CYTOCHROME P450 ENZYMES IN CHANNEL CATFISH, *ICTALURUS PUNCTATUS*, AND METABOLISM OF TESTOSTERONE BY CATFISH INTESTINAL MICROSOMES

By

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KEY TO ABBREVIATIONS

3-MC: 3-methylcholanthrene

AHH: aryl hydrocarbon hydroxylase

AhR: aryl hydrocarbon receptor

ANF: α-naphthoflavone

BNF: β-naphthoflavone

CYP: cytochrome P450

EH: epoxide hydrolase

ERM: erythromycin

GST: glutathione S-transferase

HEPES: N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

HSD: hydroxysteroid dehydrogenase

KET: ketoconazole

MET: metyrapone

PCN: pregnenolone 16α-carbonitrile

RIF: rifampicin

ST: sulfotransferase

TAO: troleandomycin

TCB: 3, 3', 4, 4'-tetrachloro biphenyl

UGT: UDP-glucuronosyltransferase

Abstract of Dissertation Presented to the Graduate School
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Intestinal cytochrome P450 provides the principal, initial source of biotransformation of ingested xenobiotics. In humans, an important cause of incomplete bioavailability is prehepatic metabolism in the GI tract, mainly by the CYP3A enzymes. The expression and properties of CYP proteins were examined along the intestine of channel catfish, *Ictalurus punctatus*, fed commercial chow or semi-purified diets. Benzo(a)pyrene hydroxylase activity was higher in proximal than distal intestine, and was stimulated by α-naphthoflavone, suggesting involvement of CYP3A. Polyclonal antibodies (IgG) generated against trout CYP3A27 reacted strongly with catfish intestinal microsomes, showing a band with MW of 59 kDa. In catfish fed with standard chow, the expression of this protein was much higher in the proximal segment than in the distal part. Testosterone 6β-hydroxylation activities were monitored as the catalytic indicator of

CYP3A, which was higher in proximal than distal intestine. The 6β-hydroxylation activities in the two segments correlated with the amount of CYP3A. Similar results were obtained with progesterone as substrate. The amount of CYP3A and steroid-6β-hydroxylation activities were lower in both segments of intestine from fish fed purified diet compared with commercial chow, but with the same trend along intestine.

Incubation of catfish intestinal or hepatic microsomes with [4-^{14}C] testosterone resulted in three major metabolites: 6β-hydroxy testosterone, androstenedione and another metabolite. The formation of this unknown metabolite requires NADPH as cofactor. Comparison of the chromatographic behavior and MS of the unknown metabolite with that of authentic testosterone derivative suggested that this metabolite corresponds to 4-androsten-3α, 17β-diol. The ratio of testosterone 6β-hydroxylation/17-oxidation was significantly higher in proximal than distal intestine.

Testosterone 6β-hydroxylation was inhibited by specific mammalian CYP3A inhibitors, ketoconazole and erythromycin, and general P450 inhibitors, metyrapone and SKF-525A, but was not affected by α-naphthoflavone. Troleandomycin, a mammalian CYP3A inhibitor, had no effect on the testosterone metabolism by catfish intestinal microsomes up to 100 μM. Dietary pretreatment of catfish with rifampicin or pregnenolone-16α-carbonitrile (PCN) did not alter the CYP3A enzyme level in proximal and distal intestine. Distal intestine from fish treated with rifampicin for 2 weeks showed significantly higher testosterone 6β-hydroxylation and 3α-oxido-reduction activities than that from control fish.

CHAPTER 1
INTRODUCTION

All organisms are exposed constantly and unavoidably to foreign chemicals, or xenobiotics, which include both man-made and natural chemicals such as drugs, industrial chemicals, pesticides, pollutants, pyrolysis products and toxins produced by molds, plants and animals. As a result of a great variety of human activities, the aquatic environment is becoming increasingly threatened by an alarming number of foreign chemicals. This pollution is a threat to the health of organisms inhabiting the waters, as well as to human consumers of such organisms. Fish populations living in highly polluted areas often have high incidences of gross pathological lesions and neoplasms, associated with elevated levels of toxic contaminants in the sediment [0]. Of most concern are xenobiotics that cannot be readily eliminated because of their lipophilicity. Biotransformation or metabolism of lipophilic chemicals to more water-soluble compounds is a requisite for detoxification and excretion. An important consequence of biotransformation is that the physical properties of a xenobiotic are generally changed from those favoring absorption (lipophilicity) to those favoring excretion in urine or feces (hydrophilicity). In addition, certain steps in the biotransformation pathway are responsible for the activation of foreign chemicals to the reactive intermediates that ultimately result in toxicity, carcinogenicity and other adverse effects. Many of the enzyme systems involved in biotransformation are also engaged in critical physiological functions such as steroid hormone biosynthesis and inactivation or fatty acid metabolism, making interactions between foreign chemicals and physiological processes possible.

The reactions catalyzed by xenobiotic-biotransforming enzymes are generally divided into two groups, called phase I and phase II. Phase I reactions involve hydrolysis, reduction, and oxidation. These reactions expose or introduce a functional group (*e.g.*, –OH, $–NH_2$, –SH or –COOH), and usually result in only a small increase in hydrophilicity. Phase II biotransformation reactions include glucuronidation, sulfation, acetylation, methylation, conjugation with glutathione (leading to mercapturic acid synthesis) and conjugation with amino acids. The cofactors for these reactions react with functional groups that are either present on the xenobiotic or are introduced/exposed during phase I biotransformation. Most phase II biotransformation reactions result in a large increase in xenobiotic hydrophilicity; hence they greatly promote the excretion of foreign compounds. Phase II biotransformation of xenobiotics may or may not be preceded by phase I biotransformation.

Xenobiotic-biotransforming enzymes are widely distributed throughout the body, and are present in several subcellular compartments. The liver is the richest source of enzymes catalyzing biotransformation reactions. These enzymes are also located in the skin, lung, gastrointestinal tract, and nasal mucosa (which can be rationalized on the basis that these are major routes of exposure to xenobiotics), as well as numerous other tissues, including kidney, heart, brain, etc. Intestinal microflora play an important role in the biotransformatiton of certain xenobiotics. The enzymes catalyzing xenobiotic biotransformation reactions are located primarily in the endoplasmic reticulum (microsomes) or the soluble fraction of the cytoplasm (cytosol), with lesser amounts in mitochondria, nuclei and lysosomes. Their presence in the endoplastic reticulum can be rationalized on the basis that those xenobiotics requiring biotransformation for urinary or

biliary excretion will likely be lipophilic and, hence, soluble in the lipid bilayer of the endoplasmic reticulum.

Phase I Enzymes

Cytochrome P450

Among the phase I biotransformation enzymes, the cytochrome P450 system ranks first in terms of catalytic versatility and the sheer number of xenobiotics it detoxifies or activates to reactive intermediates. All P450 enzymes are heme-containing proteins. The term "cytochrome P450" originates from the observation that the reduced state of the protein forms a complex with carbon monoxide that exhibits maximal absorbance at 450 nm [2]. The basic reaction catalyzed by cytochrome P450 is monooxygenation in which one atom of oxygen is incorporated into a substrate, designated RH, and the other is reduced to water with reducing equivalents derived from NADPH, as follows:

$$\text{Substrate (RH)} + O_2 + \text{NADPH} + H^+ \rightarrow \text{Product (ROH)} + H_2O + NADP^+$$

The principal catalytic cycle of cytochrome P450 is shown in Figure 1-1. The essential steps involve the following: (1) binding of the substrate, (2) reduction of the ferric (resting cytochrome P450) to the ferrous state, (3) binding of molecular oxygen to give a ferrous cytochrome P450-dioxygen complex, (4) transfer of the second electron to this complex to give a peroxoiron (III) complex, (5) protonation and (6) cleavage of the O-O bond with the concurrent incorporation of the distal oxygen atom into a molecule of water and the formation of a reactive iron-oxo species, (7) and (8) oxygen atom transfer from this oxo complex to the bound substrate, and (9) dissociation of the product. What is not clear is what steps are rate-limiting in various reactions.

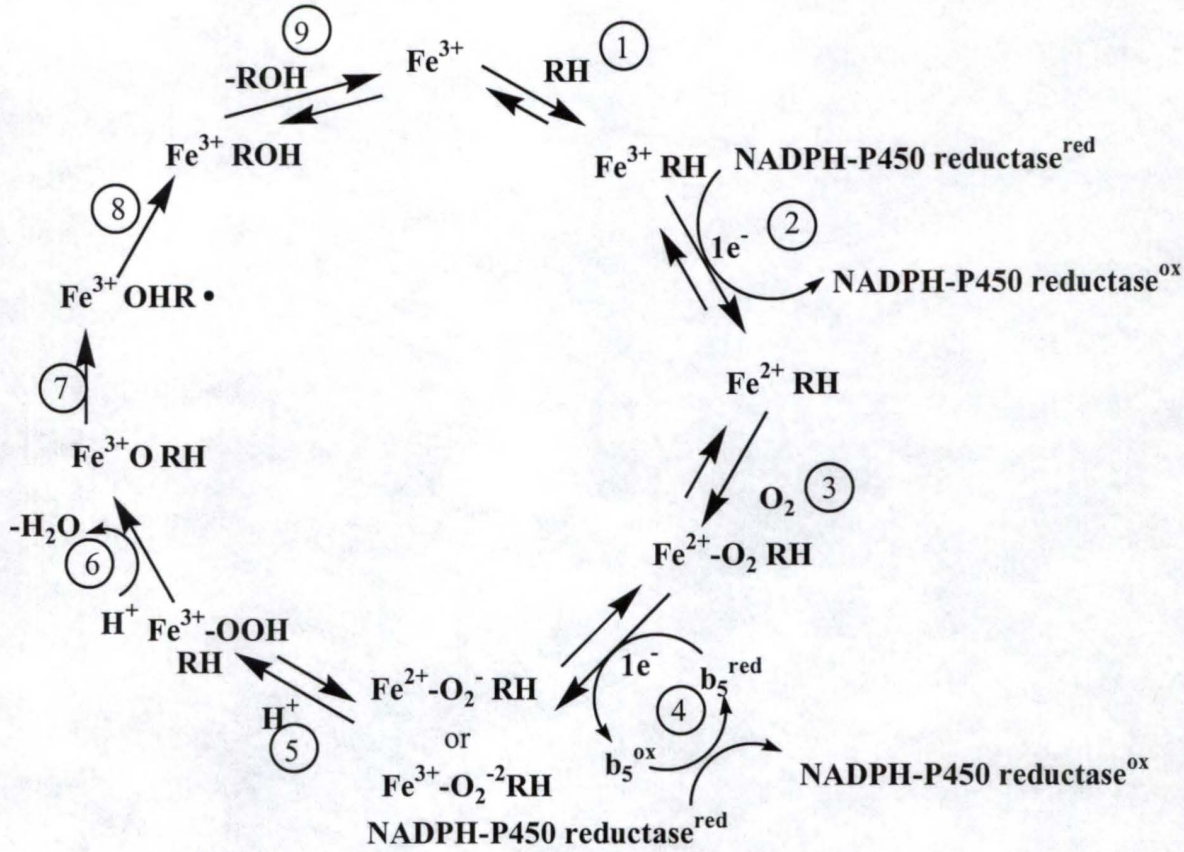


Figure 1-1. Catalytic cycle of cytochrome P450. RH: substrate; ROH: the corresponding hydroxylated metabolite. (Adapted from Guengerich, F.P., Cytochrome P450 3A4: Regulation and role in drug metabolism. *Annu. Rev. Pharmacol. Toxicol.* 39, 1-17(1999)

Cytochrome P450 monooxygenases function in the transformation of endogenous and exogenous compounds, and serve as catalysts that are significant in numerous and diverse biological pathways. The highest concentration of P450 enzymes involved in xenobiotic biotransformation is found in liver endoplasmic reticulum (microsomes), but P450 enzymes are present in virtually all tissues. The roles played by cytochrome P450 in endogenous pathways encompass the synthesis and degradation of steroids, prostaglandins, fatty acids and other biological molecules. In the transformation of foreign compounds, cytochrome P450 plays key roles in the toxicology and

pharmacology of pollutant chemicals, drugs and therapeutic agents, and in the activation and inactivation of many chemical carcinogens. The extent to which these various pathways or functions occur in different animal groups will depend to a large degree on the complement of different P450 proteins present, their catalytic function and their regulation. The P450 enzymes are encoded by a superfamily of genes. Currently, more than 800 P450s have been characterized, inclusive of the many different species of organisms that have been studied. Knowledge of these features of P450 in different species is necessary to define the general characteristics of P450s and their functions, and to indicate the evolution of these proteins. Such knowledge is also necessary to define the susceptibility of different individuals, populations or species to xenobiotic compounds, particularly those compounds whose toxicity may depend upon biotransformation. Currently, these processes are understood far better in rodent models than in wild or cultivated species that provide food and material resources. Research on mammalian cytochrome P450 continues to dominate the literature, but there is a growing recognition of its biological significance in other animals, and of our need to know the diversity and biochemistry of cytochrome P450 enzymes in these groups. The 20,000 species of fish extant represent about one-half of the known vertebrate species. The fish present extraordinary diversity, inhabiting all of the world's aquatic environments. They also present a significant source of protein for humans. The cytochrome P450 forms in fish thus acquire importance from evolutionary and toxicological standpoints.

Fish possess microsomal P450, similar to those in mammals [3]. Knowledge of the multiplicity, function and regulation of cytochrome P450 forms in fish continues to grow in importance. The first fish CYP to be cloned and sequenced was a CYP1A from

3-methylcholanthrene induced trout [4]. Recently, evidence has been presented to document that trout possess more than a single member of the 1A family [5]. A key feature of cytochrome P450 systems in both fish and mammals is their inducibility by chemical substrate for the enzymes/and by structurally related compounds. Fish respond to the same classes of xenobiotics as mammals with respect to induction of CYP1A, i.e., 3-MC, BNF, polycyclic aromatic hydrocarbons (PAHs), polyhalogenated biphenyls (PCBs and PBBs), and polychlorinated dioxins (PCDDs) and dibenzofurans (PCDFs) [6]. Fish CYP1A are induced by the above hydrocarbons given by injection, feeding or waterborne exposure. The induction can be detected by ethoxyresorufin O-deethylase (EROD) and aryl hydrocarbon (BaP) hydroxylase (AHH) activities. The molecular mechanism and cellular machinery for aromatic hydrocarbon (Ah) receptor-mediated CYP1A induction in fish appears to be similar to that of mammals, which is known to involve the following: (i) binding of the ligand to the Ah receptor, (ii) translocation of the bound receptor into the nucleus, and (iii) binding of the receptor complex to specific DNA sequences upstream of the CYP1A1 promoter (Figure 1-2). Prior to occupancy by a ligand, the inactive Ah receptor resides in the cytoplasm of target cells in a soluble complex with the heat shock protein Hsp90 (Figure 1-2). It appears that Hsp90 chaperones the AH receptor, maintains it in a ligand binding conformation, and represses its intrinsic DNA-binding activity [7]. Binding of a ligand triggers translocation of the ligand-receptor complex into the nucleus. The nuclear form of Ah receptor binds with high affinity to specific DNA enhancer sequences known as AH-responsive elements (AHRE) located in the 5'-flanking region of responsive genes. The nuclear DNA-binding complex is not a monomer but a heterodimer [8]. Several recent lines of evidence

confirm that the form of AH receptor that binds to AHREs consists of at least two proteins, the Ah receptor and ARNT (Ah-receptor-nuclear-translocator). The process by which ligand binding transforms the cytosolic Ah rceptor to its functional DNA-binding state is complicated and still poorly understood. Phosphorylation of both Ah receptor and ARNT by protein kinse C (PKC) appears to be important for generation of the functional DNA-binding complex [9]. Some inducers, for example, 3,3',4,4'-tetrachlorobiphenyl, can inhibit the catalytic activity of induced P450 [9]. In such cases analysis of catalytic activity alone might show no response, but strong induction can still be seen by immunochemical analysis of the CYP1A1 protein or hybridization studies with CYP1A1 mRNA.The fact that many of the inducers of fish P450 activities (PAHs, PCDDs, PCBs) are known aquatic pollutants has greatly stimulated research in the P450 system of fish. However, a number of studies have documented the "phenobarbital-type" inducers to be ineffective as P450 inducers in fish [11,12]. Intestines of fish are also capable of a variety of biotransformation reactions, some of which respond to dietary cytochrome P4501A (CYP1A) inducers [13]. Dietary induction studies with β-naphthoflavone, a model PAH-type inducer, in catfish indicate that under conditions of low inducer concentrations, select biotransformation activities in the intestine may equal or even exceed corresponding hepatic activities [14]. Such induction effects may potentially alter the degree and pathway of metabolism.

P450 induction has been suggested to indicate the exposure of organisms to contaminants in the environment [15]. Earlier studies on environmental induction of cytochrome P450 emphasized the analysis of catalytic activity. More recently, antibodies to the PAH-inducible cytochrome P450 from fish have been used to demonstrate

unambiguously that CYP1A forms are elevated in fish from contaminated regions [16]. Several studies with different fish species revealed correlations between the levels of induced cytochrome P450 and levels of PCBs either in the organisms or in their immediate environment.Thus, it seems that CYP1A activity or protein expression can be used to monitor the environmental pollution. Many chemical carcinogens are procarcinogens, requiring activation to a carcinogenic derivative by P450-dependent metabolic processes. Due to the predominant role that CYP1A plays in the metabolic bioactivation of environmental procarcinogens, it is not surprising that modulation of CYP1A levels and/or catalytic activity can significantly impact tumor development in fish models.

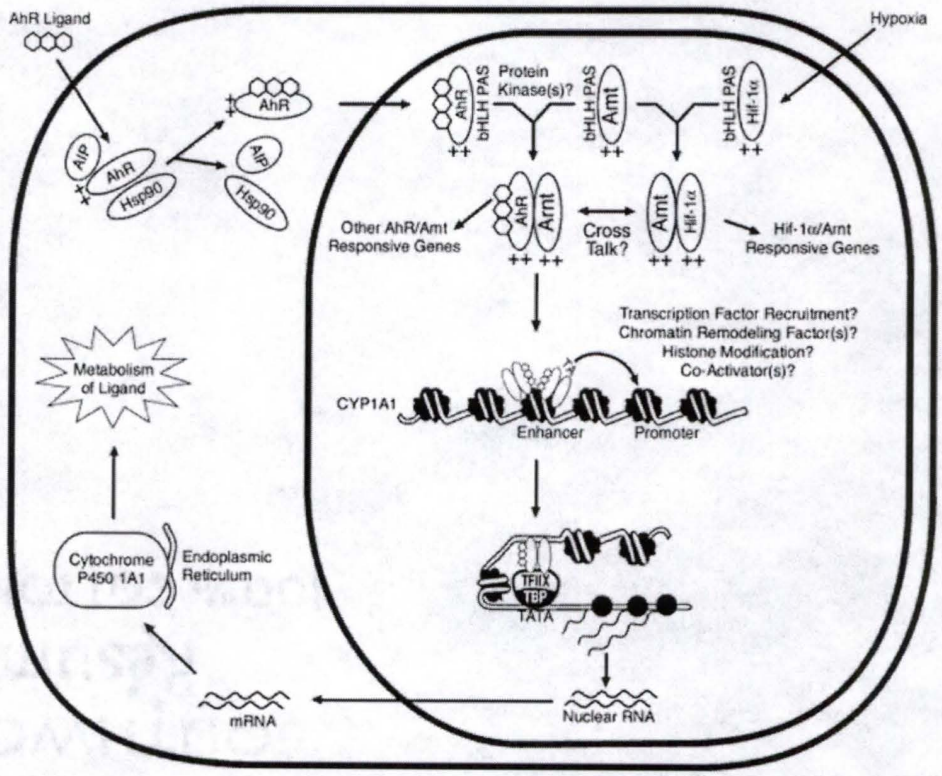


Figure 1-2. Mechanism of induction of CYP1A1 gene transcription. AhR, aromatic hydrocarbon receptor; AHRE, AH-responsive-element; ARNT, AH-receptor-nuclear translocator; Hsp90, heat shock protein 90.

Most studies of CYP in fish have focused on the PAH-inducible CYP1A subfamily. While several P450 enzymes other than CYP1A have recently been cloned and sequenced from fish, CYP2M1(previously known as LMC1) and CYP2K1 (previously named LMC2) were islated from rainbow trout liver [17,18]. CYP2M1 shows specific fatty acid hydroxylation at ω-6 position. CYP2K1 has been shown to activate aflatoxin in trout liver to its carcinogenic metabolites. The expression of CYP2K1 has been confirmed to have major sex-related differences.

The roles and regulation of CYP3A forms in fish have begun to attract growing attention. Members of the CYP3A subfamily are major constitutively expressed CYP forms in the liver and in the gastro-intestinal tract of mammals [19]. CYP3As appear to have an extraordinarily broad substrate specificity and in addition to steroids, also metabolize pro-carcinogens, therapeutic drugs and dietary chemicals [20]. Cytochrome P450 3A4 is known to catalyze the metabolism of both endogenous substrate (such as the 6β-hydroxylation of testosterone) and many important therapeutic agents, including the N-demethylation of erythromycin. Studies have indicated a significant role for human hepatic P450 3A4 in the 9,10-epoxidation of benzo(a)pyrene-7,8-dihydrodiol, forming the final carcinogen BPDE [21] (Figure 1-3). Most studies of structure, function and regulation of CYP3As have been in mammalian systems, whereas relatively little is known about CYP3A in other vertebrate groups. As a matter of fact, fish are continuously exposed to CYP3A inducers/substrates in their natural habitat as a result of food preferences and human activities. It has been shown that rainbow trout LMC5, rainbow trout P450con, scupP450A, codP450b and mammalian CYP3A (human 3A4, rat

3A1) are all immunochemically related [22]. Buhler's group reported the

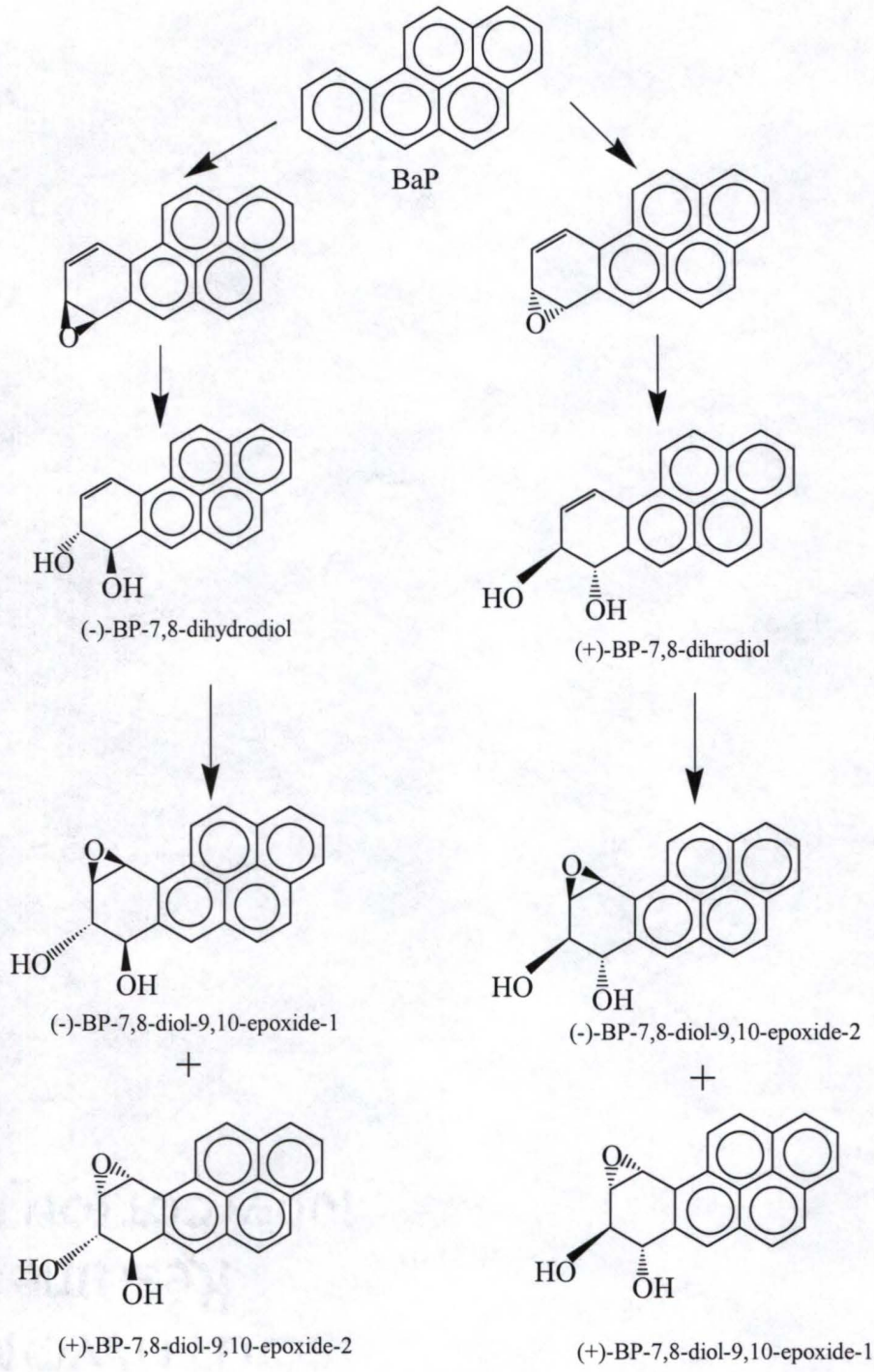


Figure 1-3. Bioactivation of benzo(a)pyrene.

first CYP3A family member, CYP3A27, in an aquatic species (rainbow trout) which encodes an LMC5-like protein [23]. The major extrahepatic expression site for CYP3A27

was upper small intestine, which also expressed smaller amounts of CYP2K1. Actually, upper small intestine has the highest expression of CYP3A27 in female trout, followed by the ovary and the liver. The high percentage of identities in alignment of CYP3A27 with other mammalian CYP3A forms suggest that there was significant sequence retention during evolutionary divergence between terrestrial and aquatic vertebrates. The fact that CYP3A proteins are present at significant levels in untreated fish implies that they are constitutively expressed and they may have important endogenous functions in fish. The substrate selectivity and the role in xenobiotic toxicity of CYP3A27 are not yet known. CYP3A30, another CYP3A subfamily protein found in aquatic species, was isolated and sequenced from killifish [24]. The sequence of CYP3A30 is 77% identical to that of CYP3A27.

CYP 3A Inhibition

The inhibition of enzyme activity is one of the major regulatory devices of living cells, and one of the most important diagnostic procedures of enzymology. Three types of enzyme kinetic inhibition patterns are commonly observed: competitive, noncompetitive, and uncompetitive. The use of chemical inhibitors is one of the common strategies employed in determining whether cytochrome P450s are involved in the hepatic and extrahepatic metabolism of drugs, xenobiotics, and endogenous compounds. Selective chemical inhibitors play an important role especially in elucidating the contribution of a particular cytochrome P450 enzyme in catalyzing the metabolism of xenobiotics [25].

