Chemical Synthesis of Six Novel 17β-Estradiol and Estrone Dimers and Study of Their Formation Catalyzed by Human Cytochrome P450 Isoforms

Aaron Yun Chen, Anthony J. Lee,† Xiang-Rong Jiang, and Bao Ting Zhu*

Department of Pharmacology, Toxicology and Therapeutics, School of Medicine, University of Kansas Medical Center, 2146 West 39th Street, Kansas City, Kansas 66160

Received June 21, 2007

Our earlier studies have shown that over 20 nonpolar 17β-estradiol metabolite peaks were detected following incubations of radioactive 17β-estradiol with human liver microsomes or recombinant human cytochrome P450 isoforms in the presence of NADPH as a cofactor. The structures of two representative nonpolar metabolites were identified earlier as dimers of 17β-estradiol linked through a diaryl ether bond between the C-3 phenolic oxygen of one molecule and the C-2 or C-4 aromatic carbon of another. Six additional putative dimers between estrone and 17β-estradiol with structures similar to the two identified ones were synthesized in this study. Using these newly synthesized estrogen dimers as reference standards, we demonstrated that incubations of human liver microsomes or various human cytochrome P450 isoforms with estrone or 17β-estradiol alone or two estrogens in combination in the presence of NADPH as a cofactor resulted in the formation of all eight estrogen dimers in varying quantities.

1. Introduction

The endogenous estrogens, such as 17β-estradiol (estradiol or E_2 [a]) and estrone (E_1), are important female gonadal hormones that have very diverse physiological and pathophysiological actions.[1] Metabolically, E_2 and E_1 undergo extensive metabolism in vivo, including oxidation and conjugation (such as glucuronidation, sulfonation, and *O*-methylation). It is known that members of the cytochrome P450 (CYP) family are the major enzymes catalyzing the NADPH-dependent oxidative metabolism of endogenous steroidal estrogens to multiple hydroxylated or keto metabolites.[2,3] There is considerable experimental evidence suggesting that some of the endogenous estrogen metabolites, such as 4-hydroxy-E_2, 15α-hydroxy-E_2, 16α-hydroxy-E_1, 16-epiestriol (16β-hydroxy-E_2), 17α-estradiol, and 2-methoxy-E_2 (an *O*-methylated product of 2-hydroxy-E_2 formed by catechol-*O*-methyltransferase), may have unique biological functions that are not associated with their parent hormones E_2 and E_1.[4,5,12] These multiple metabolic pathways not only determine the pharmacokinetic features of the endogenous estrogens in various target tissues in the body but also diversify the biological actions of endogenous estrogens in certain target sites through metabolic formation of biologically active estrogen derivatives.

Using [^{3}H]E_2 as substrate, we recently demonstrated, for the first time, the formation of over 20 nonpolar radioactive metabolites of E_2 under the commonly used NADPH-dependent conditions for human CYP enzyme-mediated oxidative metabolism of [^{3}H]E_2.[5] Some of the nonpolar metabolite peaks appeared to be preferentially formed in large quantities with certain human CYP isoforms, most notably CYP3A4 and CYP3A5.[6] Two of the major metabolites have been successfully confirmed to be E_2 dimers.[7] They are linked together through a diaryl ether bond between the C-3 phenolic oxygen of one E_2 and the C-2 or C-4 aromatic carbon of another E_2.

On the basis of the structural information derived from these two E_2 dimers, we believed that several other structurally related dimers between two E_1 molecules or between one E_2 and one E_1 could also be formed under the same in vitro metabolic conditions. To test this hypothesis, we first chemically synthesized all six additional putative dimers of E_2 and E_1 (compounds **15**−**20**, structures shown in Figure 1). The synthesis used E_2 and E_1 as starting materials and followed a four-step procedure with the Ullmann condensation reaction as a key step.[8−10] Using these newly synthesized estrogen dimers as reference standards, we then demonstrated that incubations of human liver microsomes or recombinant human CYP isoforms with E_2 and E_1 as substrates (singly or in combination) in the presence of NADPH as cofactor resulted in the formation of all eight estrogen dimers in varying quantities.

2. Results and Discussion

2.1. Chemical Synthesis of Six Estrogen−Estrogen Dimers. Using E_1 and/or E_2 as the starting material, we have synthesized six new estrogen dimers that are covalently linked together through a diaryl ether bond between a phenolic oxygen atom of one E_1 or E_2 molecule and the C-2 or C-4 aromatic carbon of another E_1 or E_2. The synthesis scheme is depicted in Figure 2. The synthetic method included four steps: (1) bromination of E_1 or E_2 to yield 4-bromo-E_1/E_2 and 2-bromo-E_1/E_2 (compounds **1**−**4**, structures shown in Figure 1); (2) protection of the C-3 phenolic hydroxyl group of the step 1 product to yield the 3-*O*-benzyl ethers of 2- or 4-bromo-E_1/E_2 (compounds **5**−**8**, structures shown in Figure 1); (3) the Ullmann condensation reaction to link C-2 or C-4 bromo-E_1/E_2 to the 3-*O* position of another E_1/E_2 to yield the 3-*O*-benzyl ethers of the dimers (compounds **9**−**14**, structures shown in Figure 1);[18] (4) removal of the protective C-3 benzyl group by hydrogenation catalyzed by Pd−C to yield the final estrogen dimers (compounds **15**−**20**, structures shown in Figure 1). In this synthetic procedure, the diaryl ether linkage (step 3), which is also commonly known as the Ullmann condensation reaction,[3,8,9] is a key step. The

* To whom correspondence should be addressed. Phone: 913-588-9842. Fax: 913-588-8356. E-mail: BTZhu@kumc.edu.

† Present address: Department of Drug Metabolism, Abbott Laboratories, Abbott Park, IL 60064.

[a] Abbreviations: E_2, 17β-estradiol; E_1, estrone; CYP, cytochrome P450; GC−MS, gas chromatography−mass spectrometry; NADPH, nicotinamide dinucleotide phosphate (reduced form); HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; HRMS, high-resolution mass spectrometry.

10.1021/jm0707323 CCC: $37.00
Published on Web 10/09/2007

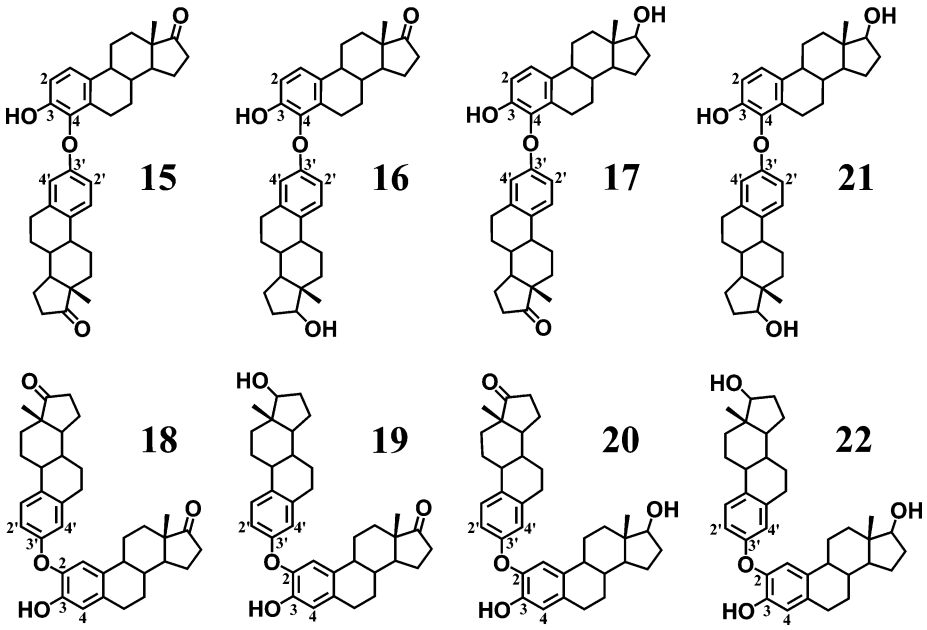

Figure 1. Chemical structures of the estrone (E_1) and 17β-estradiol (E_2) dimers. Compounds **15**−**20** are the E_1 or E_2 dimers that were chemically synthesized in the present study. The other two compounds (**21** and **22**) are the two E_2 dimers that were synthesized in our laboratory before.[6]

yields of steps 1, 2, and 4 were above 90% or even 95%, while the yields of step 3 was only about 50%. Thus, the overall yields for the synthesis of these estrogen dimers were slightly above 40%. For convenience, some relevant information concerning the synthetic procedures is given below.

