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CHARACTERIZATION OF NOVEL NONPOLAR 17β-ESTRADIOL METABOLITES FORMED BY HUMAN CYTOCHROME P450 ENZYMES

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RESEARCH ARTICLE

ABSTRACT. WE REPORT HERE OUR FINDINGS on the NADPH-dependent formation of a novel class of nonpolar estrogen metabolites by fifteen human cytochrome P450 (CYP) isoforms (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, and 4A11). A total of some 20 nonpolar radioactive metabolite peaks (designated as M1 through M20) were detected following incubations of [^{3}H]17β-estradiol with human CYP isoforms and NADPH. Some of the nonpolar metabolites were formed at varying rates with each of the 20 human CYP isoforms tested, but M15 and M16 were selectively formed only with a few CYP isoforms. CYP3A4 and 3A5 had the highest catalytic activity for the formation of M15 and M16, but CYP1A1, 2C8 and 2C9 had weak but detectable catalytic activity for their formation. Kinetic analyses showed that the apparent K_M values for CYP3A4 and CYP3A5-dependent formation of M15 and M16 ranged from 46–119 μM, and their apparent V_{MAX} values ranged from 206–276 pmol/nmol of CYP/min. Using mass and NMR spectrometric analyses, we unequivocally identified the structures of M15 and M16 to be the dimers of 17β-estradiol, which were connected together through a diaryl ether bond between a phenolic oxygen atom of one 17β-estradiol molecule and the 2- or 4-position aromatic carbon of the other 17β-estradiol. Further studies are needed to determine any biological functions that may be associated with these novel nonpolar estrogen metabolites.

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1. INTRODUCTION

THE ENDOGENOUS ESTROGENS undergo extensive metabolism in humans [1,2], such as oxidation (largely mediated by cytochrome P450 [CYP[3]] enzymes), interconversion between 17β-estradiol (E_2) and estrone, and various conjugation-deconjugation reactions. In addition, the catechol-O-methyltransferase (COMT)-mediated O-methylation of endogenous catechol estrogens to monomethyl ethers and the acyltransferase-mediated esterification of E_2 or hydroxy-E_2 metabolites with various fatty acyl-CoAs result in the formation of lipophilic estrogen derivatives that usually have much longer half-lives ($T_{1/2}$) in the body. These multiple metabolic pathways not only determine the pharmacokinetic features of the endogenous estrogens in the body and in various target tissues, but more importantly, they also diversify the biological actions of endogenous estrogens in certain target sites. For instance, 4-hydroxyestradiol is a genotoxic/mutagenic estrogen metabolite, and it also appears to have its own signal-transduction pathway that is different from the classical estrogen receptor-mediated signaling pathway [3]. This E_2 metabolite has been thought to play an important role in hormonal carcinogenesis in animal models and humans [4]. In addition, a few other hydroxylated estrogen metabolites (such as 2-hydroxyestradiol, 15α-hydroxyestradiol, and 16α-hydroxyestrone) have also been suggested to have unique biological properties [2,5]. Besides these polar estrogen metabolites, 2-methoxyestradiol [6] and E_2-17β-fatty acid esters [7] are two examples of the nonpolar estrogen metabolites that also have unique biological properties. Many studies have shown that 2-methoxyestradiol has strong growth-inhibitory, apoptotic, and antiangiogenic actions [6]. Our recent studies have shown that the naturally-occurring lipoidal E_2-17β-stearate had a strong, preferential growth-stimulatory and carcinogenic activity in the fat-rich mammary tissues over other target organs (such as the uterus and pituitary), which was much different from the parent hormone E_2 [8,9].

During our recent study to characterize the NADPH-dependent metabolism of [^{3}H]E_2 and [^{3}H]estrone by fifteen selectively-expressed human CYP isozymes, we observed that a cluster of nonpolar radioactive estrogen peaks appeared to be selectively formed in large amounts only with certain human CYP isoforms, most notably the CYP3A4 and CYP3A5 [10]. Although similar nonpolar radioactive peaks were also noted earlier by us and others when [^{3}H]E_2 or [^{3}H]estrone was incubated with human or rodent liver microsomes [11-16], further characterization of these nonpolar metabolite peaks has never been pursued mainly because there was no available evidence for the suggestion that nonpolar metabolites could be formed from steroid hormones or xenobiotics by microsomal enzymes using NADPH as a cofactor. In the present study, we characterized, for the first time, the formation of a novel class of nonpolar E_2 metabolites by certain human CYP isoforms, and also determined the structures of two representative nonpolar metabolites metabolically formed by human CYP3A isoforms.

2. MATERIALS AND METHODS

2.1. CHEMICALS AND SELECTIVELY-EXPRESSED HUMAN CYP ISOFORMS

E_2, NADPH, NADP, and glucose-6-phosphate dehydrogenase were purchased from the Sigma Chemical Co. (St. Louis, MO). N,O-bis(Trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) was obtained from Pierce Chemical Co. (Rockford, IL). [2,4,6,7,16,17-^{3}H]E_2 (specific activity of 123.0 Ci/mmol) was obtained from Perkin Elmer Life Sciences (Boston, MA). All other solvents were of HPLC grade or better, and were purchased from Fisher Scientific (Atlanta, GA).

The selectively-expressed human CYP isoforms were obtained from BD Gentest Co. (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 [10].

2.2. ASSAY OF NADPH-DEPENDENT METABOLISM OF [^{3}H]E_2 BY SELECTIVELY-EXPRESSED HUMAN CYP ISOFORMS

It is of note that the conditions used for the in vitro metabolism of E_2 leading to the formation of nonpolar estrogen metabolites were the same as those for studying the oxidative metabolism of estrogens [10]. Specifically, the reaction mixture consisted of a human CYP isoform (at 70 or 140 pmol of CYP/mL), 20 μM E_2 (containing 2 μCi of [^{3}H]E_2) in 5 μL of ethanol, 2 mM NADPH, and 5 mM ascorbic acid in a final volume of 0.5 mL of 0.1 M Tris-HCl/0.05 M HEPES buffer, pH 7.4. The enzymatic reaction was initiated by addition of a CYP isoform, and the incubations were carried out for 20 min at 37°C with periodical mild shaking. The reaction was arrested by placing the reaction tubes on ice followed immediately by addition of 10 μL of 10 mM nonradioactive E_2 (to reduce nonspecific adsorption of estrogen molecules to the test tubes) and ethyl acetate (8 mL) for extraction. The supernatants were dried under a stream of nitrogen, and the residues were analyzed by HPLC for estrogen metabolite composition. Notably, all the test tubes used in our present study were silanized as described earlier [10,17,18]. The ^{3}H-labeled E_2 substrate was re-purified with HPLC prior to its use in the metabolism experiments.

