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Ortho-hydroxylation of mammalian lignan enterodiol by cytochrome P450s from *Actinomycetes* sp.

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Abstract−An animal lignin, enterodiol (END), is known to be formed by conversion of secoisolariciresinol from flaxseed by intestinal bacteria. Thirteen bacteria strains were examined for their hydroxylation activity for END. Among them, *Streptomyces avermitilis* MA-4680 and *Nocardia farcinica* IFM10152 showed the highest hydroxylation activity for END. Reaction products profiled using GC/MS revealed that four products mono-hydroxylated in aliphatic position (Al-OH-END) and three products mono-hydroxylated in aromatic ring (Ar-OH-END) were found in *S. avermitilis* MA-4680, whereas only two Ar-OH-ENDs were detected in the case of *N. farcinica* IFM10152. From 15 mg/L of END, 900 μg/L of Al-OH-END and 210 μg/L of 4-hydroxy END (4-OH-END) were produced by *S. avermitilis* MA-4680, and 300 μg/L of 2-hydroxy END (2-OH-END) and 480 μg/L of 4-OH-END were obtained by *N. farcinica* IFM10152. To find the P450s are responsible for the substrate specificity to END, 33 P450s from *S. avermitilis* MA-4680 and 26 P450s from *N. farcinica* IFM10152 were cloned and compared with coexpression of putidaredoxin reductase (camA) and putidaredoxin (camB) from *Pseudomonas putida* as redox partners in *E. coli*. As a result, Nfa45180 showed the highest hydroxylation activity especially for ortho-hydroxylation in aromatic ring *in vivo*. The results of the docking simulation of END into the homology model of Nfa45180 explained the reason for regio-specificity of the hydroxylation. To our knowledge, this is the first report of regioselective hydroxylation of END using microorganism P450s.

Keywords: Cytochrome P450, Enterodiol, CYP154, GC/MS

INTRODUCTION

Lignan, one of phytoestogens, biosynthesized in the human body from the plant lignans uptaken through our diet, has drawn great attention due to their estrogenic, anticarcinogenic, antioxidant effects on our body [1-3]. Among such animal lignans, enterodiol (END) is known to be formed by conversion of secoisolariciresinol from flaxseed by intestinal bacteria. Flax-seed contains several plant lignans such as secoisolariciresinol ,matairesinol, isolariciresinol, and pinoresinol [4]. Secoisolariciresinol and matairesinol are known to be digested by intestinal bacteria and finally converted into the mammalian lignans enterodiol (END) and enterolactone (ENL). Subsequently, the final metabolites of such lignans are excreted in the liver of rats and humans [5]. Moreover, it has been reported that END gives rise to seven monohydroxylated metabolites upon incubation with microsomes from rat, pig, and human liver [6]. Recently, hydroxylated compounds have attracted considerable scientific interest for their health-related qualities. Hydroxylated phytoestrogens such as asdaidzein or genistein have potent antioxidant properties that contribute to their cholesterol-lowering effects, cardiovascular protection, antitumor effects, and anticarcinogenic properties [7]. Regiospecific hydroxylation of aromatic compounds by chemical synthesis is difficult and involves diverse reaction steps. The conversion of aromatic hydrocarbons into hydroxylated aromatic hydrocarbons, i.e., inserting an oxygen atom into a carbon-hydrogen bond, in a microorganism is one of the key features of oxidative metabolism of many aromatic compounds. There have been few reports about lignin hydroxylation by human P450 in *E. coli* [7]. In addition, microbial biotransformation of phytoestrogen has also been studied, aiming for regio- and stereoselective hydroxylation using cytochrome P450 from *Actinomycetes* sp. [8-11]

In this study, microorganisms participating in hydroxylations of END were screened among 13 *Streptomyces* sp., *Bacillus* sp., and *Nocardia* sp. as they are known to have several cytochrome P450 monoxygease enzymes (CYPs) that are involved in mono-hydroxylation both on aliphatic and aromatic molecules. The hydroxylation products were discovered from the extracts from whole cell reaction of *S. avermitilis* MA-4680 and *N. farcinica* IFM10152, and the structures of the products were identified by GC/MS. Finally, three CYPs from *S. avermitilis* MA-4680 and four CYPs from *N. farcinica* IFM10152 showed activity for hydroxylation of END. Especially, CYP154 (Nfa45180) from *N. farcinica* IFM10152 showed the highest activity for ortho-specific hydroxylation of END.

MATERIALS AND METHODS

1. Chemicals and Biochemicals

Enterodiol was purchased from Sigma-Aldrich Chemical Co.

