C ₃ -C ₄	0
		C ₂ -C ₃ -C ₄ -C ₅	0
		C ₃ -C ₄ -C ₅ -C ₁₀	1
		C ₄ -C ₅ -C ₁₀ -C ₁	1
		C ₅ -C ₁₀ -C ₁ -C ₂	1
		-----	3
	B	C ₁₀ -C ₅ -C ₆ -C ₇	0
		C ₅ -C ₆ -C ₇ -C ₈	4
		C ₆ -C ₇ -C ₈ -C ₉	5
		C ₇ -C ₈ -C ₉ -C ₁₀	1
		C ₈ -C ₉ -C ₁₀ -C ₅	4
		C ₉ -C ₁₀ -C ₅ -C ₆	1
		-----	15
	C	C ₁₄ -C ₈ -C ₉ -C ₁₁	46
		C ₈ -C ₉ -C ₁₁ -C ₁₂	45
		C ₉ -C ₁₁ -C ₁₂ -C ₁₃	53
		C ₁₁ -C ₁₂ -C ₁₃ -C ₁₄	58
		C ₁₂ -C ₁₃ -C ₁₄ -C ₈	61
		C ₁₃ -C ₁₄ -C ₈ -C ₉	55
		-----	318
	D	C ₁₇ -C ₁₃ -C ₁₄ -C ₁₅	42
		C ₁₃ -C ₁₄ -C ₁₅ -C ₁₆	42
		C ₁₄ -C ₁₅ -C ₁₆ -C ₁₇	25
		C ₁₅ -C ₁₆ -C ₁₇ -C ₁₃	2
		C ₁₆ -C ₁₇ -C ₁₃ -C ₁₄	27
		-----	138
Total (A+B+C+D)			474

Table IX

Summary of the Total Torsional Angle and Planarity parameter for the test steroidal inhibitors

Steroidal inhibitor (X)	Total Torsional Angle (Σ , in $^{\circ}$)	Planarity (P, $\angle$ AND - $\angle$ X, in $^{\circ}$)
AND	1166	---
CDE	858	308
DHT	857	309
146AND	719	447
ES	695	471
DHE	652	514
EQ4	596	570
EQ5	473	693
Chry	0	1166

E-2. Dependent parameter**E-2a. Cytochrome P-448 activity: ethoxyresorufin deethylation controls**

With the microsomal protein fractions isolated from rats dosed with β -naphthoflavone, a significant amount of ethoxyresorufin deethylation activity was observed in the conditions specified in *Method & Materials* section. It was also found that fractions from control rats, dosed with corn oil only, had effectively no ethoxyresorufin deethylation activity under the same conditions.

E-2b. Determination of % inhibition by steroidal test compounds

The influence of the selected steroidal inhibitors on ethoxyresorufin deethylation activities of cytochrome P-448 are shown in tables a-i (appendix B). Since the amounts of protein, cofactors and medium in every reaction were constant, the activity was expressed as nM of resorufin formed per minute per cuvette. The concentration of the substrate, ethoxyresorufin, was 1 μ M in the assay mixture while that of the various inhibitors was varied sufficiently to define the sigmoidal inhibition curve as clearly as possible. At one

extreme, DHT and 146AND required inhibition concentrations ranging from 10 to 400 μM and at the other extreme, only from 0.005 to 0.200 μM was required for chrysene.

Except for androstane, inhibition by each steroidal compound generally varied from about 10 to 80 % as the inhibitor concentration was increased. Androstane did not show any significant inhibition until inhibitor concentration reached 200 μM and 400 μM . No inhibition could be obtained for androstane beyond 400 μM because the compound simply precipitated out of the aqueous reaction medium at higher concentrations. Inhibition at each concentration of the remaining compounds was calculated by expressing the inhibited activity as a percentage of the control activity, where no compound was present. The average % inhibition values for each compound tested are given in tables j-r (appendix C).

E-2c. Relationship between steroidal concentration and % inhibition

Tables X-XVII compare % inhibition and the p (negative logarithmic) values (Appendix D) of the concentration for each steroidal compound. To illustrate the relationship between steroidal concentration and % inhibition, the p[steroidal] values are plotted against the % inhibition of ethoxyresorufin

deethylation activity for each steroidal compound in figures 8-15, except androstane. Each data point on the scatter plot is the mean of 3 experimental determinations and has an error (standard deviation) $\pm$ 1-4%. This error is due to experimental variation among microsomal fractions. Since the relative error on each point on the plots is fairly small, it becomes obvious that the relationship between pI and % inhibition is not of a straight line. Evidently, they all resemble a sigmoidal type curve.

Tables X-XVII Relationship between p[steroidal] and % inhibition for the test compounds

Table X

[3,5-cholestadiene] (μM)	p[3,5-cholestadiene] ($-\log[\text{CDE}]$)	% inhibition	$\pm$ SD
1	6.000	6	2
5	5.301	22	3
10	5.000	30	2
25	4.602	55	1
50	4.301	79.5	0.7
100	4.000	86	1

Table XI

[1-dehydrotestosterone] (μM)	p[1-dehydrotestosterone] ($-\log[\text{DHT}]$)	% inhibition	$\pm$ SD
10	5.000	13	1
25	4.602	17	1
50	4.301	30	2
100	4.000	37	2
200	3.699	58	2
400	3.398	82	3

Table XII

[1,4,6- androstatrien- 3,17-dione] (μM)	p[1,4,6- androstatrien- 3,17-dione] ($-\log[146\text{AND}]$)	% inhibition	$\pm$ SD
10	5.000	14	1
25	4.602	19	1
50	4.301	28	3
100	4.000	46	3
200	3.699	57	2
400	3.398	85	2

Table XIII

[Estrone] (μM)	p[Estrone] ($-\log[\text{ES}]$)	% inhibition	$\pm$ SD
1	6.000	22	1
5	5.301	32	3
10	5.000	45	1
25	4.602	49	1
50	4.301	53	1
100	4.000	59.3	0.1

Table XIV

[6-dehydroestrone] (μM)	p[6-dehydroestrone] ($-\log[\text{DHE}]$)	% inhibition	$\pm$ SD
1	6.000	12	1
5	5.301	18.9	0.8
10	5.000	25	1
25	4.602	33	3
50	4.301	50.8	0.7
100	4.000	85	2

Table XV

[Equilin] (μM)	p[Equilin] ($-\log[\text{EQ4}]$)	% inhibition	$\pm$ SD
0.1	7.000	1	1
0.5	6.301	23	2
2	5.699	32	2
5	5.301	45	3
10	5.000	67	1
50	4.301	85	4

Table XVI

[d-equilenin] (μM)	p[d-equilenin] ($-\log[\text{EQ5}]$)	% inhibition	$\pm$ SD
0.1	7.000	12	2
0.5	6.301	17	2
2	5.699	25	2
5	5.301	50	1
10	5.000	63.6	0.2
50	4.301	81	2

Table XVII

[Chrysene] (μM)	p[Chrysene] ($-\log[\text{Chry}]$)	% inhibition	$\pm$ SD
0.005	8.301	16	2
0.010	8.000	27	1
0.025	7.602	45	4
0.050	7.301	63.3	0.9
0.100	7.000	71	2
0.200	6.699	81	2

Figure 8 Relationship between p(CDE) and % inhibition

Figure 9 Relationship between p[DHT] and % inhibition

Figure 10 Relationship between p[146AND] and % inhibition

Figure 11 Relationship between p[ES] and % inhibition

Figure 12 Relationship between p[DHE] and % inhibition

Figure 13 Relationship between p[λ Q4] and % inhibition

Figure 14 Relationship between p[EQ5] and % inhibition

Figure 15 Relationship between p[Chry] and % inhibition

F. Discussion

F-1. Evaluation of inhibition capacity

With each test compound, a non-linear response of % inhibition is observed as a function of the pI value (figures 8, 11, 13-15). In the majority of cases a simple, sigmoid curve is obtained. These sigmoid curves are commonly observed in dose-effect relationships⁶⁶ and are characteristic of a reversible interaction of a chemical with an enzyme. Some specific examples include interactions between drugs and receptors⁶⁷, competitive drug antagonism⁶⁸ and the titration of neuroreceptors by neurotransmitters or by psychomimetic drugs.