Analysis of the nonpolar E_2 metabolites was carried out by using a new HPLC method developed for this study. This method (with run time of 70 min) selectively separated nonpolar estrogen metabolites, with all oxidative estrogen metabolites eluted as clusters within the first 10 min. The HPLC system consisted of a Waters 2690 separation module (Milford, MA), a radioactivity detector (β-RAM, INUS Systems, Inc., Tampa, FL), a Waters UV detector (Model 484), and an Ultracarb 5 ODS column (150 x 4.60 mm, Phenomenex, Torrance, CA). The solvent system for the separation of nonpolar E_2 metabolites consisted of acetonitrile, water, and methanol with a flow rate of 1.2 mL/min. The solvent gradient (acetonitrile/water/methanol) was as follows: 20 min of an isocratic elution at 30/40/30, 10 min of a convex gradient (curve number 3) to 35/30/35, 10 min of a convex gradient (curve number 3) to 40/25/35, 10 min of a convex gradient (curve number 3) to 50/20/30, 10 min of a convex gradient (curve number 3) to 60/10/30, followed by a 10-min convex gradient (curve number 3) to 65/5/30. The gradient was then returned to the initial condition (30/40/30) and held for 5 min before analysis of the next sample.

2.3. LARGE-SCALE INCUBATIONS AND HPLC ISOLATION OF NONPOLAR ESTROGEN METABOLITES M15 AND M16

For the structural identification of the two representative nonpolar estrogen metabolites M15 and M16, multiple large-scale incubations of E_2 with human CYP3A4 + b_5 were carried out. The reaction mixture consisted of CYP3A4 (200 pmol of CYP/mL), 200 μM of E_2, 10 mM $MgCl_2$, 10 mM glucose 6-phosphate, 1 mM NADP, 2 U/mL of glucose-6-phosphate dehydrogenase, and 5 mM ascorbic acid in a final volume of 60 mL of the 0.1 M potassium phosphate buffer (pH 7.4). The incubations lasted 60 min, and then the reaction mixture was extracted with 240 mL ethyl acetate. The organic layer was dried, and the nonpolar metabolites M15 and M16 were isolated to purity by using 3 HPLC steps. The first HPLC step eluted all nonpolar estrogen metabolites at the retention time between 28-37 min. The solvent gradient (acetonitrile/water/methanol) included 8 min of an isocratic elution at 16/68/16, 7 min of a concave gradient (curve number 9) to 18/64/18, 5 min of a linear gradient (curve number 6) to 30/40/30, 7 min of an isocratic elution at 30/40/40, 3 min of a linear gradient (curve number 6) to 60/1/39, and lastly 8 min of an isocratic elution at 60/1/39. The second step was mainly for the separation of M15 and M16 (retention time 43-47 min) from other nonpolar E_2 metabolites, which was the same as described earlier for the measurement of nonpolar E_2 metabolites. The last HPLC step (an isocratic elution at 40/30/30) further separated M15 (33-37 min) and M16 (37-40 min), and this step was repeated twice to yield highly-pure M15 and M16. In each of the separation steps, the HPLC trace was monitored with UV detection at 280 nm and the desired fractions were collected. The acetonitrile and methanol (part of the mobile phase composition) contained in each of the fractions were evaporated first under a mild stream of N_2, and the remaining aqueous solution was then extracted with ethyl

