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FUNCTIONAL CHARACTERIZATION OF HUMAN CYTOCHROME P450 2S1 USING A SYNTHETIC GENE-EXPRESSED PROTEIN IN *E. coli*

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Running title: Functional Characterization of human CYP2S1

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CYP and P450, cytochrome P450; Dioxin, 2,3,7,8-tetrachlorodibenzo-p-dioxin; , DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; Kpi buffer, potassium phosphate; PCR, polymerase chain reaction; PMSF, phenylmethanesulphonylfluoride

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Abstract

Human cytochrome P450 2S1 was recently identified and shown to be inducible by dioxin and hypoxia. It is highly expressed in epithelial cells of tissues that are exposed to the environment and in many tumors of epithelial origin. The biological function of CYP2S1 has not yet been determined, although its possible role in carcinogen metabolism has been suggested. In this report, we investigated its ability to metabolize carcinogens. To obtain a large quantity of active enzyme for substrate screening, we overexpressed CYP2S1 in *E. coli* (200 nmoles/ L culture), using a synthetic gene approach. High-level expression allowed us to achieve purification of CYP2S1 with high specific content and purity (16 nmoles/mg). Despite, high-level expression, we found that CYP2S1 was not readily reduced by cytochrome P450 reductase, and thus no activity was found using NADPH. However, CYP2S1's oxidative activity was supported by cumene hydroperoxide or H_2O_2, such that CYP2S1 oxidized many important environmental carcinogens, including benzo[a]pyrene, 9,10-dihydro-benzo[a]pyrene, 7,12-dimethylbenz[a]anthracene, benzo[a]pyrene-7,8-dihydrodiol, aflatoxin B1, naphthalene, and styrene, with high turnover. Most substrates tested were converted to detoxification products, except in the case of benzo[a]pyrene-7,8-dihydrodiol, which was converted into the very potent carcinogenic metabolite, 7,8-dihydrodiol-*trans*-9,10-epoxide, at a relatively efficient rate (Km=12.43±2μM, turnover=2.3 min^{-1}). This metabolite formation was also supported both *in vitro* and *in vivo* by fatty acid hydroperoxides described in the accompanying report (Bui et al., 2009). Together, these data indicate that CYP2S1 contributes to the metabolism of environmental carcinogens via an NADPH independent activity.

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Introduction

Cytochrome P450s (CYPs) are heme-thiolate enzymes functionally classified as monooxygenase (EC1.14.14.1), which catalyzes a wide spectrum of reactions, e.g. heteroatom oxygenations, carbon hydroxylations, epoxidations, and dehalogenations. P450s play critical roles in bioactivation and detoxication of a wide variety of xenobiotic substances, including environmental toxicants and therapeutic compounds, as well as participating in the endogenous metabolism of fatty acids, steroids, eicosanoids, bile acids, and fat-soluble vitamins. With the sequencing of the human genome, a total of 57 human cytochrome P450s and 58 pseudogenes have been discovered to date (http://drnelson.utmem.edu/ CytochromeP450.html). Of the 57 human cytochrome P450s, 13 remain classified as "orphans", because their functions are still largely unknown (Guengerich et al., 2005). These are P450 2A7, 2S1, 2U1, 2W1, 3A43, 4A22, 4F11, 4F22, 4V2, 4Z1, 20A1, and 27C1. The putative roles of these cytochrome P450s in carcinogenesis and toxicology warrant further study (Stark and Guengerich, 2007).

The *CYP2S1* gene was identified by a homology search in the human genome (Rylander et al., 2001) and also by a representational difference analysis (RDA) subtractive hybridization procedure to isolate novel dioxin inducible genes (Rivera et al., 2002).. Dioxin induction of CYP2S1 is mediated by the aromatic hydrocarbon receptor (AHR) and its dimerization partner, the aromatic hydrocarbon receptor translocator (ARNT) (Hankinson, 1995). Members of families 2 through 4 are not generally inducible by dioxin and PAHs. *CYP2S1* was also shown to be inducible by hypoxia via Hypoxia Inducible Factor I (Hif-1), and by all-*trans*-retinoic acid (Rivera et al., 2007; Smith et al., 2003). Moreover, elevated expression of *CYP2S1* has been detected in the smokers (Thum et al., 2006). The human *CYP2S1* gene, the sole member of the CYP2S subfamily, is located in a cluster of CYP2 family members on chromosome 19q. This

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cluster includes the *CYP2A6*, *CYP2A13*, *CYP2B6*, and *CYP2F1* genes. The proteins encoded by these genes are the closest relatives to human CYP2S1, with 47–49% sequence identity to CYP2S1 (Saarikoski et al., 2005b).

CYP2S1 is highly expressed in epithelial cells of tissues that are exposed to the environment, e.g. skin, respiratory, urinary, and gastrointestinal tracts, and expression appears to be higher than for other CYPs. In addition, CYP2S1 is highly expressed in many tumors of epithelial origin (Saarikoski et al., 2005b). Thus the level of CYP2S1 protein is increased in colorectal cancer and high expression was associated with poor prognosis (Kumarakulasingham et al., 2005). Elevated levels of CYP2S1 protein also occur in metastasic ovarian cancer (Downie et al., 2005). CYP2S1 is primarily an extrahepatic enzyme, since it is expressed in many extrahepatic tissues but very poorly in liver (Bieche et al., 2007).

Based on its mode of regulation and sites of expression, CYP2S1 was suggested to have a potential role in metabolism of carcinogens (Saarikoski et al., 2005a). However, only very limited data on the function of CYP2S1 have been published. Smith and coworkers reported that human CYP2S1 expressed in *E. coli* metabolized all-*trans*-retinoic acid to 4-hydroxy- and 5,6-epoxy-retinoic acid. However, no quantitation was reported (Smith et al., 2003). Wang and coworkers (Wang et al., 2005) expressed human CYP2S1 in Chinese Hamster Ovary (CHO) cells and insect cells, and reported that there was no increase in 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) or benzo[*a*]pyrene toxicity in the transfected CHO cells, and that the heterologously expressed CYP2S1 protein expressed in insect cells failed to metabolize nicotine and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Ingelman-Sandberg's group expressed human CYP2S1 in yeast and reported that it can metabolize naphthalene, but no identification or quantitation of products was presented (Karlgren et al., 2005). Wu and

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colleagues, however, could not detect metabolism of naphthalene or all-*trans*-retinoic acid using *E. coli* recombinant human CYP2S1, even though they obtained high-level bacterial expression of CYP2S1 using chaperon GroEL/ES (Wu et al., 2006). Thus, questions about the role of CYP2S1 have been raised in regard to carcinogen and xenobiotic metabolism, but the results have been ambiguous. In this report, we present an improved method for heterologous expression of human CYP2S1 in *E. coli*. The high-level expression that we obtained allowed us to achieve protein purification with a high yield, and provided us a reliable enzyme preparation source for analyzing and characterizing the function and catalytic activity of CYP2S1.

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Materials and Methods

Chemicals and Reagents

E. coli, LMG 194 was purchased from Invitrogen (Carlsbad, CA). The human *CYP2S1* cDNA was described previously (Rivera et al., 2002). The pBAD plasmid was purchased from Invitrogen (Carlsbad, CA). pCW'/1A2,hNPR, a pCW bicitronic IPTG-inducible plasmid containing the human P450 reductase cDNA (hNPR) was kindly provided by Dr. Fred Guengerich. All restriction digestion enzymes were purchased from New England Biolabs (Beverly, MA). Recombinant human CYP1A1, CYP2C9, CYP2C8, CYP3A4 (all containing cytochrome P450 reductase), and P450 reaction buffer mixtures containing NADP(+)/H regeneration enzymes were obtained from Biocatalytics, Inc (Pasadena, CA). Cumene hydroperoxide, hydrogen peroxide, testosterone, 6-β-hydroxytestosterone, harmaline, all-*trans*-retinoic acid, naphthalene, coumarin, and styrene were obtained from Sigma-Aldrich (St. Louis, MO). The carcinogens, aflatoxin B1, benzo[a]pyrene (BaP), 9,10-dihydro-benzo[a]pyrene (9,10-H_2-BaP), benzo[*a*]pyrene-*trans*-7,8-dihydrodiol (BaP-7,8-diol), benzo[*a*]pyrene-*r*-7,*t*-8,*t*-9,*c*-10 -tetrahydrotetrol (r7,t8,t9,c10-tetrol), 4-hydroxy benzo[*a*]pyrene (4-OH-BaP), 7,12-dimethylbenz[a]anthracene (DMBA), 7,12-dimethylbenz[a]anthracene-trans-3,4-diol (3,4-diol-DMBA), 7-hydroxy methyl-12-methylbenz(*a*)anthracene (7-OH-DMBA), 12-hydroxymethyl-7-methylbenz[*a*]anthracene (12-OH-DMBA) were purchased from the National Cancer Institute's Chemical Carcinogen Repository (Kansas City, MO). IPTG and L-arabinose and the HPLC solvents (HPLC grade) were obtained from Sigma Aldrich (St. Louis, MO). Oligonucleotides were purchased from IDT DNA (San Diego, CA). All the carcinogens were weighed out in a glove box and handled with extreme care.

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Design of a synthetic gene for CYP2S1 to optimize heterologous expression in *E. coli*

Codon and mRNA structure optimization for the *CYP2S1* gene to suit heterologous expression in *E. coli* was as follows. The amino acid sequence of CYP2S1 with N-terminal modification 4 and a C-terminal His-4 tag (see supplementary Table S1) was reverse translated by DNA2.0 software into DNA with the most abundant *E. coli* codons. The DNA sequence was then submitted to the DNA mfold server (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) to theoretically predict the secondary structure of single stranded DNA (which is used in place of single stranded RNA) and its delta G was calculated. Based on the structure and the delta G information, the *CYP2S1* DNA sequence was further optimized by replacing codon(s) in the region where base paring of more than 7 consecutive bases occured. The DNA was then refolded. The final DNA sequence (Figure S1) was sent to GENE ORACLE (Mountain view, CA) for full-length gene synthesis. After the synthetic *CYP2S1* DNA was synthesized and its sequence was confirmed, it was transferred to the pBAD vector using the Nco I and Hind III sites at the 5' and 3' ends, respectively. The synthetic *CYP2S1* DNA was also transferred into the dual-expression vector containing the human P450 reductase (*hNPR)* gene to generate pBdtacHR-cyp2S1 vector (see supplementary Fig. S2).

Expression and processing of CYP2S1

All plasmids were transformed into *E. coli* LMG 194 (Invitrogen, Carlsbad, CA) for expression. The expression trials of various constructs were carried out in 400 ml terrific broth (TB) medium plus 100 µg/ml ampicillin (amp) in 2 L flasks. The conditions were as followed. A 1 ml overnight culture at 30°C in LB (Luria-Bertani) medium + 100 µg/ml amp was transferred into 400 ml TB + 100 µg/ml amp. The culture was grown in a New Brunswick shaker (Edison,

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NJ) at 34°C at 150 RPM. After 5 hours, 0.5 mM delta amino levulinic acid (heme precursor) and 0.5 mM IPTG (inducer for the P450 reductase) were added, and the temperature was reduced to 30°C. One hour later, 0.02% of L-arabinose was added, and the growth conditions were changed to 30°C at 115 RPM, and continued for 24 to 36 hours before harvest. The culture was harvested by centrifugation at 5,000xg for 15 minutes at 4°C. The cell pellet was then resuspended in 10% of the original culture volume in 20 mM potassium phosphate buffer (Kpi), pH 7.5 containing 20% glycerol, 1 mM PMSF, and 0.1 mM DTT. Speroplasts were produced as previously described (Wester et al., 2002). The speroplasts were then resuspended in 100 mM Kpi, pH 7.5 containing 6 mM $MgSO_4$, 1 mM PMSF, and 0.1 mM DTT (4 ml for every gram of cell pellet). The resuspended speroplasts were lysed with a homogenizer at 13,000 psi pressure followed by centrifugation, to collect the CYP2S1-containing lysate. The concentration of CYP2S1 in each culture (nmol/liter culture) was measured using the Fe^{2+} CO vs Fe^{2+} difference spectrum assay of the lysate as described by Omura et al (Omura and Sato, 1964b), utilizing a Shimadzu dual beam UV/VIS spectrophotometer. The CYP2S1 content was calculated using an extinction coefficient of 91 $mM^{-1}cm^{-1}$. P450 reductase activity was estimated using cytochrome C as a substitute electron acceptor, and yields (nmol reductase/liter culture) were calculated assuming a specific activity of 3200 nmol cytC reduced/min/nmol reductase (Yasukochi and Masters, 1976).

NADPH-reduced Fe^{2+}CO vs Fe^{2+} difference assay

Bacterial cell membranes containing P450 and P450 reductase were diluted to 0.2 μM in 100 mM Kpi, pH 7.25, 20% glycerol, and 1 ml aliquots were suspended in each of two cuvets. One of the two cuvets was bubbled with CO for 30 seconds. A baseline spectrum of equal light absorption from 400-500 nm was recorded between the cuvets using a dual-beam

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spectrophotometer (Shimadzu). 1 mM of NADPH was added to both cuvets, and the difference spectrum from 400-500 nm was recorded. 50 µM of substrate was then added to both cuvets and the difference spectrum was repeated. A small amount (<1mg) of dithionite was then added as a positive control and the spectrum was recorded again.

Enzyme assay

All assays were conducted in 1.5 ml Eppendorf tubes in duplicate. For the assays using NADPH to support P450 activities, 1000 µl reactions contained 1x reaction buffer mixture (100 mM Kpi, pH 7.5, NADPH /NADP+ regenerating enzymes), 50-200 nM of recombinant CYP3A4+P450 reductase, 1A2+P450 reductase, 2C8+P450 reductase or 200 nM of CYP2S1+reductase (either non-synthetically or synthetically encoded CYP2S1), and various concentrations of substrates. The reactions were carried out at 37°C for 30 minutes to 2 hours before being terminated with 1000 µl of acetonitrile contained 2% acetic acid. The reactions were frozen in dry-ice methanol for 5 minutes, followed by centrifugation at 13,000xg for 15 minutes to remove proteins. The supernatants were then analyzed by HPLC with diode array and fluorescence detectors. For assays using cumene hydroperoxide (CHP) or H_2O_2, 150 µl reactions contained 100 mM Kpi, pH 7.5, 0.1 to 0.2 µM of CYP3A4, CYP1A1 or purified synthetically encoded CYP2S1 (see supplementary Fig S5), various concentrations of CHP, (0.1 µM to 1 mM) or H_2O_2 (0.1 µM to 10 mM), and various concentration of substrates. The reactions were carried out at 37°C for 5 minutes before being stopped with 150 µl of acetonitrile (2% acetic acid). The samples were centrifuged at 13,000xg for 15 minutes to remove protein. Supernatants were then analyzed using HPLC.