In each case, the inflection point of the sigmoid curve represents the titration of the most active binding site by the competitive inhibitor. This "end point", pI', is the inhibitor concentration necessary to saturate the binding site. The larger the pI' value for this response, the more powerful is the substrate in achieving its effect. This concept can be used to characterise the strength of the substrate for the particular biochemical function of interest. Thus the dependent parameter of inhibition potency for each test compound can be defined by this pI' value.

In a minority of cases examined, the relationship of pI to inhibition is more complex. The plots for DHT, 146AND and DHE (figures 9, 10 & 12) suggest a probable double sigmoid profile with a second characteristic pI'' value at much lower pI ranges. This indicates the occurrence of a less important, second inhibition mechanism, probably from non-specific association of one or more test molecules bound weakly at protein sites distant from the primary binding site since this binding is already occupied at much higher pI values. For present purposes, these secondary interactions are of little importance.

The pI' values, characterising the binding strength at the primary site, can be obtained by fitting a polynomial of degree 3 through the part of the curve containing the primary inflection point. The fit was achieved with computer graphics (Cricket Graphs 1.2.1) and the point of inflection is defined at this position where the second derivative becomes zero. The equations for the fitted sigmoidal curves are shown in appendix E. The pI' values obtained for each steroidal inhibitor are summarised in table XVIII together with their respective planarity parameter values.

Table XVIII

Comparison of molecular planarity of the test steroidal inhibitors and binding strength

Steroidal Inhibitor	Planarity parameter, P	Binding strength, pI'
CDE	308	4.636
DHT	309	4.438
146AND	447	4.132
ES	471	5.175
DHE	514	5.162
EQ4	570	5.059
EQ5	693	5.349
Chry	1166	7.610

F-2. Correlation of molecular planarity to inhibitor binding

By plotting the planarity parameter against the pI' values, the proposed relationship (Appendix D) between inhibitor binding and structural planarity can be assessed (figure 16). Although there is a general increase in binding affinity (pI') to cytochrome P-448 with increasing planarity of the molecule as predicted by the hypothesis, the correlation shown is rather poor and the deviations lie beyond the limits of experimental errors. Significant deviation from this straight correlation occurs for all the test compounds and it is especially pronounced for 146AND. This implies there are other factors governing the binding to cytochrome P-448 beyond the proposed structural planarity factor.

Reexamining the chemical structures (figure 6) of the selected test compounds, the general physical shapes of the compounds are fairly uniform since they all have the 4-ring steroidal backbone structure. However, they differ significantly in terms of polarity since they do not all possess the same functional groups. The functional groups on the steroidal backbone vary from none to ketones and phenols. For example, 146AND differs significantly from CDE in chemical nature. This variation appears to affect the affinity to cytochrome P-448.

Figure 16 Relationship between planarity (P) and binding affinity (pI')

The PSMO systems, including the cytochrome P-448 variant, generally convert non-polar compounds to polar compounds which are then released for excretion to the cytosol^{9 15}. Therefore, the binding site of cytochrome P-448 has a preferred interaction with non-polar entities. Relatively polar functional groups such as C=C double bonds, carbonyl and hydroxyl groups tend to reduce the affinity of molecules to cytochrome P-448. Therefore, it is highly likely that the ability of steroidal compounds to inhibit cytochrome P-448 could depend on the hydrophobicity as well as planarity, since each is known⁶⁹ to be an essential determinant for binding with protein.

F-3. Effect of molecular hydrophobicity on inhibitor binding

To demonstrate of the influence of the polarity of these test molecules, the hydrophobicity of each steroidal 4-ring system must first be deduced. Not all of the selected compounds have experimental values readily available. Furthermore, those data on experimental partition coefficients which are available in the literature are not always based on tests using the same solvent pairs. Octanol, benzene, chloroform, ether and many others are used as the organic layer, thus making it difficult to compare the values. Therefore, a consistent set of hydrophobicity parameters is only available by calculation. The Fragment Method of

calculating partition coefficients employed by Hansch & Leo⁷⁰ can be used for this type of calculation. This is an empirical, additive method in which total hydrophobicity is defined by summing the appropriate fundamental structural constants (e.g. CH_3 , CH_2 , CH , C=O). Other factors (e.g. unsaturation, chain branch) that affect partition equilibrium in the more complex solutes are also added because summation of fragments alone leads to spurious values (appendix F).

Table XIX shows the results of the hydrophobicity calculations. The correlation of hydrophobicity to the binding by the steroidal inhibitors can then be assessed by plotting the calculated hydrophobicity values of the steroidal inhibitors against their corresponding saturation points, pI' values (figure 17). Apparently, the inhibitor binding is generally proportional to hydrophobicity of the molecule. However, there are again some significant deviations. Chrysene binds far better than its hydrophobicity would predict. On the other hand, CDE binds poorly despite its high hydrophobicity.

However, these deviations appear to be in the opposite direction to the deviations in the correlation to planarity. Thus, by plotting the product of the planarity and hydrophobicity parameters against pI' , the relationship between inhibitor binding and the concerted effect of the two

properties can be observed (figure 18). Evidently, this plot shows a correlation (Appendix G) much better than comparing pI' with either planarity or hydrophobicity alone.

Table XIX

Comparison of planarity and hydrophobicity parameters of the test steroidal inhibitors

Steroidal inhibitor	Hydrophobicity (H)	Planarity (P)	H x P	Binding strength (pI')
AND	9.54	----	----	----
CDE	7.11	308	2190	4.636
DHT	2.69	309	831	4.438
146AND	1.21	447	540	4.132
ES	4.36	471	2054	5.175
DHE	3.48	514	1789	5.162
EQ4	3.35	570	1909	5.059
EQ5	3.59	693	2488	5.349
Chry	4.98	1166	5807	7.610

Figure 17 Relationship between and hydrophobicity (H) and binding affinity (pI')

Figure 18 Correlation between the concerted property of hydrophobicity and planarity ($H \times P$) and binding affinity (pI')

F-4. Concerted effect of planarity and hydrophobicity

It is apparent from these findings that the ability of steroidal molecules to inhibit cytochrome P-448 activity depends on more than one structural factor. The two which seem most important from the present study are planarity and hydrophobicity. Like many enzyme-substrate or enzyme-inhibitor interactions, the shape of the molecule which is represented here by its planarity, plays an important role. Inhibition appears to occur because the test compounds have either a fully or partially planar structure to compete with the essentially flat ethoxyresorufin for the binding site on cytochrome P-448. Such molecules, with a high degree of unsaturation, possess a large surface area and interact strongly with the binding site. Conversely, molecules with no planar region within their structures do not bind as well in the cytochrome P-448 active site, as demonstrated by the poor inhibition capability of androstane. Such molecules, with little or no unsaturation within their structures, are highly corrugated. These molecules have a relatively small contact surface area to interact with the binding site on cytochrome P-448, reducing their binding potency accordingly.

However, it has been shown in this study that planarity alone does not account for the inhibition ability of all the test compounds. There is substantial discrepancy from the

direct correlation between planarity (P) and saturation concentration, pI' (figure 16). This deviation is apparently the result of the differences in polarity among the test compounds. It was found that inhibition ability also depends upon the hydrophobicity of the molecule. The binding pocket of cytochrome P-448 is hydrophobic in nature, hence, it should preferentially interact with non-polar molecules. In general, the hydrophobic molecules do interact better than their more polar counterparts, as reflected by their better inhibition capabilities (figure 17). However, CDE and chrysene are the exceptions to this relationship.

It is likely the two parameters work in concert to govern affinity to the binding site of cytochrome P-448. However, there may be a limit to molecular size, determined by the depth of the actual binding site. Despite having a high hydrophobic character, molecules like androstane display a poor binding affinity. This indicates that non-planar molecules do not fit well into the binding pocket and the high hydrophobicity of this saturated structure has no capacity to enhance the binding affinity to cytochrome P-448. Effectively, this portion of the total hydrophobicity character is irrelevant to the binding affinity.