CYP3A enzymes are inhibited by a variety of compounds, including troleandomycin (TAO), clarithromycin, erythromycin, gestodene, ketoconazole, naringenin, and 6,7-dihydroxy-bergamottin [26]. The only common features are their

lipophilicity and relatively large molecular size. Several mechanisms of inhibition are possible, with some compounds exhibiting more than one-type, *e.g.*, erythromycin [27].

(1) Rapid reversible inhibition: Direct, rapid reversible binding of an inhibitor or its metabolite to CYP3A. Reversible inhibition has been found to result in either competitive or noncompetitive inhibition, the extent of which is determined by the relative binding constants of substrate and inhibitor for the enzyme and by the inhibitor's concentration.

(2) Formation of MI-complexes (quasi-irreversible inhibition): N-Alkyl-substituted compounds--a common feature of many CYP3A drugs--often show reversible inhibition, and an even greater effect is observed after preincubation with a metabolically competent in vitro preparation. This is due to oxidation of the inhibitor to form a nitrosoalkane species that forms a slowly reversible complex (MI-complex) with reduced heme in the CYP3A molecule. Such compounds include macrolides like TAO, oleandomycin, erythromycin, clarithromycin and roxthromycin [28]. Formation of an MI-complex may, however, be difficult to demonstrate in vitro because of its dependency on the rapid and relatively efficient generation of the causative metabolite.

(3) Irreversible, mechanism-based (suicide) inhibition: The ingestion of 6,7-dihydroxy-bergamottin, a furanocoumarin, can markedly inhibit the first-pass metabolism of CYP3A substrates. This effect was recently found to be associated with autocatalytic destruction of intestinal CYP3A both in vitro and in vivo [29]. The mechanism of suicide inhibition presumably involves CYP3A-mediated formation of a reactive

metabolite(s) that covalently binds to the enzyme in a fashion leading to its inactivation.

In mechanistic terms, reversible interactions arise as a result of competition at the CYP active site and probably involve only the first step of the CYP catalytic cycle. On the other hand, chemicals that act during and subsequent to the oxygen transfer step are generally irreversible or quasi-irreversible inhibitors. Quasi-irreversible and irreversible inhibitors require at least one cycle of the CYP catalytic process, and are thus characterized by both NADPH- and time-dependent inhibition. Experimentally, mechanisms of inhibition of inhibitors could be assessed initially by comparing their inhibitory effects obtained in the presence and absence of NADPH during a preincubation period.

Inhibitors for CYP3A have been found that are drugs, antibiotics, preservatives, poisons and toxins. Several human hepatic CYP3A substrates, erythromycin, testosterone, terfenadine, midazolam, and nifedipine mutually inhibited the metabolism of each other with complex mechanisms [30,31]. Troleandomycin (TAO) has been shown to inhibit CYP3A enzymes through both competitive inhibition and formation of MI-complex. It was found to be as effective inhibitor of CYP3A enzymes in microsomal fractions from goat and cattle and in a cell-line expressing bovine CYP3A [32]. Both human CYP1A2 and CYP3A4 play important roles in bioactivation of aflatoxin B1 (AFB1); TAO showed potent and specific inhibition of AFB1 epoxidation in CYP3A but not CYP1A2 microsomes [33]. In pharmacokinetic tests of drug bioavailability, TAO and ketoconazole have widely been used as selective inhibitors of CYP3A [34,35]. Calcium channel blockers, nicardipine, verapamil, and diltiazem were shown to inhibit human

hepatic CYP3A via, at least in part, quasi-irreversible inhibition and such findings provide a rational basis for the pharmacokinetically significant interactions reported when these calcium channel blockers were co-administered with agents that are cleared by CYP3A-mediated pathways [36].

Chemical inhibitors may also be useful in identifying the individual P450 enzymes responsible for the metabolism of xenobiotics and endogenous lipophilic compounds in non-mammalian species such as fish. Several inhibitors of mammalian P450s have been employed to inhibit fish P450s [37]. Ellipcine and α-naphthoflavone were found to inhibit benzo(a)pyrene hydroxylase activity of liver microsomes from flounder (*Platichthys flesus*) [38,39]. Aminoanthracene has been proposed as a mechanism-based inactivator of CYP1A in channel catfish [40], but its selectivity as a P450 inhibitor is not known. In a study of Miranda *et al* to evaluate chemical inhibitors of trout cytochrome P450s three monooxygenase activities, lauric acid (ω-1)-hydroxylase (LA-OH), 7,12-dimethylbenz(a)anthracene hydroxylase (DMBA-OH), and progesterone 6β-hydroxylase (PROG-OH) activities were used as functional markers for trout hepatic CYP2K1, CYP1A1, and CYP3A27, respectively [41]. At 100 μM concentration, the reversible inhibitors ketoconazole, miconazole and clotrimazole were most potent in inhibiting LA-OH activity. The global inhibitors metyrapone, chloramphenicol, and allylisopropylacetamidem had very little inhibitory effect on trout LA-OH and DMBA-OH activities. Troleandomycin, a CYP3A inhibitor in mammals, did not affect PROG-OH activity catalyzed by trout CYP3A27. None of the three enzyme activities was selectively inhibited by any of the mammalian chemical inhibitors used at a concentration of 100 μM. These results suggest that inhibition data from mammalian studies could not

be directly extrapolated to fish species and that care must be observed when mammalian P450 inhibitors are used to determine the participation of P450s in the metabolism and toxicity of xenobiotics in nonmammalian species.

CYP 3A Induction

CYP3A inducers include a broad range of steroids and antibiotics. Early studies of rat liver CYP3A enzyme induction made the important, but seemingly paradoxical, observation that both glucocorticoids (such as dexamethasone, DEX) and antiglucocorticoids (such as pregnenolone 16α-carbonitrile, PCN) induce these enzymes at the transcriptional level [42]. Both this finding and the requirement for a relatively high glucocorticoid concentration for CYP3A induction were recognized as inconsistent with the classical glucocorticoid receptor playing a major role in the CYP3A induction response. It was recently found that CYP3A genes are transcriptionally activated by foreign chemicals through a PXR-dependent mechanism [43]. PXR (pregnane X receptor), an orphan nuclear receptor, was believed to mediate the CYP3A induction. PXR, together with other four P450-regulating nuclear receptors, CAR, PPAR, LXR and FXR, share a common heterodimerization partner, retinoid X-receptor (RXR). When the ligand binds to PXR, the nuclear receptor heterodimerizes with RXR and efficiently transactivates the response elements present in CYP3A genes (Figure 1-4). Important species differences in the induction response have been described [44]. Most notably, while rat, rabbit, and human CYP3A genes are all inducible by dexamethasone, the anti-glucocorticoid PCN is an efficacious CYP3A inducer in the rat but not in humans or rabbits. By contrast, the antibiotic rifampicin is an excellent CYP3A inducer in humans and rabbits, but not in the rat. Transfection studies carried out in rat and rabbit

hepatocytes and utilizing CYP3A constructs containing DEX-responsive regulatory elements derived from rat CYP3A23, rabbit CYP3A6 and human CYP3A4 genes demonstrated that the species-specific induction responses are due to the different response element on CYP3A genes. In the case of rat CYP3A23, the dexamethasone-responsive sequence contains a DR3 motif (direct repeat, separated by 3 bp; AGTTCA-N_3 –AGTTCA) that is also present in rat CYP3A2, whereas in human CYP3A4 gene, the response element contains an unusual ER6 motif (everted repeat, separated by 6 bp; TGAACT-N_6-AGGTCT) that is conserved in human CYP3A5 and rabbit CYP3A6 [45]. Pregnane-X-receptors have recently been cloned from human, mouse, rat, rabbit and chicken. However, mouse PXR and human PXR share only ~75% amino acid sequence identity in their COOH-terminal ligand-binding domain region (vs 96% identity between their DNA-binding domains), and this apparently results in sigificant differences in ligand-binding specificities: human PXR but not mouse PXR is highly activated by compounds that preferentially induced human CYP3A genes, such as rifampicin, while mouse PXR but not human PXR exhibits the strong response to PCN that characterizes mouse CYP3A gene induction. Thus, the species-dependent ligand specificity for CYP3A induction seen *in vivo* can be explained by the corresponding ligand specificity of each species' PXR receptor. Other CYP3A inducers and PXR activators include anti-hormones belonging to several steroid classes, the organochlorine pesticide, chlordane, and various nonplanar chlorinated biphenyls [46]. Both the facts that PXR is responsive to steroids belonging to several distinct classes (prenanes, estrogens, and corticoids) and that many CYP3A enzymes catalyze steroid 6β-hydroxylation reactions suggested the

mechanism of cross-talk between PXR-dependent CYP3A induction pathways and intracellular signaling pathways involving endogenous hormones (Figure 1-5).

Pre-exposure of fish to mammalian CYP3A inducers, however, has yielded rather inconsistent results. In juvenile rainbow trout, levels of CYP3A in hepatic microsomes were slightly elevated by steroids, *i.e.*, cortisol and PCN [47]. Administration of 25 or 100 mg/kg i.p. doses of PCN to sexually immature rainbow trout caused an increase of hepatic BND (benzphetamine N-demethylase) and ECOD (7-ethoxycoumarin O-deethylase) activities but had no effect on the total P450 content or on EROD (7-ethoxyresorufin O-deethylase) activity [48]. By contrast, treatment of rainbow trout with a single *i.p.* dose of PCN at 25 mg/kg did not alter the hepatic microsomal activities of different fluorescent substrates or hepatic P450 levels [0]. Moreover, DEX or PCN treatment failed to affect hepatic CYP3A-like protein levels in rainbow trout [50,51]. These discrepencies in responsiveness to various types of mammalian CYP3A inducers reflect important differences in CYP3A regulation in different taxa.

The content of CYP3A in some teleost fish appears to be influenced by the composition of the diet, suggesting that CYP3A may be involved in the metabolism of dietary natural products as well as anthropogenic xenobiotics [52].

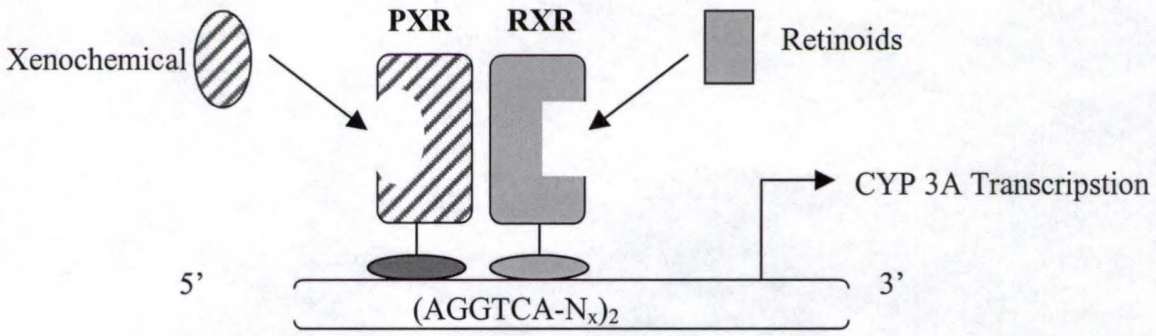


Figure 1-4. Role of PXR in CYP3A gene induction. Shown is the structure of a PXR-RXR heterodimer bound to two copies of a hexameric DNA response element based on the sequence of AGGTCA spaced by X nucleotides. The hexameric repeat can be arranged as a DR or ER motif. (Adapted from Waxman, D.J. P450 gene induction by structurally diverse xenobioticals: Central role of nuclear recptors CAR, PXR, AND PPAR. *Arch. Biochem. Biophys.* 369(1):11-23, 1999)

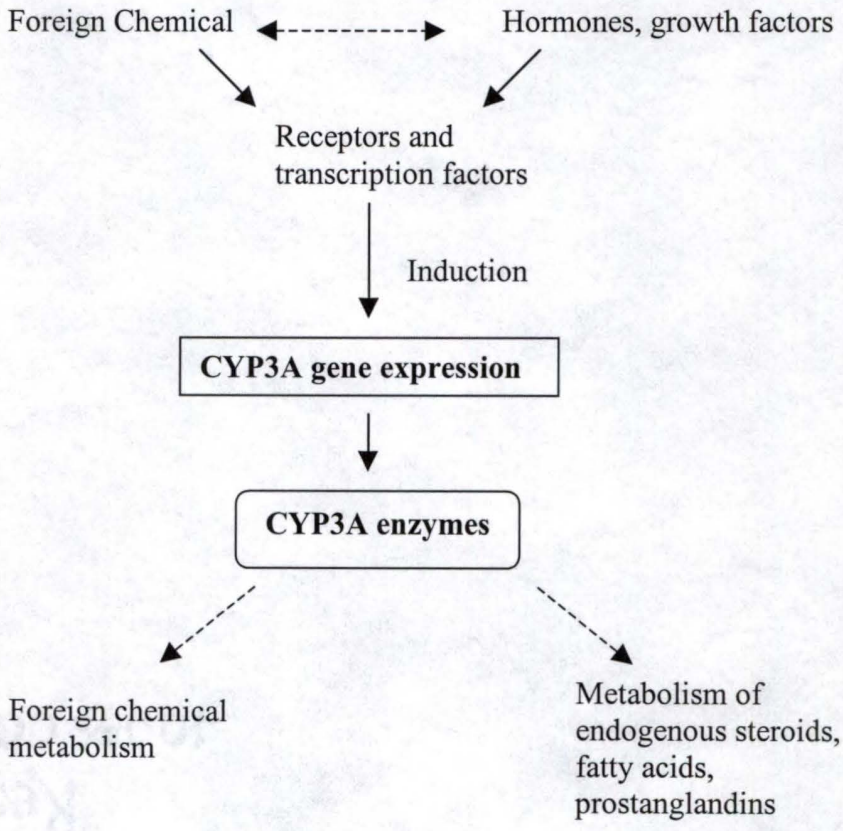


Figure 1-5. CYP3A gene induction: Cross-talk between foreign chemical and endogenous regulator pathways. (Adapted from Waxman, D.J. P450 gene induction by structurally diverse xenobioticals: Central role of nuclear recptors CAR, PXR, AND PPAR. *Arch. Biochem. Biophys.* 369(1):11-23, 1999)

CYP3A Stimulation

A unique characteristic of the CYP3A subfamily is their ability to be activated by certain compounds. Flavonoids, *e.g.*, 7,8-benzoflavone (α-naphthoflavone, α-NF), have been shown to stimulate some reactions but not others. In systems containing purified recombinant bacterial P450 3A4, positive cooperativity was seen in oxidations of several substrates, including testosterone, 17β-estradiol, amitriptyline, and most notably aflatoxin B-1 [53]. It was reported that CYP3A4-catalyzed phenanthrene metabolism was activated by 7,8-benzoflavone and that 7,8-benzoflavone served as a substrate for CYP3A4. Kinetic analyses of these two substrates showed that 7,8-benzoflavone increased the V_{max} of phenanthrene metabolism without changing the K_M and that phenanthrene decreased the V_{max} of 7,8-benzoflavone metabolism without increasing the K_M. These results suggest that both substrates (or substrate and activator) are simultaneously present in the active site. Both compounds must have access to the active oxygen, since neither phenanthrene nor 7,8-benzoflavone can competitively inhibit the other substrate. These data provide the first evidence that two different molecules can be simultaneously bound to the same P450 active site [54]. Quinidine and hydroquinidine decreased K_M and V_{max} of meloxicam hydroxylation, which was consistent with a mixed type activation. Meloxicam, in turn, decreased both K_M and V_{max} of quinidine metabolism by CYP3A4, indicating an uncompetitive inhibition mechanism [55]. These results also support the assumption that CYP3A4 possess at least two different substrate-binding sites.

The mechanism of cytochrome P450 activation has not been explored to the same depth as induction or inhibition phenomena. Enhancement of aniline para-hydroxylation

by acetone was the first reported cytochrome P450 activation interaction [56]. Based on studies with liver microsomal fractions of the dog, rabbit, mouse and rat, it was proposed that acetone affected either the formation of the peroxy anion complex of cytochrome P450 or steps beyond this (such as the formation of the oxene complex) because cumene hydroperoxide-dependent hydroxylation of aniline was stimulated by acetone [57]. Huang *et al* [58] demonstrated that the stimulatory effect of 7,8-benzoflavone on benzo(a)pyrene metabolism in rabbit liver microsomes was mediated by a different mechanism than that observed with acetone. The effect of 7,8-benzoflavone on benzo(a)pyrene metabolism was thought to be a result of enhanced interactions between cytochrome P450 and cytochrome P450 reductase. A third mechanism of activation was proposed by Johnson *et al.* [59], who reported that the stimulatory effect of α-naphthoflavone on rabbit CYP3A6 was a consequence of an allosteric effect, as shown by an increase in the P450 binding affinity for the substrate. Shou *et al.* [54] have shown that there was mutual activation between phenanthrene and 7,8-benzoflavone and suggested that the two molecules simultaneously occupy the active site, thereby altering active site geometry and oxidation efficiency. In summary, it appears that cytochrome P450 activation may occur by several mechanisms.

Epoxide Hydrolase (EH)

Carcinogenic polycyclic hydrocarbons such as benzo(a)pyrene are oxygenated in cytochrome P450 catalyzed reactions to form epoxides. Due to their electronic polarization and ring tension, epoxides are often chemically reactive. Consequently, such metabolites can bind covalently to nucleophilic groups in many tissue constituents,

including macromolecules such as RNA, DNA and proteins. Epoxide hydrolase (EC3.3.2.3) catalyzes the trans-addition of water across the oxirane ring of the epoxides to chemically less reactive transdihydrodiols [60]. The reaction is stereoselective and regioselective. Several distinct microsomal and cytosolic isoenzymes exist. Usually, microsomal epoxide hydrolase catalyze the hydration of cis-epoxide while the cytosolic EH catalyzes the hydration of trans-epoxide. Although the metabolite dihydrodiol is less toxic, it might be further metabolized by P450 to the ultimate dihydrodiol-epoxide carcinogens. A classic example is the metabolic activation of benzo(a)pyrene to 7,8-dihydrodiol-9, 10-epoxide (BPDE), which proceeds via 7,8-epoxide followed by epoxide hydrolase and another oxidation step [61]. In reactions with benzo(a)pyrene 4,5-oxide and styrene 7,8-oxide as substrates, the general trend of microsomal epoxide hydrolase activity observed was fish <amphibia <birds <rodent <larger mammals [62]. Epoxide hydrolase activity was found in several marine species, including spiny lobster, shrimp, fiddler crabs and stingray [63]. It was shown that EH activity with styrene oxide as substrate was similar in intestinal and hepatic microsomes from catfish [14].

Phase II Enzymes

Glutathione S-Transferase (GST)

The glutathione S-transferases (GST) are a ubiquitous family of isozymes whose primary functions are involved in the biotransformation and disposition of many toxic substances. The chemical function of the enzyme is to catalyze the nucleophilic addition of the thiol of glutathione (γ-L-Glu-L-CysGly) to electrophilic acceptors, the first step in

mercapturic acid biosynthesis (Figure 1-6). In addition, it is proposed that the proteins also serve as depots for the storage of toxic substances, as high capacity steroid-binding

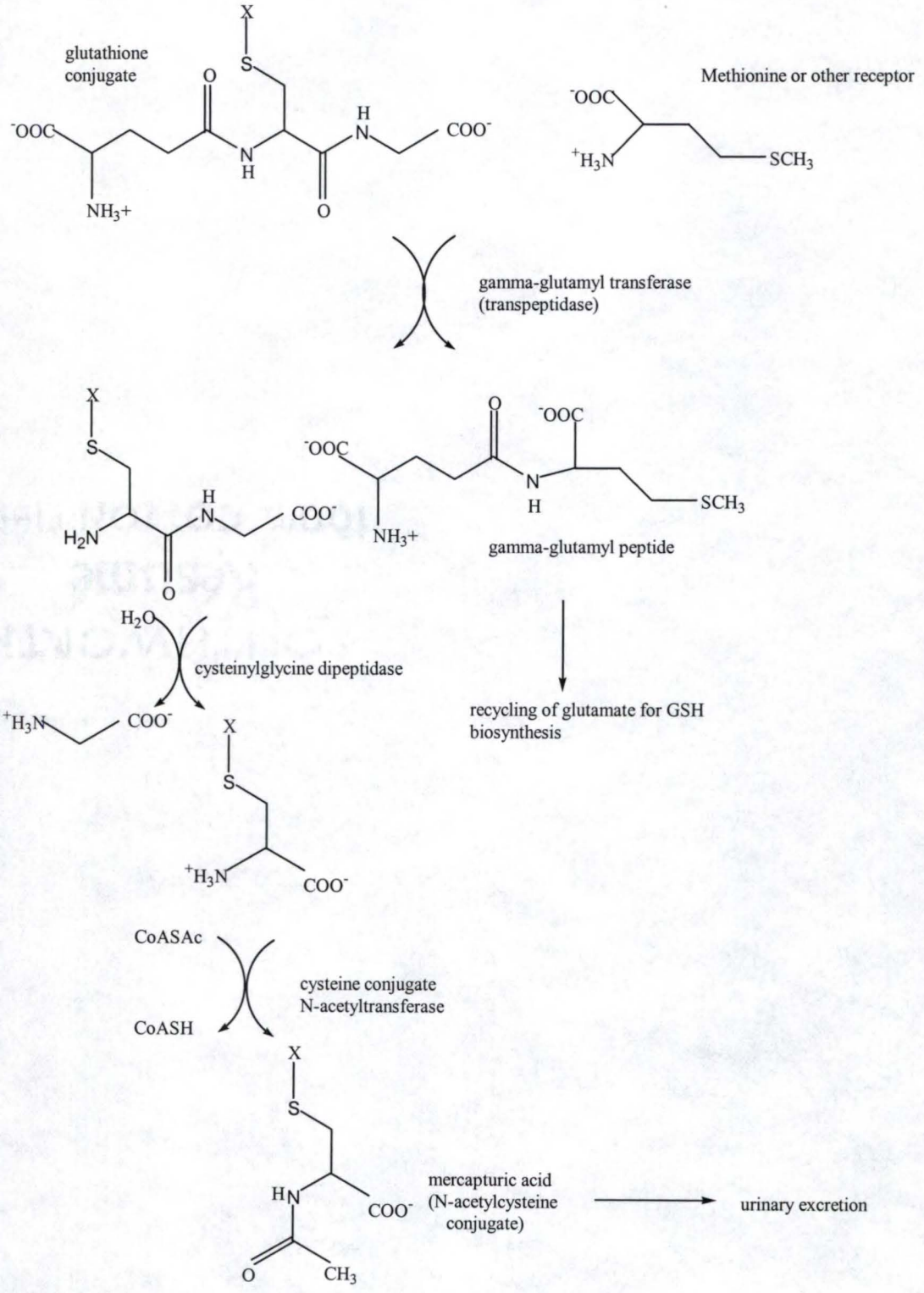


Figure 1-6. Mercapturic acid biosynthesis.

proteins, as heme-binding and transport proteins. Both the abundance of the enzymes, comprising 3 to 10% of the soluble protein in liver, and the high concentrations (5 to 10 mM) of glutathione attest to the importance of glutathione S-transferase in the maintenance of health [64].

All eukaryotic species possess multiple cytosolic and membrane-bound GST isozymes. The cytosolic enzymes are much more important and encoded by at least six distantly related gene families (designated class alpha, mu, pi, sigma, theta and zeta GST). The quaternary structure of cytosolic GSTs shows that the enzymes occur as binary combinations of subunits, including both homodimers and heterodimers. The membrane-bound GST (microsomal GST) is a trimeric protein, structurally unrelated to the cytosolic enzymes [65].

The glutathione S-transferases (GST) are an important phase II enzyme system in the detoxification of electrophilic alkylating agents. As a family of isozymes, the enzyme system is capable of handling a variety of electrophilic compounds, both from exogenous and from endogenous origins. Conjugation with GSH can generally be regarded as a detoxification pathway, although several compounds are known to be activated through this reaction [66]. The glutathione S-transferases catalyze the nucleophilic addition of GSH to electrophiles including aryl and alkyl halides, sulfate esters, phosphate and phosphorothioate triesters, nitrate esters, oxiranes, olefins, lactones, organic peroxides, disulfides and thiocyanates and quinones. The substrate selectivities exhibited by various isozymes overlap considerably but are nonetheless distinct. Most of the above reactions can be classed as simple nucleophilic displacements or Michael additions to unsaturated systems.

GSTs can be induced by a variety of chemical compounds, including conventional inducers of drug-metabolizing enzymes, such as phenobarbital, 3-methylcholanthrene, and TCDD. GST-pi has been shown to be a reliable marker for rat hepatocarcinogenesis [67].

GST was found in both marine and freshwater fish species. It has been indicated that GST usually shows higher activity than epoxide hydrolase in both hepatic and extrahepatic tissue in marine fish [63]. Rainbow trout has GST activity with CDNB(1-chloro-2,4-dinitrobenzene) in liver and intestine, while only intestine has substantial γ-glutamyl transpeptidase activity. A cluster of three GST genes, GSTA, GSTA1, and GSTA2, was isolated from marine flatfish, plaice [68]. GST-A expresses in plaice intestine as well as in liver. It was also indicated that expression of GST-A mRNA was increased in plaice intestine by pretreatment with β-naphthoflavone (BNF).

A pi-class GST was isolated from catfish intestinal mucosa with N-terminal sequence homology >63% to mammalian pi-form GST isozymes [69]. GST including this pi-class GST play an important role in the intestinal biotransformation of the epoxide and diol-epoxide metabolites of benzo(a)pyrene formed in catfish intestine.

Fish GST was also shown to be inducible by PAHs. Cytosolic GST activity towards CDNB was elevated approximately three to four-fold in intestine and liver of mummichog, collected from a creosote-contaminated site. The intestinal GST activity was even higher than liver GST, supporting the importance of intestinal metabolism of foreign compounds [70]. GST activity was slightly induced in intestinal, but not hepatic cytosol of catfish treated with BNF at 10 mg/kg diet level relative to chow controls. Yet

this induction showed no further increase with higher dose of BNF at 100 mg/kg diet [14].

Sulfotransferase (ST)

The cytosolic sulfotransferases catalyze the transfer of the sulfuryl group from 3'-phosphoadenosine 5'-phosphosulfate to nucleophiles such as alcohols, phenols, and amines. The M-form of the enzyme is thermolabile (TL form), catalyzing the sulphate conjugation of micromolar concentrations of dopamine and other phenolic monoamines. The other form, P-form, is more thermostable (TS form) and catalyzes the sulphate conjugation of micromolar concentrations of simple phenols such as p-nitrophenol. Both forms of the enzymes are particularly active in the intestinal wall but are also widespread in the body, including the platelet.