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‡This article is dedicated to Prof. Hwayong Kim on the occasion of his retirement from Seoul National Univerisity.

(St. Louis, MO, USA) and N,O-bis(trimethylsily)trifluoroacetamide for the derivatization for GC/MS analysis was obtained from Fluka (Buchs, Switzerland). All other chemicals were of the highest grade.

1-1. Bacterial Strains and Culture Condition

Streptomyces spp. including *S. avermitilis* MA-4680 were obtained from the Korea Collection for Type Cultures (KCTC, Daejeon, South Korea). Various kinds of *Bacillus* spp. were obtained from the Microbial Resources Center (SNU, South Korea). *N. farcinica* IFM10152 was provided by the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan. All microbes were cultured in an appropriate nutrient medium and under recommended culture conditions (Microbial Resources Center, SNU; Korea Collection for Type Cultures). *Streptomyces* spp. was cultured in 30 °C in R5$^-$ liquid broth. Various kinds of *Bacillus* spp. were cultured in 30 °C in Luria-Bertani (LB) medium. *N. farcinica* IFM10152 was grown in 37 °C in Bacto™ Brain Heart Infusion Broth from BD Bioscience, Sparks, Md, USA.

2. Co-expression of Cytochrome P450s and Redox Partners CamA/CamB in *E. coli* and Cell Distruption for UV Absorbance CO-binding Spectra

The 33 P450 genes from *S. avermitilis* MA-4680 and 26 P450 genes from *N. farcinica* IFM10152 were cloned into expression vector pET28a(+) and protein soluble expression was confirmed with CO binding spectra [12]. The expression vector, pETDuet-1 (Novagen) was used for the cloning of camA and camB [8]. The plasmids of both P450 and redox partners were transformed together into *E. coli* BL21(*DE3*). The transformant was grown in Luria-Bertani (LB) medium containing 25 μg/ml of kanamycin and 25 μg/ml of ampicillin at 37 °C until the cell concentration reached to 0.6 of $OD_{600\,nm}$, and isopropyl-thio-β-D-galactopyranoside (IPTG) and δ-aminolevulinic acid, which is a heme precursor, were added to a final concentration of 0.5 mM, followed by growing the cell at 30 °C for 12 hours. The recombinant cells were harvested and were resuspended in 5 ml of sonication buffer composed of 10 mM Tris-HCl (pH 7.0), 2 mM EDTA, 1 mM PMSF, and 0.01% (v/v) 2-mercaptoethanol, and disrupted by sonication. The disrupted soluble fraction was collected by centrifugation. UV absorption spectra of CO-bound recombinant CYP proteins after sodiumdithionite reduction were measured by UV/vis spectrometry (SPECTRONIC, GENESYS, MILTON ROY, USA) by scanning wavelength from 400 to 500 nm. Concentrations of each protein were measured based on CO-difference spectra using an extinction coefficient of 91 mM-1 cm-1 at 450 nm.

2-1. Reaction Condition for END Biotransformation with Wild Type *S. avermitilis* MA-4680, *N. farcinica* IFM10152 and *E. coli* BL21(DE3) Co-expressing both P450 and Redox Proteins

After being grown, the cells were harvested, washed twice with PBS buffer (pH 7.2), and resuspended in 15 ml of 100 mM phosphate buffer (pH 7.5). END dissolved in 10 mM MeOH was added into 20 ml of the cell suspension to make a final concentration of 50 mM. The mixture was shaken in 200 rpm for oxygen supply at 30 °C for 20 hours.

2-2. Extraction of Products after Biotransformation

After incubation of the cells with END, the reaction was stopped by adding the same volume of ethyl acetate (JUNSEI, Japan) and vortexed vigorously. The mixtures were centrifuged at 13,000 rpm for five minutes, and the upper organic layer was evaporated by

Table 1. Three major ions in the mass spectra of the TMS derivatives of END and its monohydroxylated products

	m/z
Enterodiol (END)	500, 410, 180
Hydroxylated in aromatic ring	588, 498, 268
Hydroxylated in aliphatic position	588, 408, 395