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Km and turnover determinations

To determine the Km of recombinant CYP2S1 for various substrates, each substrate was progressively diluted 1:3 eight times and the dilutions incubated in 150 µl reaction mixtures containing 200 nM of purified CYP2S1 and 1 mM CHP. To determine the Km of CHP and H_2O_2 for CYP2S1 supporting various substrate oxidations, seven progressive 1:3 dilutions of 1mM CHP and 10 mM H_2O_2 were used. All the reactions were terminated after 5 minutes at 37°C by the addition of 150 µl of ice-cold acetonitrile (2% acetic acid), except in the case of aflatoxin B1, where 150 µl of 100% acetonitrile was used. All samples were analyzed in duplicate. The rates of product formation were measured by HPLC using authentic standards. Turnovers were calculated as nmole product/nmole CYP2S1/minute using data from samples with the highest concentration of substrates and CHP or H_2O_2. The Km of each substrate was calculated using Prism software (San Diego, CA) with non-linear regression and the Michaelis-Menten equation [V=Vmax*S/ (Km+S)].

Analytical procedures and product identification

All samples were analyzed using HPLC. The HPLC system consisted of the Shimadzu prominence series, including the LC-20 AT prominence LC pump, DGU-20A5 degasser, CBM-20 prominence communications bus module, SPD-20A prominence UV/VIS detector, and RF-10AXL fluorescence detector (Shimadzu). A C18 reversed-phase column (Shimadzu, 4.6 mm x 250 mm, 5 µ) was used to separate the metabolite products. To separate and detect 6-β-OH-testosterone, the solvents were held at10% B from 0 to 10 minutes, increased to 90% B from 10 minutes to 25 minutes, and then held for another 5 minutes before returning to the starting condition (solvent A: H_2O, solvent B: 100% acetonitrile; flow rate 1ml/minute). The testosterone

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metabolite, 6-β-OH-testosterone, was monitored at 240 nm wavelength. To separate BaP, DMBA, or 7,8 diol-BaP and their metabolites, solvents were held at 40% B from 0 to 3 minutes, increased to 95% B from 3 minutes to 30 minutes, and then held for another 10 minutes, before returning to the starting condition (solvent A: H_2O; solvent B: 100% acetonitrile; flow rate 1ml/minute). The products were monitored from 200 nm to 500 nm with the diode array detector and with the fluorescence detector setting at 340 nm/402 nm (excitation/emission). To separate 9,10-H_2-BaP, the conditions were set as described by Kim and coworkers (Kim et al., 1998). To separate aflatoxin B1 and its metabolites, solvents were held at 20% B from 0 to 10 minutes, increased to 60% B from 10 minutes to 23 minutes, and then held for another 5 minutes, before returning to the starting condition (solvent A: H_2O, 0.1% acetic acid; solvent B: 100% acetonitrile; flow rate 1 ml/minute). The products were monitored with the diode array detector and fluorescence detector setting at 370 nm/440 nm (excitation/emission). To separate all-*trans*-retinoic acid and its metabolite, the solvents were held at 50% B from 0 to 3 minutes, increased to 90% B from 3 minutes to 20 minutes, and then held for another 10 minutes, before returning to the starting condition (solvent A: H_2O; solvent B: 100% acetonitrile; flow rate 1ml/minutes). The products were monitored at a wavelength of 340 nm.

Most products were identified using standard metabolites. However, in some cases, due to the unavailability of standards, we had to deduce the identity of products by comparing their UV spectra to those of similar metabolites, or by using information from previous reports. In the case of 9,10-H_2-BaP metabolism, we identified its product based on the comparison of its UV absorbance spectrum to that of the corresponding metabolite of BaP. In the case of aflatoxin B1's products, M1 and Q1, we used information from a previous report in which M1 is produced exclusively by CYP1A2, and Q1 is exclusively produced by CYP3A4 (Gallagher et al., 1996). In

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the case of all-*trans*-retinoic acid's 4-OH metabolite, we used information from Nadin and Murray's report (Nadin and Murray, 1999), in which CYP2C8 produces 4-OH-retinoic acid as a major product.

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Results

Expression of N-terminal modified variants of CYP2S1

Heterologous expression of recombinant human P450 enzymes in *E. coli* has proved to be challenging. Modification of the N-terminal amino acids of many human CYP enzymes is necessary in order to express them in *E. coli* (Barnes et al., 1991; Guengerich et al., 1996). However, there is no consensus N-terminal modified sequence which guarantees successful expression of human P450s in *E. coli*. Thus, we tested various N-terminal modifications which had been shown to have successful expression of P450s, with human CYP2S1 (see supplementary Table S1). CYP2S1 expression was assessed using the reduced Fe^{2+} CO vs Fe^{2+} difference assay, which not only measures the amount of P450 but also the amount of P420, the denatured form of the P450 protein. All constructs were co-expressed with cytochrome P450 reductase, and P450 measurements were performed with lysates rather than whole cells, since the former gave more accurate product identification and quantification due to lower background noise. Among the different modifications, only modification 4 was able to generate modest amounts of P450 (20-30 nmoles / liter culture), and this modification had a higher P450 to P420 ratio compared to the other modifications (see supplementary Fig. S3), although it still had a high level of P420, representing more than 70% of the total CYP2S1 protein. The incubation time and temperature of the growth conditions after inducer treatment can have great effects on the expression of P450s in *E. coli*. Various time and temperature parameters were tested with all the N-terminal modified constructs. Growth at 30°C for 24 hours provided the best conditions for modification 4 (see supplementary Fig. S3). The amount of cytochrome P450 reductase which was co-expressed with CYP2S1 was also measured using cytochrome c as substrate, and its molar concentration was at least 2-fold higher than that of CYP2S1 (data not shown).

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Construction and expression of semi-synthetic and synthetic CYP2S1.

Since modification 4 had the best expression among the modification constructs, it was chosen for further optimization of expression. The expression of several human P450s, including that of CYP2S1, has been shown to be improved upon coexpression with chaperon groEL/ES (Mitsuda and Iwasaki, 2006; Wu et al., 2006). However, in our hands, there was little improvement in expression when modification 4 was co-expressed with groES/EL (data not shown). A synthetic gene strategy that overcomes the codon bias of the *E. coli* host has commonly been used to improve heterologous expression (Potenza et al., 2007; Wardenga et al., 2008; Wu et al., 2006). In an attempt to improve expression of CYP2S1, we applied both semi-synthetic and synthetic gene strategies. The semi-synthetic cDNA (semiCYP2S1-dh) and full synthetic cDNA (syn2S1-dh) were designed based on the amino acid sequences of CYP2S1 construct 4, (CYP2S1-dh) (see supplementary Table S1 and Fig. S4). The above three cDNAs encode the same protein. The semiCYP2S1-dh cDNA was designed to reduce the delta G of mRNA folding, by introducing changes only in the first 100 bases of the gene. Whereas, the syn2S1-dh cDNA was designed to have optimized codons that fit the codon bias of *E. coli*, as well as having minimized mRNA secondary structure (Fig. 1A). The syn2S1-dh construct has the lowest delta G (-189.52 kcal/mol) and the most open secondary structure compared with native CYP2S1-dh (-245.82 kcal/mol) and semiCYP2S1-dh (-240.13 kcal/mol) (Fig. 1A). The syn2S1-dh construct also yielded the highest P450 content (>200 nmol/L culture) and the least amount of P420 (Fig. 1D). The semiCYP2S1-dh construct expressed better (50-60nmol/ L culture) than the native CYP2S1-dh construct (Figs. 1B and 1C). Our results suggest that codon

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usage and mRNA secondary structure should both be considered when designing a synthetic gene for heterologous expression.

Examination of CYP2S1 catalytic activity using NADPH.

Several compounds were examined, including some that have been reported to be substrates for CYP2S1, such as all-*trans*-retinoic acid, naphthalene, and aflatoxin B1 (Karlgren et al., 2005; Smith et al., 2003; Wang et al., 2005). Other potential substrates tested included, benzo[a]pyrene, DMBA, testosterone, methoxyresorufin, ethoxyresorufin, and methoxy-4-trifluoromethylcoumarin (MFC). All the reactions were performed with membranes containing CYP2S1 derived from various constructs (see supplementary Table S1), including the synthetic construct, plus cytochrome P450 reductase, or with membranes containing only cytochrome P450 reductase as a negative control. Even in the presence of NADPH, CYP2S1 did not form any detectable products with any of these compounds, after 2 hours of incubation (data not shown). Our failure to observe activity of CYP2S1 with NADPH agrees with the results of Wu and coworkers, who reported that CYP2S1 did not oxidize any substrate tested, including naphthalene and all-*trans*-retinoic acid (Wu et al., 2006).

Lack of interaction between CYP2S1 and P450-reductase

A plausible explanation for the lack of CYP2S1 activity *in vitro* is deficient interaction between the P450 reductase and CYP2S1. The interaction between P450 and P450 reductase has been suggested in many studies as being due to electrostatic interaction between charged amino acids, mainly involving arginine/lysine of P450 and glutamate/aspartate of P450 reductase (Nikfarjam et al., 2006; Shen and Strobel, 1993; Voznesensky and Schenkman, 1994). Fig. 2A,

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B, C show the alignment of CYP2S1 to various CYP2 family enzymes. At amino acid position 147, the human, mouse, canine and rat CYP2S1 have leucine, a neutral amino acid, whereas other CYP2s have either lysine or arginine, which are positively charged amino acids. Crespi and colleagues reported that CYP2C9*2, a slow drug metabolism allele of CYP2C9, which possesses a R144C mutation (equivalent to CYP2S1's amino acid 147) exhibited slower kinetics compared to the wild-type enzyme because of altered interaction of CYP2C9*2 with the P450 reductase (Crespi and Miller, 1997). In addition, human and canine CYP2S1 possess glutamine at position 388, whereas other CYP2s have lysine (Fig. 2B). Bernhardt and colleagues showed that when fluorescein isothiocyanate (FITC) was selectively bound to the epsilon-amino group of lysine-382 of CYP2B4 (equivalent to CYP2S1's amino acid 388), the activity of CYP2B4 was inhibited due to impaired electron transfer from the P450 reductase (Bernhardt et al., 1984). Finally, Nikfarjam and coworkers showed that K326 and/or K327 in the J-helix of P450 17α plays an important role in interaction with P450 reductase (Nikfarjam et al., 2006). The 326th amino acid of CYP2S1 is a tryptophan rather than the lysine or arginine occurring in other CYP2 family members (Fig. 2C). These differences in the CYP2S1 protein suggested that CYP2S1 may interact weakly or not at all with the P450 reductase.

In the presence of NADPH, P450 reductase transfers one electron from NADPH to heme Fe^{3+} of P450 and reduces it to Fe^{2+}. The latter has high affinity for CO as well as for oxygen. If the heme's Fe^{2+} is bound to CO, there is an increase in absorbance at 450 nm (Omura and Sato, 1964a). We utilized this characteristic of the P450 reaction cycle to examine whether the P450 reductase can transfer an electron from NADPH to CYP2S1. When NADPH was added to the CYP2S1+P450 reductase sample in the presence of CO, there was no absorbance increase at 450 nm (Fig. 3A). Electron transfer is usually more readily observed in the presence of substrate

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(Lewis and Pratt, 1998). However, when naphthalene, (which is capable of binding CYP2S1 as shown by the fact that it elicits a type I substrate-induced difference spectra, (Bui and Hankinson, data not shown) was added, there was also no increase in absorbance at 450 nm (Fig. 3A). Adding sodium dithionite, which directly reduces CYP2S1 independently of P450 reductase, increased absorbance at 450 nm (Fig. 3A). The assay was validated with CYP1A1, CYP2C9, and CYP3A4. All these P450s exhibited increased absorbance at 450 nm when NADPH was added. The absorbance at 450 nm increased even further in each case in the presence of substrate (Fig. 3B, C, and D). These data indicate that P450 reductase cannot transfer an electron from NADPH to CYP2S1, due to the lack of interaction between the two proteins, suggesting that this may be the reason why there was no detectable activity of CYP2S1 *in vitro* using P450 reductase and NADPH.

All the remaining studies in this paper were performed with purified synthetically encoded CYP2S1.

Catalytic activity of CYP2S1 with hydroperoxides.

Many P450s can utilize organic hydroperoxides to carry out oxidation of their substrates in the absence of cytochrome P450 reductase. This peroxide-dependent pathway is known as the "peroxide shunt" (Barr et al., 1996). Cumene hydroperoxide can support several P450-catalyzed reactions, including aromatic and aliphatic hydroxylation, N- or O-dealkylation, and alkene epoxidation (Barr and Mason, 1995; Griffin et al., 1980; Nordblom et al., 1976). When CYP3A4+P450 reductase was incubated with 1 mM cumene hydroperoxide in the presence of 100 μM testosterone, 6-β-OH-testosterone was detected just as it was with CYP3A4+P450 reductase incubated with testosterone and NADPH (Fig. 4A). Similarly, when CYP2S1 or

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CYP1A1+P450 reductase was incubated with 1 mM cumene hydroperoxide and benzo[a]pyrene, multiple products were detected (Fig. 4B), whereas, when the P450 reductase only sample (HR) was incubated with cumene hydroperoxide and benzo[a]pyrene, no product was detected (Fig. 4B). In addition, when CYP2S1 was heat treated at 80°C for 20 minutes, product formation was reduced by at least 80% (data not shown), thus, indicating that product formation is enzymatic and CYP2S1-dependent. 3 mM H_2O_2 also supported metabolism of BaP by CYP2S1 and CYP1A1 but not P450 reductase to multiple products (Fig. 4C). Together, these data clearly show that CYP2S1 can utilize cumene hydroperoxide and H_2O_2 to oxidize benzo[a]pyrene.

In the presence of cumene hydroperoxide, CYP2S1 oxidized benzo[a]pyrene to the 1,6 quinone, 3,6 quinone, and 6,12 quinone, with Kms and turnovers of 94±22 μM, 0.83 min^{-1}, 49±8 μM, 1.85 min^{-1}, and 73±4 μM, 3.25 min^{-1}, respectively (Fig. 5 and Table 1). At least two unknown products with absorbances at 254 nm were also detected (Fig. 5). However, no 4OH-BaP or BaP-7,8-diol was formed. The latter is a precursor of the carcinogenic metabolite, 7,8-diol-*trans*-9,10-epoxide, which is extremely reactive and can bind to macromolecules such as DNA, RNA, and protein (Gelboin, 1980). This suggests that CYP2S1 does not covert BaP into this carcinogenic metabolite. CYP2S1 (in the presence of cumene hydroperoxide) converted BaP to the above quinones at much higher rates than those reported for CYP1A1, 1A2, and 1B1 with P450 reductase and NADPH, (Kim et al., 1998). Quinone metabolites are thought to represent substrates for conjugation and detoxification pathways (Wood et al., 1975). However, some evidence suggests that the quinones can form DNA adducts when they undergo one election reduction by P450 reductase to form unstable semiquinone species (Joseph and Jaiswal, 1994).

In addition to benzo[a]pyrene, CYP2S1 could oxidize many procarcinogens in the presence of cumene hydroperoxide, including BaP-7,8-diol, 9,10-H_2-BaP, DMBA, and aflatoxin

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B1. It could also metabolize all-*trans*-retinoic acid and other compounds listed in Table 1. BaP-7,8-diol is an intermediate product of the BaP bioactivation pathway leading to the mutagenic epoxide, the 7,8-diol-*trans*-9,10-epoxide. It is formed mainly by CYP1A1 and 1B1, and these same enzymes can also metabolize it to the above epoxide (Kim et al., 1998; Shimada et al., 1999). In the presence of cumene hydroperoxide, CYP2S1 could convert BaP-7,8-diol to at least 6 products (Fig. 6). The major product was identified as BaP-*r*-7,*t*-8,*t*-9,*c*-10-tetrahydrotetrol (r7,t8,t9,c10-tetrol) (Km=12.4±2 μM, 2.3 min^{-1}) which is indicative of 7,8-diol-*trans*-9,10-epoxide formation (Fig. 6, and Table 1) (Kim et al., 1998). The turnover of r7,t8,t9,c10-tetrol formation by CYP2S1 is higher than that catalyzed by CYP1A1, 1A2, and 1B1 (Kim et al., 1998). This suggests that CYP2S1 could be important in the bioactivation of BaP-7,8-diol *in vivo*.