In the synthesis of 2 and 4-bromo-E_1 (step 1) we initially suspected that the C-17 keto group of E_1 might not be stable in the reaction. Thus, we tried to use a 1,3-dioxolane group to protect the C-17 keto group before bromination.[14,15] We later found that using E_1 directly (without the protection) for the synthesis of dimers worked even better. No appreciable amounts of the C-17-modified side products were detected when the C-17 keto group of E_1 was not protected.

It is also of note that the use of finer granules of the substrates (E_1/E_2 and *N*-bromosuccinimide) in the synthesis of 2- or 4-bromo-E_1/E_2 (step 1) would markedly facilitate the reaction. If small clumps of *N*-bromosuccinimide (NBS) were used, brief sonication of the reaction mixture would be helpful for accelerating the reaction. Also, the flask should be continuously shaken when *N*-bromosuccinimide was slowly added. Otherwise, the yields of the dibromo byproducts may increase considerably.

The purification of 2-bromo-E_1 from the mixture of 2- and 4-bromo-E_1 turned out to be a rather difficult step. We tried to use recrystallization, which was very successful in yielding 2-bromo-E_2. However, 2-bromo-E_1 just would not come out of the solvents alone without the company of 4-bromo-E_1. We tried to use a variety of organic solutions for this purpose, including some of the quite nonpolar solvents (e.g., chloroform and ethyl acetate) as well as some of the polar protic solvents (such as acetone, acetonitrile, acetic acid, ethanol, and methanol). Different combinations of those solvents were tested for crystallization. Unfortunately, none was effective enough for this purpose. Eventually, we settled on using a semipreparative HPLC method to obtain high-purity 2-bromo-E_1 (>99%) with a mobile phase consisting of methanol, water, and acetonitrile (v:v:v = 2:1:2).

It was noted earlier that the Ullmann condensation reaction usually requires quite high temperatures (up to 300 °C), a long reaction time (up to 3 days), and strong polar solvents.[16,17] These harsh conditions often also produce competitive side products such as dehalogenated arene and homocoupled diaryls. In our synthesis, in order to increase the stability of substrates and also to avoid the formation of too many side products, we chose to use relatively moderate temperatures (155−160 °C) with a longer reaction time (72 h). It turned out that the reaction proceeded slowly during the first 48 h, and the reaction appeared to further slow down between 48 and 72 h. Between 72 and 96 h, the reaction proceeded very slowly, and thus, this reaction was terminated at 72 h after it had begun, with a yield of slightly over 50%.

Although the products from the Ullmann condensation reaction could be used directly without purification for the last-step reactions, we found that a rough separation of the products using silica gel column chromatography was advantageous for simplifying the procedures in the last step. This separation step made the crystallization of the estrogen dimers in the last step easier and faster, and it also yielded estrogen dimers with much higher purity. In addition, the Pd−C catalyst that was used in the last step would have a much longer recycling life when most of the polymers and other catalysts were removed.

2.2. Formation of Nonpolar E_1 Metabolites by Human CYP Isoforms. As part of our continuing effort to expand the knowledge on the human CYP isoform-mediated formation of nonpolar estrogen metabolites, we determined in this study the nonpolar E_1 metabolites formed by 15 recombinant human CYP isoforms (CYPs 1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9*1 (Arg_{144}), 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, and 4A11) using [^{3}H]E_1 as substrate and NADPH as cofactor. The same shortened HPLC gradient condition (method B in Table 1), which was optimized in our early study for the separation of most nonpolar E_2 metabolites,[5] was used for quantifying the various nonpolar [^{3}H]E_1 metabolites.

Representative HPLC chromatographs showing the detection of nonpolar [^{3}H]E_1 metabolite peaks formed by representative human CYP isoforms are shown in Figure 3. A total of more than 20 radioactive nonpolar metabolite peaks were detected after incubations of [^{3}H]E_1 with various human CYP isoforms. For convenience of description, the major nonpolar E_1 metabolite peaks formed by human CYP isoforms were denoted as

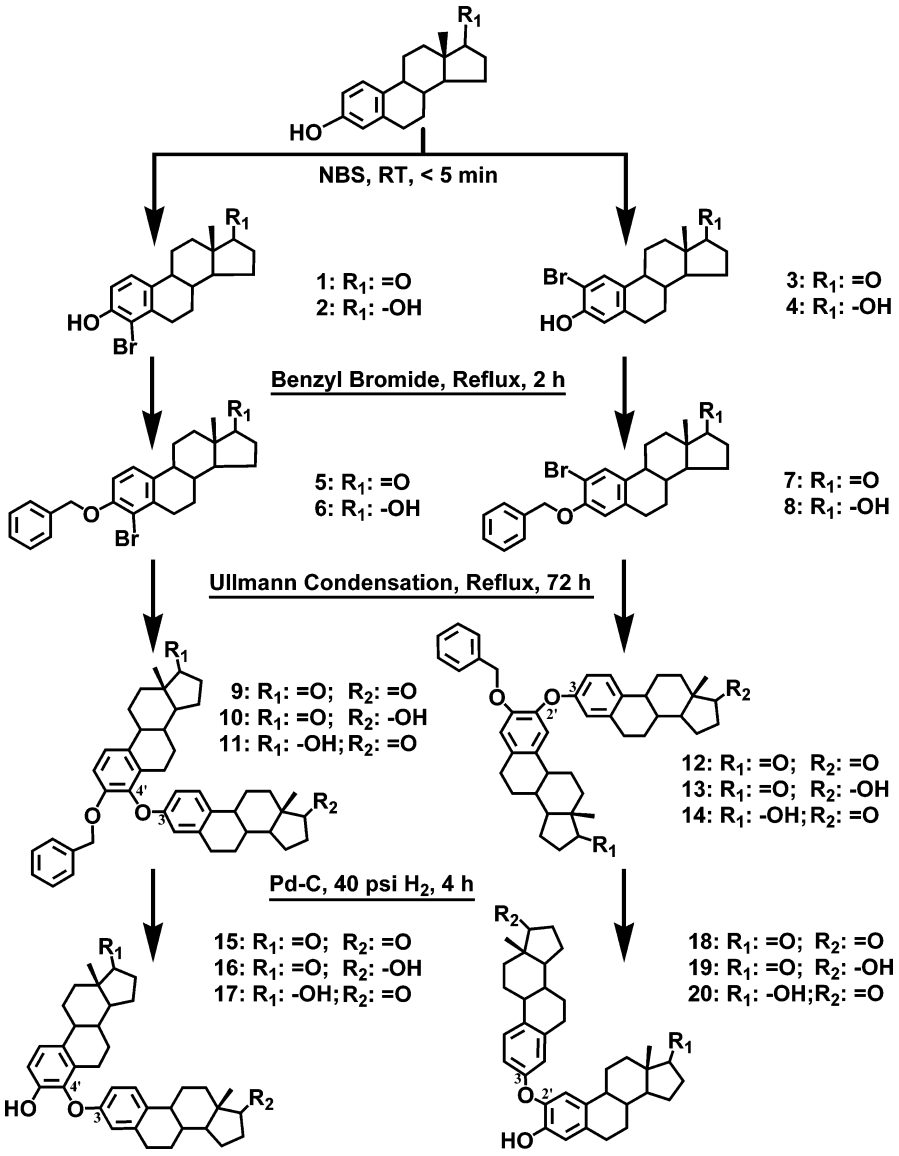

Figure 2. Overall scheme for the chemical synthesis of all estrogen dimers described in the present study.

N1 through N14 according to their elution order. The rates of their formation by different CYP isoforms are summarized in Figure 4.

Among the nonpolar estrogen metabolites detected, five of them, namely, N2, N3, N5, N6, and N7, were metabolically formed by most human CYP isoforms at varying rates (Figure 4). In comparison, N1, N4, and N8−N14 were only formed with a few of the CYP isoforms. CYP3A5 had the highest catalytic activity for the formation of N11 (65 (pmol/nmol P450)/min), followed by CYP3A4. CYPs 1A1, 1B1, 2B6, and 2C9*1 (Arg_{144}) showed weak but detectable catalytic activity for the formation of this nonpolar metabolite. All other CYP isoforms did not have appreciable catalytic activity for the formation of N11. A comparison with the retention times of the eight synthesized estrogen dimers suggested that peak N11 contained a mixture of two E_1 dimers (compounds **15** and **18**, structures shown in Figure 1). Information concerning their structural identification was described in section 2.3.