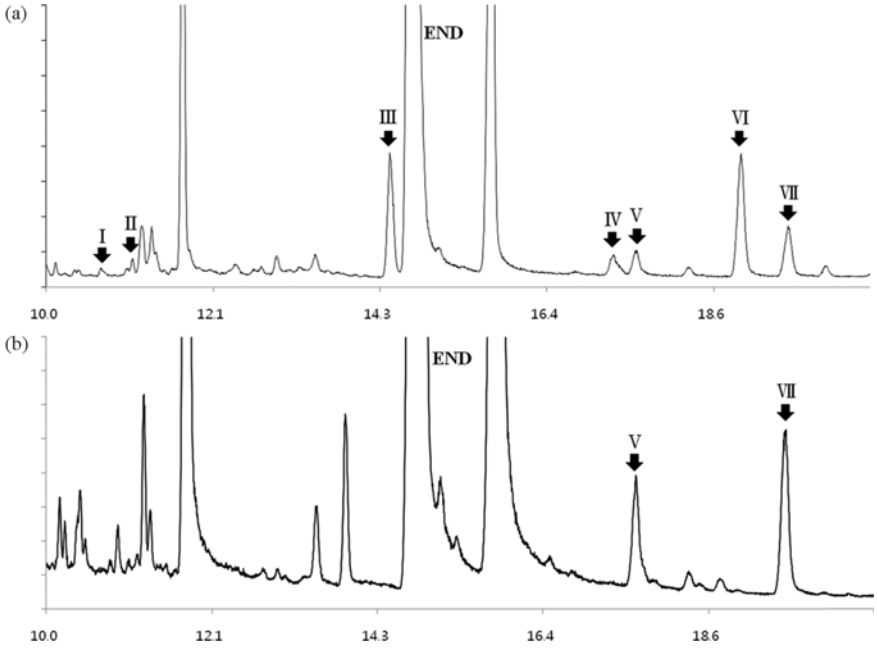

Fig. 1. GC/MS total ion current (TIC) of extract from whole cell reaction with END. (a) Seven hydroxylation products from *S. avermitlis* MA-4680 were separated. (b) Two hydroxylation products from *N. facinica* IFM10152 were shown.

vacuum concentrator (BioTron, South Korea). Subsequently, the residual was dissolved in 50 ml of methanol (MERK, Germany) and ethyl acetate for anlaysis by HPLC and GC/MS, respectively.

2-3. GC/MS Analysis

For GC/MS analysis, reaction products were converted to their trimethylsilyl (TMS) derivatives by incubating for 20 min at 70 °C with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Analysis by GC/MS was performed using a TRACE GC ULTRA gas chromatograph, coupled to an ion trap mass detector ITQ1100. The TMS-derivatives were analyzed using a nonpolar capillary column (5% phenyl methyl siloxane capillary 30 m×250 μm i.d., 0.25 μm film thickness, TR-5ms) with a linear temperature gradient (100 °C 1 min, 30 °C/min to 250 °C, hold for 10 min, 1 °C/min to 280 °C, and hold for 1 min). The injector port temperature was 230 °C. The temperature of the connecting parts was 275 °C and the electron energy for the EI mass spectra was 70 eV. Identification was performed by comparison of retention time and mass spectral data (recorded by full scan in the selected ion mode; *m/z* 50-1000) of the sample with that of authentic references.

3. Computational Methods

Nfa45180 from *N. farcinica* IFM10152 (accession number, 3109062) shows a sequence identity of 64.6% to CYP154A1 of *S. coelicolor*. The corresponding crystallographic structure [protein data bank (PDB) entry 1ODO] was chosen as structural template. The align-