When molecules successfully fit into the binding pocket, binding affinity is a result of the two parameters acting

together. Under these conditions, the planarity determines the surface area available for hydrophobic interaction between the molecules and the binding pocket. Then, the hydrophobic character of the portions of these molecules in contact with the surface will govern the strength of this interaction. Therefore, flat and hydrophobic molecules such as chrysene will bind strongly (high pI') to cytochrome P-448. Although it is less corrugated than both CDE and DHT, 146AND has a poorer binding affinity (lower pI') due to the high polarity provided by two ketone groups. Hydrophobicity can compensate the deficiency of high corrugation to increase binding affinity as shown by the fact CDE has a higher pI' than DHT or 146AND.

Aromaticity tends to flatten out the multiple-ring structures even more than simple unsaturation and, at the same time increases their hydrophobic character considerably. As a result, molecules with aromatic character (ES, DHE, EQ4 & EQ5) are about 3-10 times better in inhibiting ethoxyresorufin deethylation activity of cytochrome P-448 than non-aromatic molecules (CDE, DHT & 146AND). The binding affinity of chrysene is much higher than predicted by its hydrophobicity because the whole 4-ring system is aromatic while the remaining test compounds have either unsaturation or aromaticity in only 2 rings (A & B) on the 4-ring structure. In fact, the structure on rings C and D remains fairly

constant for rest of the test compounds: C=C unsaturation is absent in these two rings. Corrugation on rings C and D provides less effective contact and hence interaction surface area for the whole molecule in the binding site.

CDE seems to be the point of large discrepancy in every correlation in this study (figures 16, 17 & 18). Other than chrysene, it is the only test compound with no functional groups in its pure hydrocarbon structure. It is also the only compound that carries a long hydrocarbon tail at C₁₇. Since the original hypothesis assumes that only the 4-ring structure would participate in binding with cytochrome P-448, the hydrocarbon tail was not accounted for in the hydrophobicity estimation. It was not included in obtaining the degree of corrugation either because it is not rigid. The fairly rigid 4-ring structure usually has limited conformational change. Due to its flexibility, the degree of corrugation of the hydrocarbon tail could be highly variable. Nevertheless, it is evident that the hydrocarbon tail does affect the binding with cytochrome P-448. The binding affinity of CDE (pI') is lower than predicted by its concerted property of planarity and hydrophobicity. This may be explained by the possible interference by the hydrocarbon tail. The highly flexible tail would probably lie outside the binding pocket and prevent a proper fit of the rigid 4-ring structure. The essentially flat ethoxyresorufin molecule could still get in the binding

site for deethylation activity to occur. As a result, CDE appears to have a lower inhibition ability than expected.

F-5. Conclusions

Essentially, the binding affinity of steroidal inhibitors is established by the concerted property of planarity and hydrophobicity. The effects of planarity and hydrophobicity on binding affinity are not additive but multiplicative. The product of the two parameters can be called the Adhesive effect of molecules towards cytochrome P-448. Molecules with low planarity can still have considerable binding when compensated by a strong hydrophobic interaction; conversely, those with high polarity by a large interaction surface area. Binding to cytochrome P-448 will be very favourable for strongly hydrophobic and planar molecules. It appears to accept substrates in an open face binding site instead of a narrow cleft. PAH's fall precisely into this criterion thus making them an apparent substrate for cytochrome P-448. In order to refine this relationship between binding affinity and the Adhesive property of molecules, more compounds should be tested to bridge the gap between EQ5 and Chry in the correlation figures (16, 17 & 18). To achieve this, the inhibition study should be repeated using steroidal compounds with unsaturations on rings A, B and C.

This study also demonstrates that steroids can bind to cytochrome P-448 effectively. This implies that steroids can be the physiological substrate for cytochrome P-448. Since the placenta has a high level of cytochrome P-448, it is not inconceivable the enzyme is there for the purpose of steroid metabolism. The levels of β -oestradiol and other oestrogens, which have an aromatic ring in their structure, are tightly regulated by the intricate metabolic pathways within the placenta. It is possible that cytochrome P-448 is closely associated with this metabolic regulation since this study clearly shows aromatic steroids can bind to cytochrome P-448 significantly. Further investigations to support this relationship between steroids and cytochrome P-448 can be achieved in two ways. First, if similar results are obtained by repeating this same study on cytochrome P-448 isolated from the placenta, the physiological relevance of this relationship can be ascertained. Secondly, in order to demonstrate the physiological significance of cytochrome P-448, it necessary to show levels of cytochrome P-448 are induced by dosing with aromatic steroids (e.g. β -oestradiol). As a result, the excess aromatic steroids should be regulated by the induced cytochrome P-448 and would not produce any prolonged physiological effects on the tested animals.

G. References

1. R.G. Harvey, *American Scientists* 70 386 1982
2. A.H. Conney, *Cancer Research* 42 4875 1982
3. C. Ioannides, P.Y. Lum & D.V. Parke, *Xenobiotica* 14(1\2) 119 1984
4. F.P. Guengerich, *Cancer Res.* 48(11) 2946 1988
5. S.D. Black et al, *Adv.Enzymol.* 60 35 1987
6. D.R. Livingstone & J.J. Stegman, *Xenobiotica* 19(10) 1037 1989
7. B.R. Hollebone, *Drug Metab.Rev.* 17 93 1986
8. D. Kupfer, *Pharmac.Ther.* 11 469 1980
9. D.V. Parke, in *Concepts in Drug Metabolism, part B*, edited by P. Jenner & B. Testa (New York: Marcel Dekker) 1980
10. M.J. Coon, *Trans.NY Acad.Sci.* 41 41 1983
11. P.F. Hall, *Vitamins & Hormones* 42 315 1985
12. F.A. Fitzpatrick et al, *Pharmacol.Rev.* 40(4) 229 1988
13. M. Kaku et al, *J.Biochem.(Tokyo)* 96(9) 1983 1984
14. B. Bosterling et al, *Biochem.Biophys.Res.Comm.* 114(2) 8966 1984
15. A. Kappas & A.P. Alvares, *Scientific American* 232(6) 22 1975
16. W.B. Jacoby, *Trans.NY Acad.Sci.* 41 71 1983
17. H.V. Gelboin, *N.Eng.J.Med.* 309(2) 105 1983
18. N.E. Sladek & G.J. Mannering, *Biochem.Biophys.Res. Commun.* 24 668 1966
19. D.W. Nebert & M. Negishi, *Biochem.Pharmacol.* 31(14) 2311 1982
20. D.F.V. Lewis, C. Ioannides & D.V. Parke, *Biochem. Pharmacol.* 35(13) 2179 1986
21. I. Sagami et al, *Xenobiotca* 17(2) 189 1987

22. D.M. Jerina, *Drug Metab.Disp.* 11(1) 1 1983
23. S.K. Yang et al, *Arch.Biochem.Biophys.* 240(2) 456 1985
24. H.W. Strobel et al, *Basic Life Sci.* 39 61 1986
25. M.E. Manus et al, *Carcinogenesis* 4(6) 693 1983
26. D.V. Parke & C. Ioannides, *Ann.Rev.Nutr.* 1 207 1981
27. D.V. Parke & M.J. Obrebska-Parke, *Folia Medica Cracoviensia* 28(1-2) 27 1987
28. D.V. Parke, *Biochem.Soc.Trans.* 11 457 1982
29. C. Ioannides & D.V. Parke, *Biochem.Pharmacol.* 36(24) 4197 1987
30. M. Christou, *Arch.Biochem.Biophys.* 258(2) 519 1987
31. E. Bresnick, *Pharmacol.Rev.* 19 132 1986
32. K.M. Dus, *Xenobiotica* 12 745 1983
33. S.K. Yang, *Biochem.Pharmacol.* 37(1) 61 1988
34. P.J. van Bladeren et al, *J.Biol.Chem.* 259(14) 8966 1984
35. K. Schubert & K. Schade, *J.Steroid Biochem.* 8 359 1977
36. J. Fishman, *Canc.Res. (Supp)* 42 3277S 1982
37. M. Nordqvist et al, *Mol.Pharmacol.* 19 168 1981
38. J.H. van't Hoff, *Etudes de dynamique chimique*, Amsterdam: F. Muller 1884
39. L.J. Brownlee & B.R. Hollebone, *J.App.Tox.* 6(1) 61 1986
40. R.C. Bingham et al, *J.Am.Chem.Soc.* 97 1285 1970
41. R. Bucourt, *Topics in Stereochemistry* 8 159 1974
42. J.D. Dunitz, *Tetrahedron* 28 5459 1972
43. W. Klyne & V. Prelog, *Experimentia* 16 521 1960