Sulfation is one of the major phase II conjugation reactions for drugs and environmental chemicals as well as for endogenous compounds such as steroids and monoamine neurotransmitters [71]. The major physiologic consequences of the conjugation of a drug or xenobiotic with a charged sulfate moiety are increased aqueous solubility and excretion. Although the major role of sulfation is detoxification, in some instances sulfate conjugation results in the bioactivation of a compound to a reactive electrophilic species since the sulfate is such a good leaving group. The electrophile is capable of covalently binding DNA and causing a mutagenic, teratogenic, or carcinogenic response. Metabolic activation of 7,12-dimethylbenzanthacene has been demonstrated to occur by oxidation to the 7-hydroxymethyl-12-methylbenz(a)anthracene followed by sulfation and alkylation of DNA following loss of sulfate anion [72,73].

Human intestinal mucosa contains forms of phenol sulfotransferase, similar to those in other human tissues such as brain, liver, and platelet [74]. In rat hepatocyte culture, sulfotransferase expression was negatively regulated by xenobiotics such as PB-like CYP2B/3A inducers or AhR agonist CYP1A inducers [75]. In guppy and medaka after water-borne exposure to the procarcinogen 2-acetylaminofluorene(AAF), the major pathway for bioactivation was shown to be N-hydroxylation followed by sulfation. AAF-treated guppies had higher ST activity than controls, but UGT activity was reduced or unaffected by AAF exposure [76].

Sulfate conjugate was found as a metabolite of benzo(a)pyrene in an isolated perfused in situ catfish intestinal preparation [77]. The biotransformation is via oxidation by CYP1A and rearrangement of the epoxide to the phenolic metabolite. In another study with catfish, it was shown that ST activities with BaP phenols was high in intestine, suggesting that low concentrations of hydroxylated polycyclic aromatic hydrocarbon would be readily conjugated in catfish intestine.

UDP-Glucuronosyltransferase (UGT)

The UDP-glucuronosyltransferases are a group of membrane-bound proteins responsible for the transfer of the glucuronyl group from uridine 5'-diphosphoglucuronate to a large number of different nucleophilic acceptors. The enzymes are located primarily in the endoplasmic reticulum of eukaryotic cells, catalyzing the glucuronidation of a tremendous number of lipophilic molecules having nucleophilic functional groups of oxygen, nitrogen, sulfur, and carbon. Substrates for glucuronidation are typically small hydrophobic molecules that are termed aglycones (lacking carbohydrate). A wide variety of endogenous and exogenous compounds are

glucuronidated, including bilirubin, steroid hormones, bile acids, biogenic amines, fat-soluble vitamins, environmental toxins and therapeutic drugs. Phenol, dihydrodiol and quinol metabolites of polycyclic aromatic hydrocarbons are substrate for the microsomal and purified UGTs [78]. Glucuronidation is generally considered to be a detoxifying mechanism that alters the physiological and pharmacological activities of chemicals within the body. In some cases, however, covalent addition of glucuronic acid may increase the biological activity of an aglycone [79]. The UGT proteins can be conceptually divided into two domains with the amino-terminal half of the protein demonstrating greater sequence divergence between isoforms. This region apparently determines aglycone specificity. The carboxyl-terminal half, which is more conserved in sequence between different isoforms, is believed to contain a binding site for the cosubstrate UDP glucuronic acid (UDPGA).

Multiple isoforms of UGT have been found in aquatic species [80]. In a UGT study in plaice, phenol UGT activity was found to be ubiquitous in hepatic, renal, intestinal and branchial tissues, and was induced by 3-MC and Aroclor. The glucuronidation of testosterone was restricted to liver and intestine, while conjugation of bilirubin was expressed solely in hepatic tissue [81]. In the southern flounder, BaP-7,8-diol given by gavage was glucuronidated and then transported as such to liver where that was efficiently excreted into the bile. In vitro studies showed that flounder liver and intestine had similar UGT activities [82]. In channel catfish, glucuronide of BaP-9-OH was readily transported intact from the intestinal lumen to the systemic circulation. 3-OH-BaP was extensively biotransformed to BaP-3-glucuronide in intestinal mucosa [83]. UGT activities with BaP phenols were high in the catfish intestine, suggesting that low

concentrations of hydroxylated polycyclic aromatic hydrocarbon would be readily conjugated in catfish intestine. UGT activities with 3-,7- and 9-hydroxy-BaP in catfish intestine were not induced by treatment with BNF and in fish receiving the higher dose activity with 7- and 9-hydroxy-BaP was lower than in fish fed other diets. In vitro studies showed that BNF could inhibit UGT activity, suggesting the residues of BNF retained in intestinal cells after BNF treatment in diet could directly inhibit UGT activity [14]. Treatment with 3-methylcholanthrene, 10 mg/kg diet, did induce UGT in catfish intestinal microsomes [James unpublished data].

3α-Hydroxy-Steroid Dehydrogenase (3α-Oxido-Reductase)

Testosterone homeostasis is crucial for normal growth, reproduction, and development in vertebrates [84]. In teleost fish, testosterone serves as a precursor to 11-ketotestosterone and 17β-estradiol. These hormones play an important role in sexual maturation in male and female fish, respectively. More than one organ contributes to the metabolic inactivation and elimination of testosterone [85,86]. Enzymes that contribute to the metabolic elimination of testosterone include cytochromes P450, oxido-reductases, and transferases [87].

Hydroxysteroid dehydrogenases (HSDs) regulate the occupancy of steroid hormone receptors by converting active steroid hormones into their cognate inactive metabolites. HSDs belong to either the short-chain dehydrogenase/reductases (SRSs) or the aldo-keto reductases (AKRs). 3α-hydroxysteroid dehydrogenase (3α-HSD) was found in both microsomal and cytosolic liver fractions. In rodents, 3α-Hydroxysteroid dehydrogenase showed higher activities in cytosolic fraction than in microsomes using

dihydrosteroids as substrates [88,89]. By comparison, rat hepatic microsomal 3α-hydroxysteroid dehydrogenase activity was 12-fold higher than cytosolic 3α-hydroxysteroid dehydrogenase in human [90]. It was suggested that the major pathway of DHT (dihydro-testosterone) metabolism in human liver involves 3α-hydroxysteroid dehydrogenase reduction in the liver, followed by subsequent glucuronidation and clearance via the kidney [91]. Human hepatic 3α-HSD also plays a critical step in the synthesis of bile acids and is responsible for the production of 5β-cholestane-3α,7α-diol, which is a committed precursor of bile acids. In steroid target tissues, the production of 5α/5β-tetrahydrosteroids catalysed by 3α-HSD is not without consequence. In the human prostate, 3α-HSD can regulate the occupancy of the androgen receptor. It catalyses the reduction of 5α-dihydrotestosterone, a potent androgen to 5α-androstane-3α,17β-diol, a weak androgen and is positioned to regulate normal and abnormal androgen-dependent growth of this gland [92]. By contrast in the central nervous system, 3α-HSD can regulate the occupancy of the γ-aminobutyric acid $(GABA)_A$ receptor by converting 5α-dihydroprogesterone into 3α-hydroxy-5α-pregnan-20-one (allopregnanolone), a potent allosteric effector of the $GABA_A$ receptor [93,94]. In the presence of GABA, allopregnanolone will potentiate $GABA_A$-mediated chloride conductance. As a result 3α-HSD is responsible for the production of anxiolytic steroids, and decreased activity in this pathway has been implicated in the symptoms of pre-menstrual syndrome [95]. Thus 3α-HSD isoforms regulate the occupancy of both a nuclear receptor (androgen receptor) and a membrane-bound chloride-ion gated channel ($GABA_A$ receptor) and may have profound effects on receptor function [96].

Testosterone metabolites produced by juvenile and adult fathead minnows included 4-androstene-3,17-dione (androstenedione), 17β-hydroxy-5α-androstan-3-one (5α-dihydrotestosterone), 5α-androstane-3α,17β-diol (3α-androstanediol), 5α-androstane-3β,17β-diol (3β-androstanediol), 17β-hydroxy-4-androstene-3,11-dione (11-ketotestosterone), 16β-hydroxy-4-androsten-3-one (16β-hydroxytestosterone), and 6β-hydroxy-4-androsten-3-one (6β-hydroxytestosterone) [97]. Testosterone and its metabolites were eliminated from minnows in both free and conjugated form. Adult females eliminated androstanediols at a significantly greater rate than did males, suggesting higher 3-oxidoreductase activities in female fish than male.

Prehepatic Metabolism and Bioavailability

The primary function of the intestine is to absorb nutrients and water. This is achieved by mixing food with digestive enzymes to increase the contact of foodstuffs with the absorptive cells of the mucosa. In addition to this fundamental role, another function of the intestine arises from the fact that it also provides a major route for exposure to xenobiotics via food and liquid, and secondarily by swallowing inhaled xenobiotics after clearance from the tracheobronchial tree. In human and different animal species, the percentage weight of intestine is usually significantly smaller than the liver.

When a xenobiotic exhibiting systemic effects is administered orally, its fate is usually as follows: it comes into contact with the contents of the gastrointestinal system, is dissolved in intestinal juices, and then brought into contact with intestinal epithelium. It is then absorbed through the gut wall and the enterocytes lining the gut wall, and

transported by the portal veins through the liver, before reaching the systemic circulation and hence different parts of the body. When the same drug is given intravenously, it enters the systemic circulation and is distributed through out the body before reaching the liver for the first time. The extent of systemic availability is described with the pharmacokinetic term bioavailability (F). F is theoretically determined in the following way: the drug is administered to the same individual as a single dose intravenously and orally on separate occasions; drug concentrations in serum (or plasma, blood) are measured after each dose and used to determine the area under concentration curve (AUC) from the time 0 to "infinity". Absolute bioavailability of the oral dosage form is $F_{oral}=AUC_{oral}/AUC_{i.v.}$ If the oral and *i.v.* doses are unequal, a correction for the dose difference must be made. The most significant factor influencing the effect or toxicity is not necessarily the dose but rather the concentration of a xenobiotic at the site of action. The fraction of a chemical that reaches the systemic circulation is of critical importance in determining effect or toxicity. The incomplete bioavailability after oral administration may principally be a result of an incomplete absorption from the intestine or metabolism of the drug before it reaches the systemic circulation (presystemic metabolism). Presystemic metabolism can principally take place anywhere before the drug reaches the systemic circulation, *i.e.*, in intestine and in liver. The metabolism of xenobiotics before entering the systemic circulation is referred to as first-pass metabolism. This intestinal and hepatic first-pass biotransformation alters the physico-chemical properties of xenobiotics and is likely to change the bioavailability. The first-pass metabolism could substantially prevent many xenobiotics from being distributed throughout the body. However, the biotransformation could also potentially activate some xenobiotics. It has

been widely believed that the liver is the major site of such first-pass metabolism because of its size and its high content of drug-metabolizing enzymes. If large amounts of a chemical are ingested (*e.g.*, therapeutic drugs), it is usually true as the capacity of the intestinal biotransformation is likely to be overwhelmed. The compounds will be absorbed and pass to the liver, which has higher capacity for biotransformation than the intestine. However, recent clinical studies have indicated that the intestine contributes substantially to the overall first-pass metabolism of cyclosporin, nifedipine, midazolam, verapamil, and certain other drugs [98]. Some studies suggested that the role of intestinal metabolism of these drugs is quantitatively greater than that of hepatic metabolism in overall first-pass effect [99]. The contribution of intestinal enzymes to xenobiotic biotransformation is particularly important when relatively low concentrations of chemicals are present, as is normally the case for high potency drugs and environmental chemical pollutants, since the low concentrations of xenobiotics are readily metabolized in the intestine and leaving little to pass to the liver for further metabolism.

Almost all of the xenobiotic-metabolizing enzymes present in the liver also are found in the intestine, although their total amounts are generally much lower in the latter due to the lower weight of intestine relative to liver. Unlike the liver in which the distribution of P450 enzymes is relatively homogeneous, the distribution of these enzymes is not uniform either along the length of the small intestine or along the villi within a cross-section of mucosa. Longitudinal distribution of total cytochrome P450 and its activity have been measured in human intestine [100]. Both the content and activity of cytochrome P450 was higher in the proximal than that in the distal small intestine. The major enzymes catalyzing drug-metabolizing reactions in the liver and the GI tract belong

to the microsomal CYP3A subfamily. CYP3A4 is predominantly expressed in human liver and intestine, where it comprises approximately 30 to 50% of the total cytochrome P450 population in these tissues [101]. Many of the drugs with significant first-pass metabolism, like cyclosporine, midazolam, nifedipine, and terfenadine, are substrates of CYP3A. CYP3A activity is prone to induction or inhibition, which may cause clinically significant drug interactions. Whenever two or more drugs are administered concurrently, the possibility of drug interactions exists. The ability of a single CYP to metabolize multiple substrates is responsible for a large number of documented drug interactions associated with CYP inhibition. In addition, drug interactions can also occur as a result of the induction of several human CYPs following long-term drug treatment. CYP3A is highly inducible in humans by synthetic glucocorticoids (dexamethasone), macrolide antibiotics (rifampicin), and phenobarbital [102]. It has been demonstrated that an important cause of incomplete bioavailability of many drugs, which were earlier thought to be primarily poorly absorbed, is prehepatic metabolism in the GI tract, mainly by CYP3A subfamily of enzymes. Grapefruit juice, a beverage consumed by the general population, is an inhibitor of the intestinal cytochrome P450 3A4 system. A 47% reduction in intestinal CYP3A4 concentration occurs within 4 hours of the ingestion of grapefruit juice, and grapefruit juice maintains a bioavailability-enhancing effect for up to 24 hours. Grapefruit juice acts on the CYP system at the intestinal level, not at the hepatic level [103]. Drugs are not the only subgroup of xenobiotics that function as substrates for activation or deactivation by biotransformation processes, including CYP3A-catalyzed reaction. These enzymes also play a vital role in the biotransformation of such exogenous compounds as pesticides, carcinogens and other environmental pollutants. Intestinal

CYP3A4 enzymes were shown to activate dietary aflatoxin B1 to reactive metabolites that form macromolecular adducts within enterocytes [104]. It is logical for toxicologists to look for evidence of biotransformation capabilities in the first line of defense against ingested toxins or carcinogens, the intestinal mucosa.

In summary, in vitro and in vivo data have clearly demonstrated that the small intestine plays a significant role in first-pass metabolism in certain situations, especially when a small oral dose is given. The induction or inhibition of intestinal biotransforming-enzymes might potentially alter the bioavialability and metabolism pathway of the chemical exposed.

Both phase I and phase II biotransforming-enzymes have been found in fish liver and intestine. As in mammals, the major organ involved in xenobiotic metabolism in fish seems to be the liver. Yet, microsomal cytochrome P450 and cytochrome P450-dependent activities were found in extrahepatic organs in fish, *e.g.*, kidney, upper small intestine, gonad and brain [105].

Hypothesis

The hypotheses of the present project are: (1) CYP3A is constitutively expressed in catfish intestine; (2) CYP3A enzyme is expressed regionally along intestine of catfish; (3) testosterone is hydroxylated at different positions by different P450 isozymes in catfish intestinal microsomes; (4) the *in vitro* testosterone 6β-hydroxylation activity by catfish intestinal CYP3A enzymes is inhibited by mammalian CYP3A inhibitors, *e.g.*, erythromycin, troleandomycin and ketonconazole and by general P450 inhibitors, *e.g.*, metyrapone and SKF 525A, but not inhibited by specific CYP1A inhibitor, *e.g.*, α-naphthoflavone; (5) CYP3A expression in catfish intestine is under dietary modulation and is inducible by mammalian CYP3A inducers, *e.g.*, rifampicin and pregnenolone 16α-carbonitrile; (6) the intestinal CYP3A enzyme plays an important role in the biotransformation and bioavailability of both endogenous and exogenous compounds, including environmental pollutants, which the wild catfish are continuously exposed to.

CHAPTER 2
MATERIALS AND METHODS

Chemicals

[4-^{14}C]-testosterone and [4-^{14}C]-progesterone were purchased from DuPont NEN™ (Boston, MA). Authentic steroid and metabolite standards were obtained form Steraloids, Inc (Wilton, NH). Benzo(a)pyrene and 3-hydroxy benzo(a)pyrene were purchased from ChemSyn, through the NCI Chemical Carcinogen Repository. Western blotting kit was from Amersham Life Sciences, Inc. (Arlinton heights, IL). Ketoconazole and proadifen (SKF-525A) hydrochloride were gifts from Janssen Pharmaceutica, Inc. (Piscataway, NJ) and Smith Kline & French Labs (Philadelphia, PA), respectively. 2-methyl-1, 2-di-3-pyridyl-1-propanone (metyrapone) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Erythromycin, troleandomycin, tricane and NADPH were obtained from Sigma Chemical Co. (St. Louis, MO). All HPLC and microsomal preparation supplies were of the highest grade available from standard commercial sources.

Instruments

The following instruments were used for this study. The gradient HPLC system was equipped with a Beckman controller 125 solvent module, an analytical Beckman UV detector model 166 and an INUS β-RAM detector. Shimadzu UV-VIS spectrophometer model UV-160U, Perkin-Elmer fluorescence spectrometer model LS-3B

and Chromato-vue® UV detector were used. Liquid scintillation counters used were Packard Tri-Carb liquid scintillation system model 460CD and Beckman liquid scintillation system model LD 500TD. Beckman ultracentrifuge model L8-80M and DuPont Sorvall centrifuge model RC2-B were used.

Animals and Pretreatment

Groups of 4-8 catfish each (800-1300 g) were fed a commercially available Silver Cup chow (Silvercup, Nelson & Sons, Inc., Murray, UT) or a semi-synthetic purified diet (Dyets Inc., Bethlehem, PA) for at least 2 weeks. The semi-synthetic purified feed was formulated according to guidelines established for warm-water fish by the National Research Council [106], composed of casein 32%, dextrin 29.8%, cellulose 19%, soybean oil 3%, Menhaden oil 3%, gelatin 8%, salt and vitamin mix 5%, and choline chloride 0.17%.

For the respective pretreatments of fish, both control and treatment groups were acclimated to experimental conditions and maintained on purified diet at least 2 weeks prior to pretreatment. Control animals were maintained on semi-synthetic diet coated with corn oil (1 ml corn oil/100 g of diet) while for the treatment group the chemical (TCB or 3-MC) was delivered in corn oil applied as a coating on the semi-synthetic diet (1 ml corn oil/100 g of diet). Both dietary groups (control and chemical exposure) were maintained on designated experimental diets at 0.5% of fish body weight/day for specified period prior to sacrifice.

For the study of inducibility of mammalian CYP3A inducers, 0.03% (w/w) rifampicin was formulated in the semisynthetic purified diet, the treatment group were

fed the rifampicin-formulated diet at 3% of fish body weight/day for specified period of time. For the treatment of pregnenolone-16α-carbonitrile (PCN), the fish were acclimated and maintained on purified diet for at least 2 weeks prior to the surgical process for oral gavage. PCN (10mg) was dissolved in a mixture of 1 ml of 1M KCl and 0.5 ml of corn oil, and applied on 5 g of purified diet powder. Distilled water (12 ml) was added to the purified diet to form a slurry. The treated fish were fed this slurry at 0.5% of fish body weight/day by oral gavage for specified duration of exposure.

Surgical Procedures for Oral Gavage

Fish was anaesthetized with tricane (3-aminobenzoic acid ether ether, 6.4 g) in 12 gallon of water. The fish was then taken out of the water and a hole was drilled (5''/32) in the middle of nostrils. Tubings (I.D. 0.106'', O.D. 0.138'') were placed through the hole to the stomach, the other end tied to the dorsal fin. Fish was kept wet by pumping water on the gills through the surgical process (3.2 g tricane and 3.2 g pottasium bicarbonate was dissovled in 16 gallon of water).

Enzymes Preparation

Catfish were sacrificed and dissected. Intestines were removed from the stomach and rinsed thoroughly with ice-cold buffer A containing 0.25 M sucrose, 5 mM EDTA, 0.05 M Tris-Cl (pH 7.4), 0.2 mM PMSF, and 1mM dithiothreitol to remove contents. The intestine was bisected evenly into proximal and distal sections, opened, and mucosal cells removed by scraping into 10 ml of buffer A. Mucosal cells were weighed and homogenized in 4 volumes of buffer A. Washed microsomes were prepared from homogenates of each section using the procedure described by James and Little [107].

The homogenates were poured into suitably sized Sorvall polycarbonate centrifuge tubes and centrifuged at 13,300 g for 20 min at 4 °C to sediment the nuclei, cell debris and mitochondria. The supernatant containing microsomes and cytosol was transferred into polycarbonate ultracentrifuge tubes and then centrifuged at 170,000 g for 45 min at 4 °C. The supernatant was the cytosol. The microsomal pellets were resuspended in buffer A, resedimented for preparation of washed microsomes and the microsomal pellets were suspended in 0.25 M sucrose, 0.01 mM HEPES pH 7.4, 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 5%(v/v) glycerol in a volume equal to half of the weight of intestinal mucosal cells. Livers were removed, rinsed twice with fresh ice-cold buffer B (1.15% KCl, 0.05 M potassium phosphate pH 7.4, 0.2 mM PMSF), patted dry on a paper towel, weighed, minced with scissors and homogenized in 4 volumes of buffer B. The hepatic microsomes and cytosol were prepared in the same way as intestinal cytosol and microsmes. Aliquots of the microsomes were flushed with nitrogen and stored at -80°C until used in assays. Human CYP3A4 in the baculovirus system (with human NADPH-P450 reductase) was obtained from Gentest Co (Woburn, MA). Rabbit anti-trout polyclonal CYP3A27 antibody was a gift from Malin Celander in Göteberg University and was prepared as described [108,109].

Protein Assay

Lowry protein assay or Bio-Rad protein assay kit both with BSA as standard were used to determine the protein concentration of microsomes and cytosol.

Measurement of Cytochrome P450

Cytochrome P450 content was measured by the method of Omura and Sato, modified by Estabrook [110,111]. A suspension of fish intestinal or hepatic microsomes was prepared containing about 1 mg/ml protein in 0.1 M HEPES, pH 7.4 with 0.1% Emulgen 911 (Kao Atlas, Tokyo, Japan). The purpose of adding Emulgen was to solubilize the membrane-bound enzymes and prevent the suspension from settling. About 5 mg sodium dithionite was added to the microsomal suspension. The suspension was divided into two cuvettes, and the spectra recorded between 500 and 380 nm. CO was bubbled through the sample suspension, and the spectrum was recorded from 500 and 380 nm. The change in absorbance from 490 to 450 nm was noted. P450 content was calculated according to the following equation:

(Abs 450-Abs 490)/0.091=nmole P450/ml. (91 $mM^{-1}cm^{-1}$ is the absorptivity of CYP under these conditions [110])

Steroid Hydroxylation Assay

The steroid hydroxylation assay was described and modified by James and Shiverick [112]. Assay tubes contained 100 mM HEPES pH 7.4, 2 mM $MgCl_2$, 1.0 mM NADPH, 0.1 mM [^{14}C] progesterone or [^{14}C] testosterone (800,000 dpm added in 0.01 ml ethanol), and 400 μg catfish intestine microsomal protein, all in a final volume of 1 ml. For incubations with human CYP3A4, 40 pmole CYP3A4 was added. Tubes were incubated at 35°C for 10 minutes, with the exception of human CYP3A4, which was incubated at 37°C for 10 minutes. The reaction was stopped by the addition of ice-cold ethyl acetate (5 ml). The extraction was repeated and the two organic phases combined.

Anhydrous sodium sulfate was added to dry, then the extract was evaporated to dryness under nitrogen. Separation of the metabolites was achieved by TLC on LK5DF silica gel 150A precoated plates (Whatman Inc., New Jersey). The plates were developed once (progesterone assay) or three times (testosterone assay) in a solvent containing ethyl ether:toluene:methanol:acetone (70:38:0.8:1) at room temperature. Authentic standards were chromatographed on the same plate and visualized by viewing the plates under UV light. In the case of 5α-dihydro-steroids, 4-androsten-3α, 17β-diol and 4-androsten-3β, 17β-diol, the developed TLC plates were evaporated with iodine followed by spraying of 70 % methanol. Metabolite bands were located and quantified by electronic autoradiography with InstantImager™ (Packard Instrument Co., CT). The ^{14}C in each metabolite peak was corrected for blank values which usually were negligible. Various concentrations of testosterone were used (5, 10, 20, 50, 100 and 200 μM) to give K_M and V_{max} values. In the case of testosterone metabolite used for MS analysis, 120 mM non-radiolabeled testosterone and 1.1 mg catfish intestinal microsomal protein (total assay volume 11 ml) were used in the incubation. The incubation lasted for 60 min at 35°C. The 3α-reduced metabolite was isolated and recovered by TLC using the above solvent system.

Chemical Modulation of Testosterone Metabolism

At least five different concentrations of chemical inhibitors were used (1/5 to 4x IC_{50}) to calculate the IC_{50} values. Ethanol (for erythromycin and ketoconazole), acetone (for α-naphthoflavone) or DMSO (for troleandomycin) were used as the vehicle controls. The total organic solvent was kept under 2% of the total volume. Proadifen (SKF-525A)

hydrochloride and metyrapone were dissolved in distilled water. For chemicals that have been reported as CYP3A inhibitors through a MI-complex, *i.e.,* TAO, ERM and SKF-525A, the inhibitors were preincubated with catfish intestinal microsomes for 30 min at 35°C in the presence of 1.0 mM NADPH. The assay was started with the addition of testosterone and was further incubated for another 30 min under the same conditions. Testosterone concentration was 30 μM.

AHH (Aromatic Hydrocarbon Hydroxylation) Assay

The method of Nebert and Gonzalez [113] as previously optimized for catfish intestinal microsomes [14] was used. Tubes contained 0.2 M HEPES-NaOH buffer (pH 7.6), BaP 10 μM, 0.5 mg intestinal microsomes and 2 mM NADPH (added last) in a volume of 1 ml. To investigate the effect of ANF, varying concentrations of ANF (2-100 μM) were added to assay mixture from acetone solution. The volume of acetone was 1% of the total. After incubation at 35°C for 15 min, the assay was stopped by adding 1 ml ice-cold acetone. Tubes were extracted with 3 × 3 ml heptane, the pooled heptane extracts were back-extracted into 3 ml of 1 N NaOH, and the fluorescence of the NaOH extracts measured at an excitation of 392 nm, emission 513 nm.

Western Blot Analyses

Western Blot Analyses of CYP1A

Microsomal protein fractions (40 μg for intestine, 20 μg for liver), incubated in sample buffer as recommended by BioRad, were resolved in a mini gel format (BioRad) on 4% stacking gel with 8.5% resolving gel. Unstained and prestained molecular weight

standards in the range of 14,400 to 97,000 (BioRad low molecular weight range) were resolved at the same time as the SDS-treated microsomes. Gentest Supersomes™ expressing rat CYP1A were used to develop a standard curve for quantification of the antibody response. Electrophoresis was carried out using a 25 mM Tris/192 mM glycine/0.10% SDS buffer at constant voltage of 200 V. Protein was then transferred to nitrocellulose membrane at 40 V in a mini Transblot system (BioRad) using a 25 mM Tris/192 mM glycine/20% v/v methanol/pH 8.3 transfer buffer. The remaining gel was stained with Coomassie blue as an indication of transfer effectiveness.