When 9,10-H_2-BaP (a BaP derivative) was incubated with cumene hydroperoxide and CYP2S1, a major product at 15 minute retention time was detected (Fig. 7). This product has the same UV spectrum as r7,t8,t9,c10-tetrol, indicating that the 8,9-double bond was oxidized to the 8,9-diol. Thus the product is likely to be 7,8-diol-9,10-H_2-BaP (Fig. 7), which would be derived from the hydrolysis of 9,10-H_2-BaP-7,8-epoxide. Whether 9,10-H_2-BaP-7,8-epoxide is carcinogenic is not known, although a similar compound, 9,10-diol-*trans*-7,8-epoxide has week mutagenic activity (Thakker et al., 1978). The Km and rate of 7,8-diol-9,10-H_2-BaP formation were calculated based on the r7,t8,t9,c10-tetrol standard: Km = 23±6 μM, turnover = 1.6 min^{-1} (Table 1).

DMBA is a potent carcinogen that induces many types of cancer, adrenocorticolytis, and immunosuppression in mice and other species (Gao et al., 2005; Lindhe et al., 2002). Its toxicity and carcinogenicity depends on CYP1 family enzymes (Nebert and Russell, 2002). When

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DMBA was incubated with CYP2S1 and cumene hydroperoxide, 7-OH-DMBA, 12-OH-DMBA and other unknown metabolites were detected (Fig. 8). CYP2S1 converted DMBA to 7- and 12-OH-DMBA at faster rates than for any other substrate tested, 15.7 min^{-1} for 7-OH-DMBA and 26 min^{-1} for 12-OH-DMBA (Table 1). Although 7-OH and 12-OH DMBA are considered detoxification products of DMBA, these two products can be further oxidized by P450 leading to tumor induction in mice (Chou et al., 1981; Wislocki et al., 1981). Neither the carcinogenic metabolite, 1,2-epoxide-3,4-diol-DMBA, nor its precursor, 3,4-diol-DMBA, were detected (Fig. 8). Thus, CYP2S1 may play an important role in metabolism of DMBA.

All-*trans*-retinoic acid was reported by Smith and colleagues to be metabolized by CYP2S1 into the 4-hydroxy and 5,6-epoxide products (Smith et al., 2003), but we and others (Wu et al., 2006) could not repeat this observation using CYP2S1 with P450 reductase and NADPH. However, when CYP2S1 was incubated with all-*trans*-retinoic acid in the presence of cumene hydroperoxide or H_2O_2, the 4-hydroxy and other products were detected (Fig. 9), as determined by comparing CYP2S1's reaction to that of CYP2C8, since the latter has been shown to convert all-trans-retinoic acid to the 4-hydroxy product (Nadin and Murray, 1999). The Km of all-*trans*-retinoic acid for CYP2S1 was determined to be 21±0.9 μM (Table 1).

Other chemicals found to have metabolized by CYP2S1 using cumene hydroperoxide, included aflatoxin B1, naphthalene and styrene, but no activity was found with testosterone, and coumarin (Table 1).

Hydrogen peroxide has also been shown to be able to support P450-catalyzed reactions (Otey et al., 2006). We investigated whether hydrogen peroxide could support CYP2S1 activity and compared its oxidant activity to that of cumene hydroperoxide (Table 2). As described above, H_2O_2 supported oxidation of benzo[a]pyrene (Fig. 4C) and all-*trans*-retinoic acid by

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CYP2S1 (Fig. 9). Aflatoxin B1, (a mycotoxin whose mutagenicity and toxicity is dependent on P450), was also oxidized by CYP2S1 in the presence of H_2O_2 with a Km of 3500 ± 700 μM (Table 2), to products other than aflatoxin M1 and Q1, as determined by comparison of CYP2S1's reaction with those of CYP1A2 and 3A4 (Fig. 10A). At least four fluorescent products were detected in the CYP2S1 but not in the CYP1A2 and 3A4 samples (Fig. 10B). The principal carcinogenic product of aflatoxin B1 metabolism is the exo-8,9-epoxide (Gallagher et al., 1996). We could not determine whether or not CYP2S1 converts aflatoxin B1 to this epoxide. In general, hydrogen peroxide could support CYP2S1 metabolism of substrates to the same products as cumene hydroperoxide, but with considerably higher Kms and slower turnovers (Table 2). The Km for hydrogen peroxide ranged from 1 μM to 5000 μM, with the lowest Km for all-*trans*-retinoic acid and the highest for DMBA (Table 2). For cumene hydroperoxide, the Km's ranged from 1 μM to 187 μM, with the lowest Km being for all-*trans*-retinoic acid, and highest for 9,10-H_2-BaP.

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Discussion

One of the many challenges to functionally characterizing CYP2S1 and other orphan P450s is the availability of good enzyme preparations. Wu and coworkers used a system of high-expression of CYP2S1 in *E. coli*, that incorporated chaperone GroEL/ES (Wu et al., 2006). However, 50 % of the CYP2S1 was still in the form of P420, which, when present in a preparation, can undermine substrate screening. In this report, we successfully applied a synthetic gene approach to achieve a high level expression of human CYP2S1 in *E. coli* with at least 90% purity, and containing very little if any P420. The high-level of CYP2S1 expression achieved in this report will be necessary for subsequent crystallization. The synthetic gene approach for heterologous expression in *E. coli* has been used successfully in several other cases (Potenza et al., 2007; Wardenga et al., 2008; Wu et al., 2006). However, in our approach, we not only replaced the native gene's codons with the most abundant *E. coli* codons, but we also increased the delta G free energy of the mRNA structure. We demonstrated that the combination of these two factors should be considered in achieving successful heterologous expression. This synthetic gene approach could be applied to other P450s that have not been successfully expressed in *E. coli*, such as CYP2F1 (Baldwin et al., 2005).

Although high-level expression of CYP2S1 was achieved, we were unable to detect activity towards any potential substrate using NADPH and P450 reductase. We obtained evidence that the lack of NADPH–dependent of CYP2S1 activity was probably due to the lack of its interaction with P450 reductase when the two proteins were co-expressed in *E. coli*. Even when we attempted to reconstitute the purified CYP2S1 and P450 reductase utilizing L-α-1,2-dilauroyl-sn-glycero-3-phosphocholine followed a method previously described (Brian et al., 1990), we failed to demonstrate any interaction between the two proteins or NADPH-dependent

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activity (data not shown). The lack of CYP2S1 and P450 reductase interaction can be explained at least in part, by the fact that CYP2S1 does not contain certain lysine and arginine amino acids at the important 147th, 326th, and 388th positions which have been proposed to be important for the interactions of various P450s with P450 reductase (Crespi and Miller, 1997; Nikfarjam et al., 2006; Schulze et al., 2000). Althoug it is possible that N-terminal modification of the enzyme negates its interaction with the P450 reductase, this is unlikely since many mammalian P450s have been expressed in *E.coli* and none of their activities have been reported to be affected by the N-terminal modifications that are always included (Barnes et al., 1991; Guengerich et al., 1996).

The prototypical cytochrome P450 enzymes are monooxygenases that catalyze the hydroxylation or epoxidation of their substrates. To achieve this, they use molecular oxygen and reducing equivalents from NADPH, where one of the oxygen atoms is reduced to H_2O and the other is inserted in the substrate. However, there is not an absolute requirement for molecular oxygen and NADPH. The activities of some P450s, including certain microsomal P450s, do not depend on P450 reductase and NADPH. Examples include, allene oxide synthase, thromboxane synthase (CYP5A) and prostacyclin synthase (CYP8A) (Brash and Song, 1995; Yeh et al., 2007). These enzymes have been shown not to interact with P450 reductase. In addition, some P450s exhibit peroxygenase-like activity. That is, they can use peroxides as oxygen surrogates to support the oxidation of substrates. In this report, we show that CYP2S1 catalytic activity is well supported by peroxides such as hydrogen peroxide and cumene hydroperoxide. In the presence of peroxides, CYP2S1 was found to metabolize a wide variety of substrates, including many important procarcinogens, such as BaP, DMBA, BaP-7,8-diol, and aflatoxin B1, at relatively fast rates compared to other P450 enzymes involved in the metabolism of the same substrates.

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In general, CYP2S1 seems to convert most substrates tested to less toxic metabolites. Our data suggests that human CYP2S1 may play an important role in detoxification of DMBA, BaP, and aflatoxin B1. On the other hand, CYP2S1 converts BaP-7,8-diol to a very reactive metabolite, a 7,8-diol-*trans*-9,10-epoxide, which is a potent tumor initiator. The conversion of this substrate by recombinant human CYP2S1 is faster than the rate of conversion by human recombinant CYP1A1, CYP1A2, or CYP1B1 in the presence of P450 reductase and NADPH (Kim et al., 1998). This metabolism by CYP2S1 is consistent with our observation of *in vivo* toxicity of BaP-7,8-diol in mammalian cells overexpressing human CYP2S1 (Bui et al., 2009)

The mechanism by which P450s utilize cumene hydroperoxide to oxidize substrates is often quite complex. Cumene hydroperoxide can undergo both hemolytic cleavage and heterolytic cleavage in the presence of P450 (Ortiz de Montellano, 1995). When CYP2S1 was incubated with the antioxidant, butylated hydroxyanisole, its BaP-7,8-diol epoxidation activity was reduced by 50% (data not shown). This suggests that CYP2S1-catalyzed epoxidation proceeds by both a peroxidative reaction and a peroxygenative reaction, since the former incorporates an oxygen of peroxide into the substrate via heterolytic cleavage of peroxide that is not inhibitable by antioxidant, while the latter occurs via a free radical mechanism, which can be inhibited by antioxidant (Ortiz de Montellano, 1995).

Other heme proteins such as hemoglobin and myoglobin have been shown to have peroxygenase-like activity *in vitro*. For instance, hemoglobin, and myoglobin, were reported to oxidize BaP-7,8-diol using hydrogen peroxide (Catalano and Ortiz de Montellano, 1987). However, their turnover numbers (calculated using the data from the above report) are between $3x10^{-5}$ to $3x10^{-4}$ min^{-1} which are 1000 to 2000 times lower than that of CYP2S1. Also, the

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concentrations of hemoglobin and myoglobin used in the study of Catalano and Ortiz de Montellano were 50 to 100 times that of CYP2S1 used in our study. In addition, adding detergent (100 μM Tween 20) was necessary to achieve the oxidation of BaP-7,8-diol in the above cases, making the conditions not physiologically relevant. Thus, although peroxide-dependent activity may not be physiologically relevant for myoglobin and hemoglobin, it is probably relevant for CYP2S1 and other P450s, as we demonstrate in our accompanying manuscript (Bui et al., 2009).

Although cumene hydroperoxide can support the oxidative activity of CYP2S1 *in vitro*, it is not present in cells and, thus cannot serve as a cofactor for CYP2S1 *in vivo*. Hydrogen peroxide, on the other hand, occurs *in vivo* and could serve as a biological oxidizing agent to support CYP2S1 activity. Although the Km of H_2O_2 for some substrates is in the millimolar range, probably exceeding the concentration occurring under normal physiological conditions, H_2O_2 concentrations surge during oxidative stress or inflammatory responses. Since these conditions are associated with smoking-related cancer, elevated H_2O_2 could be an important contributor to BaP-7,8-diol carcinogenesis *in vivo*. Interestingly, the Km's of H_2O_2 for all-*trans*-retinoic is relatively low, corresponding more closely to those present under physiological conditions. Biological peroxides such as lipid hydroperoxides, cholesterol hydroproxide, pregnenolone 17α-hydroperoxide and progesterone 17α-hydroperoxide occur *in vivo*. Lipid hydroperoxides are capable of serving as oxygen surrogates for many P450-dependent mono-oxygenations (Anari et al., 1997; Wang and Liehr, 1994). We show in our accompanying report (Bui et al., 2009), that various lipid hydroperoxides can support CYP2S1-catalyzed oxidation of BaP-7,8-diol at faster rates than cumene hydroperoxide and hydrogen peroxide, and at the range of concentrations of fatty acid hydroperoxides that closely resemble physiological conditions. It

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is possible that CYP2S1 can also utilize these biological peroxides to oxidize other substrates *in vivo*. Thus, CYP2S1's peroxide-dependent oxidative activity could be physiologically relevant.

In summary, our data suggest that CYP2S1 can play an important role in the metabolism of various important xenobiotics as well as endogenous compounds in a peroxide-dependent fashion.

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Footnotes

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Legends for figures and tables

Table 1. Km's and turnover numbers of various substrates using purified synthetically encoded CYP2S1 (0.2 μM) and cumene hydroperoxide, CHP, (1mM). Data are represented as averages ± S.E. of duplicate determinations. Km's were determined with Prism software using nonlinear regression with one site binding mode and the V=Vmax*S/(Km+S) equation. N.D. = not determined. Turnovers were determined using the highest concentration of substrate, and are presented as nmoles of product/nmole of CYP2S1/minute.

Table 2. Km's and turnover numbers of CHP and H_2O_2 for various substrates, determined by incubation of 0.2 μM CYP2S1 and fixed concentration of substrates. Data are represented as averages ± S.E. of duplicate determinations.

Figure 1. Comparison of the hypothetical mRNA secondary structures, delta Gs, and P450 spectral characteristics expression of native CYP2S1dh, semiCYP2S1dh, and syn2S1dh. A) Structures and delta Gs were calculated at a 37°C folding temperature and 1 M Na^+ using mfold (http://mfold.bioinfo.rpi.edu/cgi-bin/dna-form1.cgi) (A). Expression of the native CYP2S1 construct (B), semiCYP2S1-dh construct (C), and syn2S1-dh construct (D) in *E. coli* LMG 194. All constructs were co-expressed with P450-reductase. Fe^{2+} CO vs. Fe^{2+} difference spectra were measured using 1:5 dilutions of lysate supernatants. The expression levels of CYP2S1, semiCYP2S1-dh, and syn2S1-dh were estimated to be (20-30 nmoles/liter culture), 50-60 (nmoles/liter culture) and 200 (nmoles/liter culture), respectively.

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Figure 2. Amino acid sequence alignment of human CYP2S1 with other CYP2 family enzymes. The highlighted amino acids are thought to be important in the interaction between the cytochromes P450 and P450 reductase.