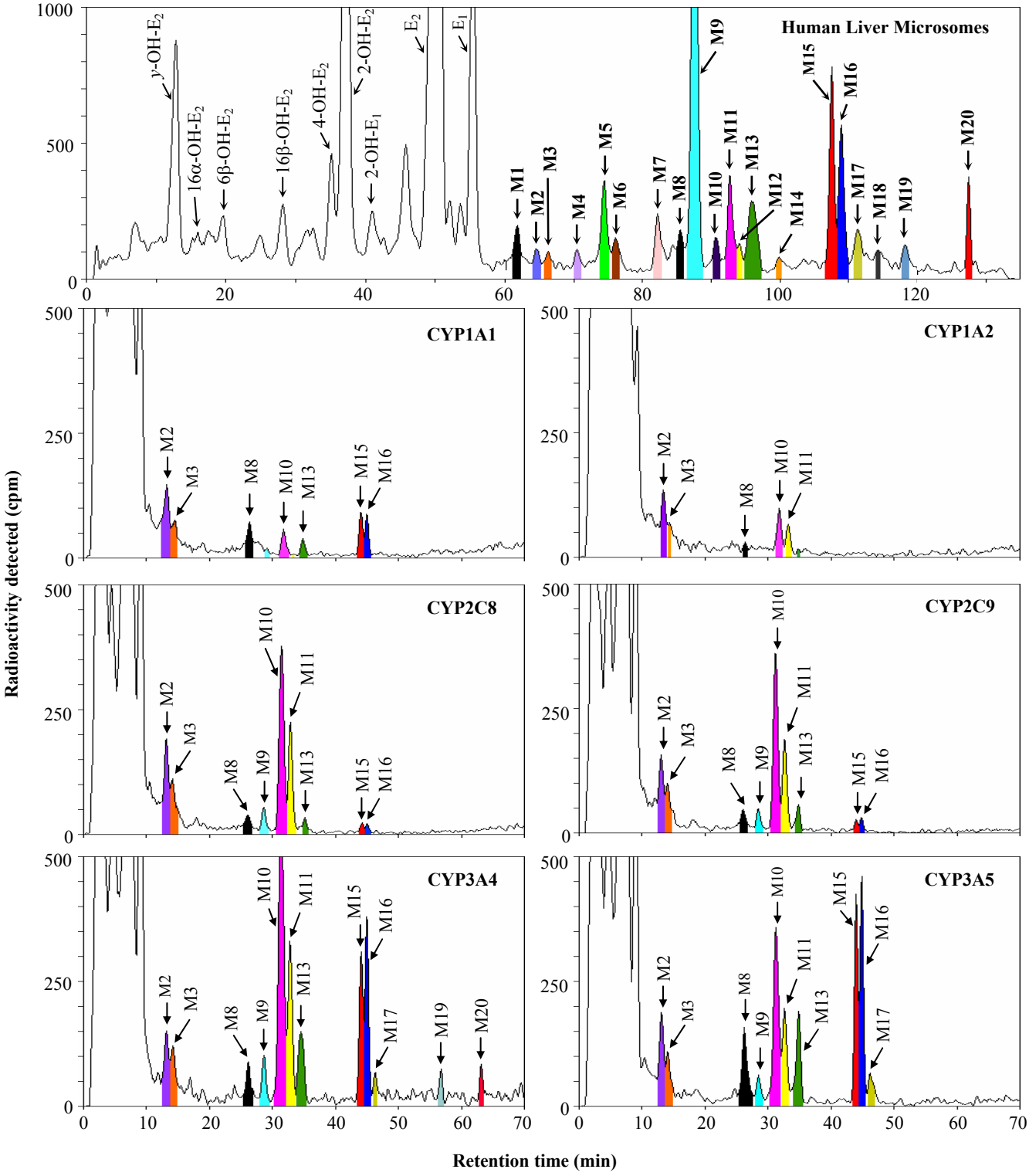

FIGURE 1. REPRESENTATIVE HPLC PROFILES FOR THE NADPH-DEPENDENT FORMATION OF NONPOLAR METABOLITES FROM [^{3}H]E_2 BY SELECTIVELY-EXPRESSED HUMAN CYP ISOFORMS OR A REPRESENTATIVE HUMAN LIVER MICROSOMAL PREPARATION. The incubation mixture consisted of a selectively-expressed human CYP isoform or human liver microsomes, [^{3}H]E_2 substrate (20 μM for CYP isoforms and 50 μM for human liver microsomes), 2 mM NADPH, and 5 mM ascorbic acid in a final volume of 0.5 mL of the buffer at pH 7.4. The formation of nonpolar estrogen metabolites was determined by HPLC as described in the Materials and Methods section.

acetate to recover the nonpolar metabolites.

2.4. STRUCTURAL ANALYSES

The UV spectra of the isolated nonpolar estrogen metabolites (dissolved in pure ethanol) were recorded on a PerkinElmer Lambda EZ 210 spectrophotometer. Mass spectra were recorded on a VG70S magnetic sector mass spectrometer. Ionization was carried out by electron impact at 70 eV. An aliquot of the ethanol solution containing the isolated M15 or M16 was used for the direct probe high-resolution mass spectrometric analysis. NMR spectra were recorded on a Varian Inova 500 spectrometer operating at a proton frequency of 500.21 MHz and carbon frequency of 125.79 MHz. Chemical shifts were given as δ values with reference to CD_3OD as an internal standard (3.30 ppm ^1H and 49.0 ppm ^{13}C). All measurements were run at 25°C using supplied pulse sequences. ^1H and ^{13}C resonance assignments were made by acquiring 1D ^1H, 1D ^{13}C, gDQCOSY, gHMQC and gHMBC datasets as required. Parameters for the two-dimensional experiments included 4000 Hz 1H (F2) spectral window collected with 2k complex data points. 256 fids (complex for the gDQCOSY) were collected for the F1 dimension. A 200-ppm F1 window for ^{13}C was used for both gHMQC and gHMBC experiments.

3. RESULTS AND DISCUSSION

3.1. QUANTIFICATION OF NONPOLAR ESTROGEN METABOLITE PEAKS FORMED WITH 15 HUMAN CYP ISOFORMS

By using a newly-developed HPLC method, we detected the formation of some 20 nonpolar radioactive metabolite peaks, in addition to a large number of polar hydroxylated or keto metabolites, following incubations of 20 and 50 μM of [^3H]E_2 with human liver microsomes in the presence of NADPH as a cofactor (a representative HPLC trace shown in FIG. 1). The formation of most of the nonpolar estrogen metabolite peaks was dependent on the presence of human liver microsomal protein, and could be selectively inhibited by the presence of carbon monoxide. Among the four cofactors (NAD, NADH, NADP, NADPH) tested, NADPH was the optimum cofactor for the metabolic formation of nonpolar E_2 metabolites in vitro, although NADH also had a weak ability to support the reactions (data not shown).

To determine whether certain human CYP isoforms have specific catalytic activity for the formation of nonpolar E_2 metabolites, we studied their metabolic formation by fifteen human CYP isoforms (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, and 4A11). We found that some 20 nonpolar radioactive metabolite peaks were detected after incubations of [^3H]E_2 with human CYP isoforms (FIG. 1). Among the nonpolar estrogen metabolites detected, M15 and M16 were only formed with a few of the CYP isoforms (FIG. 1 AND 2). CYP3A4 and 3A5 had the highest catalytic activity for the formation of M15 (27 and 33 pmol/nmol of P450/min, respectively) and also for the formation of M16 (32 and 36 pmol/nmol of P450/min, respectively). CYP1A1, 2C8 and 2C9*1 (Arg_{144}) showed weak but detectable catalytic activity for the formation of these two nonpolar metabolites. However, all other CYP isoforms did not have appreciable catalytic activity for the formation of M15 and M16 (FIG. 2).

In comparison, some of the other nonpolar estrogen metabolite peaks were detected with all fifteen CYP isoforms at varying rates (FIG. 2). Notably, the metabolite peak M9, which was detected as a quantitatively-major peak when 20 or 50 μM [^3H]E_2 was incubated with human liver microsomes (FIG. 1), was only a quantitatively-minor nonpolar peak when each of the fifteen selectively-expressed human CYP isoforms was assayed. This observation suggests that M9 likely was formed by other CYP isoform(s), or formed by non-CYP enzyme(s), or might even be formed nonenzymatically.