44. K.J. Laidler, *the Chemical Kinetics of Enzyme Reaction* Chapter 3 (Oxford Press) 1973
45. A.B. Okey et al, *Clinic.Biochem.* 19 132 1986
46. W. Dehnen, R. Tanningas & J. Roos, *Anal.Biochem.* 53 373 1973
47. L.J. Brownlee, *Chemical control of the mixed function oxidase system and its implications in determining biological hazard* M.Sc.Thesis, August 1990
48. R.D. Allison & D.L. Purich, in *Methods in Enzymology* LVI Ch.1 (Academic Press) 1979
49. M.D. Burke et al, *Biochem.Pharmacol.* 34(18) 3337 1985.
50. M.D. Burke & R.T. Mayer, *Drug Metab.Disp.* 3 245 1975
51. M.D. Burke & R.T. Mayer, *Drug Metab.Disp.* 2 583 1974
52. C.E. Phillipson et al, *Biochem.J.* 207 51 1982
53. H.F. Merk et al, *Biochem.Biophys.Res.Comm.* 148(2) 755 1987
54. K. Iwasaki et al, *Biochem.Pharmacol.* 35(21) 3879 1986
55. M.D. Burke & R.T. Mayer, *Chem.Biol.Interactions* 45 243 1983
56. D.R. Dutton & A. Parkinson, *Arch.Biochem.Biophys.* 268(2) 617 1989
57. J. Rabovsky & D.L. Judy, *Arch.Toxicol.* 63 13 1989
58. L. Jorgensen & T. Johansen, *Acta Pharmacol.Toxicol.* 53 70 1983
59. F. Plasleau et al, *Chem.Biol.Interact.* 34 279 1981
60. M.D. Burke et al, *Drug Metab.Disp.* 5 1 1977
61. D.C. Boersma, L.J. Brownlee & B.R. Hollebone, *J.App.Tox.* 6 16 1984
62. K. Pyykkö, *Acta Pharmacol.et Toxicol.* 52 39 1983
63. O.H. Lowry et al, *J.Biol.Chem.* 193 256 1951

64. *Atlas of Steroid Structure, 1 & 2*, edited by W.L. Dunn, D.A. Norton, J.F. Griffin & C.M. Weeks (Wiley) 1974 & 1984

65. *Structure Reports* 24 670, edited by J.M. Robertson 1960

66. T.A. Loomis, *Essentials of Toxicology*, Chapter 3 (Lea & Febiger) 1978

65. *Drug Disposition and Pharmacokinetics*, Chapter 13 pg 241, edited by S.H. Curry (Appleton & Lange) 1980

68. *Basic Pharmacology*, Chapter 8 pg 348, edited by K.W. Foster (Butterworths) 1986

69. P.D. Leeson et al, *J.Med.Chem.* 31 37 1988

70. C. Hansch & A. Leo, *Substituent Constants for Correlation Analysis in Chemistry and Biology* Chapter IV (Wiley) 1979

H. AppendicesAppendix A

CALCULATIONS:

a>Protein concentration (mg/mL)

$$\begin{array}{ccccccc}
 \begin{array}{c} \mu\text{g protein} \\ \text{(from graph)} \\ \hline 1000 \end{array} & & \times & & \frac{1}{\begin{array}{c} \hline \text{Vol. of} \\ \text{dilution} \\ \text{assayed (mL)} \end{array}} & & \times & & \begin{array}{c} \text{Vol. (mL) of} \\ \text{microsomal sample} \\ \text{Vol. 0.5 M NaOH (mL)} \\ \hline \text{Vol. of microsomal} \\ \text{sample (mL)} \end{array}
 \end{array}$$

For example, for a microsomal fraction where 0.3 mL of aliquots of a dilution of 0.5 mL of the sample plus 9.5 mL of 0.5 M NaOH were used, the equation will be:

$$\begin{array}{ccccccc}
 \begin{array}{c} \mu\text{g protein} \\ \hline 1000 \end{array} & \times & \frac{1}{0.3} & \times & \frac{(0.5 + 9.5)}{0.5} & = & \begin{array}{c} \mu\text{g protein} \\ \hline 15 \end{array}
 \end{array}$$

b>Ethoxyresorufin deethylation activity (nM/min/40μg)

$$\begin{array}{ccccc}
 \begin{array}{c} \text{nM resorufin formed} \\ \hline \text{minute} \end{array} & = & \begin{array}{c} \text{nM resorufin formed} \\ \hline \text{mm} \end{array} & \times & \begin{array}{c} \text{mm} \\ \hline \text{minute} \end{array}
 \end{array}$$

$$\begin{array}{ccc}
 \begin{array}{c} \text{nM resorufin formed} \\ \hline \text{mm} \end{array} & = & \text{fluorescence of resorufin}
 \end{array}$$

$$\begin{array}{ccc}
 \begin{array}{c} \text{mm} \\ \hline \text{minute} \end{array} & = & \text{slope fluorometer chart recorder}
 \end{array}$$

Appendix B

This appendix contains the raw data from the determination of the ethoxyresorufin deethylation activity of 40 μ g of microsomal protein with and without presence of steroidal inhibitors.

**TABLES a-i: INHIBITION OF ETHOXYRESORUFIN DEETHYLATION
ACTIVITY BY 9 TEST COMPOUNDS**

Table a: *Androstane (AND)*

	ACTIVITY (nM/min/40µg)			Average ±SD		%inhibition ±Δ	
CTRL	4.032	3.936	4.008	3.992	0.050	-----	
50 µM	3.931	3.911	4.032	3.958	0.065	0.85	0.024
100 µM	3.809	3.962	3.776	3.849	0.099	3.58	0.137
200 µM	3.797	3.630	3.618	3.708	0.126	7.31	0.34
400 µM	3.538	3.435	3.366	3.446	0.086	13.67	0.51
CTRL	5.209	5.079	5.010	5.133	0.068	-----	
50 µM	5.192	5.044	5.358	5.198	0.156	-1.27	0.054
100 µM	5.106	5.065	4.906	4.996	0.081	2.27	0.067
200 µM	4.919	4.728	4.741	4.796	0.106	6.57	0.23
400 µM	4.741	4.677	4.494	4.637	0.128	9.66	0.39
CTRL	5.956	6.105	6.038	6.033	0.075	-----	
50 µM	6.061	6.085	6.266	6.137	0.112	-1.17	0.036
100 µM	6.091	5.884	5.740	5.905	0.176	2.15	0.091
200 µM	5.491	5.326	5.737	5.518	0.206	8.54	0.36
400 µM	5.430	5.361	5.074	5.288	0.189	12.34	0.42

Table b: 3,5-cholestadiene (CDE)

	ACTIVITY (nM/min/40µg)			Average	±SD	%Inhibition ±A	
CTRL	4.494	4.545	4.702	4.580	0.108	-----	
1 µM	4.130	4.280	4.388	4.266	0.130	6.85	0.37
5 µM	3.481	3.514	3.362	3.452	0.080	24.62	1.15
10 µM	3.061	3.356	3.262	3.226	0.151	29.56	2.08
25 µM	2.181	1.942	2.031	2.051	0.121	55.23	4.56
50 µM	1.063	0.974	0.892	0.976	0.086	78.68	8.78
100 µM	0.725	0.625	0.629	0.660	0.057	85.59	9.41
CTRL	5.105	5.077	5.148	5.110	0.036	-----	
1 µM	4.657	4.728	4.697	4.694	0.036	8.14	0.17
5 µM	3.865	4.025	3.977	3.956	0.082	22.58	0.65
10 µM	3.643	3.717	3.703	3.687	0.039	27.83	0.49
25 µM	2.415	2.310	2.333	2.363	0.074	53.77	2.06
50 µM	1.010	1.000	1.080	1.030	0.043	79.84	3.90
100 µM	0.848	0.815	0.772	0.793	0.031	84.47	3.90
CTRL	6.072	6.011	6.270	6.118	0.135	-----	
1 µM	5.755	5.897	5.796	5.816	0.086	4.45	0.17
5 µM	5.000	4.827	4.937	4.921	0.087	19.56	0.78
10 µM	4.313	4.158	4.046	4.172	0.134	31.80	1.72
25 µM	2.871	2.624	2.551	2.682	0.168	56.16	4.76
50 µM	1.240	1.167	1.293	1.233	0.063	79.85	5.84
100 µM	0.833	0.731	0.865	0.810	0.070	86.77	9.41