Figure 3. NADPH reduced Fe^{2+} CO vs Fe^{2+} difference spectra of CYP2S1+P450 reductase (A). Light blue, is CYP2S1 + CO; Brown is CYP2S1 + CO + NADPH; Blue is CYP2S1 + CO + NADPH + naphthalene; Purple is CYP2S1 + CO + NADPH + naphthalene + dithionite. Spectra of CYP1A1+P450 reductase (B), Green is CYP1A1 + CO; Blue is CYP1A1 + CO + NADPH; Red is CYP1A1 + CO + NADPH + harmaline. Spectra of CYP2C9+P450 reductase (C), black is CYP2C9 + CO; blue is CYP2C9 + CO + NADPH; light blue is CYP2C9 + CO + NADPH + diclofenec; green is CYP2C9 + CO+ dithionite. Spectra of CYP3A4+P450 reductase (D), green is CYP3A4 +CO; red is CYP3A4 + CO + NADPH; Purple is CYP3A4 + CO+ NADPH+ diclofenic; blue is CYP3A4 + CO + NADPH + testosterone.

Figure 4. Cumene hydroperoxide and hydrogen peroxide support oxidation by CYP2S1. HPLC chromatograms of 0.15 μM CYP3A4+reductase (A) incubated with 100 μM testosterone and 1 mM cumene hydroperoxide (dark line), or with 200 μM testosterone and a NADP/NADPH regeneration mixture (gray line) for 10 minutes at 37°C. The 19-minute peak corresponds to a 6-β-OHtestoterone, detected at 245 nm. HPLC chromatograms (B) comparing activities of 0.15 μM CYP2S1 (solid black line), 0.15 μM CYP1A1 (gray line), and human reductase sample (HR, dotted line) toward 100 μM benzo[a]pyrene in the presence of 1mM cumene hydroperoxide for 10 minutes at 37°C . HPLC chromatograms (C) of 0.15 μM CYP2S1, 0.15 μM CYP1A1, and

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human reductase sample (HR) toward 100 μM benzo[a]pyrene in the presence of 10 mM H_2O_2 for 10 minutes at 37°C. Products, indicated by arrows, were detected at 254 nm.

Figure 5. Identification of CYP2S1-catalyzed products of BaP. HPLC chromatogram of 0.2 μM CYP2S1 incubated with 200 μM BaP and 1mM CHP (solid black line). Products were identified at 254 nm using authentic standards (dotted lines), 6,12-BaP-quinone (4); 3,6-BaP-quinone (3); 1,6 benzo[a]pyrene (2), 4-OH-BaP (5), and BaP-7,8-diol (1). Other products, indicated with question mark, were not identified.

Figure 6. Identification of CYP2S1-catalyzed products of BaP-7,8-diol. HPLC chromatograms of 0.2 μM CYP2S1 incubated with 133 μM BaP-7,8-diol and 1mM CHP (A), no CHP control (B) and no CYP2S1 control (C). Detection was set at 345 nm. r7,t8,t9,c10-tetrol was identified using an authentic standard (D). Other unknown products, indicated by arrows, were also detected. The product marked with *, has the same UV spectrum as r7,t8,t9,c10-tetrol, suggesting that it is its enantiomer.

Figure 7. Identification of CYP2S1-catalyzed products of 9,10-H_2-BaP. HPLC chromatogram of 0.2 μM CYP2S1 incubated with 133 μM 9,10-H_2-BaP and 1mM CHP (A), without 2S1 (C) control, or without CHP (B) control. Detection was set at 310 nm. The 7,8-diol-9,10-H_2-BaP product was identified based on the comparison of its UV-spectrum (D) to that of r7,t8,t9,c10-tetrol (E).

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Figure 8. Identification of CYP2S1-catalyzed products of DMBA. HPLC chromatogram of 0.2 µM DMBA incubated with 500 µM DMBA and CHP (solid black). Products were identified at 295 nm using authentic standards, 12-OH-DMBA (3), 7-OH-DMBA (2), and 3,4-diol DMBA (1). Other unknown products are indicated by question marks.

Figure 9. Identification of CYP2S1-catalyzed products of all-*trans*-retinoic acid (AtR). HPLC chromatograms of 0.2 µM CYP2S1 incubated with 20 µM AtR and 1 mM CHP (A) or 1 mM H_2O_2 (B), and of 0.1 µM CYP2C8+reductase incubated with 5 µM AtR and NADPH (C). Products were detected at 350nm. 4-OH-AtR was identified based on a previous report, in which CYP2C8 produces 4-OH-AtR from AtR (Nadin and Murray, 1999).

Figure 10. Identification of CYP2S1-catalyzed products of aflatoxin B1. HPLC chromatograms of 0.2 µM CYP2S1 incubated with 243 µM aflatoxin B1 and 3 mM H_2O_2 (i), of 0.2 µM CYP3A4+reductase incubated with 243 µM aflatoxin B1 and NADPH (ii), and of CYP1A2+ reductase incubated with 243 µM aflatoxin B1 and NADPH (iii). Products were detected with UV/VIS detector at 360 nm (A) and with a fluorescence detector (B). M1 and Q1 were identified based on a previous report (Gallagher et al., 1996). Unknown products are indicated by arrows.

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Table 1.

SUBSTRATE	PRODUCT	Km (μM)	Turn over (min^{-1})
Benzo[a]pyrene	1,6-dione	94±22	0.83
	3,6-dione	48.6±8	1.85
	6,12-dione	72.7±14	3.25
7,12Dimethylbenz[a]anthracene	7-OH-DMBA	219±4	15.7
	12-OH-DMBA	34±9	26
9,10-dihydro-benzo[a]pyrene	9,10H_2-BaP-7,8-dihydrodiol	22.6±6	1.6
Benzo[a]pyrene*trans*7,8-dihydrodiol	r7,t8,t9,c10-tertol	12.43±2	2.3
Aflatoxin B1	unknown	266±40	N.D.
All-*trans*-retinoic acid	4-OH-retinoic acid	20.9± 0.9	N.D.
Naphthalene	unknown	N.D.	N.D.
Styrene	unknown	N.D.	N.D.
Testosterone	No activity		
Coumarin	No acitivity		

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Table 2.

		CHP		H_20_2	
SUBSTRATE	**PRODUCT**	**Km (μM)**	**Turn over (min^{-1})**	**Km (μM)**	**Turn over (min^{-1})**
Benzo[a]pyrene (167 μM)	**1,6-dione**	**16.1±3.2**	**0.58**	**107±21**	**0.44**
	3,6-dione	**29.4±5**	**1.36**	**215±42**	**0.8**
	6,12-dione	**30.8±6**	**2.24**	**232±46**	**1.46**
7,12Dimethylbenz[a]-anthracene (133 μM)	**7-OH-DMBA**	**58.6±11**	**15.7**	**4800±950**	**15**
	12-OH-DMBA	**62.0±12**	**26**	**> 5000**	**14.8**
9,10-dihydro-Benzo[a]pyrene (50 μM)	**9,10H_2-BaP-7,8-dihydrodiol**	**188±37**	**0.55**	**60±12**	**0.13**
Benzo[a]pyrenetrans-7,8dihydrodiol (133 μM)	**r7,t8,t9,c10-tertol**	**31.6±5**	**1.1**	**1906±350**	**0.3**
Aflatoxin B1 (243 μM)	**unknown**	**8.8±2**	**N.D.**	**3500±700**	**N.D.**
Retinoic acid (20 μM)		**1.1±0.4**	**N.D.**	**1.2±0.4**	**N.D.**

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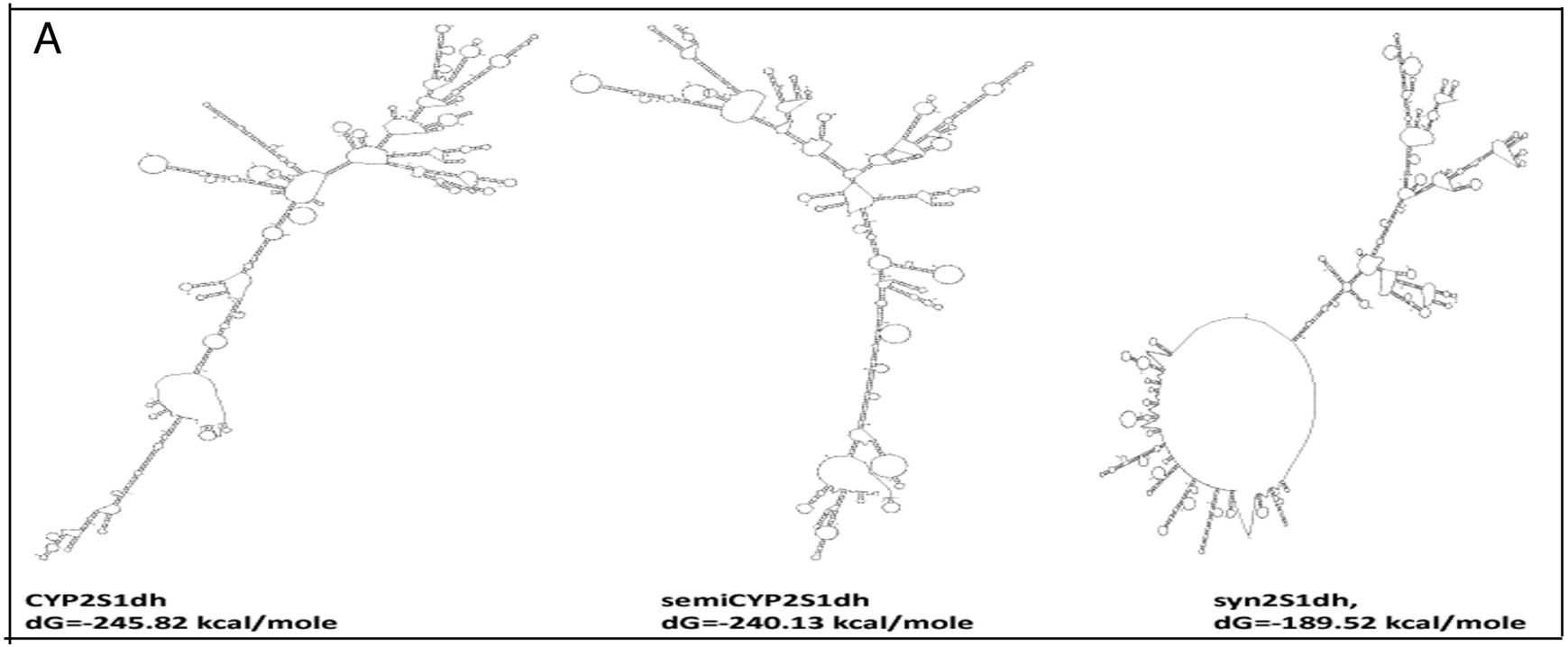
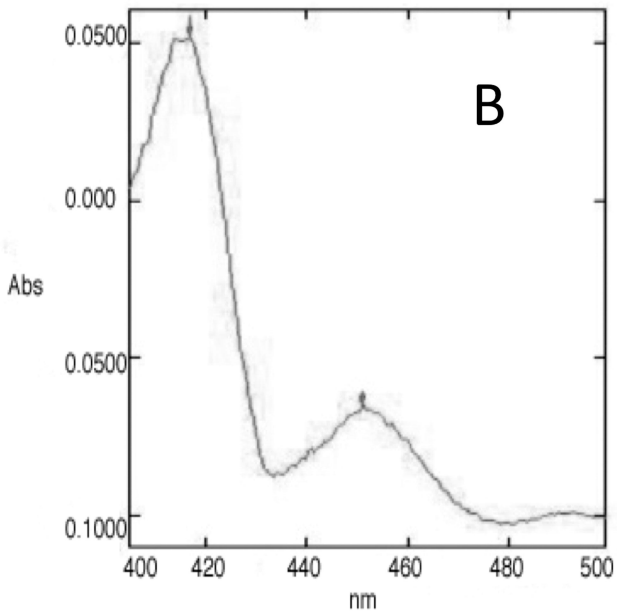
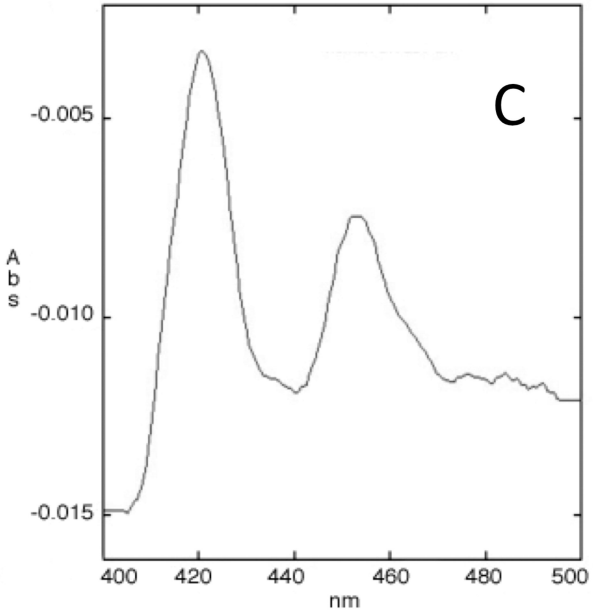
Figure 1