Notably, the formation of M15 and M16 by each of the 15 human CYP isoforms did not correlate with the overall catalytic activity for the oxidative metabolism of estrogens. For instances, although CYP1A2 had the highest catalytic activity for the formation of 2-hydroxylated metabolites from E_2 and estrone (16), we detected no appreciable activity for the formation of M15 and M16 by this CYP isoform under exactly the same in vitro reaction conditions (FIG. 1 AND 2). This observation clearly

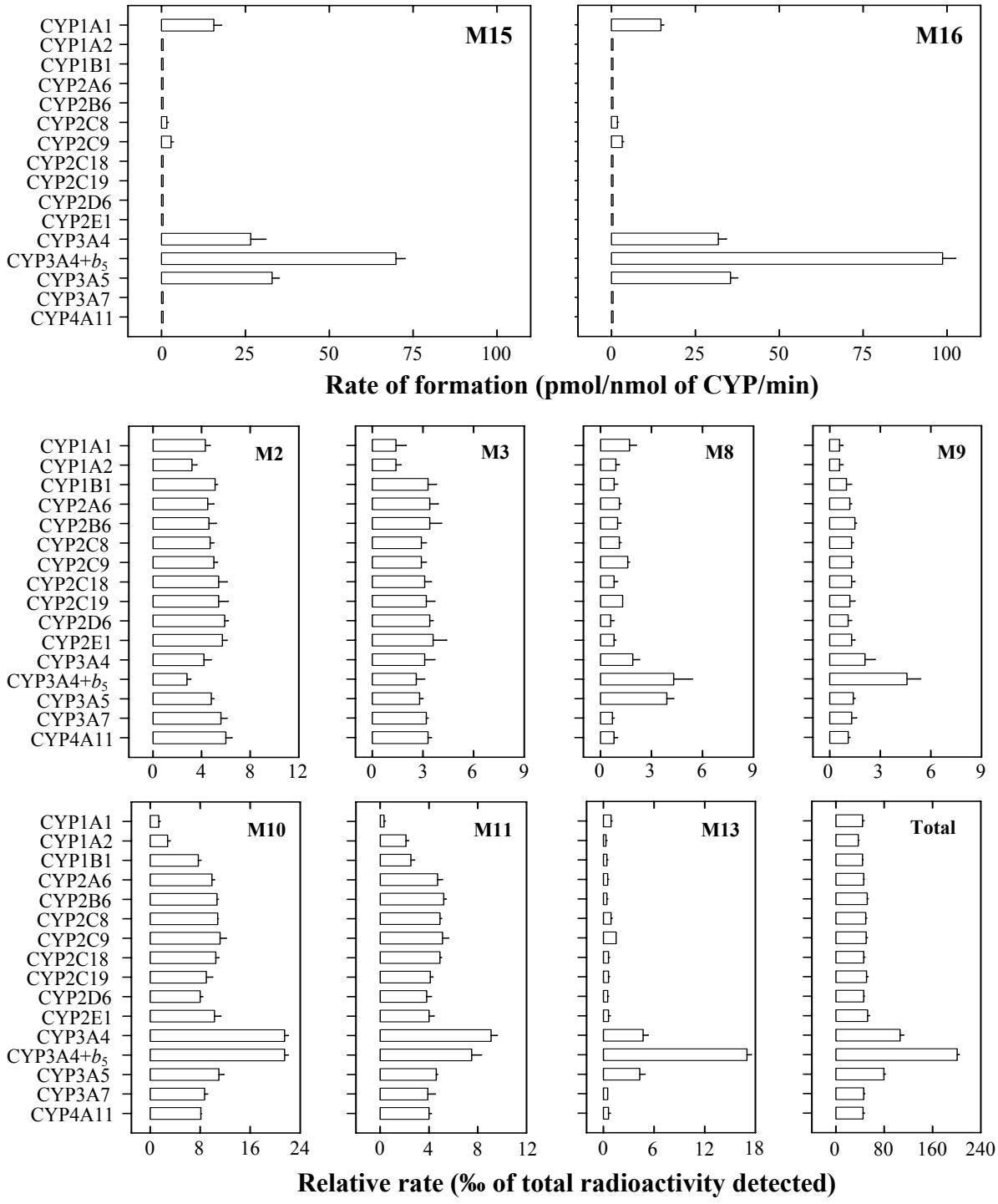

FIGURE 2. RATES OF METABOLIC FORMATION OF QUANTITATIVELY-MAJOR NONPOLAR E_2 METABOLITES BY 15 SELECTIVELY-EXPRESSED HUMAN CYP ISOFORMS. The experimental procedures were the same as described in the legend to FIG. 1. The error bars represent the standard deviation (S.D.) of triplicate determinations. Note that the absolute rates for the formation of M15 and M16 were calculated according to their identified chemical structures. For all other nonpolar metabolite peaks that their structures were not identified, the rates of their formation were expressed in relative units, i.e., "‰ of total radioactivity detected".

TABLE 1. KINETIC PARAMETERS FOR THE FORMATION OF NONPOLAR [^{3}H]E_2 METABOLITES BY SELECTIVELY-EXPRESSED HUMAN CYP3A4 OR CYP3A5.