Table c: 1-dehydrotestosterone (DHT)

	ACTIVITY (nM/min/40µg)			Average ±SD		%Inhibition ±Δ	
CTRL	4.410	4.348	4.375	4.288	0.128	-----	
10 µM	3.684	3.913	3.846	3.814	0.118	11.04	0.67
25 µM	3.648	3.609	3.587	3.615	0.031	15.70	0.60
50 µM	2.862	2.889	3.062	2.938	0.108	31.48	2.10
100 µM	2.795	2.594	2.764	2.718	0.108	36.62	2.54
200 µM	1.770	1.880	1.800	1.817	0.057	57.63	3.53
400 µM	0.594	0.645	0.564	0.601	0.041	85.98	8.43
CTRL	5.678	5.657	5.909	5.748	0.140	-----	
10 µM	4.896	5.010	5.057	4.987	0.082	13.23	0.54
25 µM	4.618	4.613	4.822	4.684	0.119	18.51	0.92
50 µM	4.140	4.181	4.218	4.180	0.039	27.29	0.92
100 µM	3.592	3.848	3.773	3.738	0.131	34.39	2.04
200 µM	2.513	2.479	2.582	2.525	0.053	56.08	2.54
400 µM	1.136	0.988	1.092	1.072	0.076	81.35	7.74
CTRL	6.572	6.958	6.694	6.741	0.197	-----	
10 µM	6.042	5.707	5.757	5.835	0.180	13.44	0.81
25 µM	5.490	5.525	5.611	5.542	0.062	17.79	0.72
50 µM	4.781	4.500	4.762	4.681	0.157	30.56	1.92
100 µM	3.998	4.197	4.244	4.146	0.131	38.49	2.34
200 µM	2.524	2.704	2.699	2.642	0.103	60.80	4.15
400 µM	1.289	1.457	1.330	1.359	0.088	79.84	7.50

Table d: 1,4,6-androstatrien-3,17-dione (146AND)

	ACTIVITY (nM/min/40µg)			Average ±SD		%Inhibition ±A	
CTRL	4.654	4.746	4.762	4.721	0.058	-----	
10 µM	4.036	3.975	4.042	4.018	0.037	14.89	0.32
25 µM	3.979	3.837	3.836	3.884	0.082	17.72	0.59
50 µM	3.559	3.674	3.262	3.498	0.212	25.90	1.89
100 µM	2.638	2.900	2.425	2.654	0.238	43.78	4.46
200 µM	2.042	2.204	2.154	2.133	0.083	54.81	2.81
400 µM	0.789	0.712	0.834	0.778	0.062	83.51	7.68
CTRL	5.736	6.022	5.847	5.868	0.144	-----	
10 µM	5.230	4.975	5.014	5.073	0.137	13.56	0.70
25 µM	4.952	4.625	4.643	4.740	0.183	19.22	1.21
50 µM	3.984	4.067	4.031	4.027	0.042	31.37	1.10
100 µM	2.961	2.867	3.162	2.997	0.151	48.94	3.67
200 µM	2.325	2.425	2.481	2.410	0.117	58.93	4.31
400 µM	1.016	0.886	0.854	0.919	0.086	84.34	9.96
CTRL	6.389	6.408	6.070	6.289	0.190	-----	
10 µM	5.641	5.522	5.418	5.527	0.112	12.12	0.61
25 µM	5.082	4.850	5.234	5.055	0.193	19.62	1.34
50 µM	4.502	4.612	4.709	4.608	0.103	26.73	1.41
100 µM	3.315	3.417	3.538	3.424	0.112	45.56	2.87
200 µM	2.533	2.835	2.762	2.710	0.157	56.91	5.02
400 µM	0.889	0.764	0.873	0.842	0.068	86.61	9.61

Resolution test chart showing patterns of horizontal and vertical lines with numerical labels: 1.0, 1.1, 1.25, 1.4, 1.6, 1.8, 2.0, 2.2, 2.5, 2.8, 3.2, 3.6, 4.0, 4.5, 5.0, 5.6, 6.3, 7.1, 8.0, 9.0, 10.

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

Table e: Estrone (ES)

	ACTIVITY (nM/min/40µg)			Average ±SD		%Inhibition ±A	
CTRL	4.348	4.455	4.375	4.393	0.056	-----	
1 µM	3.429	3.473	3.456	3.452	0.022	21.41	0.41
5 µM	3.127	3.154	2.947	3.037	0.128	30.87	1.69
10 µM	2.445	2.379	2.232	2.352	0.109	46.47	2.75
25 µM	2.202	2.231	2.186	2.206	0.023	49.78	1.15
50 µM	2.110	2.110	2.086	2.102	0.014	52.15	1.01
100 µM	1.770	1.753	1.827	1.783	0.038	59.41	2.02
CTRL	5.775	5.950	5.807	5.844	0.128	-----	
1 µM	4.598	4.569	4.359	4.509	0.130	22.84	1.16
5 µM	4.194	4.036	3.924	4.051	0.136	30.68	1.70
10 µM	3.333	3.251	3.203	3.262	0.066	44.18	1.86
25 µM	2.845	3.026	2.881	2.917	0.095	49.92	2.72
50 µM	2.681	2.805	2.652	2.713	0.081	53.58	2.77
100 µM	2.355	2.332	2.430	2.373	0.051	59.40	2.57
CTRL	6.767	6.415	6.570	6.584	0.176	-----	
1 µM	5.176	5.235	5.143	5.185	0.046	21.25	0.76
5 µM	4.177	4.221	4.418	4.272	0.128	35.11	1.99
10 µM	3.788	3.520	3.711	3.673	0.137	44.21	2.83
25 µM	3.450	3.542	3.361	3.451	0.091	47.58	2.53
50 µM	2.967	3.086	2.938	2.997	0.078	54.48	2.87
100 µM	2.633	2.724	2.701	2.686	0.047	59.20	2.61

Table 1: 6-dehydroestrone (DHE)

	ACTIVITY (nM/min/40µg)			Average ±SD		%Inhibition ±Δ	
CTRL	4.106	4.025	4.238	4.123	0.108	-----	
1 µM	3.643	3.593	3.594	3.607	0.023	12.52	0.41
5 µM	3.281	3.454	3.243	3.326	0.112	19.33	1.16
10 µM	3.117	3.152	2.937	3.069	0.115	25.57	1.63
25 µM	2.943	2.835	2.799	2.859	0.075	30.66	1.61
50 µM	1.985	1.949	2.068	2.001	0.061	51.48	2.91
100 µM	0.663	0.740	0.617	0.673	0.062	83.67	9.90
CTRL	5.969	5.856	5.882	5.902	0.050	-----	
1 µM	5.398	5.212	5.201	5.270	0.111	10.70	0.33
5 µM	4.955	4.830	4.698	4.841	0.149	17.98	0.73
10 µM	4.611	4.370	4.535	4.513	0.102	23.54	0.77
25 µM	3.858	3.551	3.795	3.795	0.219	35.70	2.42
50 µM	2.701	2.951	3.061	2.905	0.185	50.79	3.74
100 µM	0.799	0.878	0.909	0.862	0.057	85.39	6.50
CTRL	6.728	6.673	6.511	6.637	0.112	-----	
1 µM	5.942	5.733	5.656	5.777	0.147	12.96	0.55
5 µM	5.503	5.371	5.194	5.356	0.155	19.30	0.88
10 µM	4.874	5.079	5.000	4.984	0.103	24.91	0.94
25 µM	4.620	4.583	4.491	4.565	0.067	31.22	0.99
50 µM	3.128	3.558	3.289	3.325	0.217	50.10	4.12
100 µM	0.828	0.933	0.827	0.862	0.061	87.00	7.62