Notably, the metabolite peak N8, which was detected as a quantitatively major peak when [^{3}H]E_1 was incubated with human liver microsomes (data not presented), was only a quantitatively minor nonpolar peak when any of the 15 recombinant human CYP isoforms was assayed. This observation suggested that N8 likely was formed by other CYP isoform(s) or, more likely, it was formed by non-CYP enzyme(s) or formed nonenzymatically.

When the formation of N11 is used as an example, it appeared that its formation by 15 human CYP isoforms did not correlate with their overall catalytic activity for the oxidative metabolism of estrogens. For instance, CYP1A2 had a high catalytic activity for the formation of 2-hydroxylated metabolites from E_2 and E_1.[11] However, no appreciable activity for the formation of N11 by this CYP isoform was detected. This observation suggested that the overall catalytic activity of different human CYP isoforms for the oxidative metabolism of estrogens may not necessarily correlate with their ability to form certain nonpolar estrogen metabolites.

In summary, a total of over 20 nonpolar estrogen metabolite peaks were detected following incubations of [^{3}H]E_1 with human CYP isoforms using NADPH as cofactor. The N11 was only formed with a few human CYP isoforms (mainly CYP3A5), and its formation was not correlated with the overall catalytic activity of various human CYP isoforms for the oxidative metabolism of E_1.

Table 1. HPLC Mobile Phase Gradients

time (min)	solvent A (%)	solvent B (%)	solvent C (%)	curve no.
		Method A		
0	40	30	30	
20	40	30	30	1
30	30	35	35	3
40	25	35	40	3
50	20	30	50	3
60	10	30	60	3
70	5	30	65	3
		Method B		
0	68	16	16	
8	68	16	16	1
15	64	18	18	9
28	59	21	20	8
38	57	21	22	2
70	40	30	30	6
105	30	35	35	6
165	30	35	35	6
175	0	50	50	1
190	68	16	16	1
		Method C[a]		
0	68	16	16	
8	68	16	16	1
15	64	18	18	9
28	59	21	20	8
38	57	21	22	2
70	40	30	30	6
105	30	28	42	6
150	30	28	42	6
165	0	50	50	1
180	68	16	16	1

[a] Solvent A: double-distilled water. Solvent B: pure methanol. Solvent C: pure acetonitrile. In the mobile phase conditions A and B, a Phenomenex Ultracarb column was used, but in method C, a Varian Microsorb-MV 100A column was used.

2.3. Confirmation of the E_1/E_2 Dimers Formed in Vitro by Human CYP3A4 and Human Liver Microsomes. In our recent studies, two dimers of E_2 (compounds **21** and **22**, structures shown in Figure 1) were confirmed to be metabolically formed in vitro following incubations of [^{3}H]E_2 with human CYP3A4 in the presence of NADPH as cofactor.[5] Because E_1 and E_2 have the same structural frame and their only difference resides in the C-17 position, we speculated that E_1 most likely can also be metabolically activated to form the same types of dimers that have been detected for E_2. Moreover, in addition to the formation of homodimers between two E_2 or E_1 molecules, we suspected that the heterodimers between E_1 and E_2 can also be formed metabolically under the same in vitro conditions. In an effort to provide a definitive answer to these questions, we chemically synthesized all eight possible estrogen dimers (described under sections 3.3−3.6), which were used as reference standards here to help confirm the metabolic formation of these dimers in vitro.

Using two HPLC separation conditions (methods B and C, Table 1), which were modified from an HPLC condition used in our early study,[5] we demonstrated that all eight estrogen dimers could be formed in vitro following incubations of human CYP3A4 + b_5 or human liver microsomes with E_1, E_2, or E_1 + E_2 as substrate(s) in the presence of NADPH as cofactor. We chose to use the human CYP3A4 + b_5 as a representative CYP isoform for additional study because it had the highest overall catalytic activity for the formation of most of the E_1 and E_2 nonpolar metabolites in vitro, as shown in Figure 4 and previously.[5,7] Representative HPLC traces (with UV detection) for the nonpolar estrogen metabolites formed by human CYP3A4 + b_5 using E_1, E_2, or E_1 + E_2 as substrate(s) and NADPH as cofactor are shown in Figures 6 and 7. All eight nonpolar estrogen dimer peaks were clearly shown, and their retention times matched exactly the synthetic reference standards. The first and third peaks have already been unequivocally confirmed in our early study to be the 2′-E_2−3′-E_2 dimer and the 4′-E_2−3′-E_2 dimer.[5] The other six peaks matched with the six additional estrogen dimers that were newly synthesized in the present study. Moreover, when the extracts containing the metabolically formed nonpolar estrogens were analyzed with a different HPLC method that used the Ultracarb column, both UV and radioactivity detections also simultaneously detected these nonpolar peaks (although not separated well from each other) and they matched all reference standards (Figures 6 and 7).

Notably, when E_2 was used as the single substrate, 2′-E_2−3′-E_2 and 4′-E_2−3′-E_2 dimers (compounds **22** and **21**, structures shown in Figure 1) were the quantitatively predominant dimers formed by CYP3A4 + b_5 in the presence of NADPH as cofactor (parts B and E of Figure 6). The peaks of the other six dimers could also be detected, albeit not very clearly because of their small quantities. The rates of formation of all these dimers formed metabolically were summarized in Table 2 for comparison.

When E_1 was used as the single substrate, the first six nonpolar estrogen dimer peaks were generally much smaller compared to those seen when E_2 was used as substrate (parts E and F of Figure 6). As expected, the rates of formation of 2′-E_1−3′-E_1 and 4′-E_1−3′-E_1 (compounds **18** and **15**, structures shown in Figure 1) were relatively higher than the rates of formation of the other six dimers. Moreover, the rate of formation of 2′-E_1−3′-E_1 (compound **18**, structure shown in Figure 1) was higher than that of 4′-E_1−3′-E_1 (compound **15**, structure shown in Figure 1).

In the NADPH-dependent metabolism of E_1 + E_2 mediated by CYP3A4 + b_5, the rate of formation of four heterodimers, i.e., compounds **16**, **17**, **19**, and **20** (structures shown in Figure 1), was markedly increased. This was as expected because when both E_1 and E_2 were present at higher concentrations in the in vitro metabolism system, the chances for these two substrates to interact with the enzyme molecules would increase, and consequently, product formation would also increase.

Similarly, when the human liver microsomes were incubated under the same in vitro conditions where E_1, E_2, or E_1 + E_2 were used as substrates, all eight estrogen dimers were also detected (Figure 7). Overall, the formation of all eight dimers by human liver microsomes had patterns similar to those formed by human CYP3A4 + b_5. These observations suggest that CYP3A4 is among the CYP isoforms present in human liver that are largely responsible for the formation of nonpolar estrogen metabolites (including the eight dimers).