Immunodetection was carried out using monoclonal antibodies to scup CYP1A (courtesy of Dr. J.J. Stegeman). Transblotted nitrocellulose was rinsed in a 20 mM Tris, 500 mM NaCl, pH 7.5 buffer and nonspecific binding sites was blocked with 5% (w/v) dried milk in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20 for 1 hour. The membrane was washed 4 times with 20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5 buffer. The primary antibody, diluted 1:10,000 in 5% (w/v) dried milk in 20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20, was incubated with the nitrocellulose for 2 hours. The unbound antibodies were washed away and further incubated with a 1:1000 dilution of secondary antibody (rabbit anti-rat antibody conjugated to horseradish peroxidase) in blocking agent for 1 hour. After washing 4 times, the immunoreactive proteins were detected according to the Amersham Western Blotting kit for chemiluminescent detection and the protein bands were visualized by fluorography on Kodak X-OMAT AR films. Fluorograms were subsequently scanned and the protein bands were quantified by scan-analysis densitometry.

Immunochemical Analyses of CYP3A

Western blot analyses of catfish intestinal or hepatic microsomes (40 μg intestinal and 20 μg hepatic microsomal protein per lane) and standard CYP proteins were performed in discontinuous (4-8.5%) SDS acrylamide gel. The proteins were electrotransferred to 0.45 μm nitrocellulose sheet and blocked with 5%(w/v) dried milk in T-TBS buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20). After blocking, the membrane was incubated with polyclonal rabbit-anti-trout-CYP3A27 (1:1000 dilution with blocking agent) for 2 hours. The unbound antibodies were washed away in T-TBS buffer and further incubated with 1:1000 dilution of secondary antibody (donkey-anti-rabbit antibody conjugated to horseradish peroxidase) in blocking agent for 1 hour. After washing with T-TBS, the immunoreactive proteins were detected according to the Amersham Western Blotting kit for chemiluminescent detection and the protein bands were visualized by fluorography on Kodak X-OMAT AR films. Fluorograms were subsequently scanned and the protein bands were quantified by scan-analysis densitometry. Gentest Supersomes™ human CYP3A4 was the P450 standard (0.5, 1, 2, 5, 8 pmol each lane for standard curve in quantification).

HPLC Analysis of Testosterone Metabolism

The dried steroid assay extracts were redissolved in methanol and filtered through a Durapore® microporous membrane (0.45 μm pore size, Millipore). Steroid metabolites were separated on a Beckman programmable HPLC using a 5 μ Ultrasphere-ODS reverse-phase (C_{18}) analytical column (4.6 x 250 mm) fitted with a precolumn guard column (Beckman Ultrasphere ODS 4.6 x 45 mm). Elution was conducted with a mixture

of H_2O: methanol: acetonitrile 50:25:25 (v/v/v) at a flow rate of 1 ml/min. Analysis was achieved using UV (225 nm) and radiochemical detection (INUS detector). Under these conditions, 6β-hydroxy-testosterone eluted at 6.2 min, 6-dehydro-testosterone at 17.6 min, testosterone at 21.3 min, androstenedione at 23.5 min, and 4-androsten-3α, 17β-diol eluted at 33.5 min.

Mass Spectrometric Analysis

Samples were dissolved in isopropanol and analyzed via ESI (electrospray ionization)-MS and APCI (atmospheric pressure chemical ionization)-MS. Samples were injected into the HPLC system (Applied Biosystems, model 400) followed by elution with a mobile phase consisting of 1% acetic acid in 30:35:35 H_2O:MeOH:isopropanol. Finnigan MAT (San Jose, CA) LCQ was used in electrospray ionization mode. The temperatures of vaporizer and capillary for APCI/MS were 300 °C and 230 °C, respectively.

Sulfotransferase Activity Assay

3-OH Benzo(a)pyrene in methanol solution was added to assay tubes so that the final concentration would be 1 μM. The methanol was evaporated with nitrogen and 0.05 M Tris-Cl pH 7.0, 0.4% BSA, 50 μg cytosolic protein and water were added up to 0.45 ml. After 2 min pre-incubation at 35°C, the reaction was started by the addition of 20 μM 3'-phosphoadenosine-5'-phosphosulfate (PAPS) in 50 μl water and stopped after 10 min with 2 ml methanol. Methanol (2 ml) was added to blanks before PAPS. Tubes were centrifuged and 2 ml supernatant was mixed with 0.5 ml 1 N NaOH. The fluorescence of

sulfate conjugates (BaP-3-sulfate) was measured at ex294/em415 nm. The sulfate product was calculated against the standard curve of BaP-3-sulfate conjugate [14].

UDP-Glucuronosyltransferase Activity Assay

3-OH Benzo(a)pyrene in methanol solution was added to tubes so that the final concentration would be 1 μM, and the methanol was evaporated under nitrogen. To this was added 0.1 M Tris-Cl pH 7.6, 5 mM $MgCl_2$, and 50 μg microsomal protein solubilized with 0.5 mg Lubrol/mg microsomes in a final volumn of 0.4 ml. After preincubation for 2 min at 35°C, the reaction was started by adding 200 μM UDP-glucuronic acid (UDPGA) in 0.1 ml water and terminated after 30 min by addition of 2 ml methanol. Tubes were centrifuged to precipitate protein, and 2 ml of the supernatant was added to 0.5 ml 1 N NaOH. After mixing, the fluorescence was measured at ex300/em421 nm. The glucuronide product was calculated against the standard curve of BaP-3-glucuronide conjugate.

Statistical Analysis

Data are presented as the mean ± SD unless specified else. Results were analyzed by a one-way analysis of variance (ANOVA) and differences between pairs of means were tested by the student t-test. Differences with a p value of <0.05 were considered to be statistically significant unless specified else. Correlation analysis was performed using Microsoft Excel software (Microsoft, Redmond, WA).

CHAPTER 3
RESULTS

Response to Aryl Hydrocarbon Receptor Agonists

CYP1A Expression in Channel Catfish Intestine

Total P450 content, CYP1A content and aryl hydrocarbon hydroxylase (AHH) activity were measured in both the control fish, TCB-treated and 3MC-treated fish, to assess the effect of preexposure to aryl hydrocarbon receptor agonists upon the metabolic capacities of the catfish intestine. Mean P450 concentrations (between 0.02 and 0.20 nmol/mg protein) were not significantly altered with TCB or 3MC treatments (Figure 3-1) (ANOVA: $p>0.05$). CYP1A cross reactivity was not detected for either the controls or animals in the 0.5 mg TCB/kg diet treatments. CYP1A levels were variable for the 5.0 mg TCB/kg diet treatment, with values ranging from 0.14 to 24.11 pmol/mg protein (Table 3-1). Liver microsomes from catfish induced by dietary TCB (5 mg/kg diet) was used as a positive control. Composite AHH activities were 2.46 ± 1.16, 2.43 ± 1.58 and 11.35 ± 10.25 pmol/min/mg protein for the control, 0.5 and 5 mg TCB/kg diets, respectively. AHH activities of the 5.0 mg/kg treatment were not significantly greater than controls or the 0.5 mg/kg diet treatment due to the high standard deviation of the data (ANOVA: $p>0.05$). Four animals demonstrated large increases (~7 fold) in AHH activities, while 3 animals exhibited levels similar to the controls. AHH activity exhibited a strong correlation ($r^2=0.96$) with CYP1A cross reactivity in fish exposed to TCB at 5 mg/kg diets ($y=1.143x+1.026$). CYP1A was present (20.8 ± 12 pmol/mg microsomal

protein) and AHH activity was induced (26.9 ± 4.1 pmol/min/mg) in all 3MC-exposed fish (n=10). In summary, CYP1A was not constitutively expressed in catfish intestine or liver. Yet, the immunoblots of intestine microsomes from catfish treated by dietary 3-MC or TCB showed a clear band crossing-reacting with the anti scup CYP1A antibody (Figure 3-2 and Figure 3-3). Table 3-1 shows the CYP1A amount in intestinal microsomes from control fish and fish pretreated by 3-MC (10 mg/kg diet) or TCB (5 mg/kg diet) for 10 days. CYP1A induction did not response at the pretreatment level of 0.5 mg TCB/kg diet (not shown). The results indicated that catfish intestine CYP1A is inducible by AhR agonists and the AHH activity is highly correlated to CYP1A amount (Figure 3-4), suggesting intestinal CYP1A can be used both as a biochemical and an exposure biomarker.

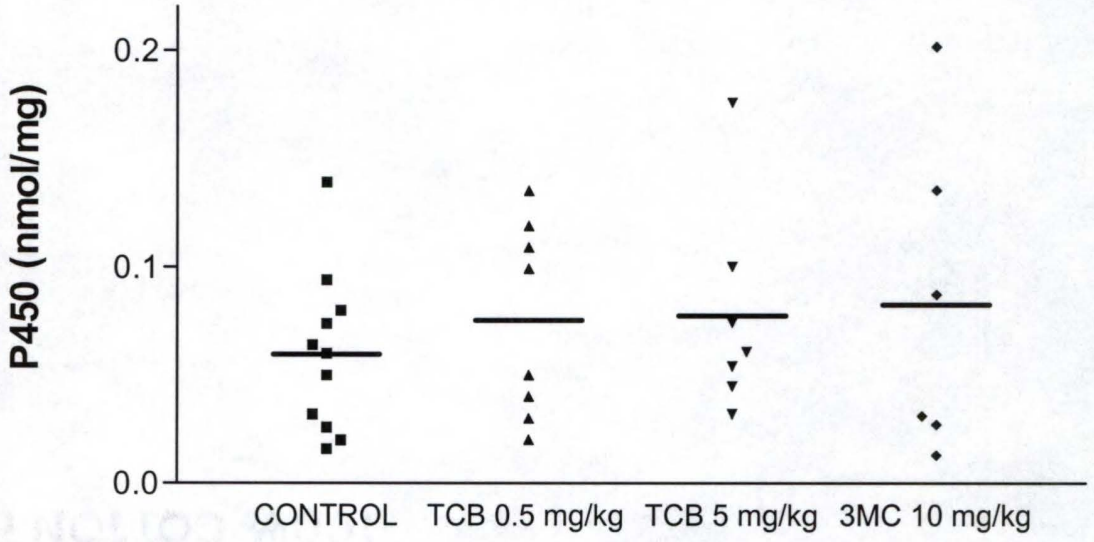
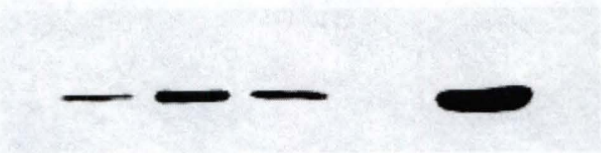


Figure 3-1. Intestinal P450 content in control and treated fish. The scatter graph shows the P450 content in intestinal microsomes from control catfish (n=11) or animals exposed for 10 days to 0.5 mg TCB/kg (n=8), 5 mg TCB/kg (n=6) or 10mg 3-MC/kg diet (n=6). The horizontal bars indicate the mean values for each group.



(1) (2) (3) (4) (5)

Figure 3-2. Intestinal and hepatic CYP1A in control and fish treated with 3MC or TCB.
40 μg intestinal (Lane1-4) and 20 μg hepatic (Lane5)
microsomal protein were in each lane:
(1)-(3) Three individual fish pretreated with 10 mg 3-MC/kg diet;
(4) Control fish;
(5) Fish pretreated with 5 mg TCB/kg diet.

(1) (2) (3) (4) (5)

Figure 3-3. Intestinal and hepatic CYP1A in control and fish treated with TCB.
40 μg intestinal (Lane 1-4) and 20 μg hepatic (Lane 5) microsomal
protein were in each lane:
(1)-(4) Four individual fish pretreated with 5 mg TCB/kg diet;
(5) Fish pretreated with 5 mg TCB/kg diet.

Table 3-1. Intestinal CYP1A level in control and treated fish.

Pretreatment	**Intestinal CYP1A pmol/mg microsomal protein**
Control (n=10)	< D.L.
3-MC (10 mg/kg diet) (n=10)	20.86 ± 12.85
TCB (5 mg/kg diet) (n=4)	14.49 ± 7.73

D.L.: detection limit.

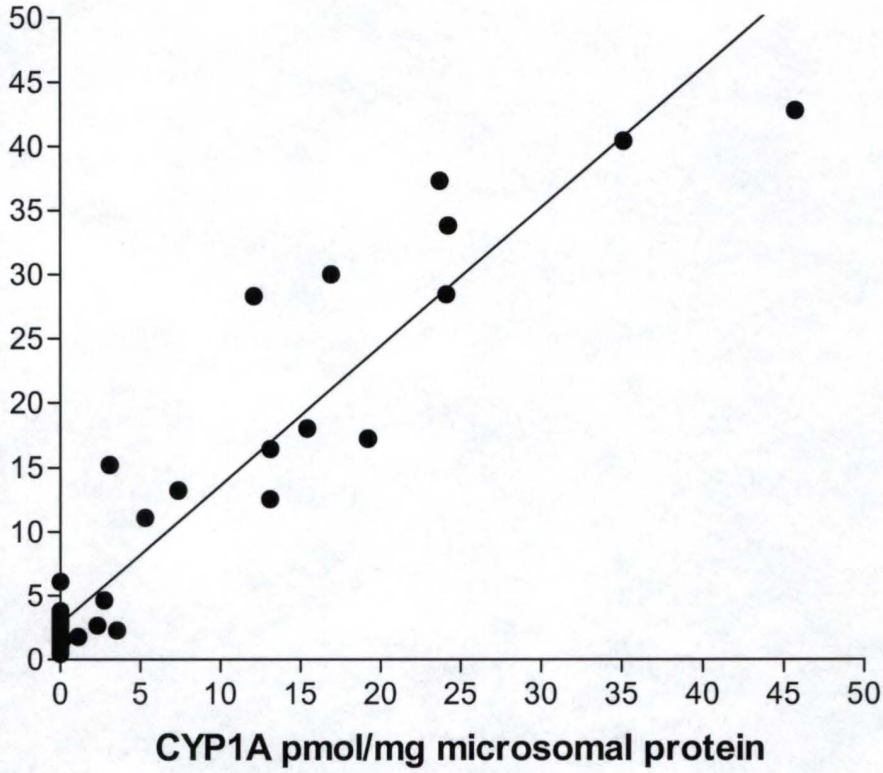


Figure 3-4. Intestinal CYP1A content and AHH activity. This graph shows that CYP1A content, pmol/mg microsomal protein correlated in a linear fashion ($r=0.947$, $p<0.001$) with AHH activity in intestinal microsomes of catfish from control, exposed to 0.5 and 5 mg TCB/kg and 10 mg 3MC/kg diets for 10 days. The CYP1A content was determined by cross-reactivity with a scup CYP1A monoclonal antibody and quantified relative to a rat CYP1A standard curve.

UDP-Glucuronosyltransferase Expression in Catfish Intestine

UGT activity was determined in control and 3MC treated fish. Intestinal microsomal UGT activity was significantly higher than that in control fish (Figure 3-5). In addition, the UGT activity correlates with the AHH activity, showing a r^2 of 0.75 (Figure 3-6).

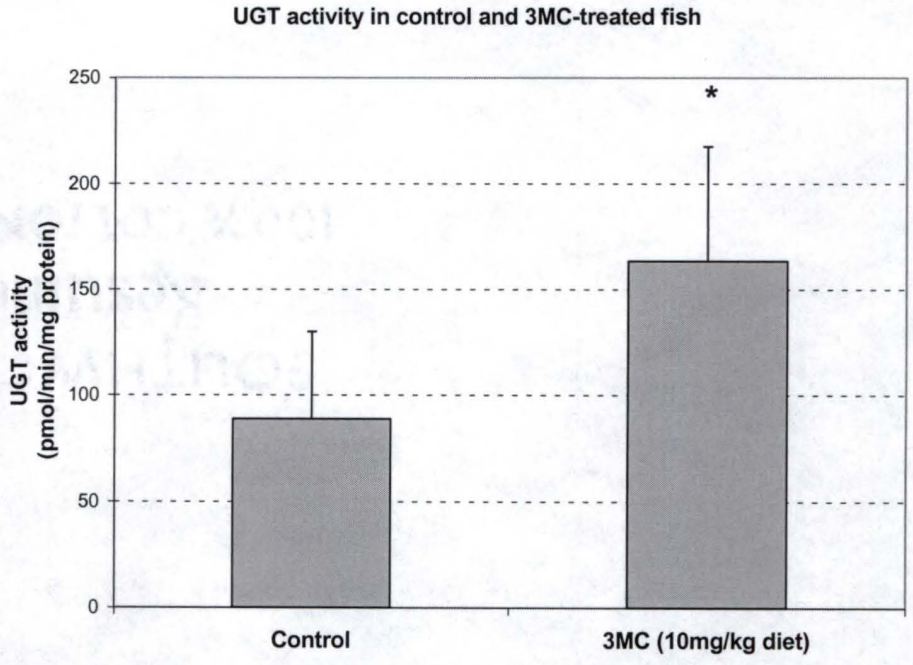


Figure 3-5. UGT activity in control (n=12) and 3MC treated (n=11) fish.

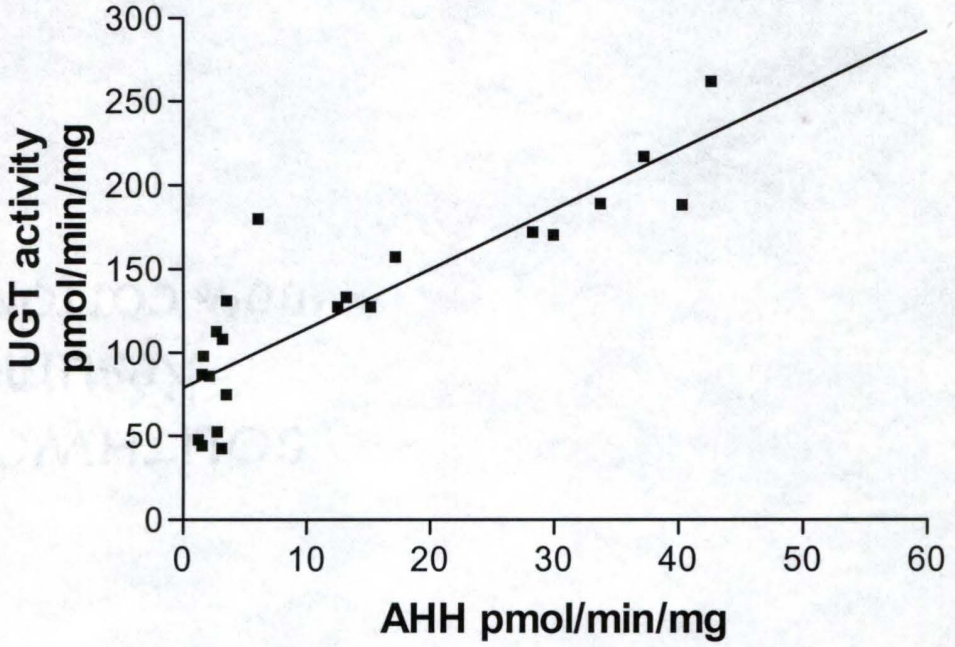


Figure 3-6. Intestinal microsomal AHH and UGT activities. This graph shows that AHH, pmol/min/mg microsomal protein correlated in a linear fashion ($r=0.866$, $p<0.001$) with UGT activity in intestinal microsomes of catfish from control, exposed to 0.5 and 5 mg TCB/kg for 10 days.

Function and Expression of CYP3A and Testosterone Metabolism

TLC Analyses of Testosterone Metabolism by Catfish Intestinal Microsomes

Our results indicate that catfish intestinal microsomes hydroxylate testosterone and progesterone in a regioselective and stereospecific manner. Shown in Figure 3-7 is a representative chromatogram of the [^{14}C]-testosterone metabolism profile exhibited by catfish intestinal microsomes. 4-Androsten-3α, 17β-diol, 6β-hydroxytestosterone and androstenedione were identified as the three major metabolites of testosterone by their cochromatography with anthentic standards. There were also trace amounts of 11α-hydroxytestosterone shown (Figure 3-8). Recombinant CYP3A4 in a baculovirus system coexpressing NADPH-P450 reductase (Supersomes™) only hydroxylated testosterone at 6β-position. Similarly, progesterone was hydroxylated or reduced by catfish intestinal microsomes to give 3α-hydroxypregn-4-en-20-one, 6β-hydroxy-, 17α-hydroxy-, and 16α-hydroxytestosterone as metabolites (Figure 3-8 and 3-9). There were reduced amounts of 6β- and 17α-hydroxylated metabolites and absolutely no 3α-reduced metabolite formed for both steroids as substrates without NADPH under the same assay conditions (not shown). Figure 3-10 depicts the Lineweaver-Burk plots for metabolism of testosterone to the three metabolites (see above) by catfish intestinal microsomes. Table 3-2 shows the apparent K_M and V_{max} values of these three metabolism pathways from testosterone. The lower K_M value of testosterone-6β-hydroxylation than those of the other two metabolites indicates that this may be the most important physiological pathway because of the low physiological concentration of the substrate.

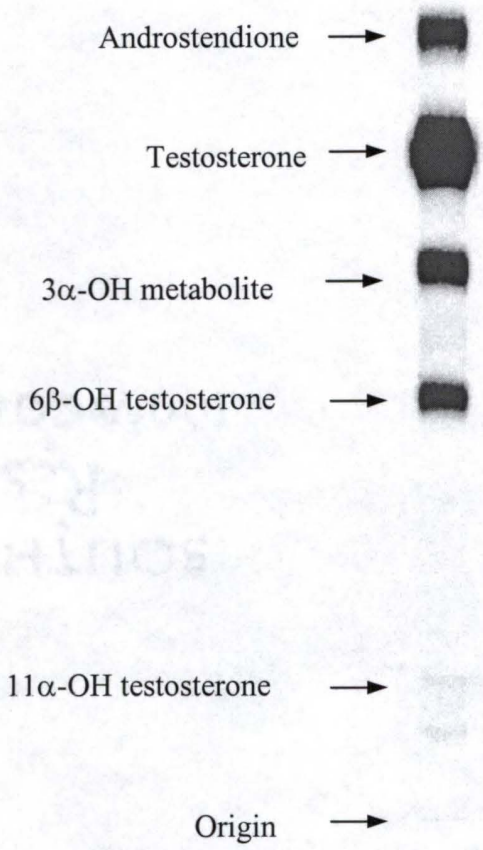


Figure 3-7. TLC of testosterone metabolism by catfish intestinal microsomes.

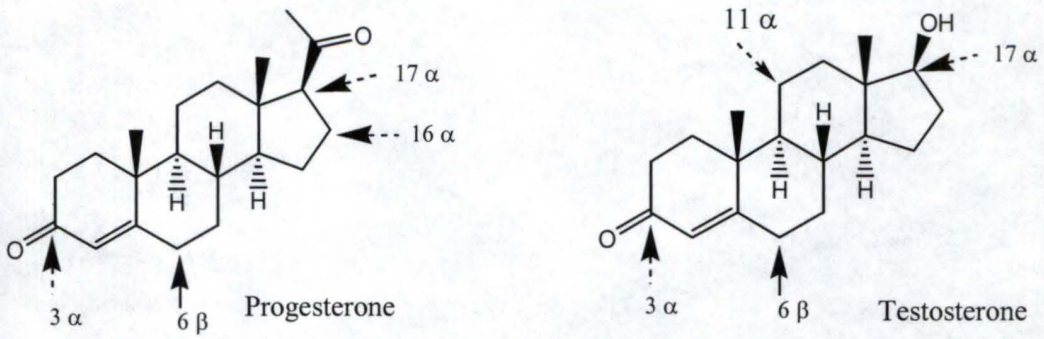


Figure 3-8. Progesterone and testosterone metabolism positions by catfish intestinal microsomes.

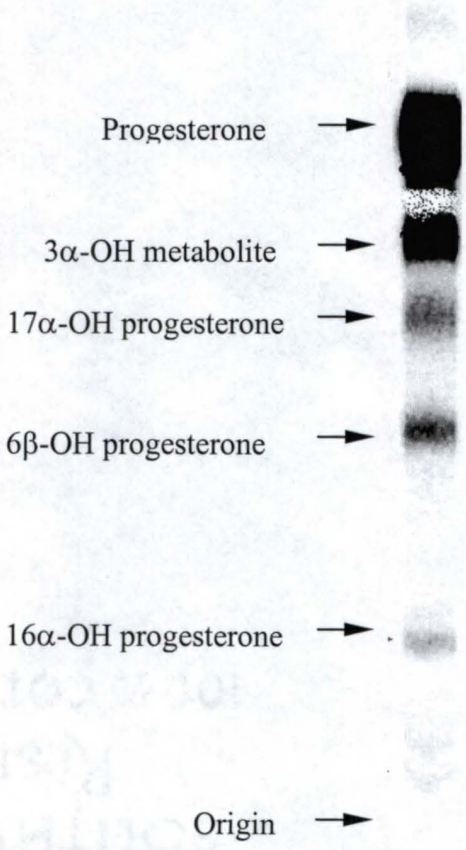


Figure 3-9. TLC of progesterone metabolism by catfish intestinal microsomes.