Table g: *Equilin* (EQ4)

	ACTIVITY (nM/min/40µg)			Average	±SD	%Inhibition (%)	
CTRL	4.207	4.375	4.453	4.345	0.126	-----	
.1 µM	3.685	3.667	3.613	3.655	0.037	15.88	0.62
.5 µM	3.441	3.420	3.519	3.460	0.052	20.37	0.90
2 µM	3.109	2.853	3.333	3.098	0.241	28.69	3.06
5 µM	2.305	2.595	2.325	2.408	0.162	44.57	4.29
10 µM	1.369	1.404	1.521	1.431	0.080	67.06	5.69
50 µM	0.512	0.474	0.487	0.491	0.019	88.70	6.00
CTRL	5.042	5.359	5.202	5.201	0.159	-----	
.1 µM	4.192	4.290	4.307	4.263	0.062	18.03	0.81
.5 µM	4.082	4.074	3.885	4.014	0.112	22.83	1.33
2 µM	3.454	3.525	3.495	3.491	0.035	32.87	1.33
5 µM	2.940	2.978	3.191	3.036	0.135	41.62	3.12
10 µM	1.533	1.771	1.600	1.635	0.123	68.57	7.25
50 µM	0.815	0.838	0.740	0.798	0.032	84.66	8.00
CTRL	6.555	6.735	6.546	6.612	0.106	-----	
.1 µM	5.290	5.333	5.641	5.421	0.191	18.01	0.92
.5 µM	4.819	4.968	5.088	4.958	0.134	25.01	1.08
2 µM	4.224	4.593	4.473	4.430	0.188	33.00	1.93
5 µM	3.322	3.527	3.530	3.460	0.119	47.67	2.40
10 µM	2.351	2.214	2.137	2.234	0.108	66.21	4.26
50 µM	1.147	1.156	1.342	1.215	0.111	81.62	8.79

Table h: *d-Equilenin (EQ5)*

		ACTIVITY (nM/min/40µg)			Average ±SD		%Inhibition ±Δ	
CTRL		4.612	4.887	4.832	4.777	0.145	-----	
.1	µM	4.313	4.286	4.235	4.278	0.039	10.45	0.41
.5	µM	4.186	4.135	3.932	4.084	0.134	14.50	0.92
2	µM	3.382	3.550	3.643	3.525	0.132	26.21	1.78
5	µM	2.347	2.233	2.383	2.321	0.078	51.41	3.29
10	µM	1.643	1.690	1.917	1.750	0.146	63.37	7.21
50	µM	0.961	0.885	0.997	0.948	0.057	80.16	7.25
CTRL		5.714	5.842	5.678	5.745	0.086	-----	
.1	µM	5.092	5.007	5.030	5.043	0.043	12.27	0.29
.5	µM	4.700	4.925	4.728	4.784	0.123	16.73	0.68
2	µM	4.505	4.433	4.465	4.466	0.036	22.26	0.51
5	µM	2.941	2.711	2.899	2.850	0.122	50.39	2.91
10	µM	2.122	1.998	2.150	2.090	0.081	63.62	3.42
50	µM	1.142	1.205	1.257	1.201	0.058	79.09	5.00
CTRL		6.268	6.011	6.075	6.118	0.134	-----	
.1	µM	5.122	5.284	5.428	5.278	0.153	13.73	0.70
.5	µM	4.888	4.912	5.045	4.948	0.085	19.12	0.75
2	µM	4.546	4.468	4.630	4.548	0.081	25.66	1.02
5	µM	3.169	3.068	3.171	3.136	0.059	48.75	1.98
10	µM	2.161	2.368	2.129	2.219	0.130	63.72	5.13
50	µM	0.993	1.048	1.094	1.045	0.051	82.92	5.86

Table 1: Chrysene (Chry)

	ACTIVITY (nM/min/40µg)			Average	±SD	%Inhibition (A)	
CTRL	4.134	4.127	3.946	4.069	0.106	-----	
.005 µM	3.363	3.625	3.531	3.506	0.133	13.83	0.88
.010 µM	3.066	2.967	2.914	2.962	0.046	27.20	1.13
.025 µM	2.227	2.222	2.465	2.305	0.139	43.35	3.74
.050 µM	1.583	1.367	1.440	1.463	0.110	64.04	6.48
.100 µM	1.020	1.086	1.064	1.057	0.033	74.03	4.23
.200 µM	0.695	0.687	0.604	0.662	0.050	83.73	8.51
CTRL	5.382	5.215	5.564	5.387	0.174	-----	
.005 µM	4.287	4.411	4.447	4.382	0.084	18.66	0.96
.010 µM	3.778	3.876	3.918	3.857	0.072	28.39	1.44
.025 µM	2.911	2.675	2.728	2.702	0.037	49.85	2.29
.050 µM	2.141	1.926	2.040	2.036	0.107	62.21	5.28
.100 µM	1.679	1.661	1.504	1.615	0.096	70.03	6.42
.200 µM	1.085	0.940	0.977	1.001	0.075	81.42	8.73
CTRL	6.389	6.321	6.157	6.289	0.119	-----	
.005 µM	5.485	5.272	5.215	5.324	0.142	15.43	0.70
.010 µM	4.508	4.657	4.785	4.650	0.138	26.06	1.27
.025 µM	3.787	3.798	3.407	3.640	0.265	42.13	3.86
.050 µM	2.253	2.296	2.334	2.294	0.041	63.52	2.34
.100 µM	1.961	1.729	1.847	1.846	0.116	70.65	5.78
.200 µM	1.190	1.430	1.352	1.324	0.122	78.95	8.77

Appendix C

This appendix contains the mean % inhibition values of the ethoxyresorufin deethylation reaction by the test compounds at various concentrations.

**TABLES j-r: % INHIBITION OF ETHOXYRESORUFIN O-ETHYLATION
ACTIVITY BY 9 TEST COMPOUNDS**

Table j: *Androstane (AND)*

[Inhib.] (μ M)	% INHIBITION			MEAN	$\pm$ SD
50	0.00	0.00	0.00	0.00	
100	0.00	0.00	0.00	0.00	
200	7.31	6.57	8.54	7.47	1.00
400	13.67	9.66	12.34	11.89	2.04

Table k: *3,5-cholestadiene (CDE)*

[Inhib.] (μ M)	% INHIBITION			MEAN	$\pm$ SD
1	6.85	8.14	4.49	6.49	1.85
5	24.62	22.58	19.56	22.25	2.55
10	29.56	27.83	31.80	29.73	1.99
25	55.23	53.77	56.16	55.05	1.20
50	78.68	79.84	79.85	79.46	0.67
100	85.59	84.47	86.77	85.61	1.15

Table l: *1-dehydrotestosterone (DHT)*

[Inhib.] (μ M)	% INHIBITION			MEAN	$\pm$ SD
10	11.04	13.23	13.44	12.57	1.33
25	15.70	18.51	17.79	17.33	1.46
50	31.48	27.29	30.56	29.78	2.20
100	36.62	34.39	38.49	36.50	2.05
200	57.63	56.08	60.80	58.17	2.41
400	85.98	81.35	79.84	82.39	3.20

Table m: *1,4,6-androstatrien-3,17-dione (146AND)*

[Inhib.] (μ M)	% INHIBITION			MEAN	$\pm$ SD
10	14.89	13.56	12.12	13.52	1.39
25	17.72	19.22	19.62	18.85	1.00
50	25.90	31.37	26.73	28.00	2.96
100	43.78	48.94	45.56	46.09	2.62
200	54.81	58.93	56.91	56.58	2.06
400	83.51	84.34	86.61	84.82	1.60

Table n: *Estrone (ES)*

[Inhib.] (μ M)	% INHIBITION			MEAN	$\pm$ SD
1	21.41	22.84	21.25	21.83	0.88
5	30.87	30.68	35.11	32.22	2.50
10	46.47	44.18	44.21	44.95	1.31
25	49.78	49.92	47.58	49.09	1.31
50	52.15	53.58	54.48	53.40	1.18
100	59.41	59.40	59.20	59.34	0.12