	CYP3A4		CYP3A5	
	V_{MAX} [1]	K_M (μM)	V_{MAX} [1]	K_M (μM)
M1	N.D.	N.D.	75	107
M2	N.D.	N.D.	N.D.	N.D.
M3	646	2075	N.D.	N.D.
M4	116	409	N.D.	N.D.
M5	50	183	133	783
M6	32	58	91	330
M7	200	579	293	906
M8	714	790	211	149
M9	N.D.	N.D.	57	257
M10	N.D.	N.D.	N.D.	N.D.
M11	229	283	166	248
M12	278	552	217	556
M13	50	85	67	75
M14	15	53	49	388
M15	142 [276][3]	119	119 [232][3]	46
M16	106 [206][3]	84	111 [216][3]	50
M17	25	61	32	46
M18	21	62	39	425
M19	26	105	10	98
M20	29	97	41	502

NOTE: The experimental procedures were the same as described in the legend to FIG. 3. The K_M and V_{MAX} values were obtained by using the non-linear regression program of the Prism software (GraphPad Software, Inc., San Diego, CA).
[1] The unit for V_{MAX}: (% of total radioactivity detected) x (μM E_2)/20 min.
[2] N.D. denotes "not determined".
[3] Since the structures of M15 and M16 were identified in the present study, the absolute V_{MAX} values (pmol/nmol of CYP/min) were also calculated and shown for reference.

suggested that the overall catalytic activity of different human CYP isoforms for the oxidative metabolism of E_2 did not necessarily correlate with their ability in contributing to the formation of nonpolar estrogen metabolites. It is apparent that only certain human CYP isoforms (such as CYP3A4 and 3A5) were involved in the formation of nonpolar estrogen metabolites.

We also determined the apparent K_M and V_{MAX} values for the CYP3A4 and 3A5-dependent formation of several nonpolar E_2 metabolites (FIG. 3 AND TABLE 1). As shown in FIG. 3, the curve shapes for the formation of some nonpolar metabolites roughly followed the Michaelis-Menten curve patterns. However, the formation of most of nonpolar estrogen metabolites by CYP3A4 or CYP3A5 did not reach complete plateaus even at the highest E_2 substrate concentration tested (150 μM). Taking M15 and M16 as an example, CYP3A5 had relatively higher affinities (lower K_M values; 46.0 and 50.0 μM, respectively) for their formation than did CYP3A4 (118.8 and 83.7 μM, respectively). However, the apparent V_{MAX} values of CYP3A5 and CYP3A4 for their formation were comparable (206.0-275.9 pmol/nmol of CYP/min). Accordingly, CYP3A5 had ~2-fold higher turnover numbers (V_{MAX} /K_M) for the formation of M15 and M16 than did CYP3A4. It is of note that when these kinetic parameters were compared against the kinetic parameters for the 2- and 4-hydroxylation of E_2 [10], CYP3A4 and 3A5-dependent formation of M15 and M16 had comparable affinities (i.e., similar K_M values) but slightly lower capacities (V_{MAX} values).

In summary, some 20 nonpolar estrogen metabolite peaks were detected following incubations of [^{3}H]E_2 with certain human CYP isoforms and NADPH. M15 and M16 were selectively formed with a few human CYP isoforms (mainly CYP3A4 and CYP3A5). The CYP3A4 and 3A5-dependent formation of M15 and M16 followed Michaelis-Menten curve patterns. The formation of these two representative nonpolar estrogen metabolites by human CYP isoforms was not correlated with their overall catalytic activity for the oxidative metabolism of E_2.

3.2. STRUCTURAL IDENTIFICATION OF TWO REPRESENTATIVE NONPOLAR ESTROGEN METABOLITES M15 AND M16

To rule out whether any of these nonpolar E_2 metabolites are catechol estrogen monomethyl ethers or estrogen-17-fatty acid esters, our HPLC