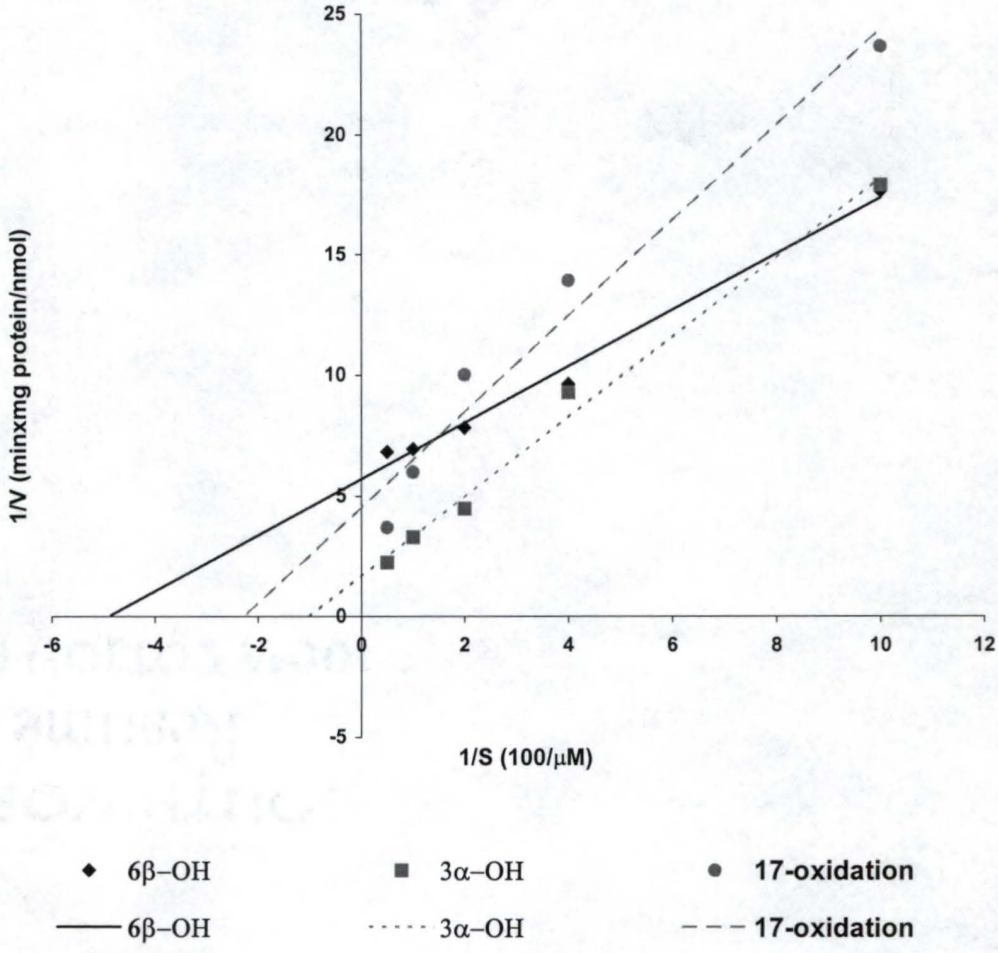


Figure 3-10. Lineweaver-Burk plot of testosterone metabolism by catfish intestinal microsomes

Table 3-2. Kinetic analysis of testosterone metabolism by catfish intestinal microsomes.

	6β-hydroxylation	3α-reduction	17-oxidation
K_M (μM)	20.4	100.6	44.4
V_{max} (pmol/min/mg protein)	175.4	608.9	223.4

Expression of CYP3A along Catfish Intestine

As shown in Figure 3-11, the polyclonal antibodies (IgG) generated against trout CYP3A27 reacted strongly with catfish intestinal microsomes, showing a band with molecular weight of 59 kDa. The rabbit-anti-trout CYP3A27 antibody also recognizes human CYP3A4, which was used in CYP3A quantification. Human CYP3A4 has molecular weight of 54 kDa. The CYP3A-like protein was expressed constitutively in catfish intestine. In both groups of fish fed either commercially available chow or semisynthetic purified diet, the expression of this protein was much higher in the proximal segment than in the distal part (Figure 3-12). A breakdown product with MW 44 kDa has been found which also cross-reacted with the rabbit-antitrout CYP3A27. In addition, the intestinal CYP3A amount in fish fed chow was higher than those fed purified diet (Figure 3-12). This trend is more obvious in the proximal section of intestine rather than distal part, probably due to the low expression in distal intestine from both groups of fish.

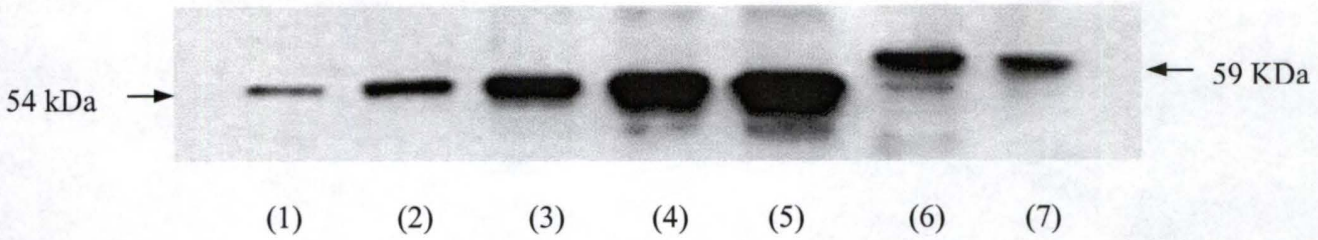


Figure 3-11. Western blot of hCYP3A4 and catfish intestinal CYP3A.
Lane (1)-(5): 0.5, 1, 2, 3.5, 5 pmol hCYP3A4;
(6) 20 μg proximal intestinal microsomes from catfish fed purified diet;
(7) 20 μg distal intestinal microsomes from catfish fed purified diet.

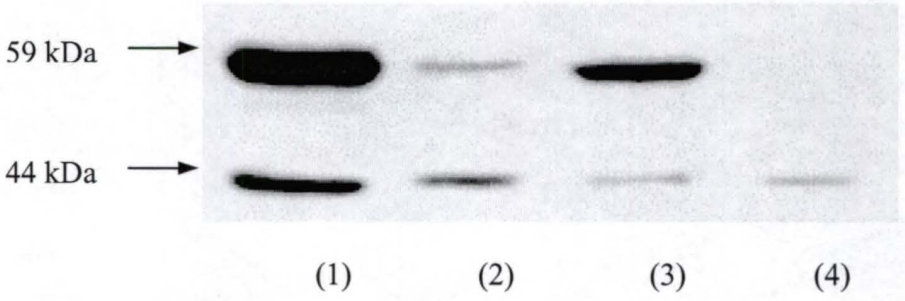


Figure 3-12. Western blot of CYP3A in catfish intestine. Each lane has 20 μg intestinal microsomal protein. (1) proximal intestinal from fish fed chow; (2) distal intestine from fish fed chow; (3) proximal intestine from fish fed purified diet; (4) distal intestinal from fish fed purified diet.

Regional Expression and Dietary Effects on Intestinal CYP3A

Testosterone hydroxylation activities by catfish intestinal microsomes and CYP3A amount are summarized in Table 3-3. Testosterone 6β-hydroxylation activities were significantly higher in the proximal segment in catfish intestine than in the distal part for both fish groups whether fed on chow or purified diet (Figure3-13). In addition, in the proximal half of the catfish intestine, the CYP3A catalytic indicator, testosterone 6β-hydroxylation activity, was significantly higher in fish fed chow than those fed semi-purified diet (Table 3-3 and Figure 3-13). This trend was not observed in the distal part of the intestine. The total metabolism of testosterone in the proximal segment was slightly higher but not significantly different from the values found in distal intestine (Figure 3-14). We have also studied the effect of diet on the CYP3A expression in the catfish intestine. It indicated that the amount of CYP3A protein was lower in the intestine of catfish that were fed purified diet than those fed with commercial chow, but the two groups show the same trend of expression along the intestine (Table 3-3). Testosterone 6β-hydroxylation activities, the CYP3A catalytic indicator, correlated with the CYP3A amount shown by immunoblotting ($r=0.88$) (Fig. 3-15). On the contrary, the testosterone 17-oxidation and the formation of the reduced metabolite, 4-androsten-3α, 17β-diol, showed poor correlation with the CYP3A protein amount. The ratio of testosterone 6β-hydroxylation over 17-oxidation was much higher in proximal intestine, ranging from 1 to 4, than that in distal section, which is approximately 0.8 (Figure 3-16). This suggests that the percentage of CYP3A enzyme in total P450 content was significantly higher in the proximal part than that in the distal intestine. These results demonstrated that a CYP3A-like protein, related to CYP3A27, was expressed at higher concentrations in the

proximal than in the distal segment of catfish intestine. In addition, the expression of this CYP3A-like protein was modulated by the diet.

TABLE 3-3. CYP3A expression and catalytic activities along catfish intestine

Diet	Testosterone Metabolism		CYP3A
	6β-OH	Total Metabolism	Enzyme Amount
	pmol/min/mg protein		pmol/mg protein
Chow (n=4)			
Proximal	262.8±80.3 [a,b]	986.9±363.4	101.0±31.1[a,b]
Distal	88.6±15.6	622.3±225.5	32.3±22.8
Purified (n=8)			
Proximal	158.4±32.6 [a]	687.6±107.0	52.5±6.9[a]
Distal	104.1±38.1	466.1±180.8	21.6±15.2

[a] significantly higher than the corresponding distal values by one tailed student t-test for paired samples: $p<0.01$.

[b] significantly different from the corresponding proximal values for purified diet group by single factor ANOVA: $p<0.01$.

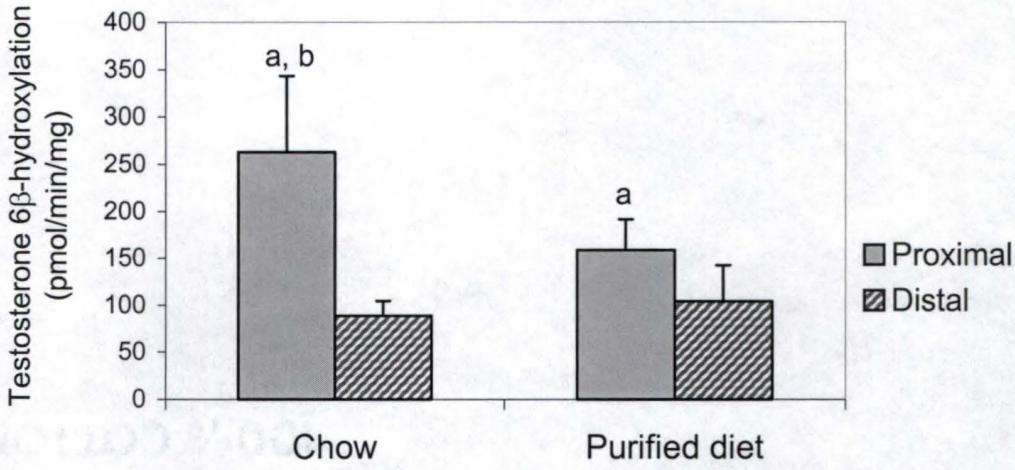


Figure 3-13. Testosterone 6β-hydroxylation activities in proximal and distal intestine of fish fed chow or purified diet.

[a] significantly higher than the corresponding distal values by one tailed student t-test for paired samples: $p<0.01$.
[b] significantly different from the corresponding proximal values for purified diet group by single factor ANOVA: $p<0.01$.

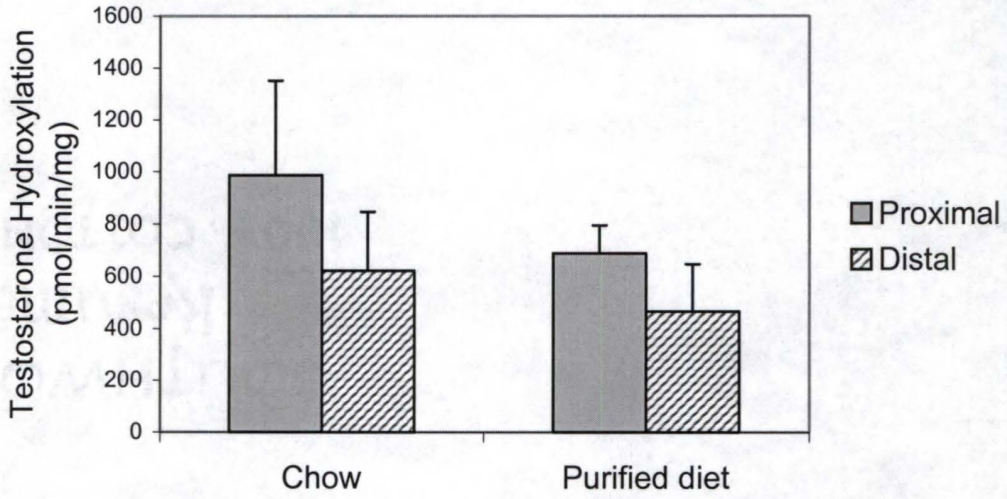


Figure 3-14. Testosterone metabolism activities in proximal and distal intestine of fish fed chow or purified diet.

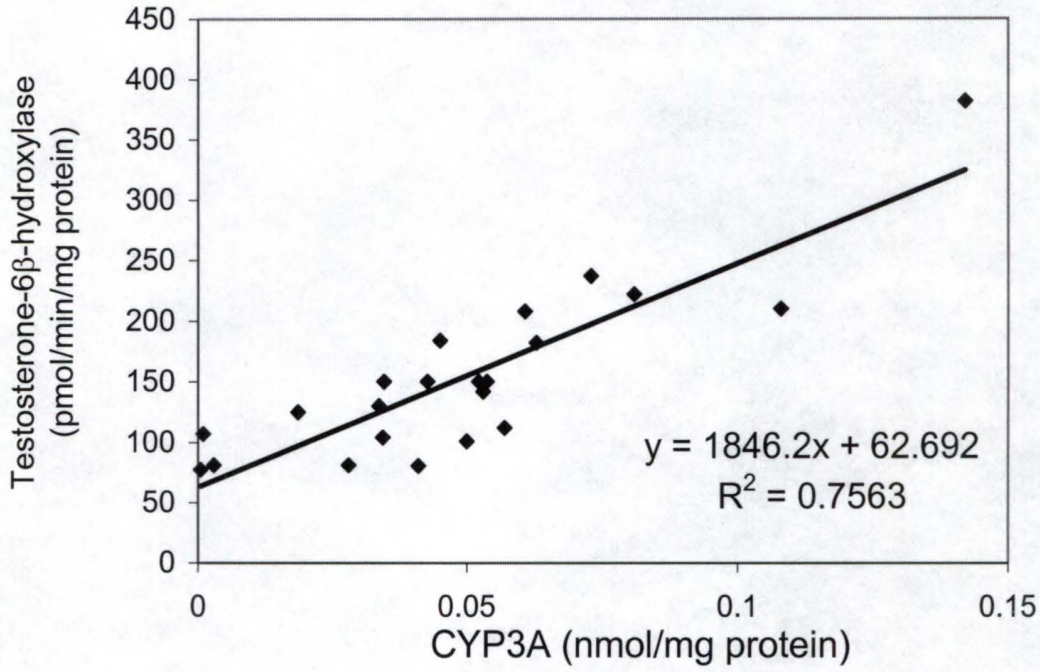


Figure 3-15. Correlation between testosterone 6β-hydroxylation and CYP3A enzyme amount.

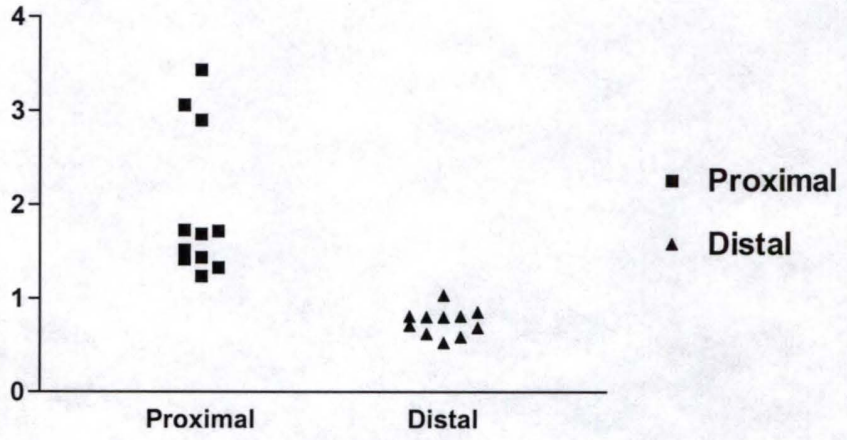
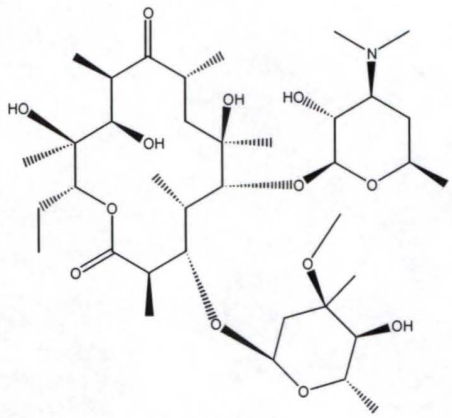


Figure 3-16. Ratio of testosterone 6β-hydroxylation/17-oxidation in proximal and distal intestine of control catfish.

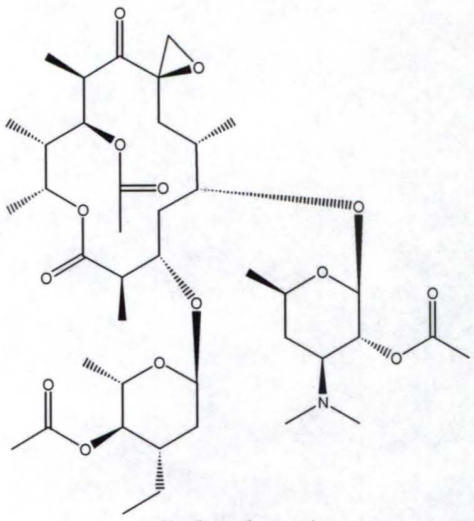
Effects of Modulators on CYP3A Activities

The chemical structures of the six CYP3A modulators used in our study are shown in Figure 3-17. The three mammalian quasi-irreversible CYP3A inhibitors, troleandomycin, erythromycin and SKF 525A, have an N-alkylated amine required to form the metabolite-intermediate complex. Surprisingly, the selective CYP3A inhibitor for mammals, troleandomycin, showed no inhibition of formation of any of the three testosterone metabolites by catfish intestinal microsomes (Figure 3-18). Figure 3-19 and 3-20 are representative TLC analyses, showing chemical inhibition of testosterone metabolism activities. Erythromycin, ketoconazole, metyrapone and SKF-525A inhibited testosterone 6β-hydroxylation to different extent {Figure 3-21, 3-22, 3-23, 3-24). Figure 3-25 summarizes the effects of four CYP inhibitors, erythromycin, ketoconazole, metyrapone and SKF-525A, on the metabolism of testosterone. All four chemicals showed strong inhibition of testosterone 6β-hydroxylation. The inhibitory effects of CYP3A-mediated testosterone 6β-hydroxylation were: ketoconazole > metyrapone > SKF-525A > erythromycin as the inhibitory potency decreased (Table 3-4). None of the four inhibitors showed significant effect on the testosterone 3-oxidoreduction or 17-oxidation. Only ketoconazole exhibited a concentration dependent inhibition of the formation of androstenedione (Figure 3-21 and 3-24). Yet the IC_{50} value of ketoconazole for testosterone 6β-hydroxylation was almost 10^5-fold smaller than that for the 17-oxidation to form androstenedione (Table 3-4). The compound α-naphthoflavone had no significant effect on testosterone 6β-hydroxylation activities (Figure 3-26). At 100 μM α-naphthoflavone concentration, which is more than three times the substrate concentration

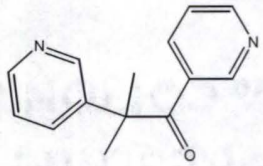
(30 μM), testosterone 6β-hydroxylation still had 91% activity left in comparison to control (Figure 3-26).



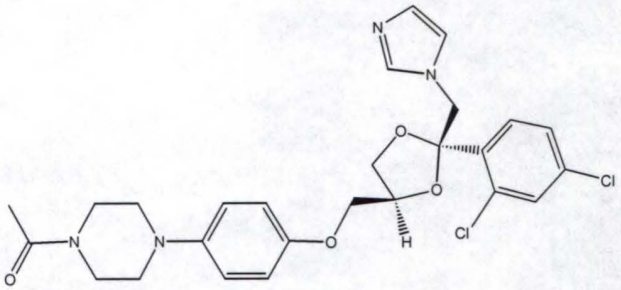
Erythromycin



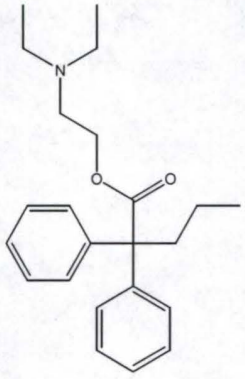
Troleandomycin



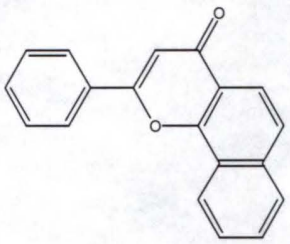
Metyrapone



Ketoconazole



SKF 525A (Proadifen)



α-naphthoflavone

Figure 3-17. Chemical structures of mammalian CYP3A modulators. Five inhibitors (erythromycin, troleandomycin, metyrapone, ketoconazole and SKF 525A) and one enhancer (α-naphthoflavone) are shown.

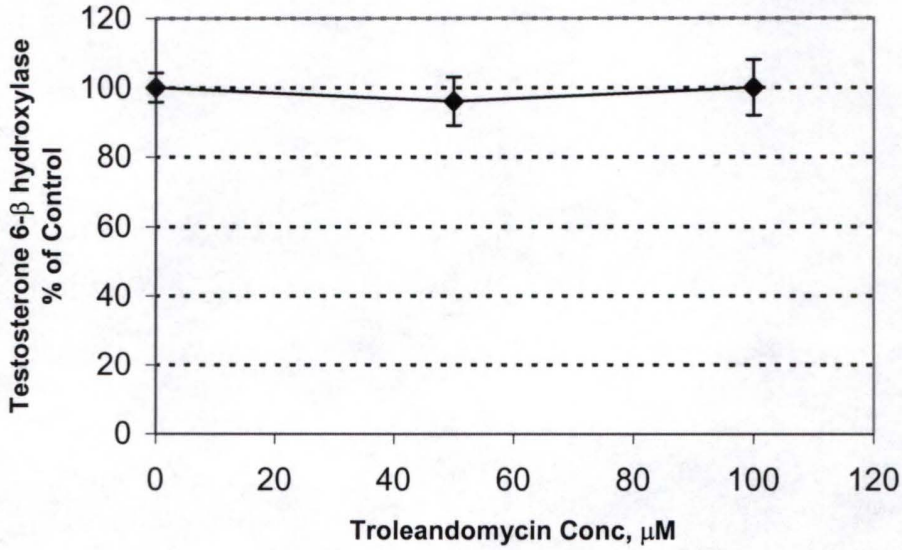


Figure 3-18. Effect of testosterone 6β-hydroxylation by addition of troleandomycin.

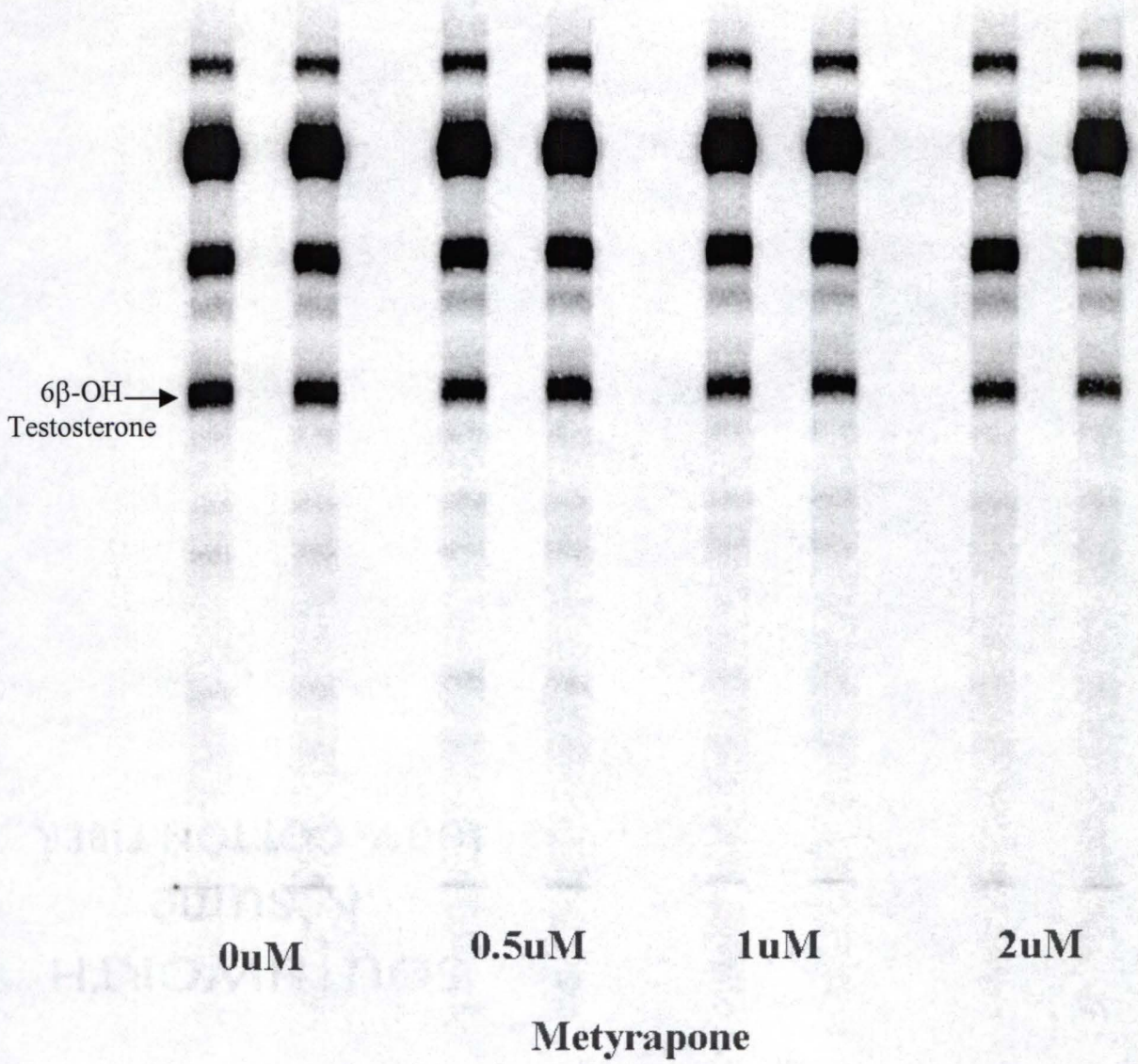


Figure 3-19. TLC of inhibition of testosterone metabolism by metyrapone.