Table o: *C-dehydroestrone (DHE)*

[Inhib.] (μ M)	% INHIBITION			MEAN	$\pm$ SD
1	12.52	10.70	12.96	12.06	1.20
5	19.33	17.98	19.30	18.87	0.77
10	25.57	23.54	24.91	24.67	1.04
25	30.66	35.70	31.22	32.52	2.76
50	51.48	50.79	50.10	50.79	0.69
100	83.67	85.39	87.00	85.35	1.67

Table p: *Equilin* (EQ4)

[Inhib.] (μM)	% INHIBITION			MEAN	$\pm$ SD
0.1	15.88	18.03	18.01	17.31	1.24
0.5	20.37	22.83	25.01	22.74	2.32
2	28.69	32.87	33.00	31.52	2.45
5	44.57	41.62	47.67	44.62	3.03
10	67.06	68.57	66.62	67.42	1.02
50	88.70	84.66	81.62	84.99	3.55

Table q: *d-Equilenin* (EQ5)

[Inhib.] (μM)	% INHIBITION			MEAN	$\pm$ SD
0.1	10.45	12.27	13.73	12.15	1.64
0.5	14.50	16.73	19.12	16.78	2.31
2	26.21	22.26	25.66	24.71	2.14
5	51.41	50.39	48.75	50.18	1.34
10	63.37	63.62	63.72	63.57	0.18
50	80.16	79.09	82.92	80.72	1.98

Table r: *Chrysene* (Chry)

[Inhib.] (μM)	% INHIBITION			MEAN	$\pm$ SD
0.005	13.83	18.66	15.43	15.97	2.46
0.010	27.20	28.39	26.06	27.21	1.17
0.025	43.35	49.85	42.13	45.11	4.15
0.050	64.04	62.21	63.52	63.26	0.94
0.100	74.03	70.03	70.65	71.57	2.15
0.200	83.73	81.42	78.95	81.37	2.39

Appendix D

The linear relationship between the independent parameter (planarity, P) and the dependent parameter (inhibition capacity, PI') is justified as follows.

$$\text{Observed rate} = \frac{V_{\max} [S]}{(1 + [I]/K_i) K_m + [S]}$$

Assuming that $[S] \ll K_m$, therefore,

$$\text{Observed rate} = \frac{V_{\max} [S]}{(1 + [I]/K_i) K_m}$$

$V_{\max}$, $[S]$ and K_m are held constant during the assay, thus $[I]/K_i$ becomes the only variable.

$$\text{Theoretical rate} = A.e^{-\Delta G^*/RT}$$

where ΔG^* is the only variable. Since the Observed rate is equivalent to the Theoretical rate, the variables must be related. In order to test for a linear relationship between these two non-linear forms, take the logarithm of the observed variable and equate power series expansions of both forms. Thus for the observed form, the general algebraic series from Taylor's expansion is:

$$\therefore \frac{1}{(1 - x)} = \sum x^n \quad \{n = 0 \text{ to } \infty\}$$

$$\therefore \frac{1}{(1 - (-\ln [I]/K_i))} = 1 + (-\ln [I]/K_i)^1 + \dots$$

For the theoretical form, the Taylor's expansion to first order is:

$$\therefore e^x = \sum \frac{x^n}{n!} \quad \{n = 0 \text{ to } \infty\}$$

$$\therefore e^{-\Delta G^*/RT} = 1 + (-\Delta G^*/RT)^1/1! + \dots$$

Thus, to the first order, the forms are identical in their expanded series and the variables can therefore be equated;

$$-\ln [I]/K_1 = -\Delta G^*/RT$$

$$\therefore pI' = f(\Delta H^* - T\Delta S^*)$$

Assuming the enthalpic component is constant so only the entropic component (i.e. planarity, P) is variable.

Therefore, $pI' = f(P)$ is a linear relationship.

Appendix E

The polynomial equations of the curves fitted for the sigmoid portions of the pI-%inhibition plots (figures 6-13) are shown as follows. The double derivatives of each function is deduced in order to obtain the point of inflection, hence, the binding strength, pI'.

$$y' = \frac{dy}{dx}, \quad y'' = \frac{dy^2}{d^2x}$$

3,5-cholestadiene (CDE)

$$y = 69.961x^3 - 972.94x^2 + 4431.3x - 6549.4 \quad r^2 = 0.998$$

$$y' = 209.883x^2 - 1945.88x - 4431.3$$

$$y'' = 419.776x - 1945.88$$

Point of inflection is when $y'' = 0$,

$$\therefore 419.776x = 1945.88$$

$$pI' = x = 4.636$$

1-dehydrotestosterone (DHT)

$$y = 80.521x^3 - 1072.1x^2 + 4712.9x - 6814.8 \quad r^2 = 1.000$$

$$y' = 241.563x^2 - 2144.2x + 4712.9$$

$$y'' = 483.126x - 2144.2$$

$$\text{Set } y'' = 0, \text{ then } pI' = x = 4.438$$

1,4,6-androstadien-3,17-dione (146AND)

$$y = 97.784x^3 - 1212.0x^2 + 4945.7x - 6602.2 \quad r^2 = 1.000$$

$$y' = 293.352x^2 - 2424.0x + 4945.7$$

$$y'' = 586.704x - 2424.0$$

$$\text{Set } y'' = 0, \text{ then } pI' = x = 4.132$$

Estrone (ES)

$$y = 15.411x^3 - 239.27x^2 + 1209.2x - 1948.3 \quad r^2 = 0.978$$

$$y' = 46.233x^2 - 478.54x + 1209.2$$

$$y'' = 92.466x - 478.54$$

$$\text{Set } y'' = 0, \text{ then } pI' = x = 5.175$$

6-dehydroestrone (DHE)

$$y = 12.747x^3 - 197.39x^2 + 988.34x - 1625.3 \quad r^2 = 1.000$$

$$y' = 38.241x^2 - 394.78x + 988.34$$

$$y'' = 76.482x - 394.78$$

$$\text{Set } y'' = 0, \text{ then } pI' = x = 5.162$$

Equilin (EQ4)

$$y = 4.7015x^3 - 71.357x^2 + 322.24x - 353.96 \quad r^2 = 0.978$$

$$y' = 14.1045x^2 - 142.714x + 322.24$$

$$y'' = 28.209x - 142.714$$

$$\text{Set } y'' = 0, \text{ then } pI' = x = 5.059$$

d-equilenin (EQ5)

$$y = 8.2945x^3 - 133.11x^2 + 669.12x - 993.81 \quad r^2 = 0.981$$

$$y' = 24.8835x^2 - 266.22x + 669.12$$

$$y'' = 49.767x - 266.22$$

$$\text{Set } y'' = 0, \text{ then } pI' = x = 5.349$$

Chrysene (Chry)

$$y = 14.335x^3 - 327.28x^2 + 2440.8x - 5892.4 \quad r^2 = 0.996$$

$$y' = 43.005x^2 - 654.56x + 2440.8$$

$$y'' = 86.01x - 654.56$$

$$\text{Set } y'' = 0, \text{ then } pI' = x = 7.610$$

Appendix F

The hydrophobicity parameter is calculated by the Fragment method used to deduce partition coefficients for each steroidal 4-ring system. The partition coefficients are calculated by adding together the appropriate fragment constants. The fragment constants used are listed in the table below.

General fragment (f)	Fragment constants
aliphatic CH ₃	0.89
aliphatic CH ₂	0.66
aliphatic CH	0.43
aliphatic C	0.20
aromatic CH ϕ	0.35
aromatic C ϕ	0.13
aromatic C' ϕ (ring fusion carbon)	0.22
F _b (bond factor)*	-0.09
secondary aliphatic OH	-1.86
OH ϕ (aromatic hydroxyl)	-0.44
C=O	-1.90
F ₌ (double bond factor)	-0.55
F ₌ ^c (factor for double bond conjugated to ϕ)	-0.42

*For a multiple ring system with n carbon-carbon bonds (C-C or C $\equiv$ C bond counts as 1 carbon-carbon bond), [n-1] x F_b is the bond factor contribution.

This calculation method⁷⁰ for the hydrophobicity partition coefficient retains constant fragment values (f) for the fundamental structural elements and then looks for other factors (F) that affect the partition equilibrium in the more complex solutes where summation of fragments alone leads to spurious results.