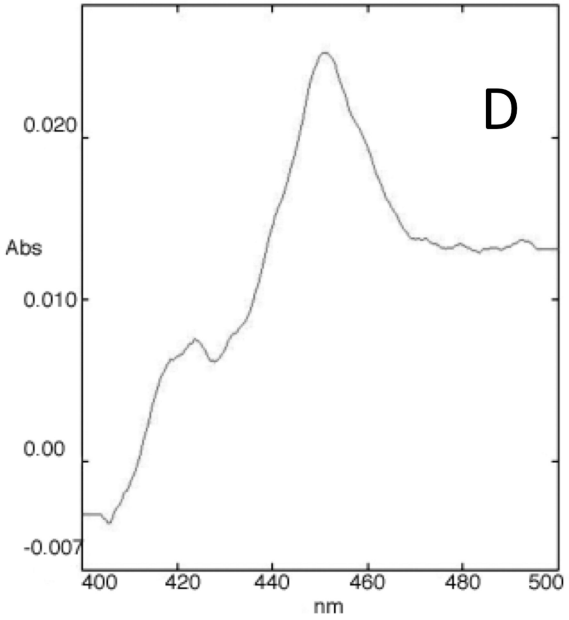


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Figure 2

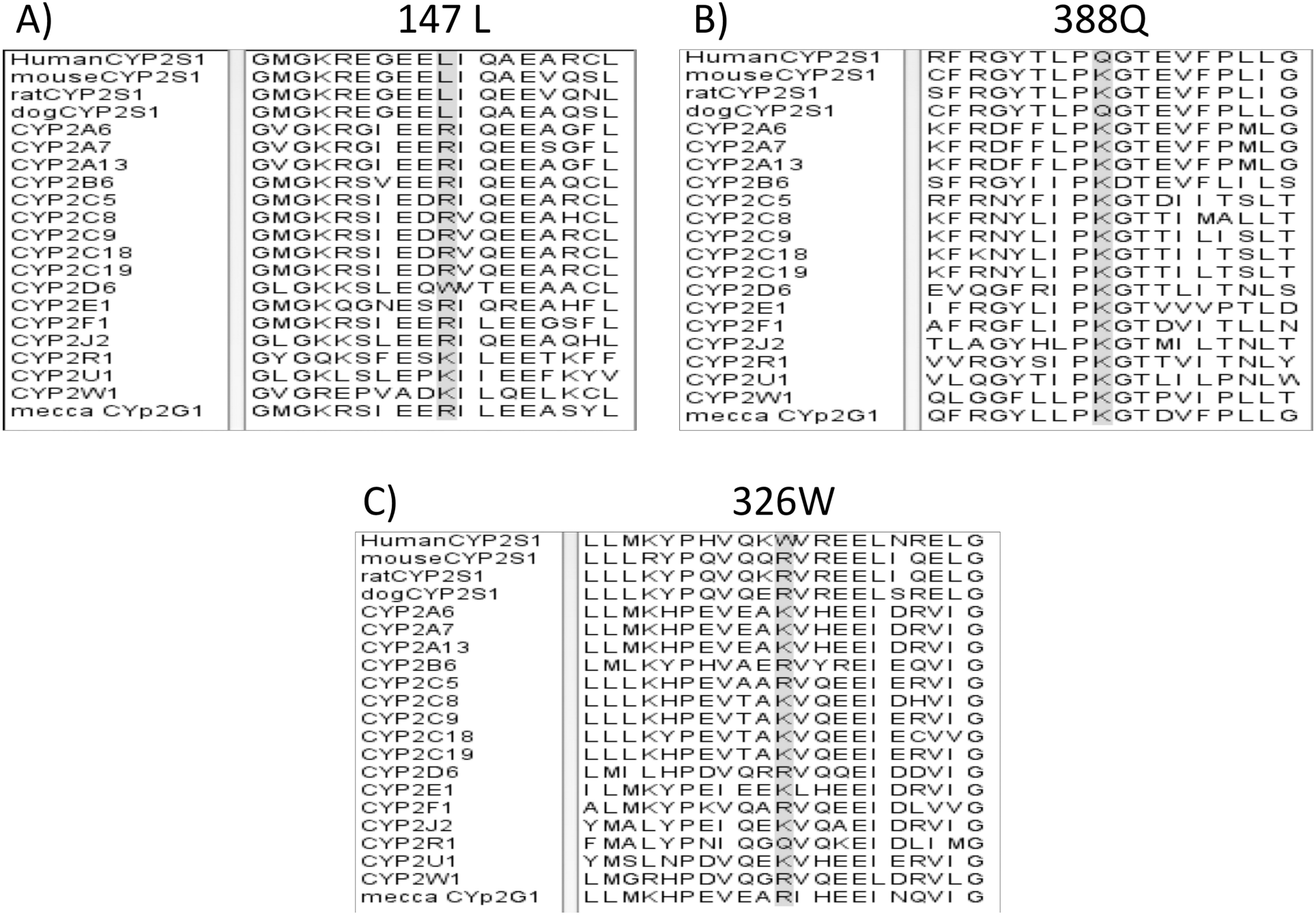

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Figure 3

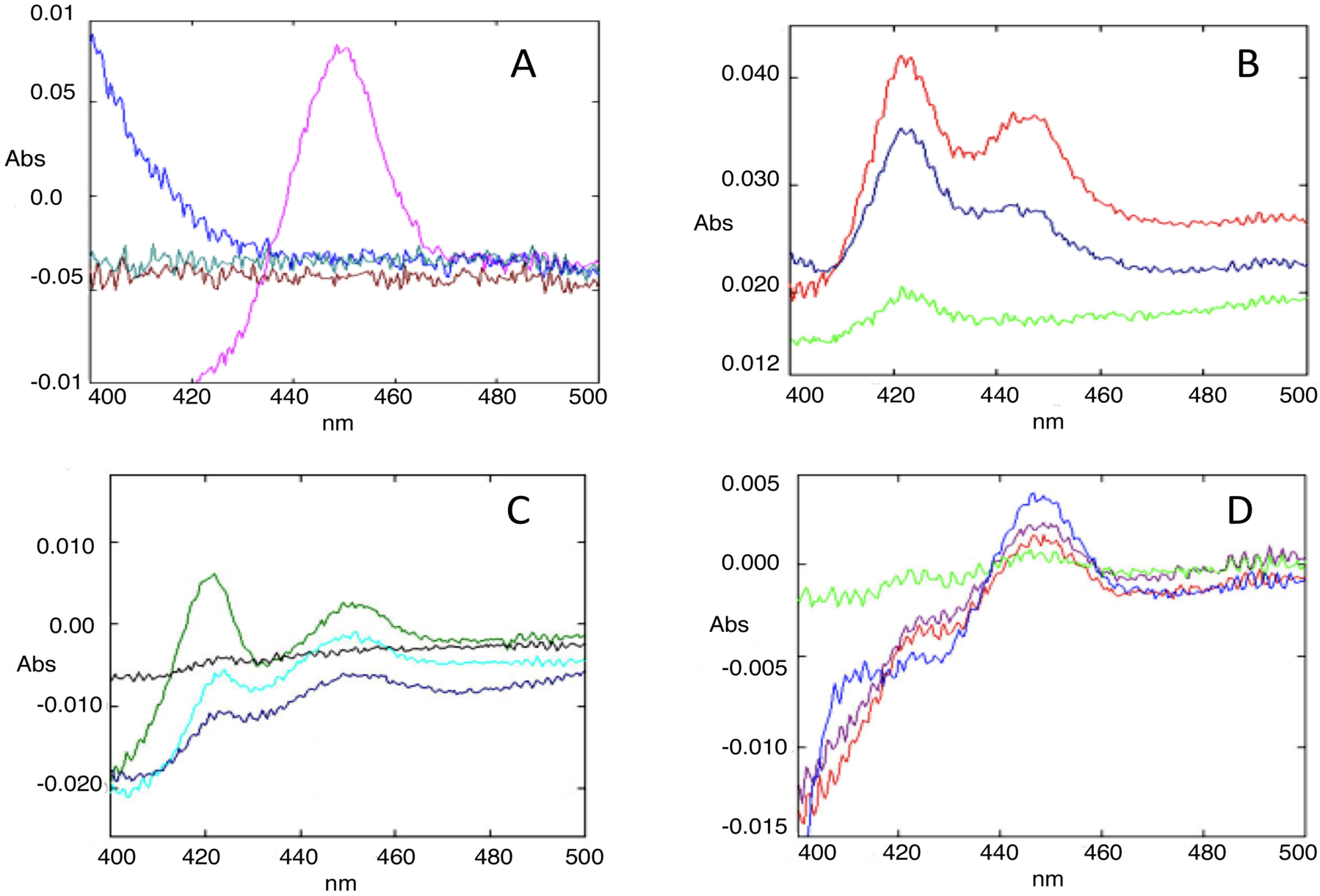

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Figure 4

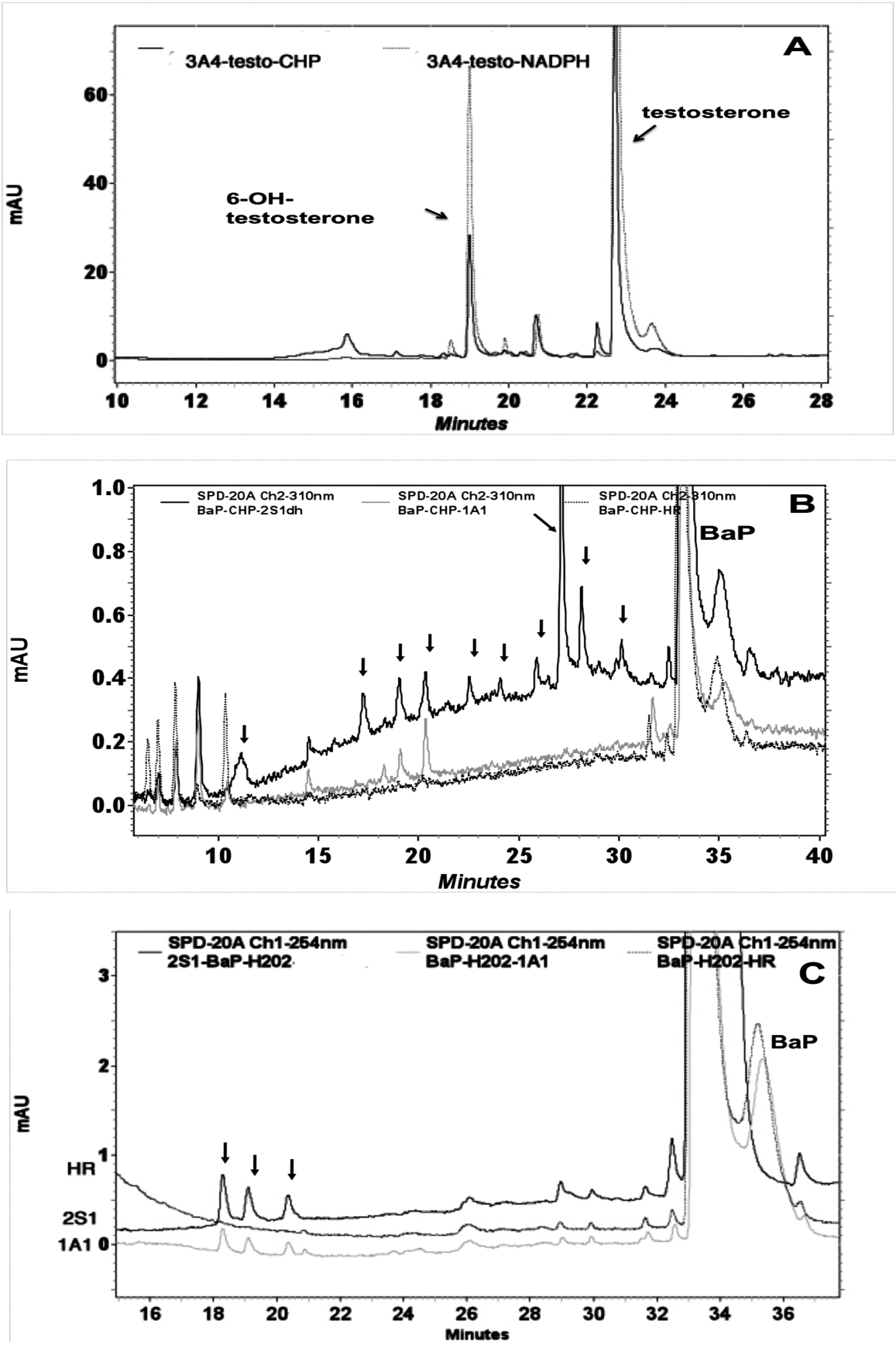

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Figure 5

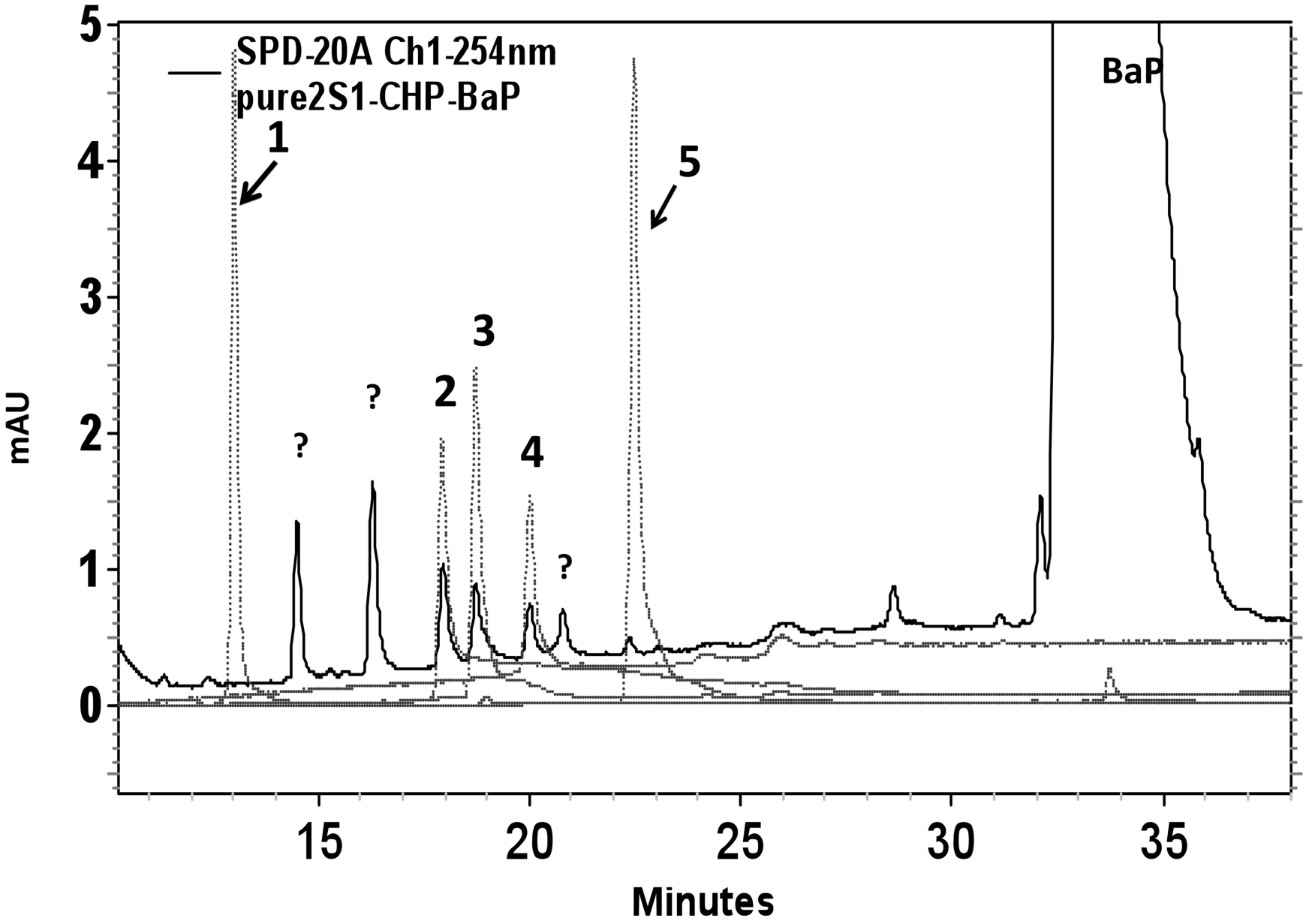

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Figure 6

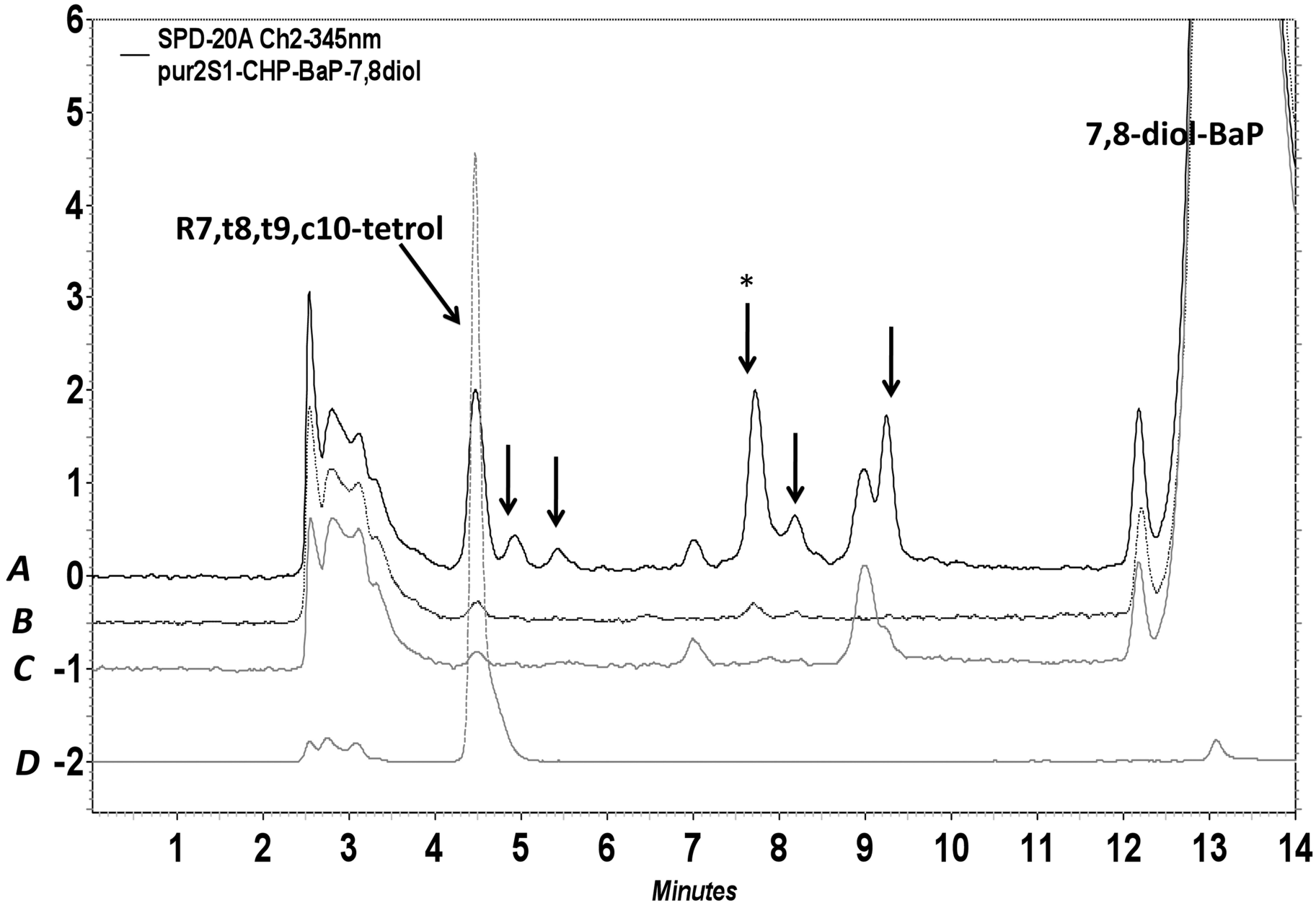

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Figure 7

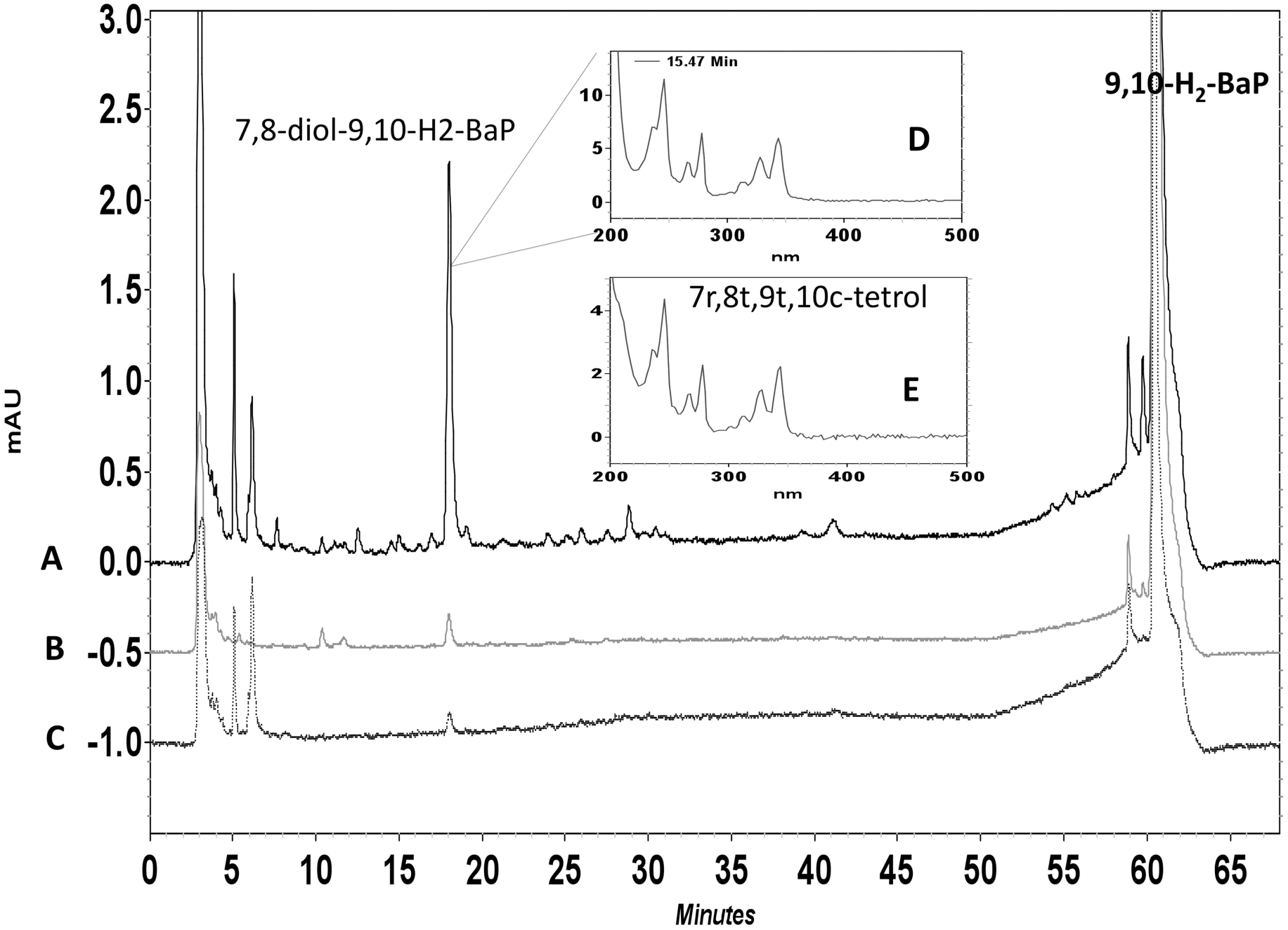

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Figure 8

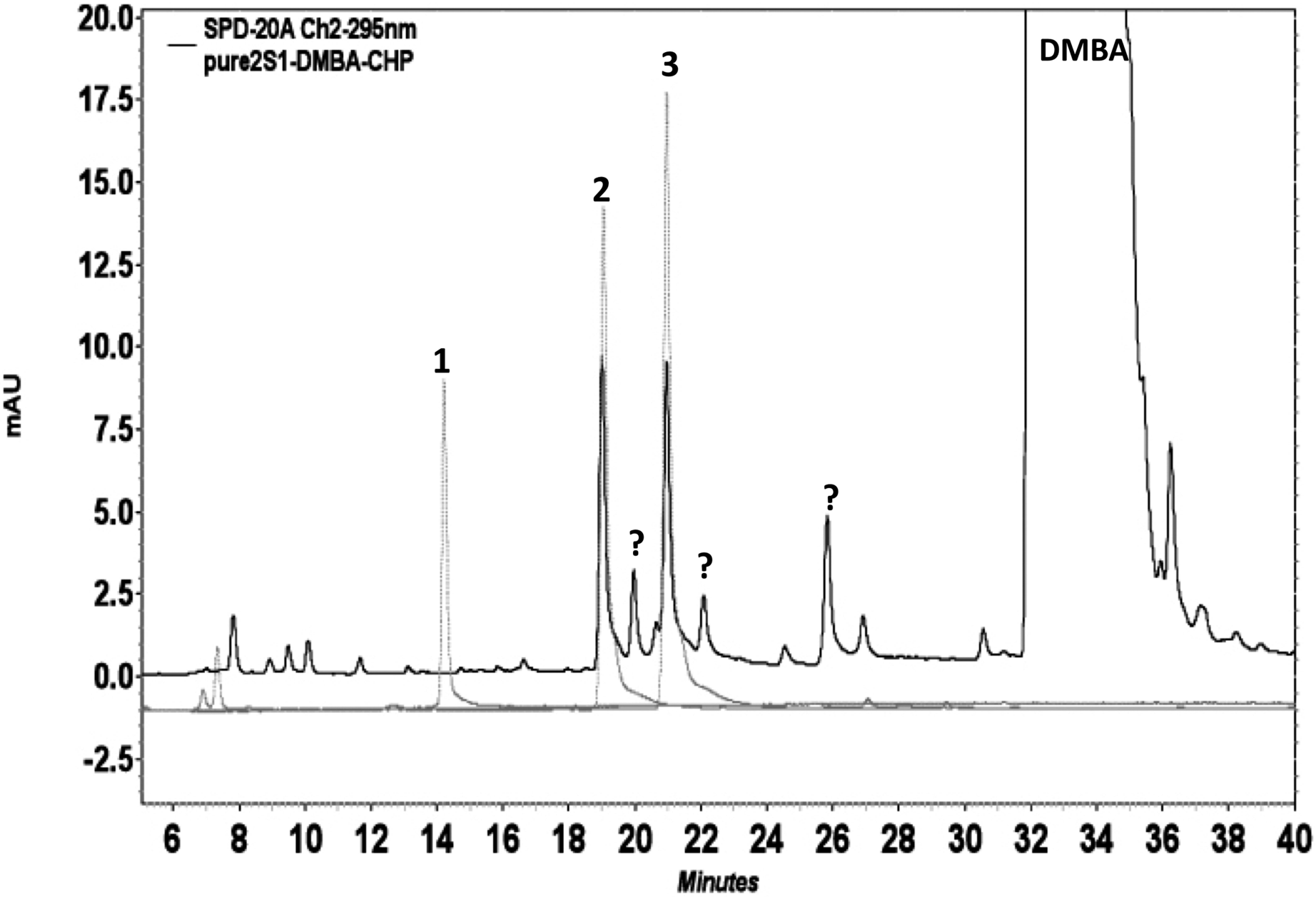

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Figure 9

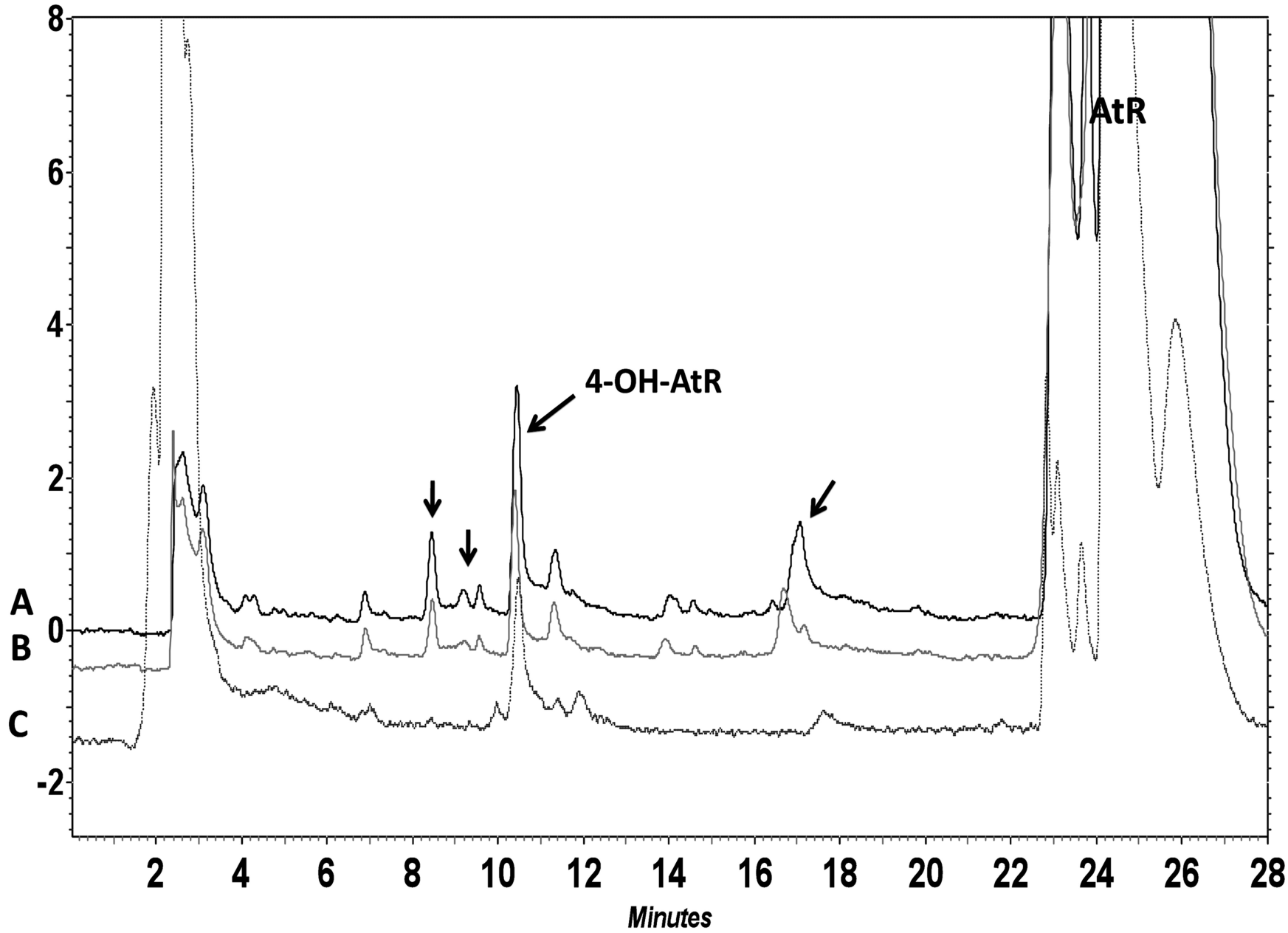

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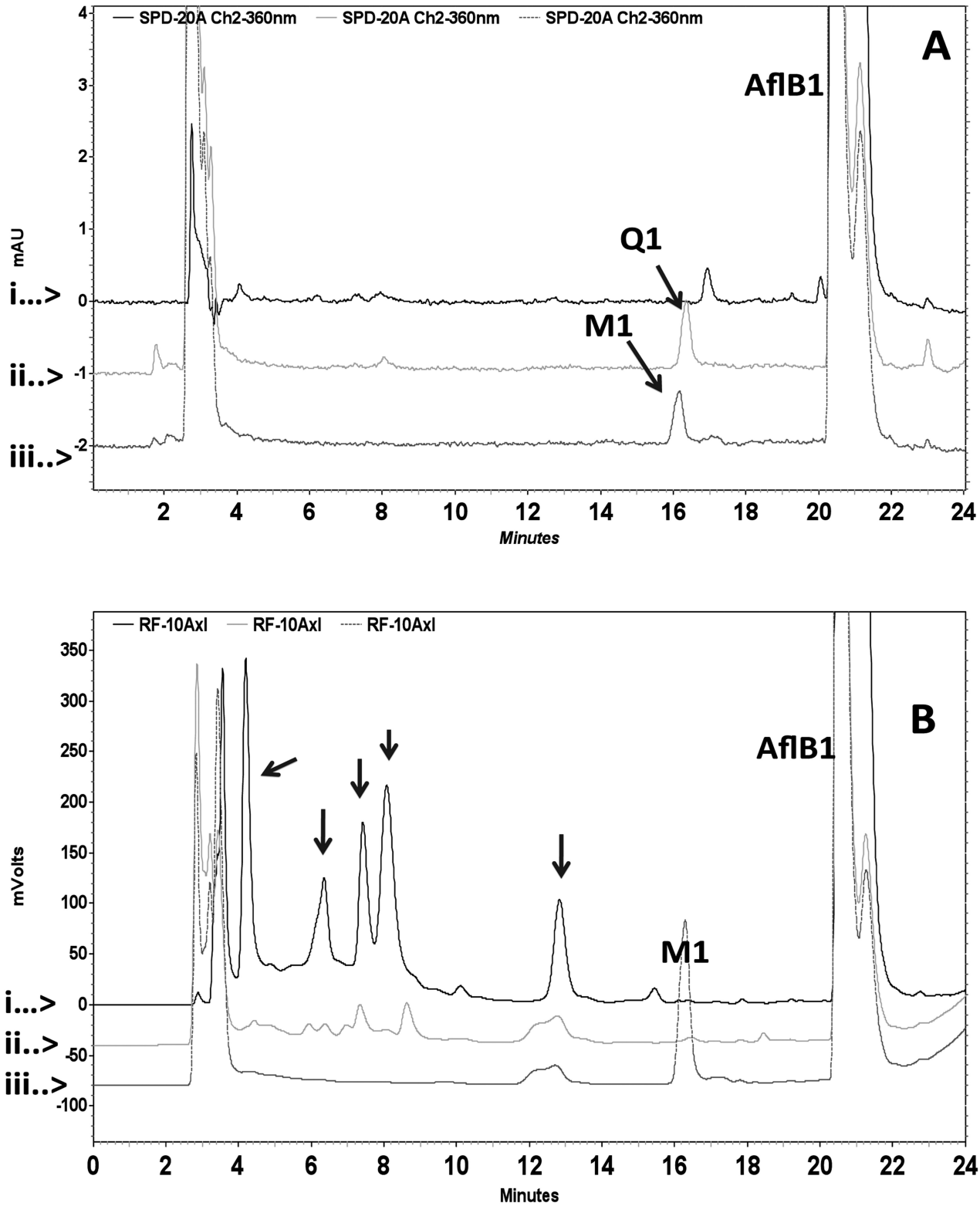
Figure 10



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Supplemental Material

N-terminal modifications of CYP2S1

N-terminal modifications (Table S1) were introduced into the native CYP2S1 cDNA construct by PCR-based mutagenesis using 5' primers containing a Bsa I site (which produces a Nco I compatible cohesive end) and 3' primers containing a Hind III site (see Table S2). A C-terminal 4-histidine tag was introduced using a 3' primer containing sequences encoding 4 histidine residues and Hind III (Table S2). Pfx High-Fidelity DNA polymerase (Invitrogen) was used for PCR amplification at an annealing temperature of 52°C for the first 8 cycles, followed by 25 cycles with an annealing temperature of 57°C. The PCR products were purified using the Qiagen PCR purification kit (Qiagen, Valencia, CA). The purified products were digested with Bsa I and Hind III and ligated with the pBAD-His A vector at the Nco I and Hind III sites. The constructs containing the modifications were verified by sequencing.

Construction of a dual-expression vector for co-expression of CYP2S1 and human P450 reductase, (hNPR)

The dual-expression vector has a *pBAD* promoter that is inducible by L-arabinose, and a *pTac* promoter that is inducible by IPTG. The *CYP2S1* gene was inserted under the control of the *pBAD* promoter and the *hNPR* (human P450 reductase) gene was inserted under the control of the *tac* promoter (Fig S2). The vector was created as follows. The CYP1A2 cDNA was removed from the pCW'/1A2,hNPR plasmid using Xba I and BamH I digestion, followed by re-ligation, to generate pCW-hNPR. The region of pCW-hNPR containing the *tac* promoter and *hNPR* was then amplified with a 5' primer containing a Nde I site and a 3' primer containing a Sph I site using pfx DNA polymerase. This PCR product was cloned into PCR4blunt topo vector (Invitrogen, Carlsbad, CA) for sequencing. It was then transferred into the pBAD-CYP2S1

plasmid using Nde I and Sph I sites. The final construct was named pBdtacHR-h2S1 vector (Fig. S2).

Purification of CYP2S1 from the synthetic gene construct

The use of detergent to solubilize P450s from membranes can be disadvantageous, since detergent can interfere with downstream uses of the enzyme, such as crystallization (Wester et al., 2002). Wester and colleague showed that use of high salt (500 mM Kpi) dissociated a N-terminal truncated CYP2C5 from the membrane without using any detergent. We applied a similar approach for the purification of CYP2S1 as follows. *E. coli* membranes were prepared as previously described (Wester et al., 2002) from LMG 194 cells carrying the pBAD-syn2S1dH construct which expresses the N-terminal modified 4 (see Table S1). The membranes were resuspended