It is of note that in recent years there is an increased interest in studying the metabolic formation and biological functions of certain nonpolar estrogen metabolites such as 2-methoxy-E_2[4,18] and estrogen-17β-fatty acid esters.[19−22] Studies of the naturally occurring estrogen-17β-fatty acid esters have suggested that these endogenous estrogen derivatives have preferential mitogenic and tumorigenic effects in the fat-rich mammary tissue compared the other target sites, such as the uterus and pituitary in the female rats.[21,22] In comparison, E_2 had a significantly stronger effect than E_2-17β-fatty acid esters in stimulating the growth and/or tumorigenesis of the pituitary and uterus in these animals.[22] The nonpolar estrogen dimers represent a new class of nonpolar estrogen metabolites. When the relative rates for the in vitro formation of various nonpolar estrogen

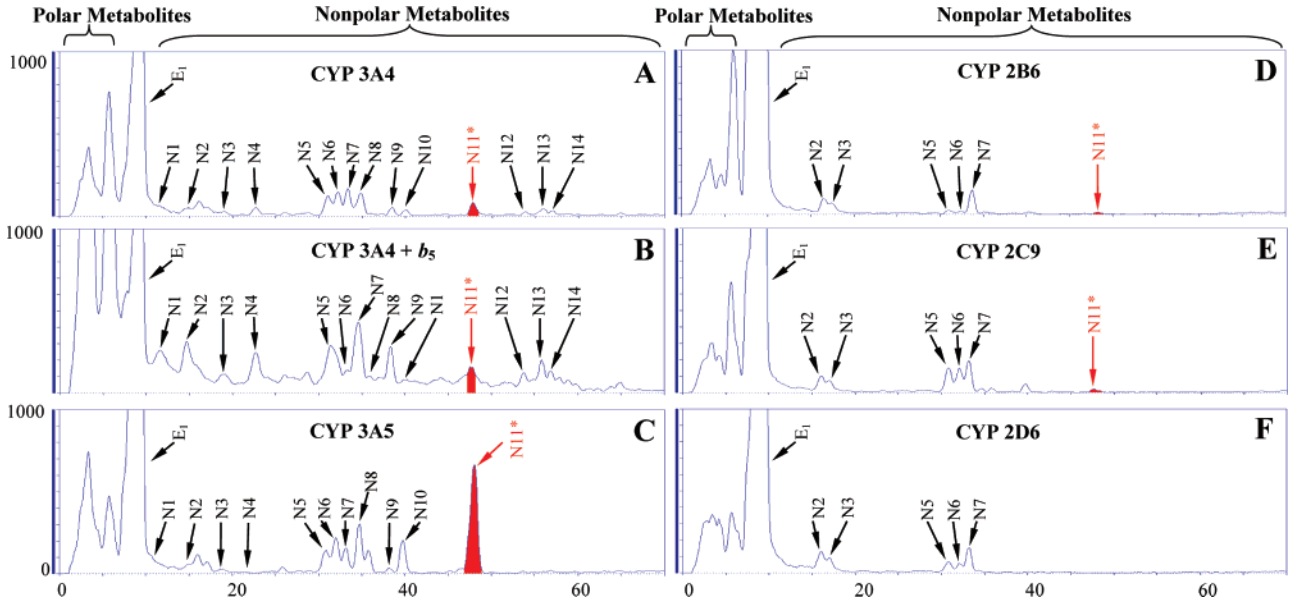

Figure 3. Representative HPLC traces of metabolites of E_1 by six representative CYP isoforms using NADPH as a cofactor. The separation was carried out by using the mobile phase method A on a Phenomenex Ultracarb column (radioactivity detection). E_1 was applied as substrate, and different CYP isoforms were used as indicated in each figure. All the polar metabolites were eluted within the first 8 min and indicated by a brace of each trace. The peak N11 with an asterisk was the mixture of two dimers of E_1. The quantitatively predominant peaks were labeled by numbers, and their peak areas were calculated for comparison (data are summarized in Figure 4).

metabolites were compared with the polar estrogen metabolites,[2,3,5,6,11] we were surprised to realize that the overall rates of formation of the former metabolites by human liver microsomes or CYP isoforms were comparable to the overall rates of formation of the latter metabolites. Given the high stability and lipophilicity of these metabolically formed nonploar estrogen metabolites, they are expected to have very long half-lives in vivo and they may also have a preferential biological activity in the fat-rich mammary tissues, in a similar way as known for the endogenous estrogen-17β-fatty acid esters.[21,22]

It has been known for years that covalently attaching a bulky nonpolar side chain to the C-7α or C-11β positions of an E_2 molecule is an effective strategy to produce high-affinity antiestrogens for therapeutic use in humans.[15,23] It was reported recently that different types of nonpolar estrogen dimers, which were chemically linked together and have different structures compared with the biologically formed ones described in the present study, have varying degrees of binding affinity for the estrogen receptors and also have cytotoxicity in a murine skin cancer cell line.[24] Given these intriguing earlier studies, it is tempting to suggest that some of the nonpolar estrogen metabolites formed by human CYP isoforms may be able to function as endogenously formed "selective estrogen receptor modulators" (SERMs). Certainly, more studies are needed to determine whether these nonpolar estrogen dimers are formed in vivo and also to determine what are their potential biological functions.

3. Materials and Methods

3.1. Chemicals and Reagents. E_1 and E_2 were purchased from Steraloids (Newport, RI). NADPH and ascorbic acid were obtained from Sigma-Aldrich (St. Louis, MO). [2,4,6,7 -^{3}H(N)]E_1 (specific activity of 65.5 Ci/mmol) and [2,4,6,7,16,17-^{3}H]E_2 (specific activity of 123.0 Ci/mmol) were obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA). NBS, potassium carbonate (K_2CO_3, anhydrous), benzyl bromide, anhydrous 4-picoline (4-methylpyridine), copper(II) oxide (CuO), anhydrous sodium sulfate (Na_2SO_4), and 10% palladium on carbon (Pd−C) were of ACS grades and purchased from ACROS (through Fisher Scientific, Atlanta, GA). Acetonitrile, chloroform-*d* (isotope D, 99.6%), dimethyl sulfoxide-d_6 (isotope D, 99.9%), *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), methanol-d_4 (isotope D, 99.96%), scintillation cocktail (ScintiVerse LC), and other solvents (HPLC grade or better) were purchased from Fisher Scientific (Atlanta, GA).

The human liver microsomes (HLM HG03) and the 15 selectively expressed human CYP isoforms (1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, and 4A11) were obtained from BD Gentest (Woburn, MA). These human CYP isoforms were expressed in insect cells that were selectively transfected with a baculovirus expression system containing the cDNA for each of the desired human CYP isoforms. The total microsomal protein concentration, CYP content, CYP reductase activity, cytochrome b_5 content, and the specific catalytic activity for the marker substrate(s) for each expressed CYP isoform were listed in our recent study.[11]

3.2. Equipment. High-resolution mass spectra (HRMS) were recorded by using a VG 70S analytical mass spectrometer. Methanol solution or powder samples of the synthesized chemicals were used for direct-probe HRMS. Gas chromatography−mass spectrometry (GC−MS) spectra were obtained by using the Agilent Technology model 6890N GC system, with a model 5973 mass selective detector. BSTFA with 1% TMCS was used as a derivatization reagent for GC−MS analysis. The nuclear magnetic resonance (NMR) spectra were recorded on a Varian Inova 300 or 400 spectrometer. Chemical shifts were given as δ values with reference to deuterium solvent.

The HPLC system used for separation of various estrogen metabolites consisted of a Waters 2690 separation module (Waters, Milford, MA), a radioactivity detector (β-RAM; IN/US Systems, Inc., Tampa, FL), a Waters UV detector (model 484), and an Ultracarb 5 ODS column (150 mm × 4.60 mm; Phenomenex, Torrance, CA) or a Microsorb-MV 100A column (300 mm × 3.9 mm; Varian, Palo Alto, CA). The detailed solvent gradients used for the simultaneous separation of both polar and nonpolar estrogen metabolites on an Ultracarb column and those used for the selective separation of the eight estrogen dimers on a Microsorb-MV 100A column are described later in section 2.7.