The hydrophobic fundamental fragments include the aliphatic carbons (CH_3 , CH_2 , CH & C in the table) where the hydrogens are already accounted for within each fragment constant. Carbon atoms multiply bonded to a hetero-atom (e.g. $\text{C}=\text{O}$) and hetero-atom functional groups (e.g. OH) are polar fundamental fragments which decrease the overall partition coefficient of the molecule.

The double bond factor (F_{u}) accounts for the lowering of partition coefficient since polarity introduced by unsaturation increases the accommodation of water relative to octanol. On the other hand, it is only localised polarity that favours accommodation by water, and therefore, conjugation would be expected to reduce the "polarity" effect of unsaturation (F_{u}^{c}). The convenient way to use this is to first consider the structure as saturated and then add the appropriate double bond factor.

Using the n-alkane series, it was found experimentally that the partition coefficient does not increase linearly with the chain length. Apparently, lower values are observed for carbon fragments in the larger molecules as compared to the smaller ones. This deviation is ascribed to the chain flexibility, which becomes apparent only after the second and higher bonds are added to a chain. In the traditional "flickering cluster" approach to hydrocarbon solvation, this flexibility might reduce the degree of order in the solvation shell; therefore, the bond factor (F_{b}) has a negative sign, and is taken (n-1) times where n is the number of bonds.

For aromatic structures, a different set of fundamental carbon fragments are used: CH ($\text{CH}\phi$), C ($\text{C}\phi$) and ring fusion C ($\text{C}'\phi$). Their partition coefficients can be obtained the same way as in the aliphatic structures but it no longer requires the adding of double bond factors because the unsaturation is already accounted for in this set of aromatic fragment constants.

Androstane (AND)

Partition coefficient =

$$\begin{aligned}
 & 11 f[\text{CH}_2] + 4 f[\text{CH}] + 2 f[\text{CH}_3] + 2 f[\text{C}] + (19-1) F_b \\
 & = 11(0.66) + 4(0.43) + 2(0.89) + 2(0.20) + 18(-0.09) \\
 & = 9.54
 \end{aligned}$$

3,5-cholestadiene (CDE)

Partition coefficient =

$$\begin{aligned}
 & 7 f[\text{CH}_2] + 7 f[\text{CH}] + 2 f[\text{CH}_3] + 3 f[\text{C}] + 2 F_{\text{=}} + (19-1) F_b \\
 & = 7(0.66) + 7(0.43) + 2(0.89) + 3(0.20) + 2(-0.55) + 18(-0.09) \\
 & = 7.11
 \end{aligned}$$

1-dehydrotestosterone (DHT)

Partition coefficient =

$$\begin{aligned}
 & 6 f[\text{CH}_2] + 7 f[\text{CH}] + 2 f[\text{CH}_3] + 3 f[\text{C}] + f[\text{C=O}] + \\
 & f[\text{OH}] + 2 F_{\text{=}} + (21-1) F_b \\
 & = 6(0.66) + 7(0.43) + 2(0.89) + 3(0.20) + (-1.90) \\
 & + (-1.86) + 2(-0.55) + 20(-0.09) \\
 & = 2.69
 \end{aligned}$$

1,4,6-androstatrien-3,17-dione (146AND)

Partition coefficient =

$$\begin{aligned}
& 4 f[\text{CH}_2] + 8 f[\text{CH}] + 2 f[\text{CH}_3] + 3 f[\text{C}] + 2 f[\text{C=O}] \\
& + 3 F_{\text{=}} + (21-1) F_{\text{b}} \\
& = 4(0.66) + 8(0.43) + 2(0.89) + 3(0.20) + 2(-1.90) \\
& + 3(-0.55) + 20(-0.09) \\
& = 1.21
\end{aligned}$$

Estrone (ES)

Partition coefficient =

$$\begin{aligned}
& 6 f[\text{CH}_2] + 3 f[\text{CH}] + f[\text{CH}_3] + f[\text{C}] + 3 f[\text{CH}\phi] \\
& + f[\text{C}\phi] + 2 f[\text{C}'\phi] + f[\text{OH}\phi] + f[\text{C=O}] + (15-1) f[F_{\text{b}}] \\
& = 6(0.66) + 3(0.43) + (0.89) + (0.20) + 3(0.35) \\
& + (0.13) + 2(0.22) + (-0.44) + (-1.90) + 14(0.09) \\
& = 4.36
\end{aligned}$$

6-dehydroestrone

Partition coefficient =

$$\begin{aligned}
& 4 f[\text{CH}_2] + 5 f[\text{CH}] + f[\text{CH}_3] + f[\text{C}] + 3 f[\text{CH}\phi] \\
& + f[\text{C}\phi] + 2 f[\text{C}'\phi] + f[\text{OH}\phi] + f[\text{C=O}] \\
& + F_{\text{=}}^{\text{c}} + (15-1) F_{\text{b}} \\
& = 4(0.66) + 5(0.43) + (0.89) + (0.20) + 3(0.35) \\
& + (0.13) + 2(0.22) + (-0.44) + (-1.90) \\
& + (-0.42) + 14(-0.09) \\
& = 3.48
\end{aligned}$$

Equilin (EQ4)

Partition coefficient =

$$\begin{aligned}
 & 5 f[\text{CH}_2] + 3 f[\text{CH}] + f[\text{CH}_3] + 2 f[\text{C}] + 3 f[\text{CH}\phi] \\
 & + f[\text{C}\phi] + 2 f[\text{C}'\phi] + f[\text{OH}\phi] + f[\text{C}=\text{O}] \\
 & + F_{\text{a}} + (15-1) F_{\text{b}} \\
 & = 5(0.66) + 3(0.43) + (0.89) + 2(0.20) + 3(0.35) \\
 & + (0.13) + 2(0.22) + (-0.44) + (-1.90) \\
 & + (-0.55) + 14(-0.09) \\
 & = 3.35
 \end{aligned}$$

d-equilenin (EQ5)

Partition coefficient =

$$\begin{aligned}
 & 4 f[\text{CH}_2] + f[\text{CH}] + f[\text{CH}_3] + f[\text{C}] + 5 f[\text{CH}\phi] \\
 & + 3 f[\text{C}\phi] + 2 f[\text{C}'\phi] + f[\text{OH}\phi] + f[\text{C}=\text{O}] + (10-1) F_{\text{b}} \\
 & = 4(0.66) + (0.43) + (0.89) + (0.20) + 5(0.35) \\
 & + 3(0.13) + 2(0.22) + (-0.44) + (-1.90) + 9(-0.09) \\
 & = 3.59
 \end{aligned}$$

Chrysene (Chry)

Partition coefficient =

$$\begin{aligned}
 & 12 f[\text{CH}\phi] + 6 f[\text{C}\phi] \\
 & = 12(0.35) + 6(0.13) \\
 & = 4.98
 \end{aligned}$$

Appendix G

The equation for the linear correlation function between the concerted property of hydrophobicity and planarity (HxP) and binding affinity in figure 16 is:

$$y = 0.00064585x + 3.7730 \quad r^2 = 0.950$$

By substituting x values (HxP) into the above equation, the predicted binding affinity, PI'_{calc} can be obtained. The following table summarises the results of PI'_{calc} , comparing to the PI'_{obs} values obtained in the inhibition study.

Compound	H x P	PI'_{calc}	PI'_{obs}	Deviation $ PI'_{calc} - PI'_{obs} $
CDE	2190	5.187	4.636	0.551
DHT	831	4.310	4.438	0.128
146AND	540	4.122	4.132	0.010
ES	2054	5.100	5.175	0.075
DHE	1789	4.928	5.162	0.234
EQ4	1909	5.006	5.059	0.053
EQ5	2488	5.380	5.349	0.031
Chry	5807	7.523	7.610	0.087

By inspection, CDE appears to be an obvious outlying point. If point CDE is excluded from the set, a second linear correlation function can be obtained again by computer graphics (Cricket Graph 1.2.1) with a better correlation coefficient:

$$y = 0.00064543x + 3.8527 \quad r^2 = 0.994$$

END

04.03.92

FIN