in 500 mM Kpi, pH 7.5, 20%glycerol, 0.5 mM PMSF, and 10 mM 2-mecaptoethanol buffer (buffer A). The high salt concentration of this buffer was used to release CYP2S1 from the membranes. After incubation for 3 hours at 4°C, the sample was centrifuged at 105,000g for 90 minutes using a Beckman ultracentrifuge (Beckman optima, LE, 80K). The supernatant containing CYP2S1 was collected and applied directly to a metal-ion-affinity resin, Ni-NTA agarose (Qiagen, Chatsworth, CA), which was equilibrated with buffer A. Approximately 1 ml of resin was used per 90 nmoles of P450. The resin was then washed with 50 ml of buffer A, followed by a subsequent wash with 2 volume equivalents of 100 mM Kpi, pH7.5, 100 mM sodium chloride, 20% glycerol, 1mM PMSF, and 10 mM 2-mercaptoethanol (buffer B). Subsequent washes were performed with 5 column volumes each of buffer B containing 50 mM glycine, and then a 20 mM imidazole was added to reduce the nonspecific binding of undesired proteins to the resin. The bound CYP2S1 were then eluted at a rate of 0.5 ml /min using 10 mM Kpi, pH 7.5, 500 mM NaCl, 20% glycerol, 10 mM 2-mercaptoethanol, 1

mM PMSF, and 200 mM imidazole. Fractions of 1ml volume were collected. The presence of CYP2S1 in the eluted fractions was checked using the reduced Fe^{2+} CO vs Fe^{2+} difference assay and western blot using our previously described CYP2S1 antibody (Rivera et al., 2007). The result indicated that there was still a significant amount of CYP2S1 attached to the membrane (lane 2, Fig. S5A) after incubation in 500 mM Kpi for 3 hours, and there was a little CYP2S1 in the elution fractions, as assessed by Western blot analysis and the reduced Fe^{2+} CO vs Fe^{2+} difference assay (data not shown).

We then attempted to purify CYP2S1 using detergent , as follows. The membranes were solubilized with 300 mM Kpi, pH 7.5, 20% glycerol, 1.0 % CHAPS, 0.1 mM DTT, and 0.1 mM EDTA buffer (buffer C) for 3 hours with stirring. The mixture was then centrifuged at 105,000 g for 30 minutes. The supernatant was applied to a Ni-NTA agarose column, which had been equilibrated with 10 column volumes of buffer C plus 20 mM imidazole (buffer D). The resin was washed with 10 volume of buffer D, followed by elution at a flow rate of 0.5 ml/min with buffer D plus 200 mM imidazole. Fractions of 1 ml aliquots were collected into tubes containing 10 µl of 100 mM EDTA. The presence of CYP2S1 in each fraction was determined by the reduced Fe^{2+} CO vs Fe^{2+} difference assay and SDS-PAGE. The eluted fractions were pooled and dialyzed 3 times against 100 volumes of 100 mM Kpi, pH 7.5, 20% glycerol, 0.1 mM DTT, and 1mM EDTA, to remove CHAPs.

More than 90% of the CYP2S1 was solubilized with the use of 1% CHAPs. The CHAPs-solubilized preparation was then applied to a NTA column. CYP2S1 was then eluted with 200 mM imidazole and the eluate contains high P450 concentration (up to 20 µM), as judged by the orange color of the fraction and by the P450 content. The elution factions contained a major band at ~ 52 KD (Fig. S5B). The elution fractions were pooled and dialyzed, and the specific

concentration of CYP2S1 was estimated to be 16 nmole/mg protein, based on the reduced Fe^{2+} CO vs Fe^{2+} difference assay, and the Bio-Rad protein assay. Furthermore, CYP2S1 was free of P420 (Fig. S5C). Thus, successful purification of CYP2S1 required 1% CHAPs.

Table and Figures