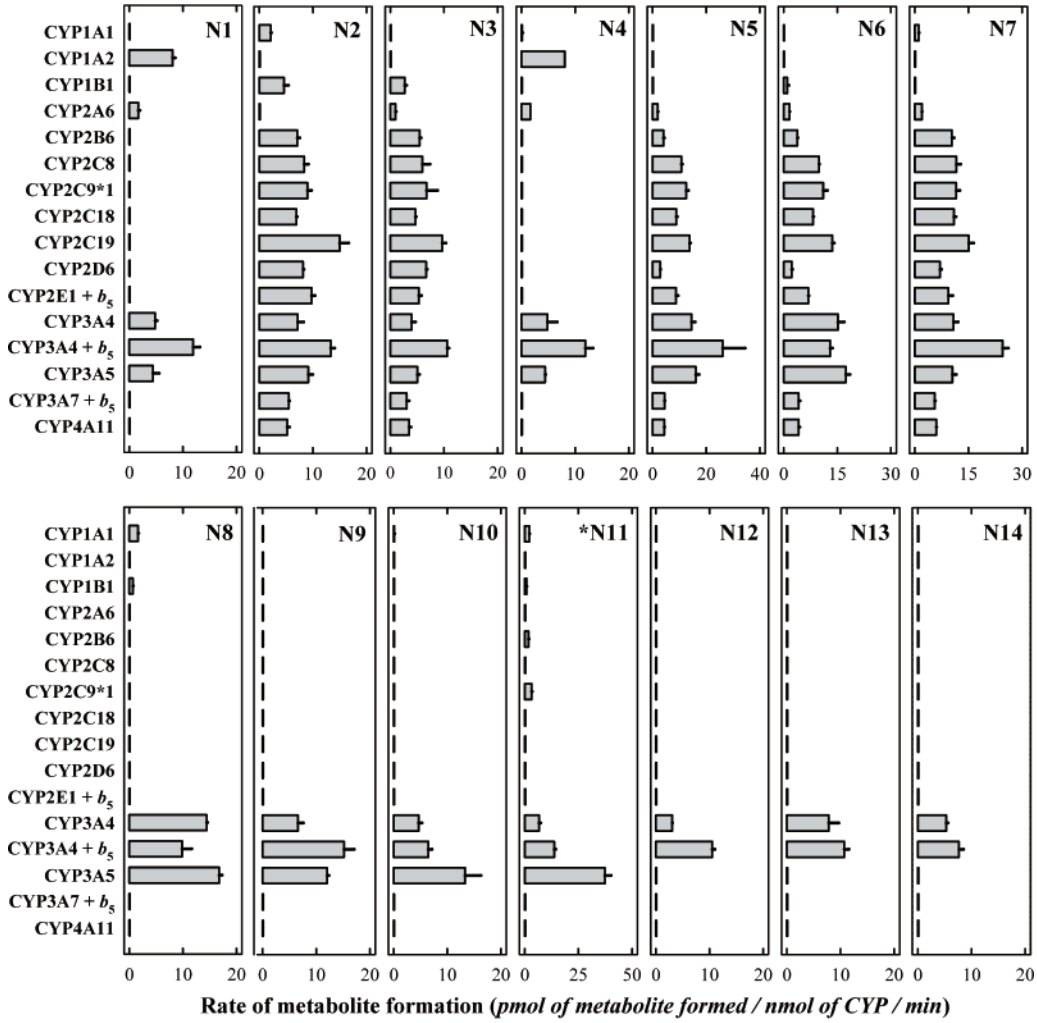

Figure 4. Rate of formation of nonpolar E_1 metabolites catalyzed by 16 human CYP isoforms. The unit is (pmol/nmol CYP)/min. Peak N11 was a mixture containing two E_1 dimers (compounds **15** and **18**, structures shown in Figure 1).

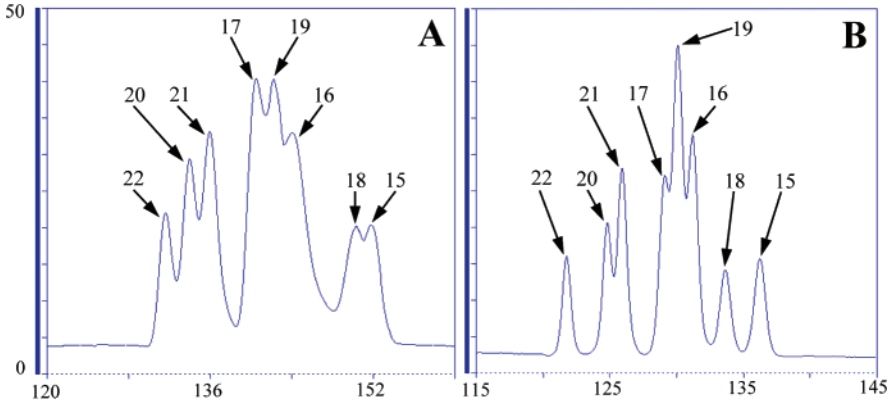

Figure 5. HPLC separation of the reference standards of the eight estrogen dimers using two different columns and two different mobile phase conditions. Both HPLC traces were obtained by using a UV detector: (A) HPLC trace showing the partial separation of eight estrogen dimers by using method B coupled with a Phenomenex Ultracarb column; (B) HPLC trace showing a better separation of the eight estrogen dimers using the HPLC method C on a Varian Microsorb-MV 100A column.

3.3. Chemical Synthesis of 2-Bromo-E_1/E_2 and 4-Bromo-E_1/E_2 (Compounds 1−4). E_1 (5 mmol) was suspended evenly in chloroform (50 mL), into which fine granules of NBS (5 mmol) were then added as the bromine donor. The mixture was shaken until it appeared to be a colorless transparent solution. The reaction usually took only less than 5 min if the NBS is in fine granules. The solution was then dried in a rotary evaporator to yield solids. Upon crystallization of the dried crude products that were redissolved in pure ethanol, 4-bromo-E_1 (compound **1**, structure shown in Figure 1) was first separated as white powder. 2-Bromo-E_1 (compound **3**, structure shown in Figure 1) was obtained by using a semipreparative HPLC. This step of the reaction was rather complete, and the yields for compounds **1** and **3** were between 45% and 48%. GC−MS analysis of its trimethylsilylated (TMS) product showed two molecular ions with masses (*m*/*z*) of 420 and 422 (purity >95%).

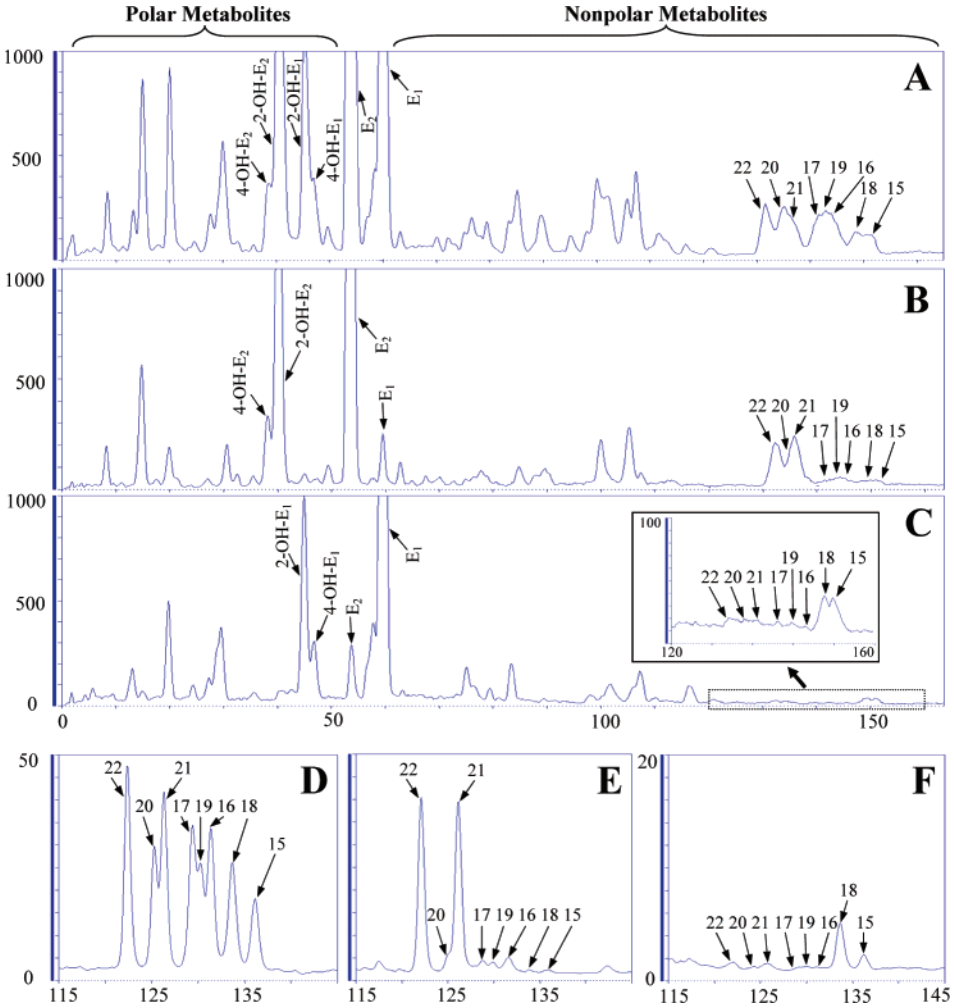

Figure 6. Representative HPLC traces showing the formation of nonpolar estrogen metabolites in which CYP3A4 + b_5 was used as the enzyme. $E_1 + E_2$ (A), E_2 (B), or E_1 (C) was used as the substrate. (A−C) Radioactivity detection was used. CYP3A4 at 140, 70, and 70 pmol/mL was used to produce panels A, B and C, respectively. The separation was carried out by using the mobile phase method B on a Phenomenex Ultracarb column (radioactivity detection). Panels D−F are the results from UV detection of the same metabolite extracts shown in panels A−C, respectively. Here, the mobile phase method C and a Varian Microsorb column was applied. Note that larger-scale in vitro reactions (e.g., 2 mL in total volume) were used in order to produce clearer HPLC traces (with UV detection) of all eight estrogen dimer peaks.