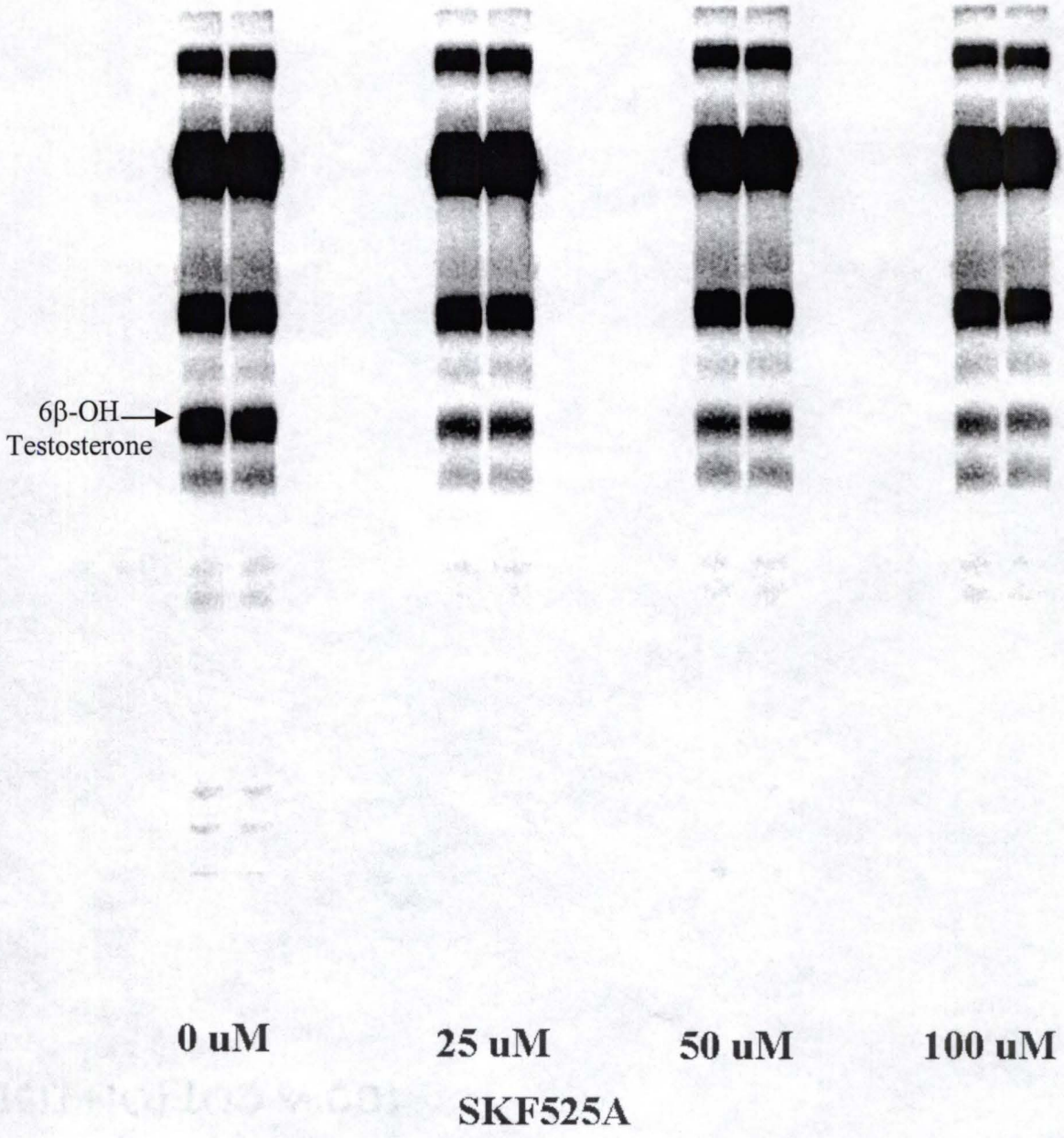


Figure 3-20. TLC of inhibition of testosterone metabolism by SKF-525A

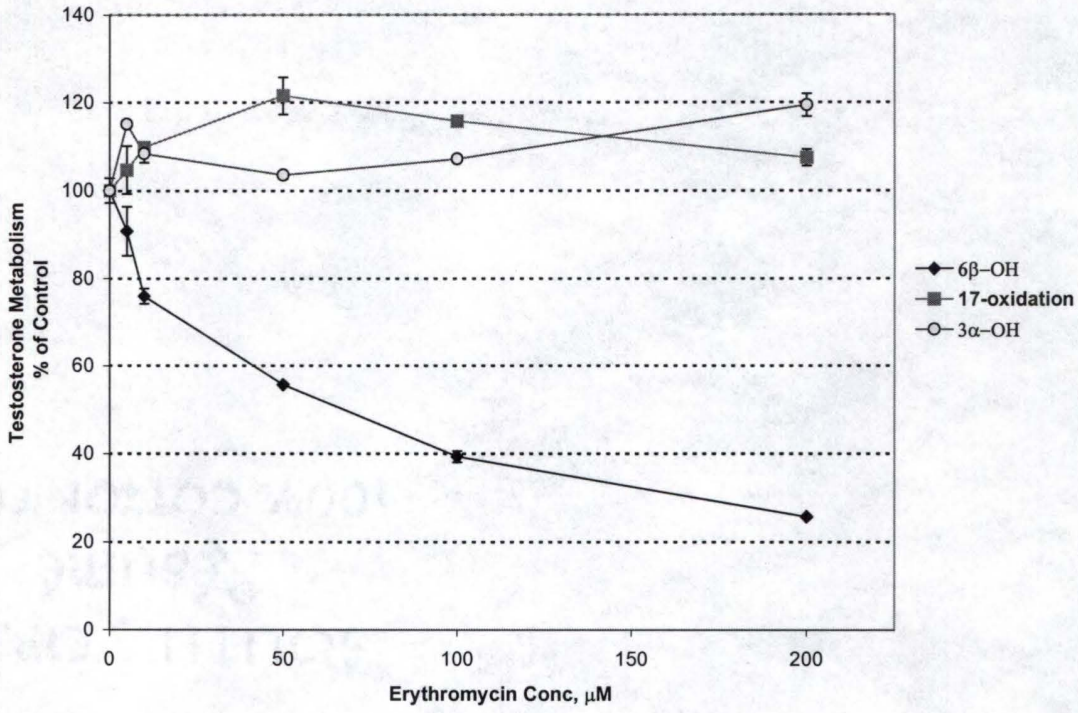


Figure 3-21. Inhibition of testosterone metabolism by erythromycin.

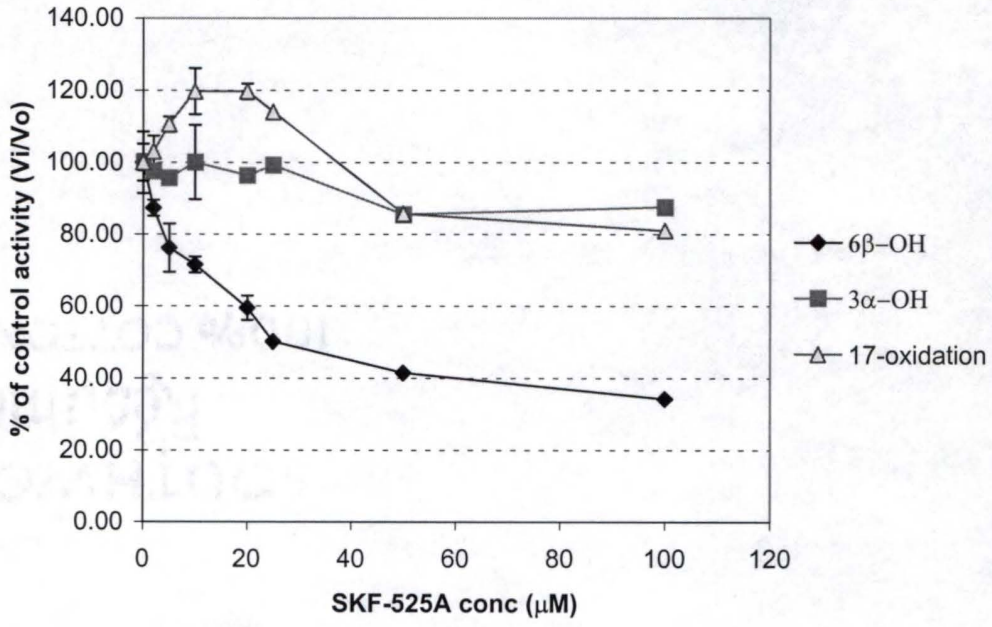


Figure 3-22. Inhibition of testosterone metabolism by SKF-525A.

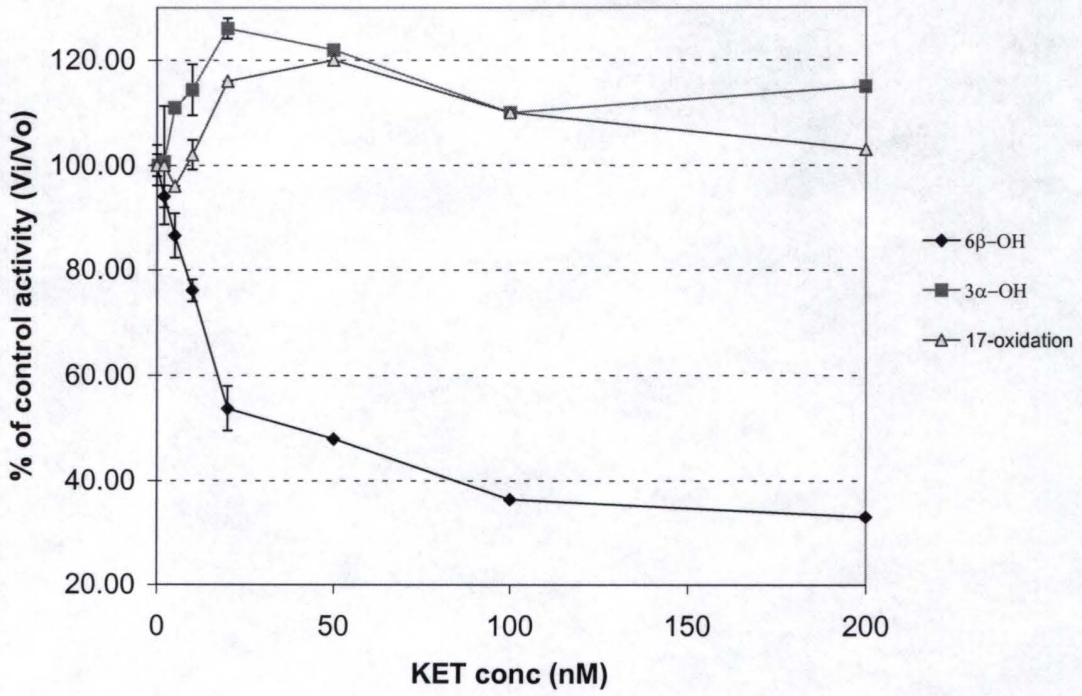


Figure 3-23. Inhibition of testosterone metabolism by ketoconazole.

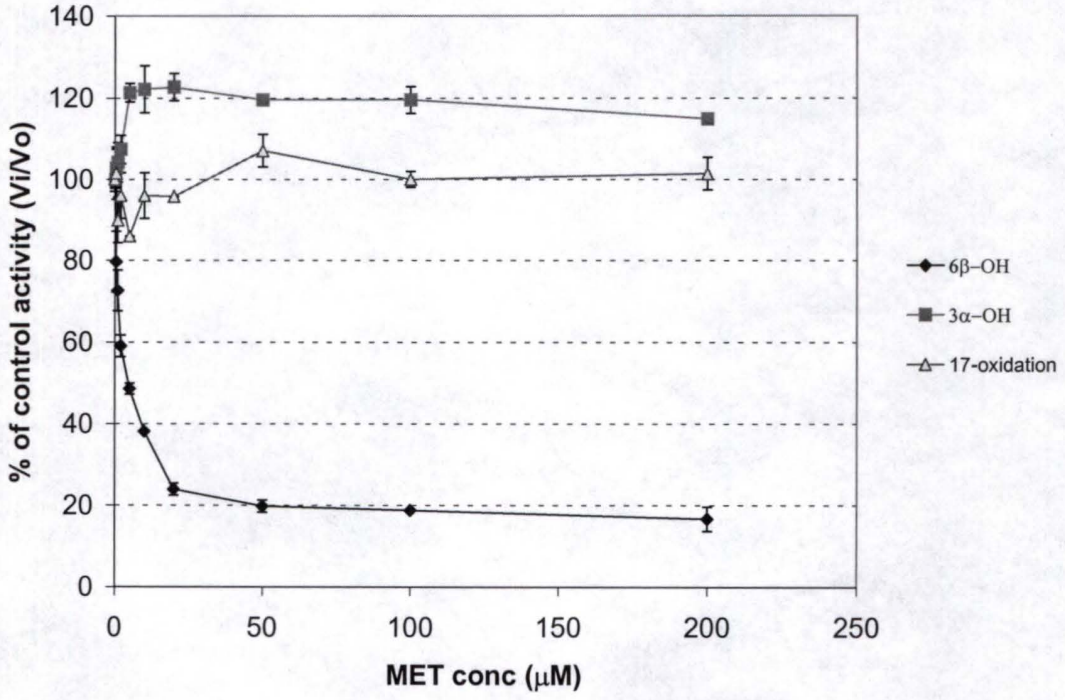


Figure 3-24. Inhibition of testosterone metabolism by metyrapone

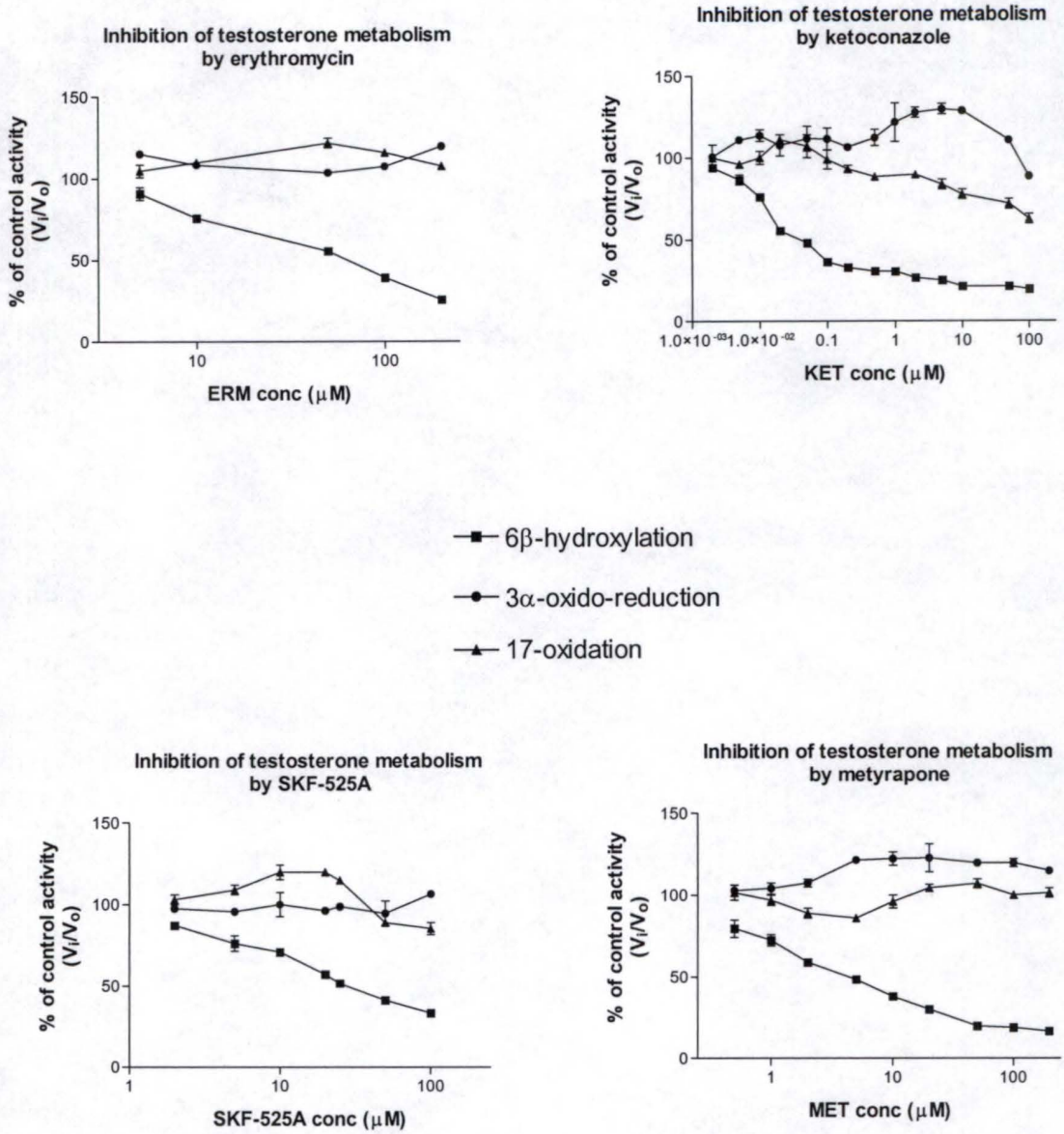


Figure 3-25. Determination of IC_{50} of testosterone 6β-hydroxylation by ERM, KET, SKF-525A and MET. Abbreviations: ERM, erythromycin; KET, ketoconazole; MET, metyrapone.

Table 3-4. IC_{50} values of the four inhibitors on testosterone hydroxylation.

Chemical	IC_{50} (μM)	
	6β-hydroxylation	17-oxidation
Ketoconazole	0.0404	>500
Metyrapone	4.48	n.r
SKF-525A	32.1	n.r
Erythromycin	53.8	n.r

n.r. : no inhibition observed up to 200 μM of the inhibitor concentration

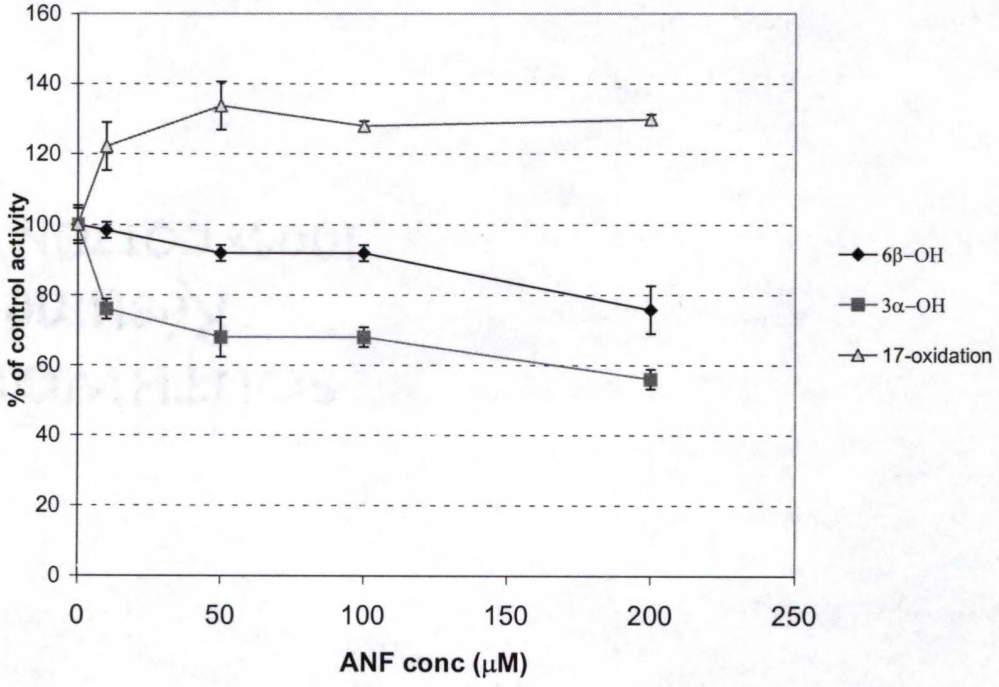


Figure 3-26. Modulation of testosterone metabolism by α-naphthoflavone.

Modulation of AHH Activities

AHH activity was enhanced by the addition of α-naphthoflavone (Figure 3-27). Maximal enhancement was achieved at 20 μM.

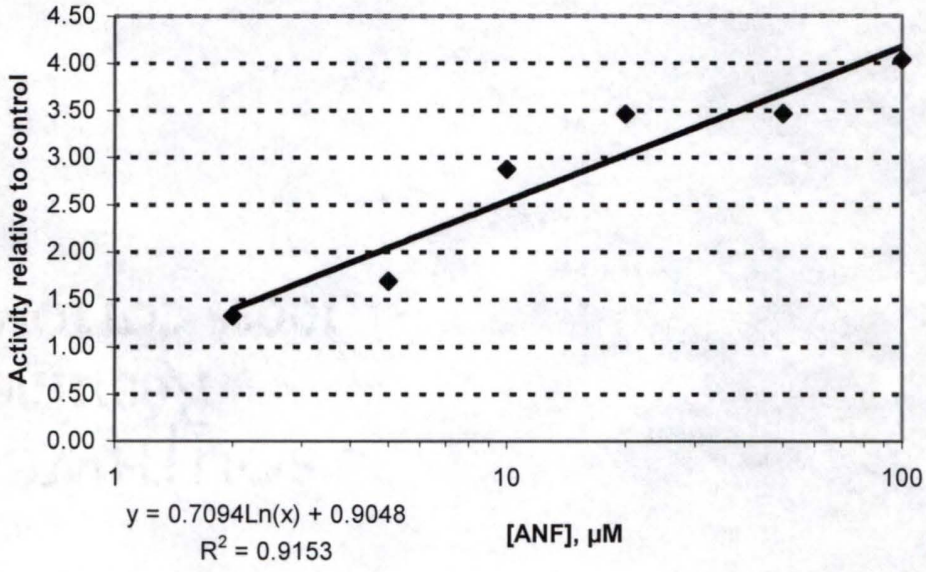


Figure 3-27. Stimulation of AHH activity by α-naphthoflavone.

Identification of Major Testosterone Metabolite

HPLC Analysis of Testosterone Metabolism by Catfish Intestinal Microsomes

A typical HPLC chromatogram of the ethyl acetate extracts obtained after incubation of [^{14}C] testosterone with NADPH and catfish intestinal microsomes is shown in Figure 3-28. Testosterone and its hydroxylated metabolites showed peaks around 240 nm on UV spectrum. However, 4-androsten-3α,17β-diol has maximal absorbancy at 212 nm. The UV detector was set at 225 nm to avoid interference by the mobile phase. The unknown metabolite isolated with TLC gave a peak at 33.5 min on radiochemical detection, similar to the 4-androsten-3α,17β-diol standard by UV detection (Figure 3-29). As the molar absorptivity of 4-androsten-3α,17β-diol is much lower than that of testosterone or its hydroxylated metabolites due to the lack of a 3-keto group to conjugate with the C^4=C^5, it is not surprising that signal of the unknown metabolite from assay extract was below detection limit on UV detector. To further prove the identity of the unknown metabolite, [^{14}C] testosterone assay extract was mixed with 4-androsten-3α,17β-diol standard. The mixture was dried under nitrogen and reconstituted in methanol. Figure 3-30 indicated the UV and radiochemical identities of the mixture of [^{14}C] testosterone assay extract with 4-androsten-3α,17β-diol standard. The standard and the unknown metabolite eluted at the same time of 33.5 min on UV and radioactivity detection respectively. Thus, testosterone was converted to 6β-OH testosterone, androstenedione and 4-androsten-3α,17β-diol by catfish intestinal microsomes. The HPLC profile also provided valuable information on the identification of another metabolite, 6-dehydrotestosterone (17β-hydroxy-4,6-androstadiene-3-one) (Figure 3-28, 3-30). The retention time of this 6-dehydrotestosterone metabolite matched that of the

authentic standard. HPLC analysis of [^{14}C] testosterone incubation with catfish intestinal cytosol was also performed by radioactivity detection (not shown). No direct formation of the 3α-reduced metabolite was observed in incubation with catfish intestinal cytosol.

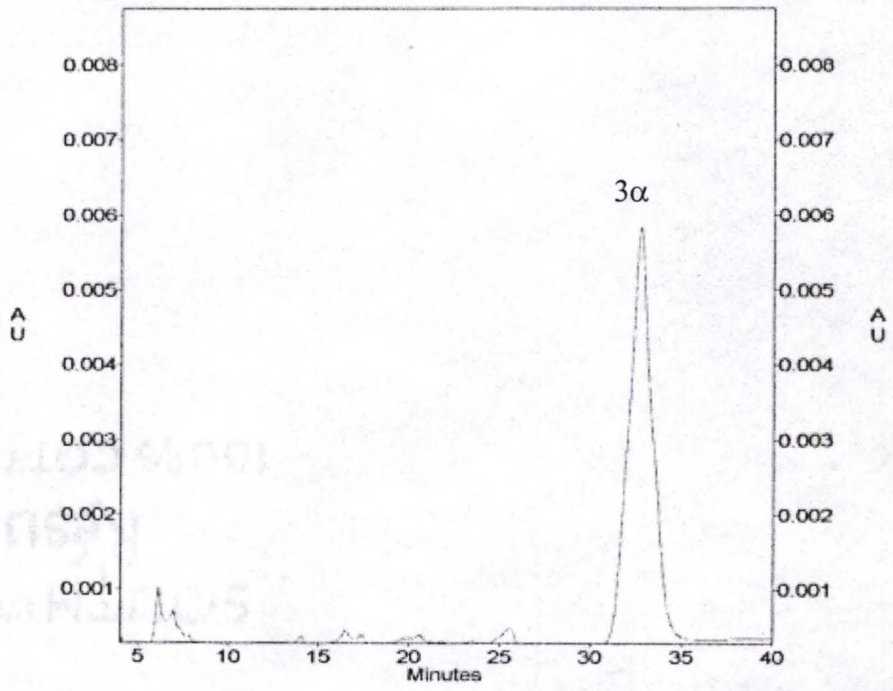


Figure 3-28. HPLC (UV detection) profile of 4-androsten-3α,17β-diol standard.

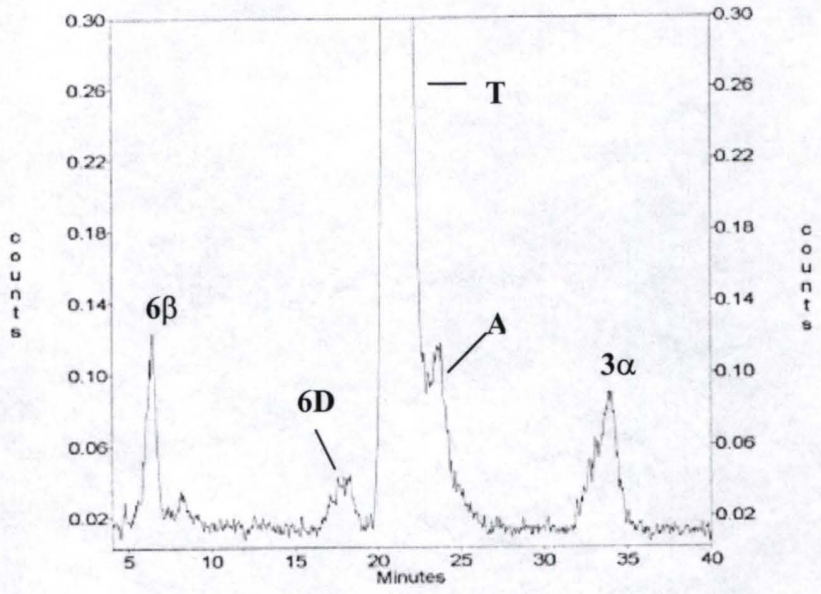


Figure 3-29. HPLC (radiochemical detection) of [C^{14}] testosterone metabolism catalyzed by catfish intestinal microsomes. Catfish intestinal microsomes (0.4 mg) was used; substrate concentration 30 μM.
Abbreviations: 6β, 6β-hydroxy-testosterone; 6D, 6-dehydrotestosterone or 17β-hydroxy-4,6-androstadiene-3-one; T, testosterone; A, androstenedione; 3α, 3α-hydroxytestosterone or 4-androsten-3α,17β-diol.