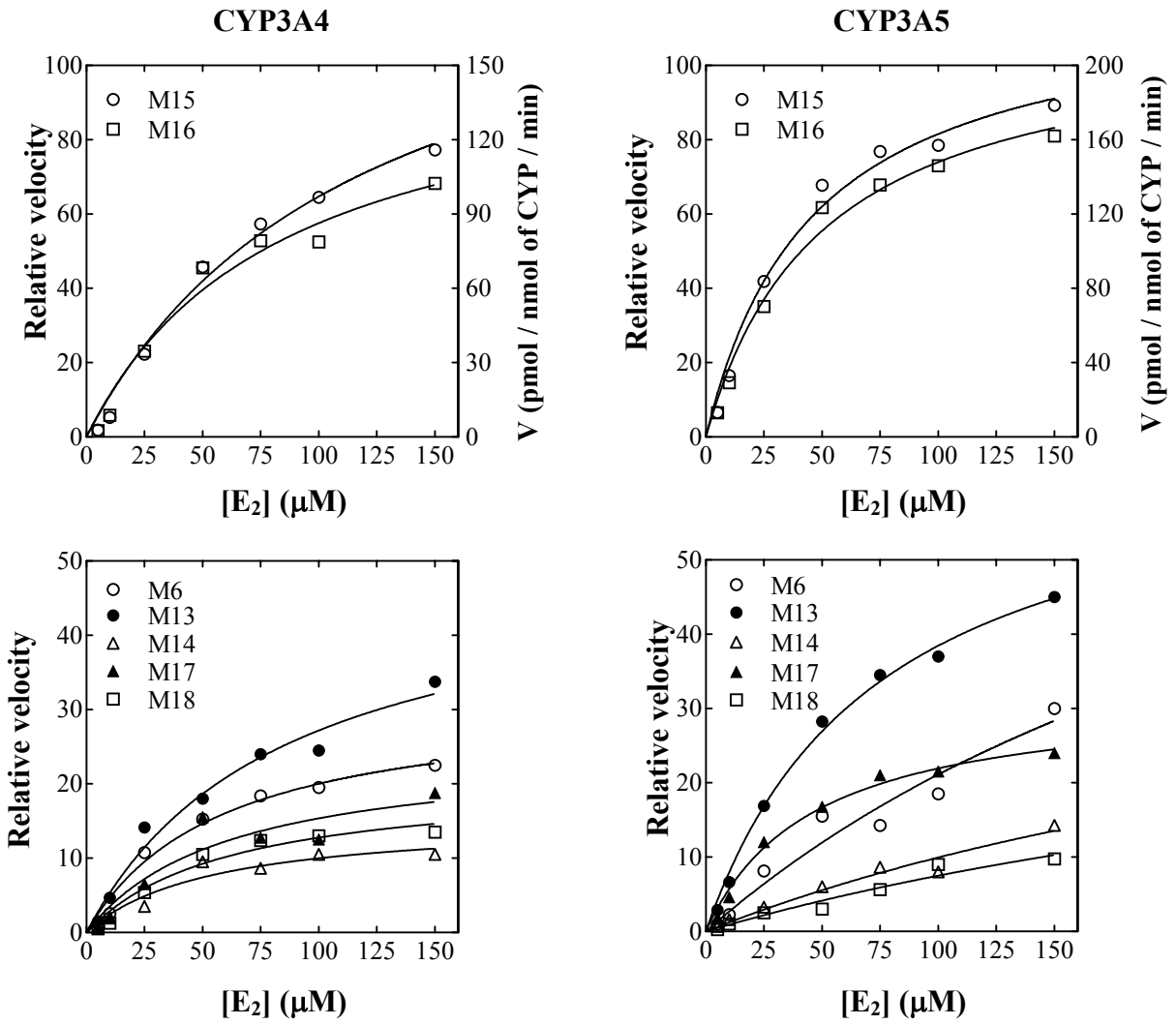

FIGURE 3. MICHAELIS-MENTEN CURVES FOR THE FORMATION OF REPRESENTATIVE NONPOLAR [^{3}H]E_2 METABOLITES BY HUMAN CYP3A4 (LEFT PANELS) OR CYP3A5 (RIGHT PANELS). The experimental procedures were the same as described in the legend to FIG. 1, except that varying concentrations of E_2 (5, 10, 25, 50, 75, 100, and 150 μM) were used. The K_M and V_{MAX} values were determined by using the non-linear regression program of the Prism software (GraphPad Software, Inc., San Diego, CA). Note that since the structures for most of the nonpolar metabolite peaks (except M15 and M16) are still not known, only a relative unit (‰ of total radioactivity detected) was used to express the relative velocity for the formation of each of the major nonpolar metabolite peaks. For M15 and M16, the absolute rates (pmol/mg protein/min) of their formation were also shown (upper panels, right ordinates) for reference.

analyses showed that the retention times of these nonpolar estrogen metabolites did not match any of the known nonpolar estrogen metabolites, thus indicating that they were a new group of nonpolar estrogen metabolites. As part of our ongoing effort to provide definitive structural information for these nonpolar metabolites, we have chosen to determine, in the present study, the chemical structures of two representative nonpolar estrogen metabolites, M15 and M16. We first produced large amounts of M15 and M16 by using multiple batches of large-scale incubations (at a 60-mL final volume) consisting of 200 μM E_2, human CYP3A4 (co-expressed with cytochrome b_5), and an NADPH-generating system. Following the enzymatic reactions, all nonpolar estrogen metabolites were extracted and then M15 and M16 were separated with our HPLC system using three different solvent gradients (details of the