Compound 1: ^{1}H NMR (400 Hz, $CDCl_3$) 7.189 (1H, d, J = 8.4), 6.872 (1H, d, J = 8.4), 5.553 (1H, s), 2.968 (2H, dd, J = 18.0, 6.4 Hz), 2.376 (2H, dd, J = 6.4, 4.0 Hz), 0.901 (3H, s). HRMS: 348.0725 (calculated) and 348.0723 (observed).

Compound 3: ^{1}H NMR (400 Hz, $CDCl_3$) 7.342 (1H, s), 6.760 (1H, s), 5.293 (1H, s), 2.838 (2H, m), 2.507 (2H, dd, J = 14.8, 8.4 Hz), 0.909 (3H, s). HRMS: 348.0725 (calculated) and 348.0713 (observed).

The same method was used to produce 4-bromo-E_2 (compound **2**, structure shown in Figure 1) with E_2 as the starting material. After 4-bromo-E_2 was separated from the reaction mixture, the solution was evaporated to dryness and then redissolved in acetonitrile with mild sonication. 2-Bromo-E_2 (compound **4**, structure shown in Figure 1) was then crystallized from the ethanol solution. The structure and purity of both compounds were confirmed by using GC−MS analysis of their TMS products (double molecular m/z = 494 and 496), and each had a purity of >95%.

3.4. Synthesis of 3-*O*-Benzyl Ether of 2-Bromo-E_1/E_2 or 4-Bromo-E_1/E_2 (Compounds 5−8). The sample of 2- or 4-bromo-E_1/E_2 (1.0 mmol) was first dissolved in acetonitrile (150 mL), followed by addition of anhydrous potassium carbonate (10 mmol) and benzyl bromide (M:M = 1:1 with bromo-E_1 or bromo-E_2). Under nitrogen gas protection, the reaction mixture was refluxed with stirring for 2 h. After filtration of the mixture, the colorless 3-*O*-benzyl ethers of the starting materials could be readily obtained by crystallization from acetonitrile. The yields of this step were 90−92%. If pure products are not necessary, the whole reaction mixture can be dried in a rotary evaporator and then in a vacuum desiccator. The resulting powder can be used directly for the next synthetic step.

Compound 5: ^{1}H NMR (400 Hz, $CDCl_3$) 7.483 (2H, d, J = 6.4 Hz), 7.386 (2H, t, J = 8.0), 7.313 (1H, m), 7.190(1H, d, J = 8.8 Hz), 6.781(1H, d, J = 8.4 Hz), 5.152 (2H, s), 0.898 (3H, s), 3.065 (2H, dd, J = 18.4, 6.0 Hz), 2.514 (2H, dd, J = 19.2, 9.6 Hz). HRMS: 438.1194 (calculated) and 438.1181 (observed).

Compound 7: ^{1}H NMR (400 Hz, $CDCl_3$) 7.469 (2H, t, J = 7.6 Hz), 7.390 (2H, t, J = 8.0 Hz), 7.258 (1H, s), 7.676 (1H, s), 5.114 (1H, s), 0.906 (3H, s), 2.829 (2H, dd, J = 9.2, 4.4 Hz), 2.504 (2H, dd, J = 19.2, 9.2 Hz). HRMS: 438.1194 (calculated) and 438.1193 (observed).

GC−MS analysis of the 3-*O*-benzyl ethers of 4- or 2-bromo-E_2 (compounds **6** and **8**, structures shown in Figure 1) showed that the molecular masses matched the calculated mass values (double molecular m/z= 512 and 514) for these compounds and their purity was >95%.

3.5. Synthesis of 3-*O*-Benzyl Ethers of the Estrogen Dimers (Compounds 9−14).[13] Each of the products (compounds **5**− **8**, at 1.0 mmol) from the last synthetic step was dissolved in small amount of 4-picoline (10 mL). E_1 or E_2 (1.0 mmol) was then added as cosubstrate depending on which target estrogen dimers would be synthesized. Cupric oxide (40 mg, 0.5 mM) was added as

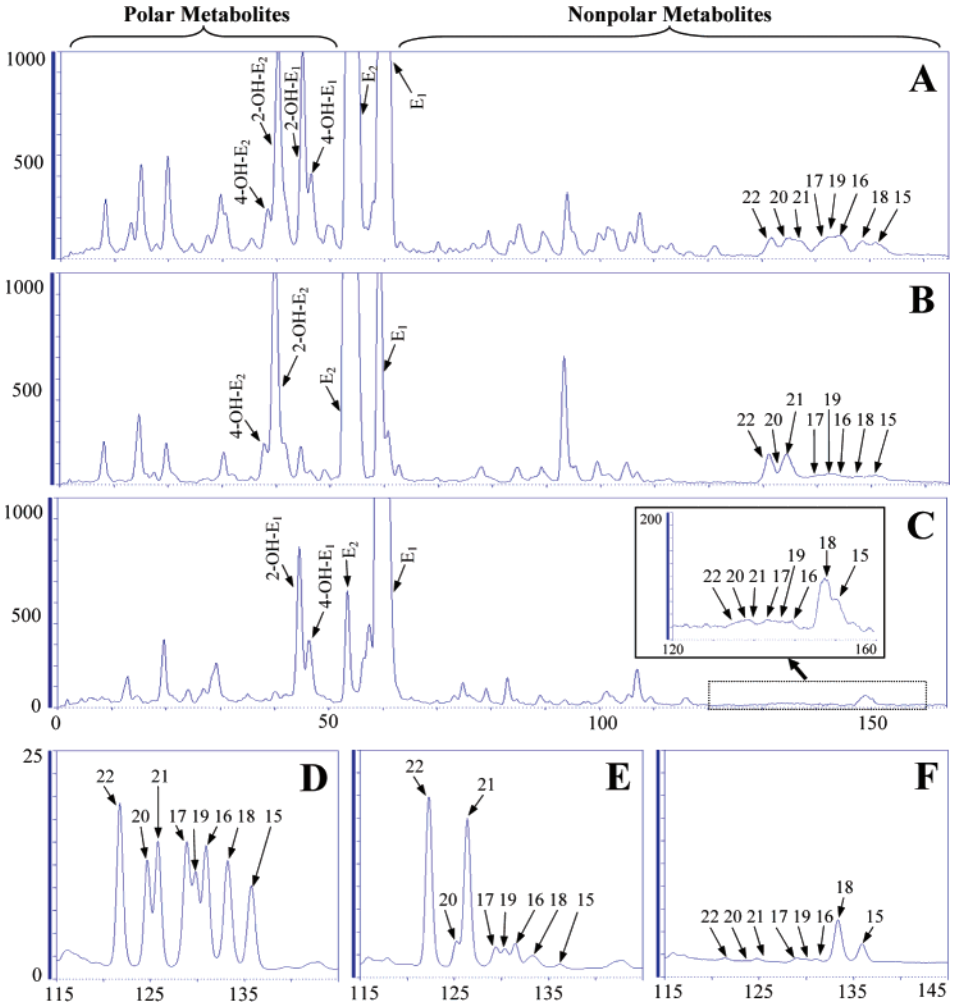

Figure 7. Representative HPLC traces showing the formation of nonpolar estrogen metabolites. Human liver microsomes were used as the enzyme. $E_1 + E_2$ (A), E_2 (B), or E_1 (C) was used as the substrate. (A−C) Radioactivity detection was used. Human liver microsomes at 4, 2, and 2 mg/mL were used to produce panels A, B, and C, respectively. The separation was carried out by using the mobile phase method C on a Phenomenex Ultracarb column (radioactivity detection). Panels D−F are the results from UV detection of the same metabolite extracts shown in panels A−C, respectively. Here, the mobile phase method C and a Varian Microsorb column were used. Note that larger-scale in vitro reactions (e.g., 2 mL in total volume) were used in order to produce clearer HPLC traces for UV detection of all eight estrogen dimer peaks.