Table S1. N-terminal modifications. Top sequences are the CYP2S1 modified N-terminal sequences (the amino acid changes are in bold). The bottom sequence is that of the native protein. Modification 1: replacement with a N-term modification previously used for CYP2C9 and CYP2C8 expression in *E. coli* (our unpublished data). Modification 2: replacement with more hydrophobic amino acids and deletion of very hydrophilic amino acids. Modification 3: replacement with the N-terminal sequence of P450-17α1 (Barnes et al., 1991). Modification 4: deletion of the 23 amino acids after the methionine, changing the second glycine to alanine, and incorporating four histidines at the C-terminus.

1. Modification #1

MALVVLVLLLLLLFL-----LALSGTRARGHLPPGPTPLPLLGNLLQLRPGALYSGLMR
MEATGTWALLLALALLLLLTLALSGTRARGHLPPGPTPLPLLGNLLQLRPGALYSGLMR

2. Modification #2

M-Av-v-ALLLALALLLLLTLALSGTRARGHLPPGPTPLPLLGNLLQLRPGALYSGLMR
ME_AT_G_T_W_ALLLALALLLLLTLALSGTRARGHLPPGPTPLPLLGNLLQLRPGALYSGLMR

3. Modification #3

MALLLAVFLVLALALLLLLTLALSGTRARGHLPPGPTPLPLLGNLLQLRPGALYSGLMR
MEATGTWALLLALALLLLLTLALSGTRARGHLPPGPTPLPLLGNLLQLRPGALYSGLMR

4. Modification #4

M----------ATRARGHLPPGPTPLPLLGNLLQLRPGALYSGLMR
MEATGTWALLLALALLLLLTLALSGTRARGHLPPGPTPLPLLGNLLQLRPGALYSGLMR

Table S2. Primers for N-terminal modifications of CYP2S1

Modification	5'-primer	3'-primer
#1	**1**-TATAGGTCTCCCATGGCTCTGGTTGTTCTGGTTCTGCTGCTGCTGCTG **2**-GTTCTGCTGCTGCTGCTGCTGTTCCTGCTGGCGCTGTCCGGGACCAGG	ATTGCCAAGCTTCATCTGGTCTGCGTGGTGGAG
#2	TATAGGTCTCCCATGGCTGTTGTTGCACTG CTGCTGGCGCTGGCGCTG	ATTGCCAAGCTTCATCTGGTCTGCGTGGTGGAG
#3	TATAGGTCTCCCATGGCTCTGCTGCTGGCAGTTTTCCTGGTTCTGGCGCTGGCGCTGCTC	ATTGCCAAGCTTCATCTGGTCTGCGTGGTGGAG
#4	TATAGGTCTCCCATGGCTACCAGGGCCCGAGGCCACCTGCCCCCC	ATTGCCAAGCTTCAGTGGTGATGGGAGTGGGACGGACTTGCAGCTGGAAG
semiCYP2S1-dh	**1**-TATAGGTCTCCCATGGCTACTCGTGCTCGAGGTCATCTGCCGCCGGGTCCGAC **2**-TCATCTGCCGCCGGGTCCGACCCCGCTACCACTGCTGGGTAACCTCCTGCAGCTAC	ATTGCCAAGCTTCAGTGGTGATGGGAGTGGGACGGACTTGCAGCTGGAAG

Figure S1. Amino acid and DNA sequences of synthetic CYP2S1 (syn2S1)

MATRARGHLPPGPTPLPLLGNLLQLRPGALYSGLMRLSKKYGPVFTIYLGPWRPVVVLVGQEAVREALGGQAEEFSG
RGTVAMLEGTFDGHGVFFSNGERWRQLRKFTMLALRDLGMGKREGEELIQAEARCLVETFQGTEGRPFDPSLLLAQA
TSNVVCSLLFGLRFSYEDKEFQAVVRAAGGTLLGVSSQGGQTYEMFSWFLRPLPGPHKQLLHHVSTLAAFTVRQVQQ
HQGNLDASGPARDLVDAFLLKMAQEEQNPGTEFTNKNMLMTVIYLLFAGTMTVSTTVGYTLLLLMKYPHVQKWVREE
LNRELGAGQAPSLGDRTRLPYTDAVLHEAQRLLALVPMGIPRTLMRTTRFRGYTLPQGTEVFPLLGSILHDPNIFKH
PEEFNPDRFLDADGRFRKHEAFLPFSLGKRVCLGEGLAKAELFLFFTTILQAFSLESPCPPDTLSLKPTVSGLFNIP
PAFQLQVRPT**HHHH**

ATGGCTACTCGTGCGCGTGGCCATCTGCCGCCGGGTCCGACTCCGCTGCCGCTGCTGGGTAACCTGCTGCAGCTGCG
TCCGGGTGCACTGTATTCCGGCCTGATGCGCCTGTCTAAAAAGTACGGCCCGGTATTTACCATCTACCTGGGTCCGT
GGCGTCCGGTTGTGGTACTGGTTGGTCAAGAAGCTGTTCGTGAGGCTCTGGGCGGTCAAGCAGAGGAATTTTCCGGT
CGTGGCACCGTAGCGATGCTGGAGGGCACCTTTGACGGTCACGGTGTTTTCTTCTCCAACGGTGAACGTTGGCGTCA
ACTGCGCAAATTCACCATGCTGGCGCTGCGCGATCTGGGTATGGGCAAACGCGAAGGCGAAGAACTGATCCAAGCGG
AAGCTCGCTGTCTGGTAGAAACCTTTCAGGGTACTGAAGGCCGTCCGTTTGATCCGTCCCTGCTGCTGGCTCAGGCT
ACCTCTAACGTTGTTTGTTCCCTGCTGTTCGGTCTGCGTTTCTCTTACGAAGACAAAGAGTTCCAGGCGGTTGTTCG
CGCTGCTGGTGGCACTCTGCTGGGTGTTTCCTCTCAGGGTGGCCAGACTTATGAAATGTTCTCTTGGTTCCTGCGTC
CGCTGCCGGGTCCGCACAAACAGCTGCTGCACCACGTATCTACTCTGGCGGCGTTTACTGTGCGTCAGGTACAGCAA
CACCAGGGCAACCTGGACGCATCCGGTCCGGCTCGTGATCTGGTAGACGCATTTCTGCTGAAAATGGCACAGGAAGA
ACAGAACCCGGGTACTGAATTTACTAACAAGAACATGCTGATGACCGTTATCTACCTGCTGTTCGCAGGCACCATGA
CCGTTAGCACTACCGTGGGTTACACTCTGCTGCTGCTGATGAAATATCCGCACGTTCAGAAGTGGGTTCGCGAGGAA
CTGAACCGTGAGCTGGGCGCTGGTCAGGCACCGAGCCTGGGCGATCGTACCCGTCTGCCGTACACTGACGCGGTGCT
GCATGAAGCTCAGCGTCTGCTGGCTCTGGTTCCGATGGGCATCCCGCGTACTCTGATGCGCACTACCCGCTTCCGCG
GTTACACCCTGCCGCAGGGCACCGAGGTATTCCCGCTGCTGGGCTCTATTCTGCATGACCCGAACATCTTCAAACAC
CCGGAGGAGTTTAACCCGGATCGTTTCCTGGATGCTGATGGTCGTTTCCGTAAACACGAAGCATTCCTGCCGTTCTC
TCTGGGCAAACGTGTTTGTCTGGGCGAAGGTCTGGCTAAAGCAGAACTGTTCCTGTTTTTCACCACCATCCTGCAAG
CGTTCAGCCTGGAATCCCCGTGTCCGCCAGATACTCTGTCCCTGAAGCCGACTGTTTCCGGTCTGTTTAACATTCCG
CCGGCATTCCAACTGCAGGTTCGTCCGACCGACCTGCATTCCACCACCCAAACCCGTTAA

Figure S2. Vector Map of pBdtacHR-h2S1 construct.