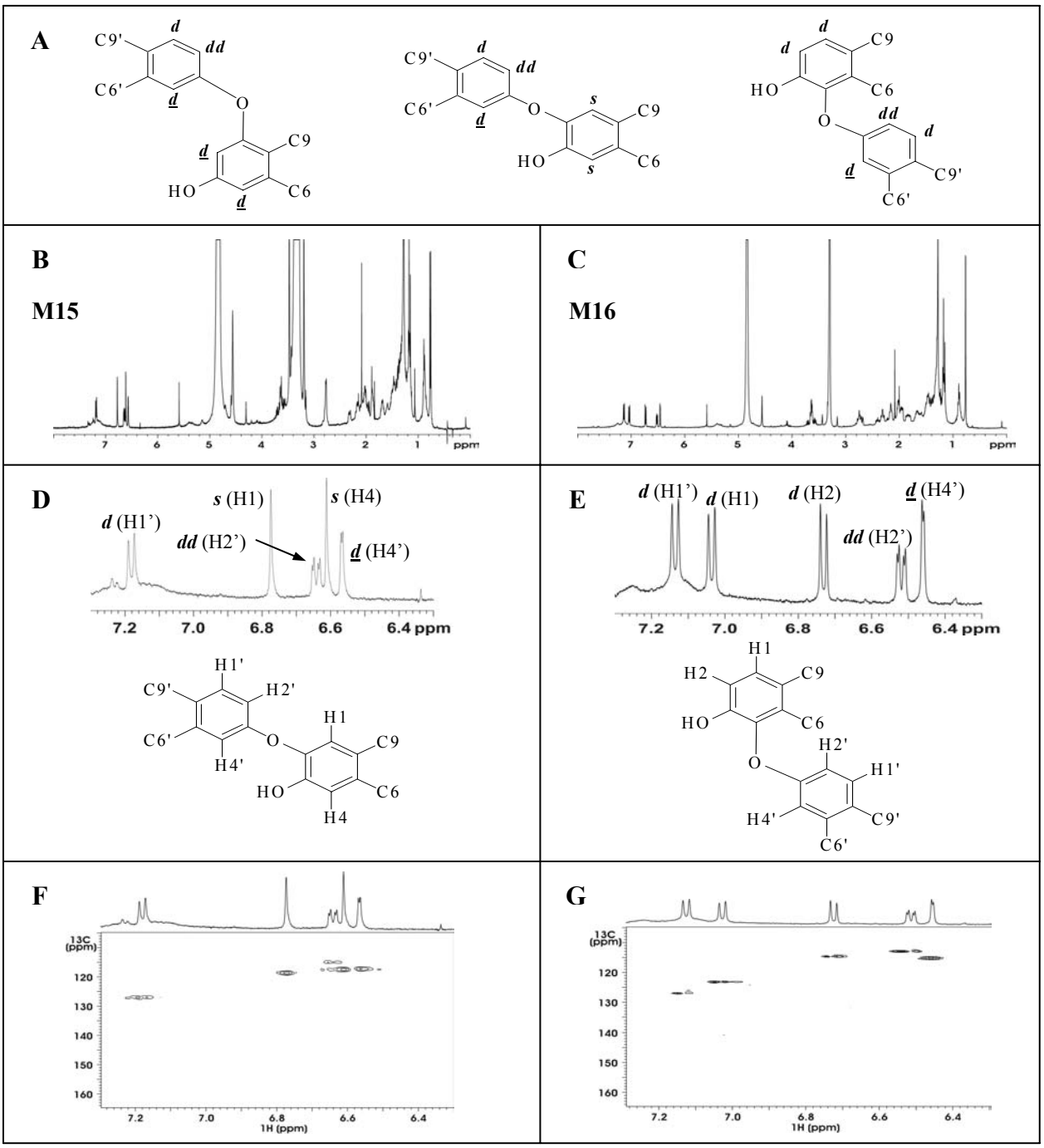

FIGURE 4. STRUCTURAL DETERMINATION OF M15 AND M16 BY NMR SPECTROMETRIC ANALYSES. The NMR spectra were recorded on a Varian Inova 500 spectrometer operating at a proton frequency of 500 MHz and a carbon frequency of 125 MHz. Chemical shifts were given as δ values with reference to CD_3OD as an internal standard. d, doublet; d, doublet with smaller coupling constant; dd, doublet of doublet; s, singlet.

A: Partial structures (only showing the aromatic rings of two E_2 molecules) of three possible E_2 dimers with a diaryl ether bond. Their theoretical ^1H NMR peak patterns for various aromatic protons are labeled.

B: ^1H NMR spectrum of M15.

C: ^1H NMR spectrum of M16.

D: ^1H NMR spectrum (aromatic region) of M15 and peak assignments with the suggested structure.

E: ^1H NMR spectrum (aromatic region) of M16 and peak assignments with the suggested structure.

F: gHMQC (aromatic region) spectrum of M15.

G: gHMQC (aromatic region) spectrum of M16.

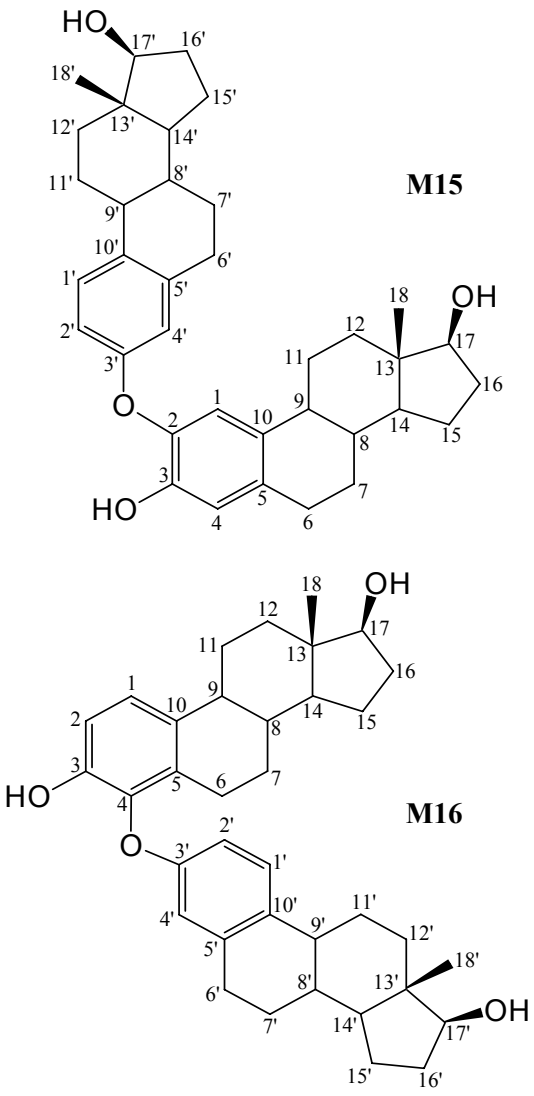

FIGURE 5. THE IDENTIFIED CHEMICAL STRUCTURES OF M15 AND M16.

HPLC gradients were provided in the Methods section) to yield sufficient amounts of highly-pure M15 and M16.

The structures of M15 and M16 were first analyzed by using various spectrometric analyses. UV spectrometric analysis showed that M15 and M16 had the absorption peaks (281 nm and 278 nm, respectively) that were highly similar to those of E_2 and 2-hydroxy-E_2 (282 nm and 289 nm, respectively). FT-IR spectrometric analysis of M15 and M16 in a dichloromethane solution indicated the absence of the ester or carbonyl group(s) in their structures. Initial LC/MS analysis of the dansyl derivatives of the metabolically-formed M15 and M16 showed that each had a specific peak with a m/z of 777 (as M + H). This initial information suggested that the molecular weight of M15 is 542, based on the following calculation: 777 (M + H) - 1 (H) - 234 (a dansyl group) = 542. Additional mass spectrometric analysis using a direct-probe approach confirmed that the molecular weights of both M15 and M16 were 542. Becasue the molecular weight of E_2 is 272, a molecular weight of 542 would give a possibility that M15 and M16 might be certain form(s) of E_2 dimers ($C_{36}H_{46}O_4$). High-resolution mass spectrometric analysis confirmed that the precise molecular weights of M15 and M16 were 542.3400 and 542.3397, respectively, which automatically yielded the same chemical formula ($C_{36}H_{46}O_4$, calculated molecular weight of 542.3396), consistent with the suggestion that M15 and M16 are E_2 dimers.

To probe the number of hydroxyl groups present in each of the molecules, a small amount of metabolically-formed M15 or M16 (following three sequential steps of isolation with HPLC) was incubated with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) at 65ºC for 30 min to convert it into the corresponding trimethylsilyl (TMS)-derivative. It is known that addition of a TMS group will increase the molecular weight by 72. After TMS derivatization, both compounds showed a molecular weight of 758 (which equals to 542 + 72 × 3), thereby indicating the presence of three hydroxyl groups in each of the compounds. To further determine whether these hydroxyl groups are attached at aliphatic or aromatic carbons, benzyl ether derivatives of M15 and M16 were prepared by their reactions with benzyl bromide in an acetonitrile solution in the presence of potassium carbonate. It is known that only the phenolic hydroxyl group (but not the aliphatic hydroxyl group) can be benzylated by reaction with benzyl bromide under the alkaline conditions devised. The mass spectrometric analyses of the benzyl ether derivatives of both M15 and M16 showed a molecular weight of 632, indicating the presence of only one phenolic hydroxyl group in either M15 or

M16. This finding indicates that one of the phenolic hydroxyl groups of the two original E_2 molecules was involved in the covalent linkage. Taken into consideration all the above information from spectrometric analyses and chemical derivatization experiments, it was quite certain that M15 and M16 were dimers of E_2, with a diaryl ether bond between the C-3 oxygen of one E_2 molecule and an aromatic carbon (at 1, 2, or 4-position) of another E_2 molecule.