Table 2. Rates of Metabolic Formation of Eight Nonpolar Estrogen Dimers Catalyzed by CYP3A4 + b_5 and Human Liver Microsomes

	enzyme: CYP3A4 + b_5 ((pmol/nmol of CYP)/min)			enzyme: human liver microsomes ((pmol/mg protein)/min)		
dimer	substrate $E_1 + E_2$	substrate E_2	substrate E_1	substrate $E_1 + E_2$	substrate E_2	substrate E_1
15	12.3	0.8	2.4	8.5	1.2	3.9
16	21.9	3.1	0.4	11.3	4.0	0.6
17	22.6	2.1	0.4	12.2	4.5	0.9
18	19.8	5.6	7.1	11.5	4.3	9.0
19	13.7	3.7	0.6	7.8	3.3	0.5
20	26.1	52.3	1.4	10.8	26.9	0.8
21	16.8	4.8	0.5	9.3	4.5	0.3
22	28.8	49.2	1.6	14.0	31.3	1.2

catalyst. The whole reaction mixture was heated to 155−160 °C with constant stirring under nitrogen for 72 h.

After the mixture was cooled to nearly room temperature, ethyl acetate (200 mL) was added to dilute the reaction mixture, which was then filtered. The resulting filtrate was washed with 1 N HCl (50 mL) three times to remove 4-picoline until the aqueous washing phase appeared to be light-brown. The organic layer was dried with anhydrous Na_2SO_4 and concentrated in a rotary evaporator. The product mixture appeared to be a brown oily liquid. The product was then roughly purified by going through a silica gel column with a mixture of hexane and ethyl acetate (v:v = 2:1) as the mobile phase to remove most of the polymer impurities. The eluent was dried and then placed in a vacuum desiccator. Compound **9** (a representative 3-*O*-benzyl ether of the estrogen dimers) was purified by using an HPLC system with an isocratic mobile phase containing methanol, water, and acetonitrile (v:v:v = 2:1:2) and a C_{18} preparation column (Alltech, Apollo C_{18} (5 μm), 250 mm × 10 mm). NMR and HRMS analyses of this product were performed to confirm its structure. The yield of this step was 50−55%.

Compound 9: ^{1}H NMR (400 Hz, $CDCl_3$) 7.255−7.220 (2H, m), 7.174−7.150 (1H, m), 7.089−7.050 (2H, m), 6.883 (1H, d, J = 8.8 Hz), 5.229 (1H, s), 5.016 (2H. s), 2.542−2.475 (2H, m), 0.920 (6H, s). HRMS: 628.3563 (calculated) and 628.3563 (observed).

3.6. Synthesis of Various Estrogen Dimers (Compounds 15−20). Each of the products from the preceding steps (i.e., compounds **9**−**14**, structures shown in Figure 2) was redissolved in ethanol in

a Parr flask in the presence of Pd−C (500 mg) as catalyst. The mixture was kept shaken under hydrogen at 40 psi for 4 h. Then most of the catalyst was removed after filtering through four layers of the regular filter papers. The filtered catalyst was ready for reuse after washing with pure ethanol. The solution was then concentrated in a rotary evaporator to a final volume of approximately 20 mL. The residue was then filtered using a 0.22 μm filtration membrane to remove the remaining catalyst. The filtrate was collected into a glass bottle and placed in a hood for slow crystallization to yield the white powdered estrogen dimers. To obtain a small amount of pure product, an HPLC system with an isocratic mobile phase containing methanol, water, and acetonitrile (v:v:v = 2:1:2) and a C_{18} preparation column (Alltech, Apollo C_{18} (5 μm), 250 mm × 10 mm) was employed to purify each dimer from the mixture. The yield of this step was 95−98%.

Compound 15: ^{1}H NMR (400 Hz, DMSO-d_6) 7.102 (1H, d, J = 8.8 Hz), 6.947 (1H, d, J = 8.8 Hz), 6.840 (1H, d, J = 8.0 Hz), 6.516 (1H, s), 6.423 (1H, s), 2.710−2.611 (2H, m), 0.799 (6H, s). HRMS: 538.3083 (calculated) and 538.3077 (observed).

Compound 16: ^{1}H NMR (400 Hz, MEOH-d_4) 7.141 (1H, d, J = 8.4 Hz), 7.052 (1H, d, J = 8.4 Hz), 6.749 (1H, d, J = 8.4 Hz), 6.529 (1H, d, J = 8.8 Hz), 6.464 (1H, d, J = 4.0 Hz), 3.650 (1H, d, J = 8.8 Hz), 2.817−2.743 (3H, m), 2.503−2.434 (3H, m), 0.867 (3H, s), 0.770 (3H, s). HRMS: 540.3240 (calculated) and 540.3255 (observed).

Compound 17: ^{1}H NMR (400 Hz, $CDCl_3$) 7.160 (1H, d, J = 8.4 Hz), 7.087 (1H, d, J = 8.7 Hz), 6.846 (1H, d, J = 8.4 Hz), 6.618 (1H, m), 5.116 (1H, s), 3.660 (2H, m), 2.713 (2H, dd, J = 14.8, 8.4 Hz), 0.887 (3H, s), 0.757 (3H, s). HRMS: 540.3239 (calculated) and 540.3228 (observed).

Compound 18: ^{1}H NMR (400 Hz, DMSO-d_6) 7.346−7.226 (2H, m), 6.886 (1H, s), 6.735 (1H, s), 6.618 (2H, dd, J = 8.8, 3.2 Hz), 6.568 (1H, d, J = 2.4 Hz), 4,146 (1H, s), 2.830 (1H, m), 0.891 (6H, s). HRMS: 538.3083 (calculated) and 538.3092 (observed).

Compound 19: ^{1}H NMR (400 Hz, MEOH-d_4) 7.306 (1H, m), 7.198 (1H, m), 7.076 (1H, d, J = 8.8 Hz), 6.686 (1H, s), 6.546 (2H, s), 6.469 (1H, s), 3.557 (1H, t, J = 8.0 Hz), 2.380 (2H, dd, J = 19.2, 9.2 Hz), 0.805 (3H, s), 0.667 (3H, s). HRMS: 540.3240 (calculated) and 540.3258 (observed).

Compound 20: ^{1}H NMR (400 Hz, $CDCl_3$) 7.216 (1H, s), 7.187 (1H, s), 6.847 (1H, s), 6.732 (1H, s), 6.732 (4H, m), 5.269 (1H, s), 3.656 (1H, t, J = 8.7 Hz), 2.459 (2H, dd, J = 15.6, 10.4 Hz), 0.898 (3H, s), 0.749 (3H, s). HRMS: 540.3239 (calculated) and 540.3236 (observed).

3.7. Methods for Studying the Estrogen Dimers Formed in Vitro by Human Liver Microsomes and CYP Isoforms. 3.7.1. In Vitro Metabolism Conditions. The conditions used for the in vitro metabolic formation of nonpolar [^{3}H]E_1 and [^{3}H]E_2 metabolites were the same as those used in the past for studying the NADPH-dependent oxidative metabolism of estrogens.[6,7,11] Specifically, the reaction mixtures consisted of the desired amount of human liver microsomes (at a final concentration of 1 mg of protein/mL) or human CYP isoforms (containing 70 or 140 pmol of CYP/mL), 50 μM E_1 and E_2 as substrate (singularly or in combination), 2 μCi of radioactive E_1 and/or E_2, 2 mM NADPH, and 5 mM ascorbic acid in a final volume of 500 μL of 0.1 M Tris-HCl/0.05 M HEPES buffer, pH 7.4. The enzymatic reaction was initiated by addition of human liver microsomes or a CYP isoform, and the incubation was carried out at 37 °C for 20 min with periodic mild shaking. The reaction was then arrested by placing the reaction tubes in ice-cold water, followed immediately by extraction with 5 mL of ethyl acetate. The supernatant was transferred to another set of test tubes and dried under a stream of nitrogen. The resulting residue was redissolved in 100 μL of methanol, and an aliquot (50 μL) was injected into an HPLC system coupled with radioactivity and UV detection for analysis of estrogen metabolite composition.