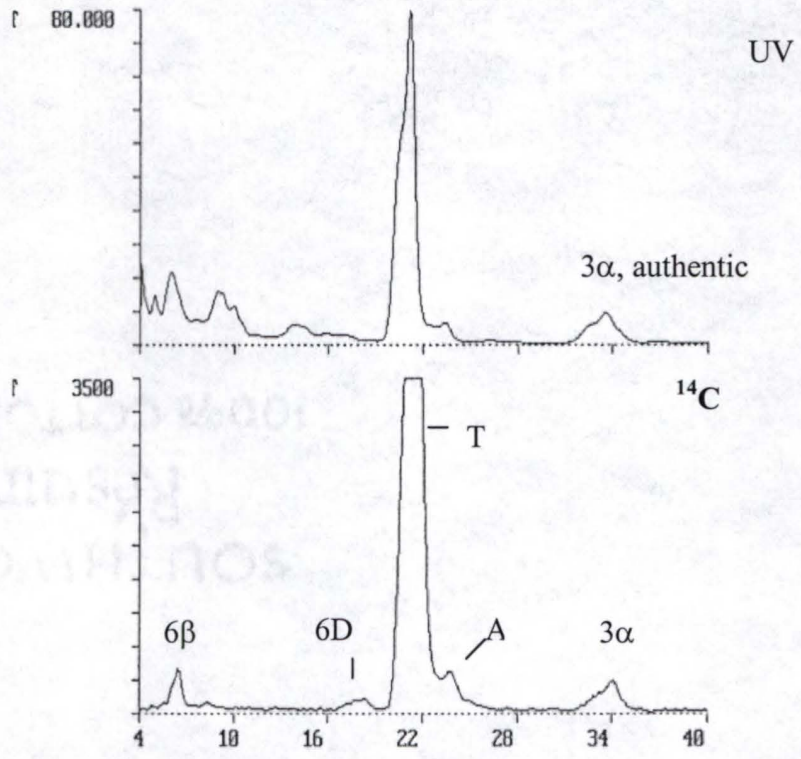


Figure 3-30. HPLC analysis of mixture of 4-androsten-3α,17β-diol and [^{14}C] testosterone assay extract. Abbreviations: 6β, 6β-hydroxy-testosterone; 6D, 6-dehydrotestosterone or 17β-hydroxy-4,6-androstadiene-3-one; T, testosterone; A, androstenedione; 3α, 3α-hydroxy-testosterone or 4-androsten-3α,17β-diol.

Mass Spectrometric Analysis of Testosterone Metabolite

To confirm the structure of metabolite to be 4-androsten-3α,17β-diol, the metabolite was isolated and subjected to mass spectroscopy. ESI-MS did not provide efficient ionization of the sample. With APCI-MS, both the 4-androsten-3α, 17β-diol standard and the testosterone metabolite gave related m/z 255 and 273 ions and m/z 289, with only very low abundance of the expected m/z 291$[M+H]^+$ ion. Among the three daughter ions, m/z 273 is the most intense ion, followed by m/z 255 and m/z 289. The (+)APCI-MS/MS daughter spectra of all three of these ions from the testosterone metabolite matched those obtained from the 4-androsten-3α, 17β-diol standard (Figure 3-31, 3-32, 3-33). Attempts to increase the yield of m/z 291 ions were not successful. The m/z 255 and 273 ions are probably due to $[M+H-2H_2O]^+$ and $[M+H-H_2O]^+$ ions of the 4-androsten-3α, 17β-diol (MW 290). It is possible that the MW 290 steroid is thermally labile, eliminating a H_2O molecule forming a MW 272 compound which is then ionized. The m/z 289 is likely the $[M+H]^+$ ion of another steroid, which is probably testosterone (Figure 3-34).

In summary, identification of the 3α-reduced testosterone metabolite was based on a complete agreement of its chromatographic pattern with that of the authentic steroid during chromatography on the reverse phase HPLC column and on TLC in the system ethyl ether: toluene: methanol: acetone (70:38:0.8:1) and on a perfect agreement between the mass-spectrum of the three daughter ions (m/z 255, 273, and 289) of the testosterone metabolite with that of the authentic steroid (Figure 3-31, 3-32, 3-33).

In vitro metabolism of testosterone by catfish intestinal microsomes is summarized in figure 3-35.

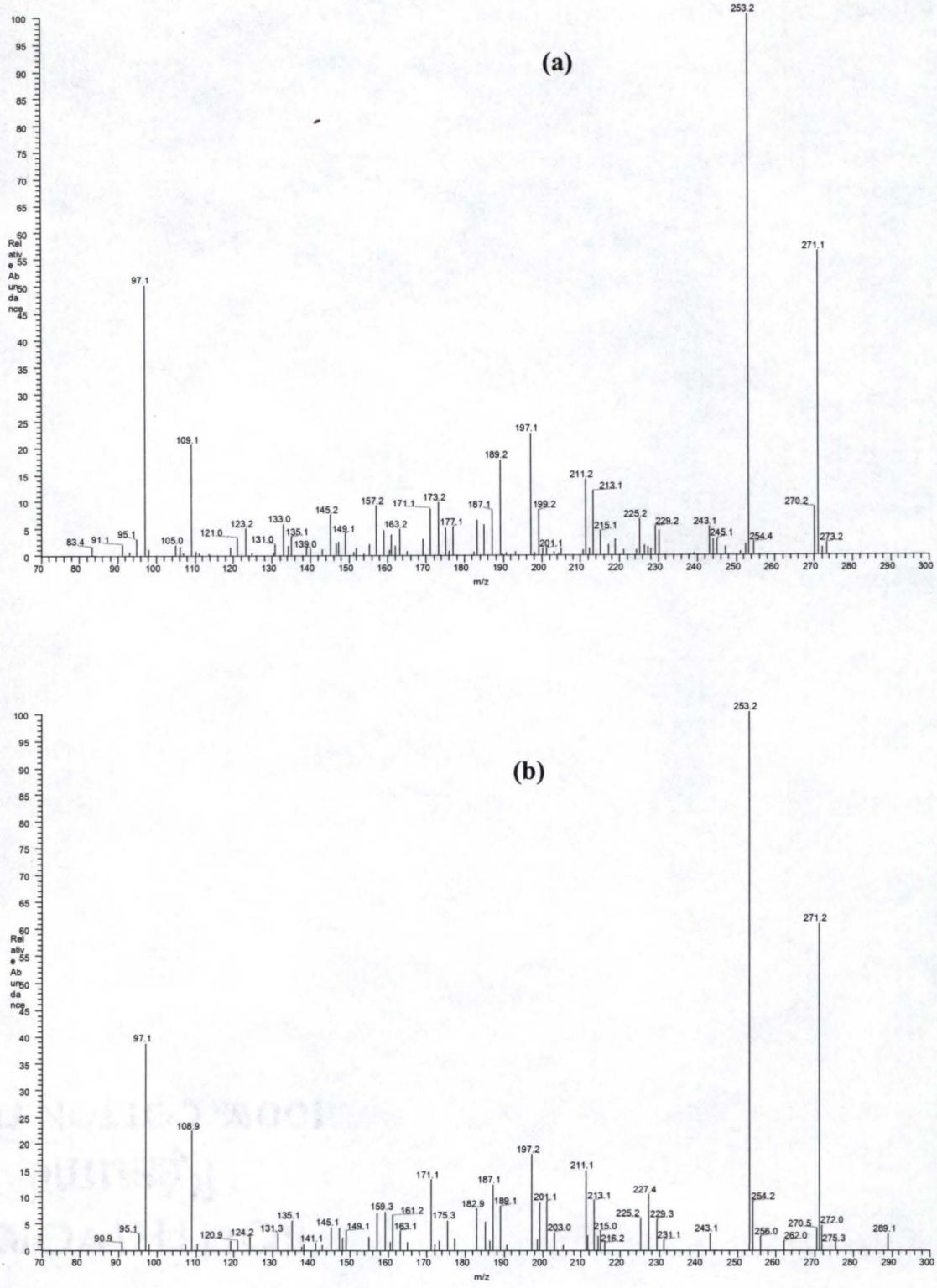
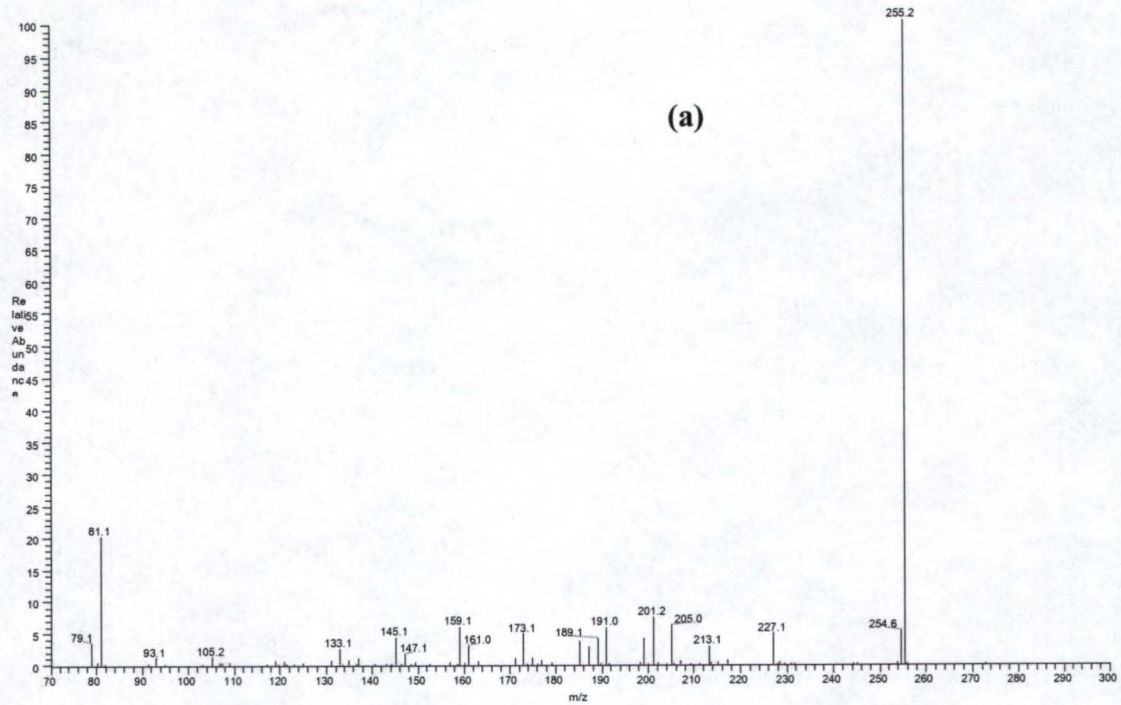


Figure 3-31. (+)APCI-MS/MS daughter spectra of m/z 289 ions. The spectra are from (a) authentic 4-androsten-3α,17β-diol; (b) metabolite isolated from testosterone incubation.



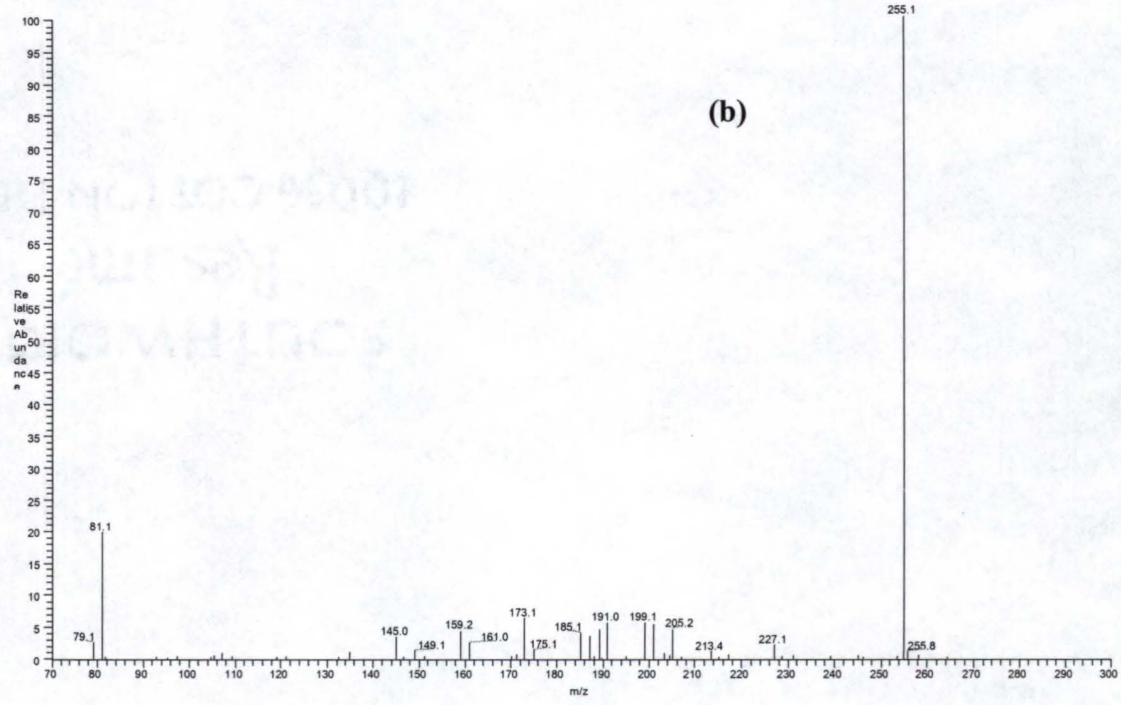


Figure 3-32. (+)APCI-MS/MS daughter spectra of m/z 273 ions. The spectra are from (a) authentic 4-androsten-3α,17β-diol; (b) metabolite isolated from testosterone incubation.

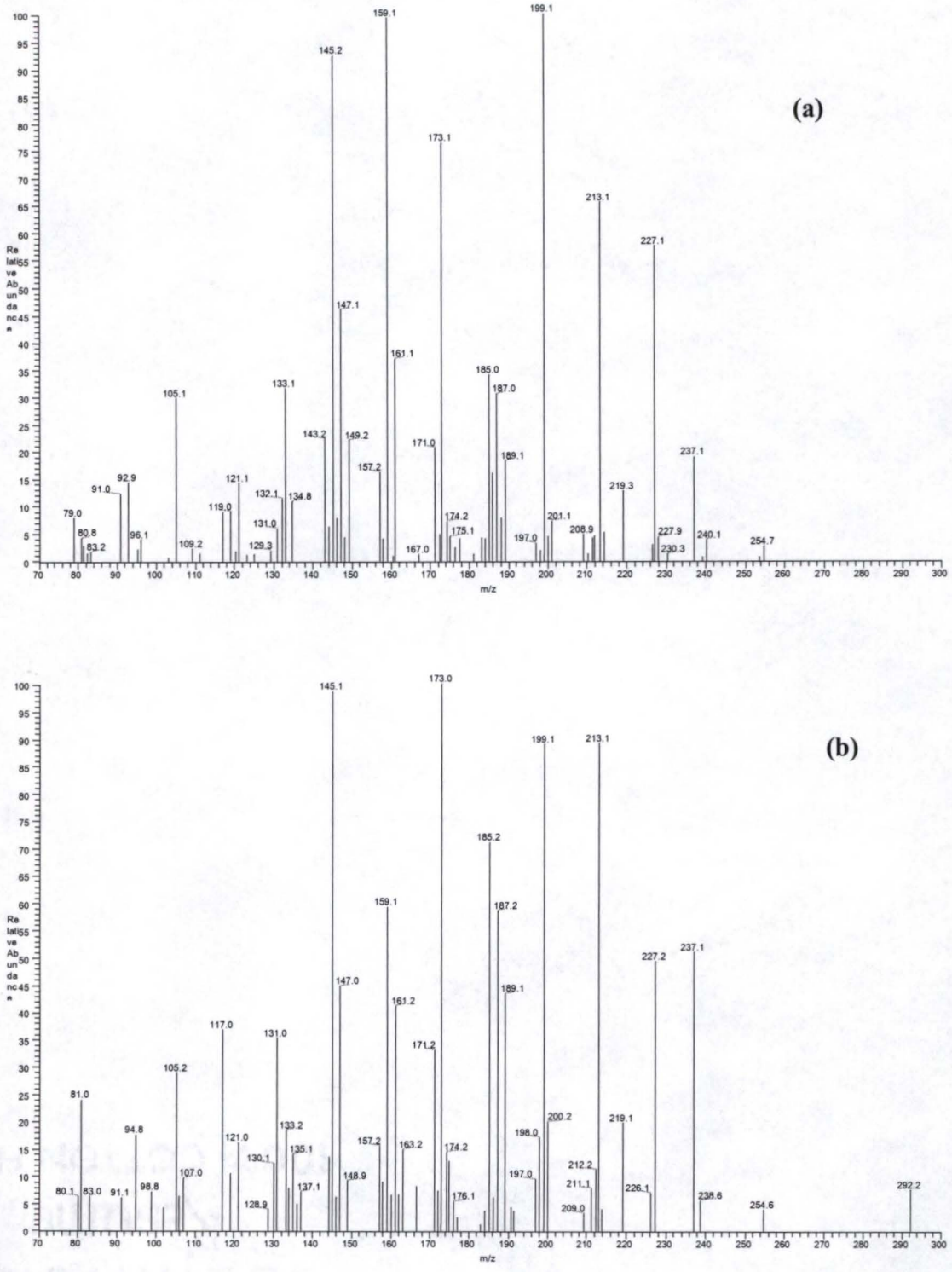


Figure 3-33. (+)APCI-MS/MS daughter spectra of m/z 255 ions. The spectra are from (a) authentic 4-androsten-3α,17β-diol; (b) metabolite isolated from testosterone incubation.

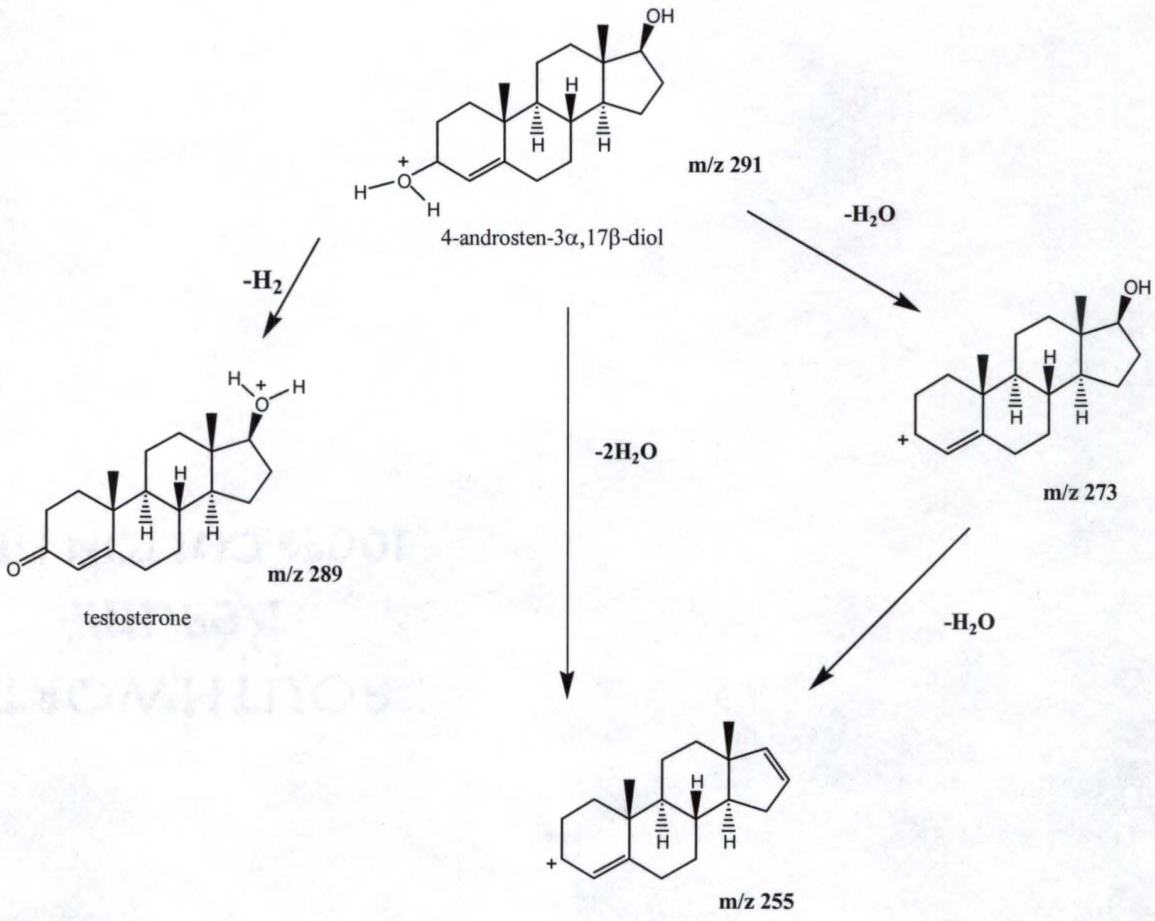


Figure 3-34. Fragmentation of 4-androsten-3α,17β-diol in APCI-MS.

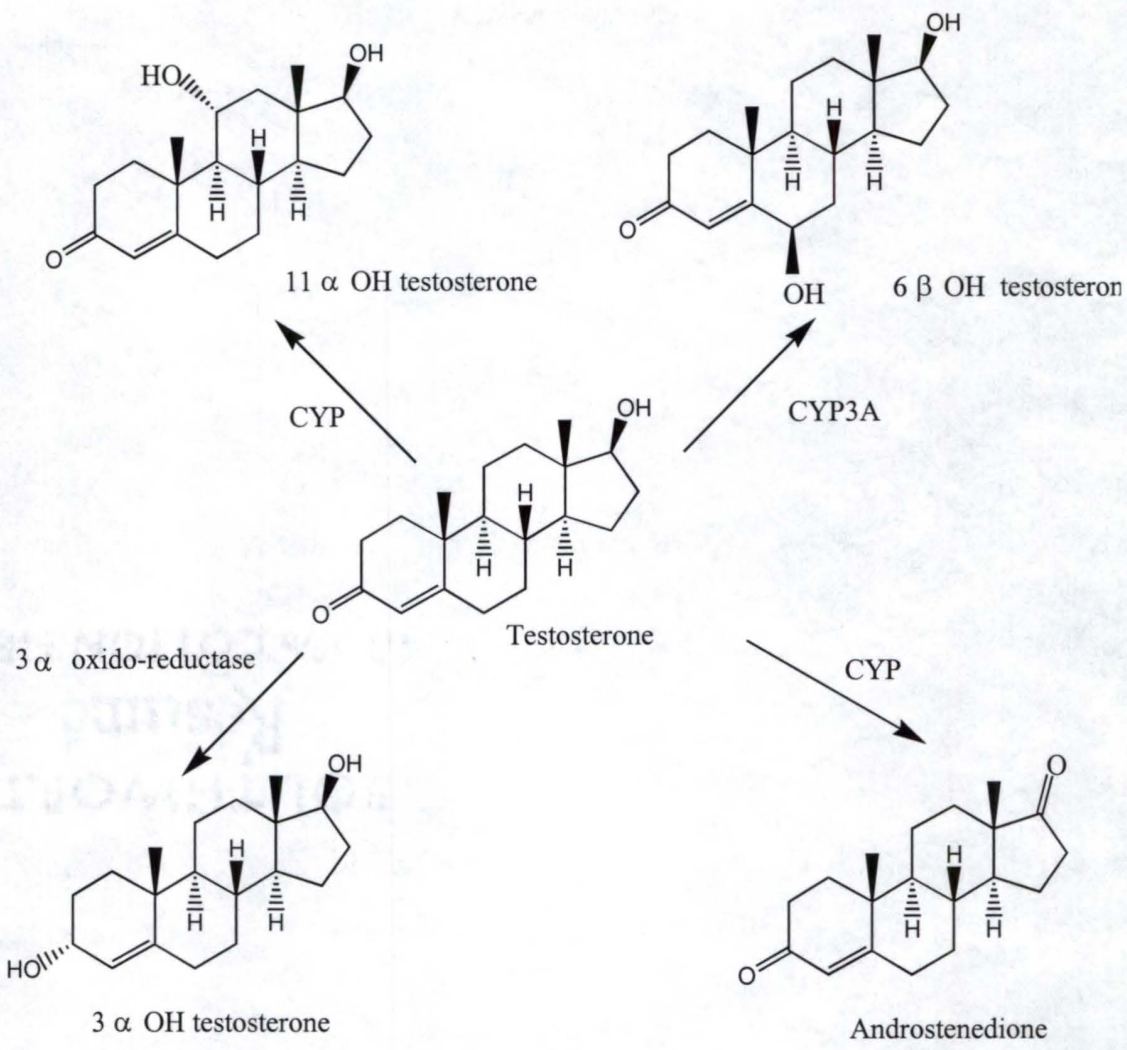


Figure 3-35. *In vitro* metabolism of testosterone by catfish intestinal microsomes.

Regional Expression of 3α-Hydroxysteroid Dehydrogenase in Catfish Intestine

Table 3-5 summarizes the in vitro testosterone metabolism from catfish fed semi-synthetic diet or commercial chow. Both groups of catfish showed higher testosterone 3α-oxido-reduction in proximal intestine than distal. In proximal intestine, testosterone 6β-hydroxylation, but not 3α-oxido-reduction, was significantly higher in fish fed commercial chow than those fed semi-synthetic diet (Table 3-5). Our previous work has indicated that testosterone 6β-hydroxylase in catfish intestinal microsomes was mediated by CYP3A-like enzymes. From the above results, we found that diet may play a more significant part in CYP3A expression than expression of 3α-hydroxysteroid dehydrogenase in catfish intestine, especially in the proximal section. The formation of 4-androsten-3α, 17β-diol from testosterone by catfish intestinal microsomes was not affected by the two general cytochrome P450 inhibitors, metyrapone and SKF-525A.

Table 3-5. Testosterone metabolism by intestinal microsomes from catfish fed with chow or semi-synthetic purified diet

Diet	Testosterone Metabolism			
	6β-OH	3α-reduction	17-oxidation	total metabolism
	pmol/min/mg protein			
Chow (n=4)				
Proximal	262.8±80.3 [a,b]	346.1±210.5	285.8±119.5 [aa,b]	989.9±363.5
Distal	88.6±15.6	292.7±220.7	154.1±42.7	622.3±225.5
Purified (n=5)				
Proximal	158.4±26.3 [a]	325.4±83.9 [a]	117.0±15.7	691.8±13.6 [aa]
Distal	108.8±44.4	162.0±38.1	136.6±56.4	481.6±158.6

[a] significantly higher than the corresponding distal values by one tailed student t-test for paired samples: $p<0.01$.