Final definitive evidence for the confirmation of M15 and M16 as E_2 dimers was obtained from multiple NMR analyses, which included the 1D ^{1}H, 1D ^{13}C, gDQCOSY, gHMQC, and gHMBC. Experimentally, various NMR spectra of E_2 were obtained first and all proton and carbon peaks were correctly assigned to the appropriate atoms. Note that the ^{1}H NMR peaks for the aromatic protons of E_2 could be recorded as two doublets and one doublet of doublet because of the meta-coupling between H-2 and H-4, giving two doublet peaks with different coupling constants. The ^{1}H NMR spectra (full scale) for M15 and M16 were then obtained and are shown in FIG. 4B AND 4C, respectively. Overall, the ^{1}H NMR spectra of M15 and M16 were quite similar to the spectrum of E_2, except for the aromatic region (δ 6~8). Because only very small amounts of metabolically-formed M15 and M16 were available, a few additional peaks coming from impurities and solvents were also shown in the NMR spectra. However, these additional peaks could be rationally excluded by analyzing their 2-dimensional NMR spectra. M15 showed two doublets, two singlets, and one doublet of doublet centered at 6.568 (d, J = 2.5 Hz, 1H, for H-4'), 6.613 (s, 1H, for H-4), 6.641 (dd, J = 8.5 and 2.5 Hz, 1H, for H-2'), 6.775 (s, 1H, for H-1), and 7.182 (d, J = 8.5 Hz, 1H, for H-1') ppm (δ) in the region of the aromatic protons (FIG. 4D), with equal areas, thus indicating the existence of five aromatic hydrogens in the molecule. It is noteworthy that theoretically, only three possible E_2 dimers with a diaryl ether bond may be formed, and their expected ^{1}H NMR peak patterns are depicted in FIG. 4A for reference. Through comparison, it was apparent that M15 was a dimer of E_2 linked together through a diaryl ether bond between the C-3 oxygen atom of one E_2 molecule and the 2-position carbon of another E_2 molecule (FIG. 5, UPPER PANEL). This structure was further verified through analysis of the gHMQC (FIG. 4F) and gHMBC spectra for its aromatic hydrogens (H-1 correlated with C-2, 3, 5, 9; H-4 correlated with C-2, 3, 6, 10; H-1' correlated with C-3', 5', 9'; H-2' correlated with C-3', 4', 5', 10'; and H-4' correlated with C-2', 6', 10'). In the same ways, the structure of M16 was verified (its proton NMR and gHMQC shown in FIG. 4E AND 4G).

Recently, we have synthesized small amounts of M15 and M16 using E_2 as the starting material [19]. The synthesis included 4 steps: (i) synthesis of 2- or 4-bromoestradiol from E_2; (ii) protection of the C-3 phenolic hydroxyl group of the 2- or 4-bromoestradiol; (iii) the Ullmann condensation reaction between the phenol-protected bromo-E_2 and the E_2 potassium salt under our modified reaction conditions (with a 41% product yield); and (iv) removal of the C-3 benzyl group by catalytic hydrogenation. The chemically-synthesized M15 or M16, when mixed together with the metabolically-formed M15 or M16 and then injected into our HPLC system, had the same retention time as a single HPLC peak. In addition, the ^{1}H and ^{13}C NMR spectra (i.e., the 1D ^{1}H, 1D ^{13}C, gDQCOSY, gHMQC, and gHMBC datasets) of the metabolically formed and chemically-synthesized M15 and M16 matched perfectly with each other.

Lastly, it is of considerable interest to note that we recently have conducted a pilot study aimed at probing the possible presence of M15 and M16 in humans. Urine samples from non-pregnant women during their ovulation period were collected, hydrolyzed with glucuronidase and sulfatase, extracted with ethyl acetate, and then separated by silica gel column chromatography. A mass peak of m/z 542 was detected in the urine sample extracts, and further analysis of this mass peak by using high resolution mass spectrometry gave an exact mass of 542.3400 (note that the calculated mass for M15 and M16 is 542.3396). This observation gives the possibility that M15 and M16 is formed in the human body and present in the urine. Further studies are currently underway in our laboratory to confirm their formation in vivo and also to understand any biological functions that may be associated with these nonpolar metabolites.

4. CONCLUSIONS

We demonstrated, for the first time, that a novel class of nonpolar E_2 metabolites were formed by certain human CYP enzymes using NADPH as a cofactor. Among some 20 nonpolar E_2 metabolite peaks detected, M15 and M16 were only selectively formed with a few of the human CYP isoforms (namely CYP3A4, CYP3A5, CYP1A1, CYP2C8, and CYP2C9). The formation of these two representative nonpolar estrogen metabolites by human CYP isoforms did not correlate with their overall catalytic activity for the oxidative metabolism of E_2. The structures of the metabolically-formed M15 and M16 were unequivocally identified to be the dimers of E_2, linked together through a diaryl ether bond between a phenolic oxygen atom of one E_2 molecule and the 2- or 4-position aromatic carbon of another E_2. Further studies are warranted to determine any biological functions associated with these novel nonpolar estrogen metabolites.

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ABBREVIATIONS USED

E_2, 17β-estradiol; CYP, cytochrome P450; NADP and NADPH, nicotinamide dinucleotide phosphate and its reduced formed, respectively; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; gDQCOSY, gradient-enhanced double quantum filtered correlated spectroscopy; gHMQC, gradient-enhanced heteronuclear multiple quantum coherence; gHMBC, gradient-enhanced heteronuclear multiple bond correlation.

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