3.7.2. HPLC Analytical Conditions. In order to separate various estrogen metabolites formed in vitro for quantification, different mobile phase conditions had to be used. The HPLC methods combined the use of three solvents in complex gradients, and the three basic solvents included doubly distilled water (solvent A), pure methanol (solvent B), and pure acetonitrile (solvent C). In our early studies, we have developed method A to quickly elute all polar metabolites in the first 10 min as clusters (without much separation) and then selectively separate most of the nonpolar metabolites within the next 60 min. This method was also used in the present study for the elution of various nonpolar metabolites formed with E_1 as enzymatic reaction substrate. In addition, we have optimized a reported HPLC method,[5] which was also developed in our lab, to reach the best separation of the eight nonpolar dimers of interest (method B in Table 1).

It is of note that the HPLC methods A and B could not fully separate all eight chemically synthesized nonpolar estrogen dimers on an Ultracarb column (Phenomenex, 15 cm × 4.6 mm, 5 μm, ODS). Therefore, we further modified the separation conditions by using a different mobile phase condition (method C in Table 1) on a different HPLC column (Microsorb-MV 100A column, Varian Analytical Instruments). With this modified HPLC elution condition, all eight synthetic dimers could be better resolved in a single HPLC run (a representative HPLC trace is shown in Figure 5). This method enabled us to simultaneously quantify the formation of all eight estrogen dimers formed by human liver microsomes or CYP isoforms. Last, it is also of note that while method C in combination with a Microsorb-MV 100 A column produced a better separation of the eight synthetic nonpolar estrogen dimers, it had a rather low efficacy in separating various polar estrogen metabolites (in the first 60 min of elution).

Acknowledgment. This study was supported by a grant from the American Cancer Society (Grant No. RSG-02-143-01-CNE). Part of the work described in this paper was completed when the authors were at the University of South Carolina, Columbia, SC.

Supporting Information Available: The GC−MS separation conditions[25] and representative chromatographs of compounds **1**−**4**, **6** and **8** are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Cavalieri, E.; Frenkel, K.; Liehr, J. G.; Rogan, E.; Roy, D. Estrogens as endogenous genotoxic agents−DNA adducts and mutations. *J. Natl. Cancer Inst., Monogr.* **2000**, *27*, 75−93.
(2) Lee, A. J.; Kosh, J. W.; Conney, A. H.; Zhu, B. T. Characterization of the NADPH-dependent metabolism of 17β-estradiol to multiple metabolites by human liver microsomes and selectively expressed human cytochrome P450 3A4 and 3A5. *J. Pharmacol. Exp. Ther.* **2001**, *298*, 420−432.
(3) Lee, A. J.; Mills, L. H.; Kosh, J. W.; Conney, A. H.; Zhu, B. T. NADPH-dependent metabolism of estrone by human liver microsomes. *J. Pharmacol. Exp. Ther.* **2002**, *300*, 838−849.
(4) Zhu, B. T.; Conney, A. H. Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis* **1998**, *19*, 1−27.
(5) Lee, A. J.; Zhu, B. T. NADPH-dependent formation of polar and nonpolar estrogen metabolites following incubations of 17β-estradiol with human liver microsomes. *Drug Metab. Dispos.* **2004**, *32*, 876−883.
(6) Lee, A. J.; Sowell, J. W.; Cotham, W. E.; Zhu, B. T. Chemical synthesis of two novel diaryl ether dimers of estradiol-17β. *Steroids* **2004**, *69*, 61−65.
(7) Zhu, B. T.; Lee, A. J. NADPH-dependent metabolism of 17β-estradiol and estrone to polar and nonpolar metabolites by human tissues and cytochrome P450 isoforms. *Steroids* **2005**, *70*, 225−244.
(8) Weingarten, H. Mechanism of the Ullmann condensation. *J. Org. Chem.* **1964**, *29*, 3624−3626.
(9) Williams, A. L.; Kinney, R. E.; Bridger, R. F. Solvent-assisted Ullmannether synthesis. Reactions of dihydric phenols. *J. Org. Chem.* **1967**, *32*, 2501−2505.
(10) Ullmann, F. Another representation method of phenyl acid. *Chem. Ber.* **1904**, *37*, 853−854.
(11) Lee, A. J.; Cai, M. X.; Thomas, P. E.; Conney, A. H.; Zhu, B. T. Characterization of the oxidative metabolites of 17β-estradiol and estrone formed by 15 selectively expressed human cytochrome p450 isoforms. *Endocrinology* **2003**, *144*, 3382−3398.

(12) Kabat, G. C.; O'Leary, E. S.; Gammon, M. D.; Sepkovic, D. W.; Teitelbaum, S. L.; Britton, J. A.; Terry, M. B.; Neugut, A. I.; Bradlow, H. L. Estrogen metabolism and breast cancer. *Epidemiology* **2006**, *17*, 80−88.
(13) Harrington, P. M.; Singh, B. K.; Szamosi, I. T.; Birk, J. H. Synthesis and herbicidal activity of cyperin. *J. Agric. Food Chem.* **1995**, *43*, 804−808.
(14) Leese, M. P.; Hejaz, H. A.; Mahon, M. F.; Newman, S. P.; Purohit, A.; Reed, M. J.; Potter, B. V. A-ring-substituted estrogen-3-*O*-sulfamates: potent multitargeted anticancer agents. *J. Med. Chem.* **2005**, *48*, 5243−5256.
(15) Jiang, X. R.; Sowell, J. W.; Zhu, B. T. Synthesis of 7α-substituted derivatives of 17β-estradiol. *Steroids* **2006**, *71*, 334−342.
(16) Smith, K.; Jones, D. A superior synthesis of diaryl ethers by the use of Ultrasound in the Ullmann reaction. *J. Chem. Soc., Perkin Trans. 1* **1992**, 407−408.
(17) Palomo, C.; Palomo, C.; Oiarbide, M.; Lopez, R.; Gomez-Bengoa, E. Phosphazene P-4-Bu-t base for the Ullmann biaryl ether synthesis. *Chem. Commun.* **1998**, *19*, 2091−2092.
(18) Zhu, B. T.; Conney, A. H. Is 2-methoxyestradiol an endogenous estrogen metabolite that inhibits mammary carcinogenesis? *Cancer Res.* **1998**, *58*, 2269−2277.
(19) Hochberg, R. B.; Pahuja, S. L.; Zielinski, J. E.; Larner, J. M. Steroidal fatty acid esters. *J. Steroid Biochem. Mol. Biol.* **1991**, *4*, 577−585.
(20) Hochberg, R. B. Biological esterification of steroids. *Endocr. Rev.* **1998**, *19*, 331−338.
(21) Mills, L. H.; Lee, A. J.; Parlow, A. F.; Zhu, B. T. Preferential growth stimulation of mammary glands over uterine endometrium in female rats by a naturally occurring estradiol-17β-fatty acid ester. *Cancer Res.* **2001**, *61*, 5764−5770.
(22) Mills, L. H.; Lee, A. J.; Zhu, B. T. Naturally-occurring estradiol-17β-fatty acid ester, but not estradiol-17β, preferentially induces the development of mammary tumor in female ACI rats. *Proc. Am. Assoc. Cancer Res* **2003**, *44*, 835−836.
(23) Zhang, J. X.; Labaree, D. C.; Hochberg, R. B. Nonpolar and short side chain groups at C-11beta of estradiol result in antiestrogens. *J. Med. Chem.* **2005**, *48*, 1428−1447.
(24) Berube, G.; Rabouin, D.; Perron, V.; N'Zemba, B.; Gaudreault, R. C.; Parent, S.; Asselin, E. Synthesis of unique 17β-estradiol homodimers, estrogen receptors binding affinity evaluation and cytocidal activity on breast, intestinal and skin cancer cell lines. *Steroids* **2006**, *71*, 911−921.
(25) Adlercreutz, H.; Kiuru, P.; Rasku, S.; Wahala, K.; Fotsis, T. An isotope dilution gas chromatographic−mass spectrometric method for the simultaneous assay of estrogens and phytoestrogens in urine. *J. Steroid Biochem. Mol. Biol.* **2004**, 92, 399−411.

JM0707323