[aa] significantly higher than the corresponding distal values by one tailed student t-test for paired samples: $p<0.05$.

[b] significantly different from the corresponding proximal values for purified diet group by single factor ANOVA: $p<0.01$.

Catfish Intestinal CYP3A Inducibility Studies

CYP3A enzyme amount in proximal and distal intestine was measured in control fish and fish treated with rifampicin (RIF) for 2 or 4 weeks or with PCN for 1 or 3 weeks. The CYP3A quantification was achieved by immunoblotting using Supersomes® human CYP3A4 as standard. Figure 3-36 shows a typical Western blot of intestinal microsomes from control and treated fish. There is no significant difference in intestinal CYP3A level between control and any treated groups, either in the proximal or distal segment (Figure 3-37).

On the contrary, it seemed that testosterone 6β-hydroxylation and 3α-oxido-reduction activities, but not 17-oxidation were statistically significantly higher in distal intestine from fish treated with rifampicin for 2 weeks than control fish (Table 3-6).

(1) (2) (3) (4) (5) (6) (7) (8)

Figure 3-36. Western blot of intestinal CYP3A from control and PCN treated fish. Intestinal microsomal protein (40 μg) was in each lane.
(1) and (4): fish treated with PCN for 3 weeks;
(2) and (3): fish treated with PCN for 1 week;
(5)-(8): control fish.

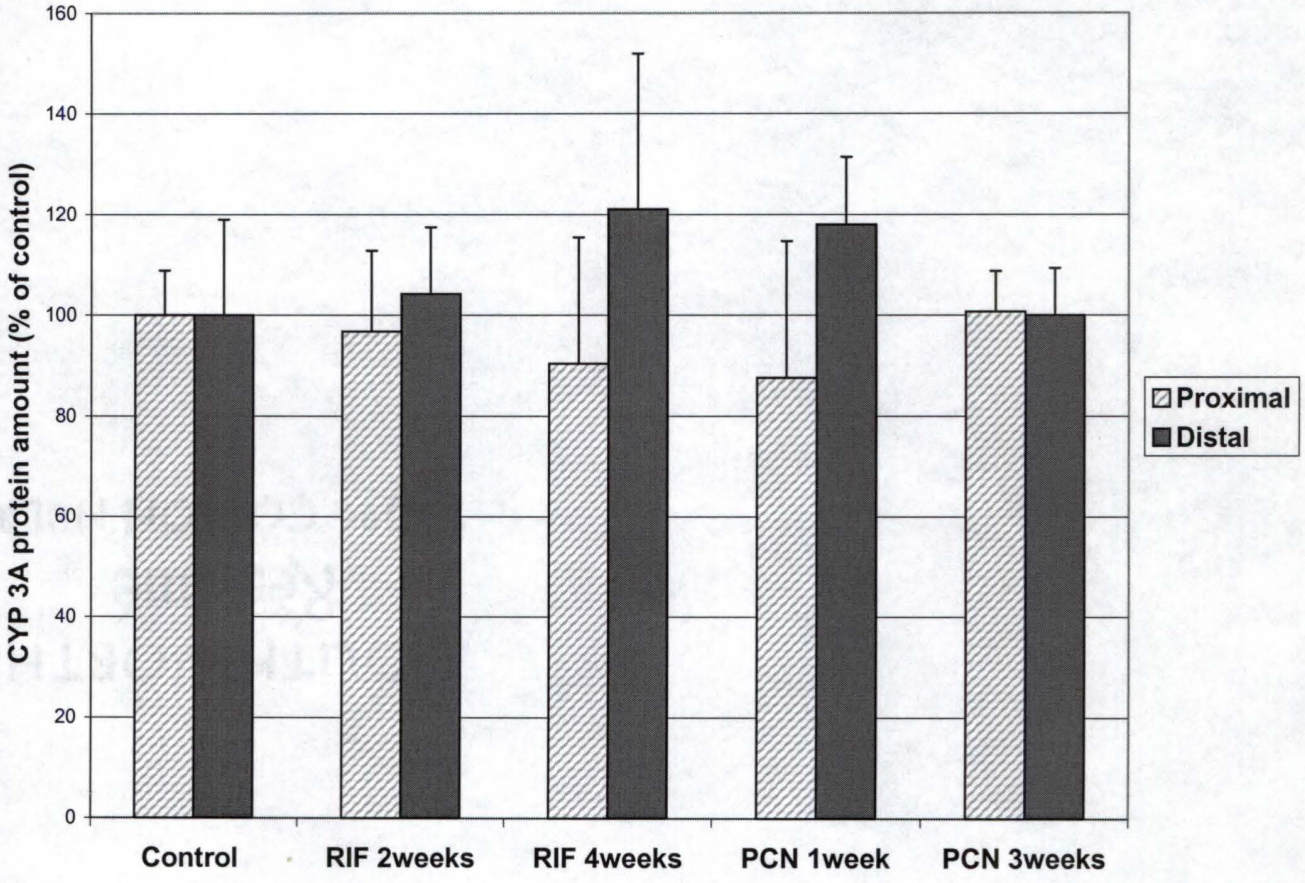


Figure 3-37. Intestinal CYP3A expression in control or fish treated with RIF or PCN. Both proximal and distal intestinal CYP3A protein levels are shown in fish treated with rifampicin (n=4) or PCN (n=3) compared with control fish (n=4).

Table 3-6. *In vitro* testosterone metabolism activities in proximal and distal intestine from control fish and fish treated with rifampicin for two weeks.

		6β-hydroxylation	3α-oxido-reduction	17-oxidation	Total Metabolism	Ratio (6β-OH/17-oxidation)
		(pmol/min/mg protein)				
Proximal	Control	107.5±8.66	156.8±48.7	73.1±3.75	395±41.7	1.47±0.15
	RIF treatment	108.1±11.4	142.5±26.3	74.3±3.75	370±40.7	1.46±0.01
Distal	Control	40.6±5.54	66.2±12.9	50.0±2.04	178.1±19.6	0.82±0.11
	RIF treatment	61.3±11.6*	83.7±9.24**	65.6±18.1	231.8±0.01*	0.95±0.12

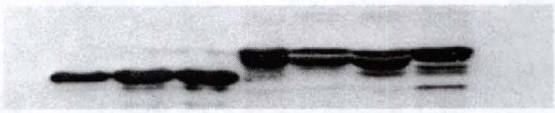
*: $p<0.01$
**: $p<0.05$

Catfish Hepatic CYP3A Expression

Figure 3-38 indicates Western blotting results of CYP3A expression in catfish liver. More than one band in catfish hepatic microsomes cross-reacted with polyclonal antiCYP3A27 antibody. There were individual differences in the CYP3A bands but no sex differences can be concluded from Western blotting results (Figure 3-39).

PCN (10 mg/kg body weight), but not rifampicin (10 mg/kg) pretreatment of catfish resulted in a slight increase in hepatic CYP3A amount (Table 3-7).

Although PCN and rifampicin pretreatment caused a slight increase in testosterone 6β-hydroxylation activities, it is not statistically different ($p>0.05$) probably due to high intra-group variation caused by small group size. However, both PCN and rifampicin pretreatment resulted in significant increases in 3α-oxido-reduction and testosterone total metabolism activities (Figure 3-40).



(1) (2) (3) (4) (5) (6) (7)

Figure 3-38. Western blot of hCYP3A4 and hepatic microsomes from control fish and fish pretreated with RIF or PCN (10 mg/kg). Lane (1)-(3) has 3, 5, 7 pmol human CYP3A4; lane (4)-(7) are catfish hepatic microsomes, 40 μg per lane. Lane (4) and (5) are from control fish; lane (6) from fish pretreated with rifampicin for 2 weeks; lane (7) from fish treated with PCN for 1 week. Lane (5) and (6) were from female fish; lane (4) and (7) were from male fish.

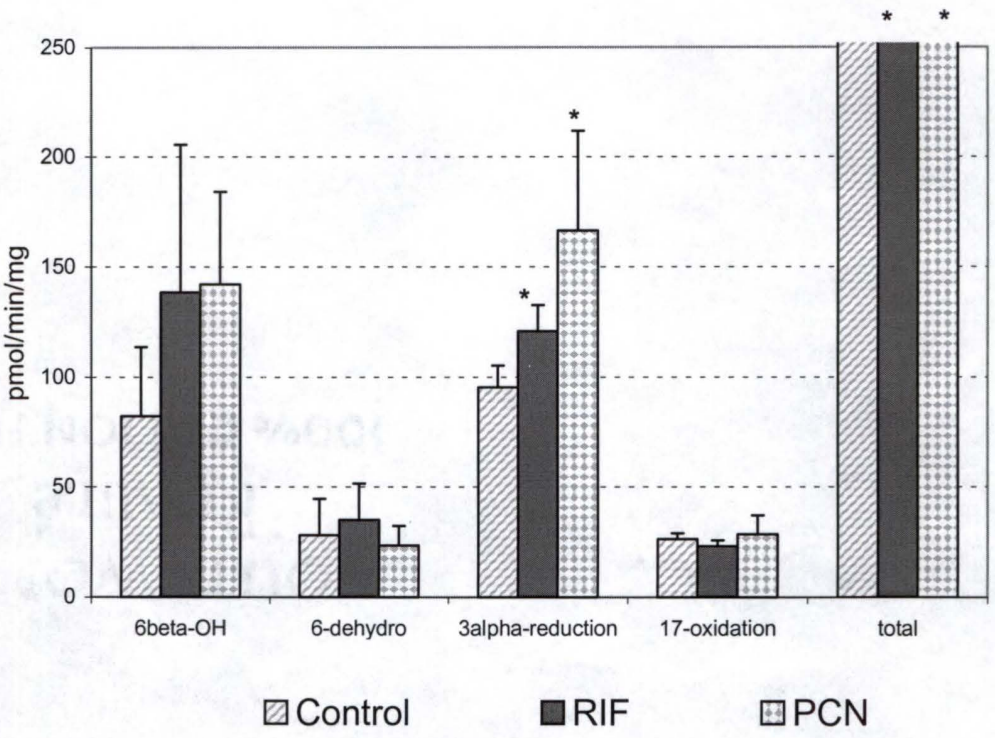
(1) (2) (3) (4) (5) (6) (7) (8)

Figure 3-39. Western blot showing cross-reactivity of catfish hepatic microsomes against a polyclonal antibody to trout CYP3A27. Lane 1-8 has 40 μg hepatic microsomes protein per lane. Lane (1) (2): from control fish; lane (3), (4), (5): from fish pretreated with rifampicin (10 mg/kg) for 2 weeks; lane (6): from fish pretreated with rifampicin (10 mg/kg) for 3 days; lane (7) and (8): from fish pretreated with PCN (10 mg/kg) for 1 and 3 weeks, respectively. (1), (3), (4) and (7) were from female fish; (2), (5), (6) and (8) were from male fish.

Table 3-7. Hepatic CYP3A expression in control and treated fish.

Treatment	Body Weight	Liver Weight	Protein Yield	CYP3A
	(g)	(g)	(mg/g)	(pmol/mg)
Control (n=4)	1032.8 ± 167.5	20.96 ± 0.26	10.01 ± 2.30	178.96 ± 15.98
RIF (n=4)	1303.5 ± 116.8	23.16 ± 3.44	8.73 ± 2.70	169.02 ± 33.01
PCN (n=3)	1767.3 ± 712.5	28.61 ± 14.31	8.13 ± 1.92	205.76 ± 15.40*

*: $p<0.05$



***: p<0.05.**

Figure 3-40. *In vitro* testosterone metabolism activities by hepatic microsomes of fish from control, rifiampicin (RIF) and PCN pretreated groups.

CHAPTER 4
DISCUSSION

Induction of CYP1A and UGT in Catfish Intestine

Intestinal AHH activity and CYP1A content were not affected by the 0.5 mg tetrachlorobiphenyl (TCB)/kg-diet pretreatment and showed induction, but great variability, with the 5.0 mg TCB/kg diet. The consistency of AHH activities at near-control values for the 0.5 mg TCB/kg-diet dose, and the lack of detectable CYP1A, suggest that this dosage may be insufficient to induce intestinal AHH activity when fed at approximately 0.5% of body weight. Conversely, detectable CYP1A content and AHH activities ranging from control levels to more than 12-fold higher with the 5 mg/kg diet dosage indicate that TCB is capable of intestinal induction. Previous studies have demonstrated dose-dependent induction and inhibition of fish hepatic CYP1A catalytic activities by TCB with induction at low and high doses while inhibition occurs only at high doses [13,114]. The differential response seen in the catfish intestine is most likely differential induction resulting from factors associated compound ingestion and bioavailability. Direct correlation of AHH activity with CYP1A content for individual animals suggests that the variability was not related to TCB inhibitory effects.

Administration by gavage of 10 mg/kg 3MC yielded induction of CYP1A in all fish, though again a variable response was observed. This variability may be due to different uptake by intestine orally, or to individual genetic difference between fish.

UGT activity was also induced by 3-MC treatment. The induction of UGT correlates with the induced AHH activity (r^2=0.75). The UGT expression in catfish intestine showed similar inducibility as CYP1A by AhR agonists. At least two isoforms (UGT1A6 and UGT1A9) of human UGTs have been shown to be induced by AhR inducers [115]. The AhR agonist 3-MC is a bifunctional inducer, inducing both phase I (CYP1A) and phase II (UGT) enzymes in catfish intestine

In vitro Testosterone Metabolism by Catfish Intestinal Microsomes

Steroid hydroxylation reactions catalyzed by cytochrome P450s serve numerous physiological functions including catabolism of cholesterol to bile acids, activation of vitamin D_3, and the biosynthesis of all major classes of steroid hormones [116]. Steroid hormones are subject to site-specific hydroxylation reactions catalyzed by many, but not all of the more than 20 distinct P450 enzymes [117]. Studies carried out using hepatic P450s purified from rodent and human resources have established that individual P450 enzymes exhibit unique patterns of steroid hormone hydroxylation. These patterns can be both characteristic of individual P450s and diagnostic of the identity and purity of isolated P450 preparations. Hydroxysteroid metabolite patterns can also be useful in monitoring the relative concentrations of individual P450 forms present in microsomal fractions that simultaneously express multiple cytochrome P450.

Our present results show that testosterone was hydroxylated at 6β- and 17α-, and to a lesser extent, 11α-positions by catfish intestinal microsomes. The metabolism of testosterone is concentration-dependent on both testosterone and the cofactor NADPH. The K_M of testosterone 6β-hydroxylation was lower than those of the other two major metabolites, indicating that this may be the most important physiological pathway

because of the low physiological concentration of the substrate. Another major metabolism pathway of testosterone by catfish intestinal microsomes is 3α-reduction, probably via 3α-hydroxysteroid dehydrogenase. 3α-Hydroxysteroid dehydrogenase catalyzes the reversible interconversion of hydroxy and carbonyl groups at position 3 of the steroid nucleus. In mammalian tissues, 3α-hydroxysteroid dehydrogenase works in concert with 5α- and 5β-reductase to convert 5α/5β-dihydrosteroids into 5α/5β-tetrahydrosteroids [118,119]. Human 3α-hydroxysteroid dehydrogenase plays an important role in steroid hormone metabolism and action [96]. In rodents, 3α-hydroxysteroid dehydrogenase showed higher activities in cytosolic fraction than in microsomes, using dihydrosteroids as substrates [88,89]. Our results showed that the 3α-oxido-reduction of testosterone by catfish intestinal microsomes is NADPH-dependent and there is no direct formation of 4-androsten-3α, 17β-diol observed using intestinal cytosol (not shown).

CYP3A Expression in Catfish Intestine

Small intestinal cytochromes P450 provide the principal, initial source of biotransformation of ingested xenobiotics. The P4503A subfamily has been demonstrated to be particularly prominent in the human small intestine [19,100]. An important cause of incomplete bioavailability of many drugs is prehepatic metabolism in the GI tract, mainly by the CYP3A subfamily of enzymes. Although most previous studies of CYP in fish have focused on the PAH-inducible CYP1A subfamily, CYP3A in fish is clearly an important constitutive enzyme. The CYP3A-like enzyme has good cross-reactivity with the polyclonal antibody rabbit-anti-trout CYP3A27, showing the same molecular weight

of 59 kDa as trout hepatic CYP3A27 [120,47,23]. This CYP3A-like protein was expressed in untreated catfish intestine at significant levels. Both this fact and results showing that two steroids and benzo(a)pyrene could be metabolized by intestinal microsomes from control fish imply that the CYP3A enzyme is constitutively expressed, and it may have important endogenous functions in catfish intestine. This CYP3A protein was expressed gradually along catfish intestine, showing the highest amount at the proximal end. This longitudinal distribution is similar to the expression of most CYP enzymes in mammalian intestine [100]. Correlation between testosterone 6β-hydroxylation activities and immunochemically measured CYP3A content (r^2=0.79) suggests that testosterone 6β-hydroxylation is a good catalytic marker of CYP3A enzymes in fish species as well as in mammals.

Chemical Inhibition of *in vitro* Testosterone Metabolism

Chemical inhibition of a CYP enzyme can go through one or more than one of the following three mechanisms: (1) reversible inhibition; (2) formation of MI-complex; (3) irreversible mechanism-based (suicide) inhibition [27]. In the present study, we used two global CYP enzyme inhibitors (metyrapone, SKF-525A) and three specific mammalian CYP3A inhibitors (ketoconazole, erythromycin and troleandomycin) [26,121]. Among these five inhibitors, metyrapone and ketoconazole are categorized as reversible inhibitors. A reversible inhibitor (or its metabolite) binds to a CYP enzyme in a direct, rapidly reversible way. Reversible inhibition is either competitive or noncompetitive, the extent of which is determined by the relative binding constants of substrate and inhibitor for the enzyme and by the inhibitor's concentration [27]. SKF-525A and troleandomycin

are classified as quasi-irreversible inhibitors of mammalian P450. The oxidation of these two N-alkyl-substituted compounds forms a nitroso derivative, which binds tightly to the ferrous iron of P450 heme to form the MI complex. Erythromycin inhibits human CYP3A in both competitive reversible and quasi-irreversible way. The formation of an inhibitory cytochrome P450•Fe^{2+}-metabolite complex can easily be detected in spectral analysis of a peak at 456 nm [28,32,122]. Preincubation was performed in our studies for quasi-irreversible inhibitors to examine the possibility that these inhibitors may be metabolized by catfish intestinal microsomal enzymes to products that form complex with the CYP3A enzyme. Surprisingly, we found that TAO, the selective CYP3A inhibitor for mammals, showed no inhibition of formation of any of the three metabolites produced by catfish intestinal microsomes. This is in accordance with the literature reports of the lack of inhibitory effect of TAO on trout monooxygenase [41]. A possible explanation for the lack of inhibitory effect of TAO is the inability of catfish CYP3A to form a MI complex with TAO. CYP3A in catfish intestine may be inactive in catalyzing TAO N-demethylation, an obligatory step in the formation of the nitroso intermediate. SKF-525A, also an amine and a quasi-irreversible inhibitor of mammalian P450, was found to inhibit CYP3A-mediated testosterone 6β-hydroxylation even without preincubation with the inhibitor. We concluded that the inhibition of testosterone 6β-hydroxylation caused by SKF-525A may be the result of competitive reversible inhibition. Further kinetic study is needed to verify this assumption. Testosterone 6β-hydroxylation activities were inhibited by erythromycin, ketoconazole, metyrapone and SKF-525A, but not affected by α-naphthoflavone (Figure 3-21, 3-22, 3-23, 3-24, 3-25, 3-26). The inhibition by erythromycin, ketoconazole, metyrapone and SKF-525A was

specific toward 6β-hydroxylation, only ketoconazole showed slight inhibition of testosterone 17-oxidation at high concentration of the inhibitor. In summary, the results of the chemical inhibition studies showed which of the reversible and quasi-irreversible inhibitors of mammalian P450s are effective inhibitors of catfish P450. α-Naphthoflavone, the specific CYP1A inhibitor, did not affect CYP3A-mediated testosterone 6β-hydroxylation activities. The inhibitory potency of testosterone 6β-hydroxylation is: ketoconazole > metyrapone > SKF-525A > erythromycin. Troleandomycin is inactive toward catfish intestinal CYP3A enzymes. Thus, caution must be observed in the use of mammalian P450 inhibitors as probes for the involvement of P450 in the metabolism and toxicity of chemical in fish.

Stimulation of AHH Activities by α-Naphthoflavone

Our results suggest that CYP3A is important in biotransformation of benzo(a)pyrene in fish that was not exposed to an inducer of CYP1A. No CYP1A was detected in control fish [123], but benzo(a)pyrene was metabolized to fluorescent metabolites, and this metabolism was stimulated by α-naphthoflavone. Previous studies showed that α-naphthoflavone inhibited CYP1A-dependent metabolism of benzo(a)pyrene [38]. Others have shown that α-naphthoflavone can stimulate CYP3A-dependent monooxygenase activity with some, but not all substrates [53,55,124,125,126,127]. In this regard, the CYP3A like protein in catfish intestine appears to behave similarly to other CYP3A proteins in that α-naphthoflavone stimulates activity with some, but not all, substrates [127].

Inducibility of Catfish Intestinal CYP3A

In contrast to mammals, very little is known about the inducibility of the CYP3A enzymes in fish. Interestingly, it has been found that the pretreatment of rainbow trout with 3,4,5,3',4',5'-hexachlorobiphenyl (1 mg/kg) significantly increases the progesterone 6β-hydroxylase activity of trout liver with no effect on CYP3A levels [128]. Our results indicate that the CYP3A has a lower expression in the intestine of catfish fed purified diet than those fed chow. This finding correlates with the fact that the hepatic CYP3A content in teleost fish was under dietary modulation [52].

Mammalian CYP3A enzymes are highly inducible by the synthetic glucocorticoid (dexamathasone), macrolide antibiotic (rifampicin), synthetic steroid (pregnenolone 16α-carbonitrile) and phenobarbital. Yet, the two mammalian CYP3A inducers we used in catfish study, *i.e.* rifampicin and PCN, failed to induce intestinal CYP3A protein level at the dose of 10 mg/kg body weight/day. While fish treated with rifampicin for two weeks showed significantly higher testosterone 6βhydroxylation and 3α-oxido-reduction activities in their distal intestine compared to that in control fish, the 6β-OH/17-oxidation ratio was not changed, again indicating no significant induction of CYP3A.

Identification of 3α-Reduced Metabolite of Testosterone

The present study demonstrated that, in addition to hydroxylation, testosterone was also reduced at the 3-keto position to form 4-androsten-3α, 17β-diol by catfish intestinal microsomes. The latter pathway is not likely to be a P450-mediated process as it is not affected by any of the cytochrome P450 inhibitors used in this study, *i.e.,* metyrapone, SKF-525A, ketoconazole, erythromycin, α-naphthoflavone. These 3α-

reduction activities were mainly in the microsomes rather than in cytosolic fraction, which is similar to that of human liver [90]. Although testosterone 6β-hydroxylation has the lowest K_M value, 3α-oxido reduction has the highest V_{max} (Table 3-2), suggesting that the 3α-oxido reduction may play an important role in endogenous function. Another metabolic pathway for testosterone is 6-dehydrogenation. It was shown that 6-dehydro testosterone was formed directly from testosterone by CYP3A isozymes that catalyze 6β-hydroxylation of testosterone in rat liver microsomes; the formation of 17β-hydroxy-4,6-androstadiene-3-one was enhanced by pretreatment with phenobarbital, pregnenolone 16α-carbonitrile, and dexamethasone [129]. Both 6β-hydroxylation and 6-dehydrogenation are likely mediated by CYP3A isozymes in catfish intestine [130].

The (+)APCI-MS/MS daughter spectra of three major daughter ions from the testosterone metabolite matched those obtained from a 4-androsten-3α, 17β-diol standard. The m/z 255 and 273 may be the $[M+H-2H_2O]^+$ and $[M+H-H_2O]^+$ ions, respectively, of the MW 290 steroid or they may be the $[M+H-H_2O]^+$ and $[M+H]^+$ ions, respectively, of a MW 272 decomposition product or thermally degraded MW 290 steroid. Similarly, the m/z 289 may be the $[M+H]^+$ ion of a MW 288 compound (*e.g.*, testosterone), the $[M+H-H_2O]^+$ ion of an MW 306 compound or be due to thermal dehydrogenation of the MW 290 steroid during the vaporization (220 °C and 300 °C) process. The daughter spectra from the testosterone metabolite are almost identical to those obtained from the authentic steroid indicating they are most likely the same structures.

Methods commonly used for identification of steroid hormone metabolites include high-performance liquid chromatography (HPLC), thin layer chromatography

(TLC) and gas- or liquid-chromatography-mass spectrometry (GC-MS and LC-MS). Our identification process of this 3α-reduced testosterone metabolite suggested that a combination of these methods is perhaps the ideal way to identify a steroid hormone metabolite definitively in laboratories that have access to the specialized equipment required.

In mammalian tissues, 3α-hydroxysteroid dehydrogenase works in concert with 5α- and 5β-reductases to generate the 3α,5α- and 3α,5β-tetrahydroxysteroids, respectively, thereby acting as a molecular switch in steroid hormone activation [131]. Our finding of high activities of 3α-hydroxysteroid dehydrogenase in catfish intestinal microsomes and activities of 5α-steroid reductase in intestinal cytosol suggested that these two reductases could work in concert *in vivo*. The biological significance of this steroid metabolism pathway in fish is not very well known.

Catfish Hepatic CYP3A Expression

The reason for the inducibility of PCN in hepatic CYP3A enzyme amount quantified by densitometric scans of immuno blots, but lack of effect on the CYP3A-mediated testosterone 6β-hydroxylation activities is still unknown. Whether a larger dose of PCN or rifampicin would have resulted in both CYP3A enzyme amount and its catalytic activities remains to be determined.

CHAPTER 5
SUMMARY AND CONCLUSIONS

In conclusion, the present study showed that a CYP3A-like protein was expressed in catfish intestine. It is expressed gradually along intestine, being the highest at the proximal end. Testosterone 6β-hydroxylation is a good biomarker for this CYP3A-isozyme and this catalytic activity could be inhibited by P450 inhibitors, metyrapone, ketoconazole, SKF-525A and erythromycin. Intestinal CYP3A expression and its catalytic activities were under dietary modulation, being higher in fish fed commercial chow than those fed semisynthetic purified diet. The mammalian CYP3A inducers, rifampicin and PCN, were not able to induce CYP3A in catfish intestine at the dose of 10 mg/kg fish body weight/day. In addition, testosterone was metabolized to 4-androsten-3α, 17β-diol and androstenedione by catfish intestinal microsomes. The former 3α-oxido reduction process is likely mediated by 3α-hydroxysteroid dehydrogenase. The activity of 3α-hydroxysteroid dehydrogenase was mainly in the microsomal fraction in catfish intestine, which was similar to that of human rather than rodents.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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