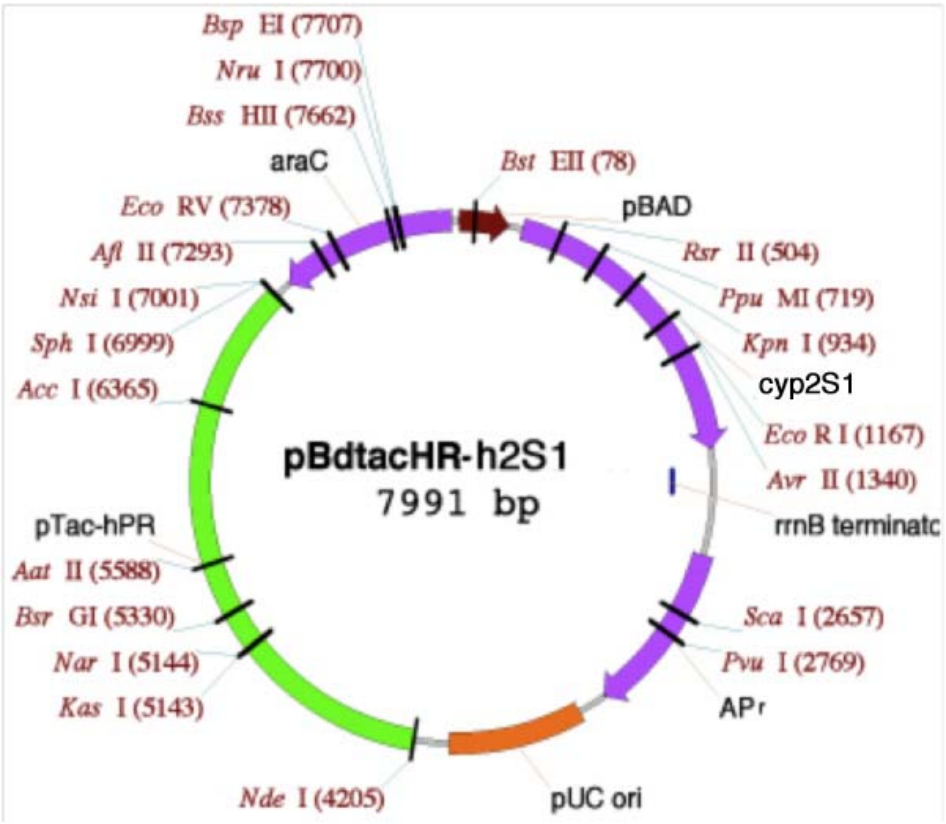


Figure S3. Expression of CYP2S1 modification 1 (A), 2 (B), 3 (C), and 4 (D) in *E. coli*. All N-terminal modification constructs were co-expressed with P450-reductase in strain LMG 194. Fe^{2+} CO vs Fe^{2+} difference spectra were measured using a 1:5 dilution of lysate supernatant in 100 mM Kpi, 20% glycerol.

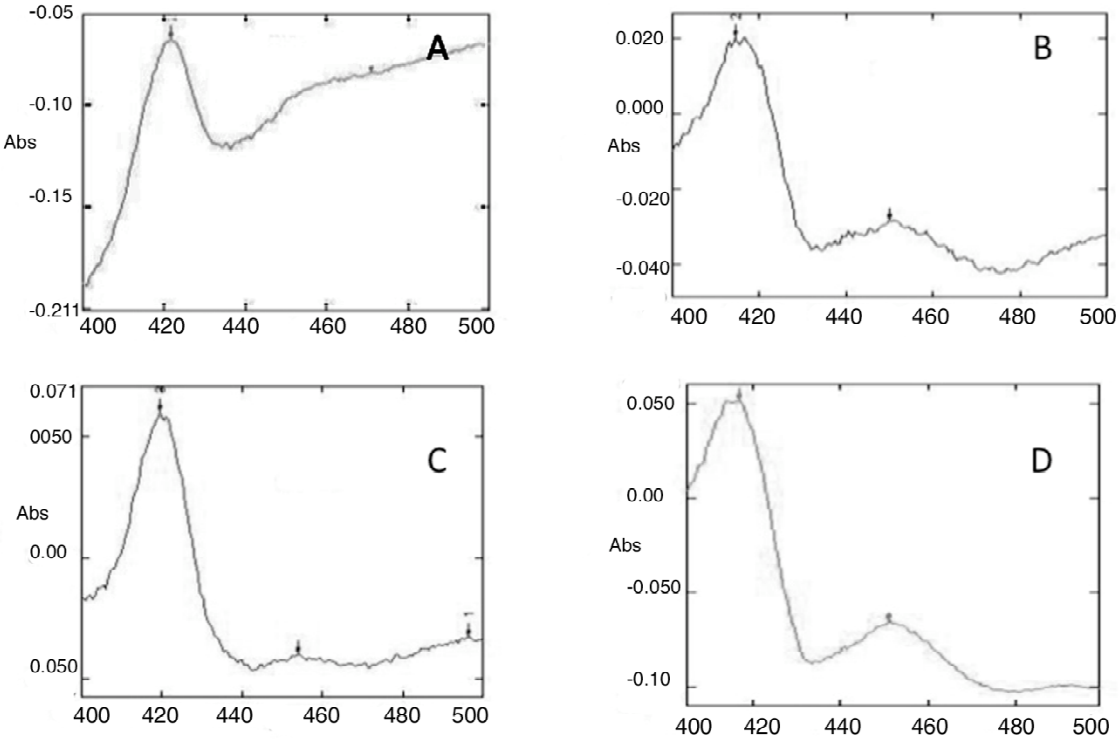


Figure S4. Modifications introduced into the CYP2S1 cDNA to optimize codon usage in *E. coli* and minimize mRNA secondary structure. Nucleotide changes are indicted with letters. CYP2S1-dH cDNA represents CYP2S1 constructs 4 (table 1) with native DNA sequence. semiCYP2S1-dH cDNA represents CYP2S1 construct 4 with some modifications at the 5' end of the gene. syn2S1-dh cDNA represents CYP2S1 construct 4 with a fully synthetic DNA containing nucleotide optimized for *E. coli* expression.

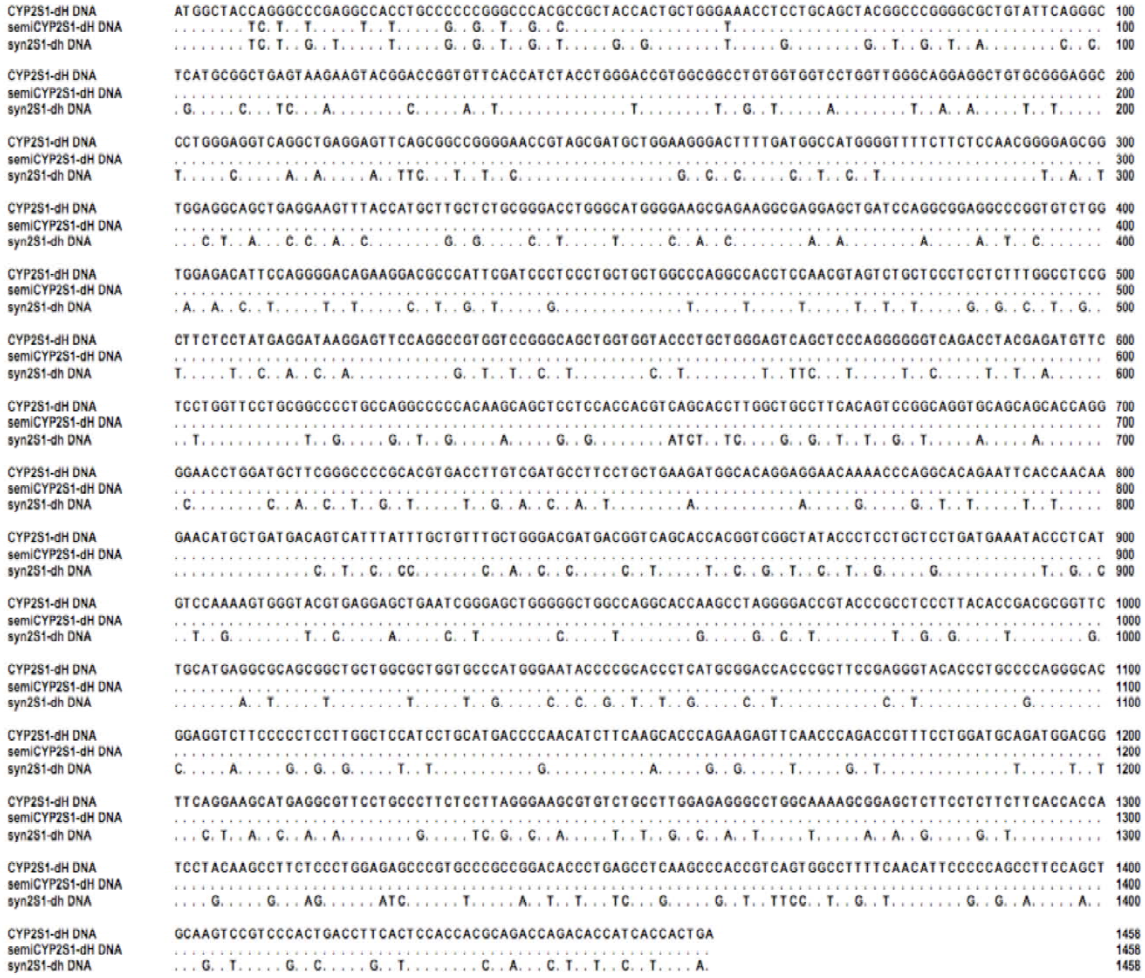
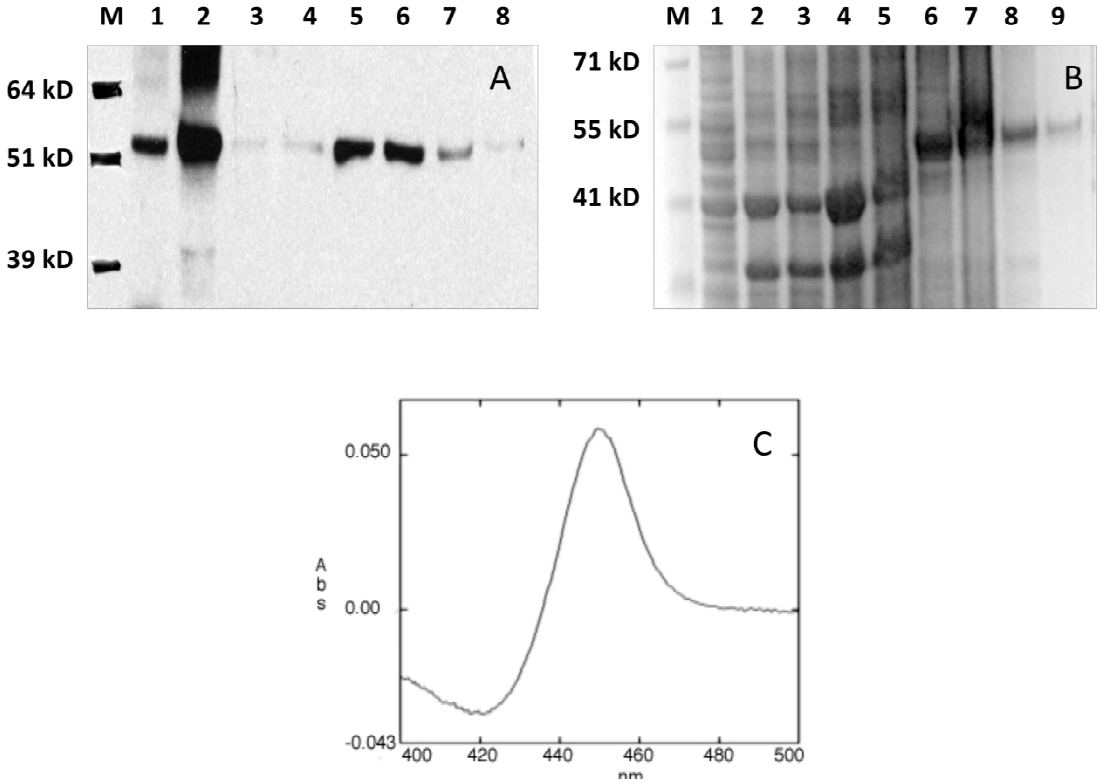


Figure S5. Purification of synthetic CYP2S1 using a Ni^{2+} nitriloacetic acid (NTA) column with and without detergent. Western blot of CYP2S1 purified without detergent (A) Lane 1- lysate, lane 2- ultracentrifuged membrane, lane 3- ultracentrifuged supernatant, lane 4- column flow through, line 5-8- elution fractions 1 to 4. SDS-PAGE of synthetic CYP2S1 purification with 1% CHAPs (B) Lane 1-lysate, lane 2-detergent solubilized membrane, lane 3- ultracentrifuged supernatant, lane 4-ultracentrifuged pellet, lane 5- column flow through, lane 6-9-elution fractions 1 to 4. Reduced Fe^{2+} CO vs Fe^{2+} difference spectrum of dialyzed purified CYP2S1 measured using a 1:20 dilution (C). The P450 concentration is 16 nmole/ mg protein.



References

Barnes HJ, Arlotto MP and Waterman MR (1991) Expression and enzymatic activity of recombinant cytochrome P450 17 alpha-hydroxylase in Escherichia coli. *Proc Natl Acad Sci U S A* **88**(13):5597-5601.

Rivera SP, Wang F, Saarikoski ST, Taylor RT, Chapman B, Zhang R and Hankinson O (2007) A novel promoter element containing multiple overlapping xenobiotic and hypoxia response elements mediates induction of cytochrome P4502S1 by both dioxin and hypoxia. *J Biol Chem* **282**(15):10881-10893.

Wester MR, Stout CD and Johnson EF (2002) Purification and crystallization of N-terminally truncated forms of microsomal cytochrome P450 2C5. *Methods Enzymol* **357**:73-79.