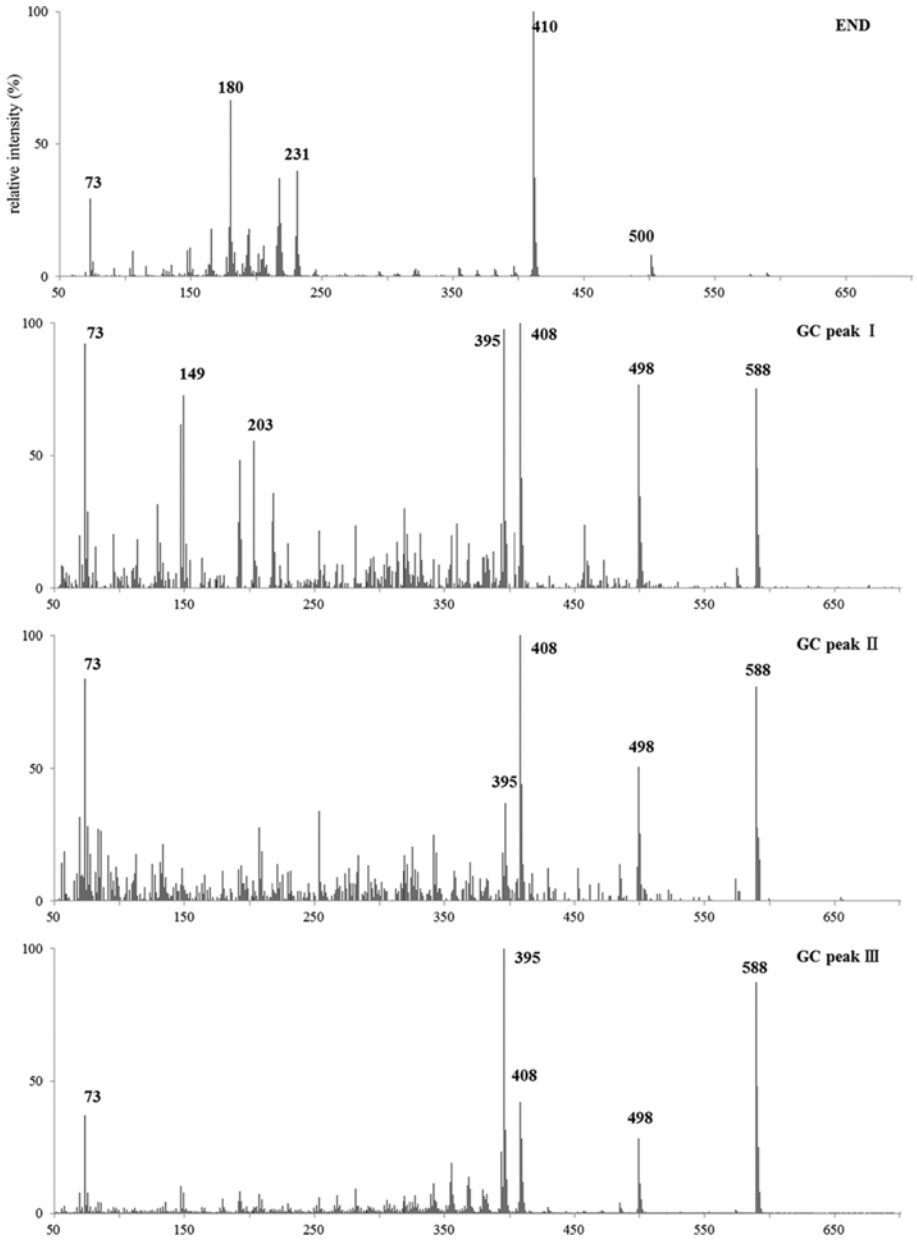

Fig. 2. Mass spectra of hydroxylated END (TMS derivatives). The GC peak numbers refer to Fig. 1. There were four Al-OH-ENDs and three Ar-OH-ENDs. The *m/z* value of three highest ions at GC peaks I, II, III and VI in Fig. 1(a) were 395, 408 and 588 corresponding to GC/MS fragment pattern of Al-OH-ENDs. And, the *m/z* value of major ions of fragmented Ar-OH-ENDs were equal to the *m/z* value of three highest ions at GC peaks IV, V and VII in Fig. 1(a) as 268, 498 and 588.

ment was generated with ClustalW 1.83 [13] and SPEM [14]. A model was generated by Modeller 9.4 [15] with hetero-atom containing mode. The coordinates of END for docking were generated manually and energetically optimized using the MM + force field using Chem3D Ultra 8.00. AutoDock (version 3.00) was applied for docking of END into the homology model of Nfa45180 [15]. 1000 docking runs were carried out, and the minimum energy value was −1.43 kcal/mol.

RESULTS AND DISCUSSION

1. Whole Cell Reaction of END by *S. avermitilis* MA-4680 and *N. farcinica* IFM10152

Recently completed genome sequences of a few *Actinomycetes* sp. strains revealed that more than a dozen of CYP monooxygenases are present in their genome. The CYP enzymes are known to be involved in various hydroxylation steps of primary and secondary metabolites [8]. Based on this observation, *Streptomyces* sp., *Bacillus* sp., and *Nocardia* sp. were examined for their ability to convert END into their corresponding hydroxylated products (Table S1). Among the strains examined, two strains showed hydroxylation activities for END. The products of *S. avermitilis* MA-4680 and *N. farcinica* were separated by GC and their structures were identified by mass spectrometry. Various hydroxylation products were observed and they were divided into two major groups, hydroxylated ENDs at aliphatic moiety and aromatic moiety. From the previous studies on the microsomal metabolism of END and ENL [6] and on the urinary metabolites of lignans in humans (Jacobs

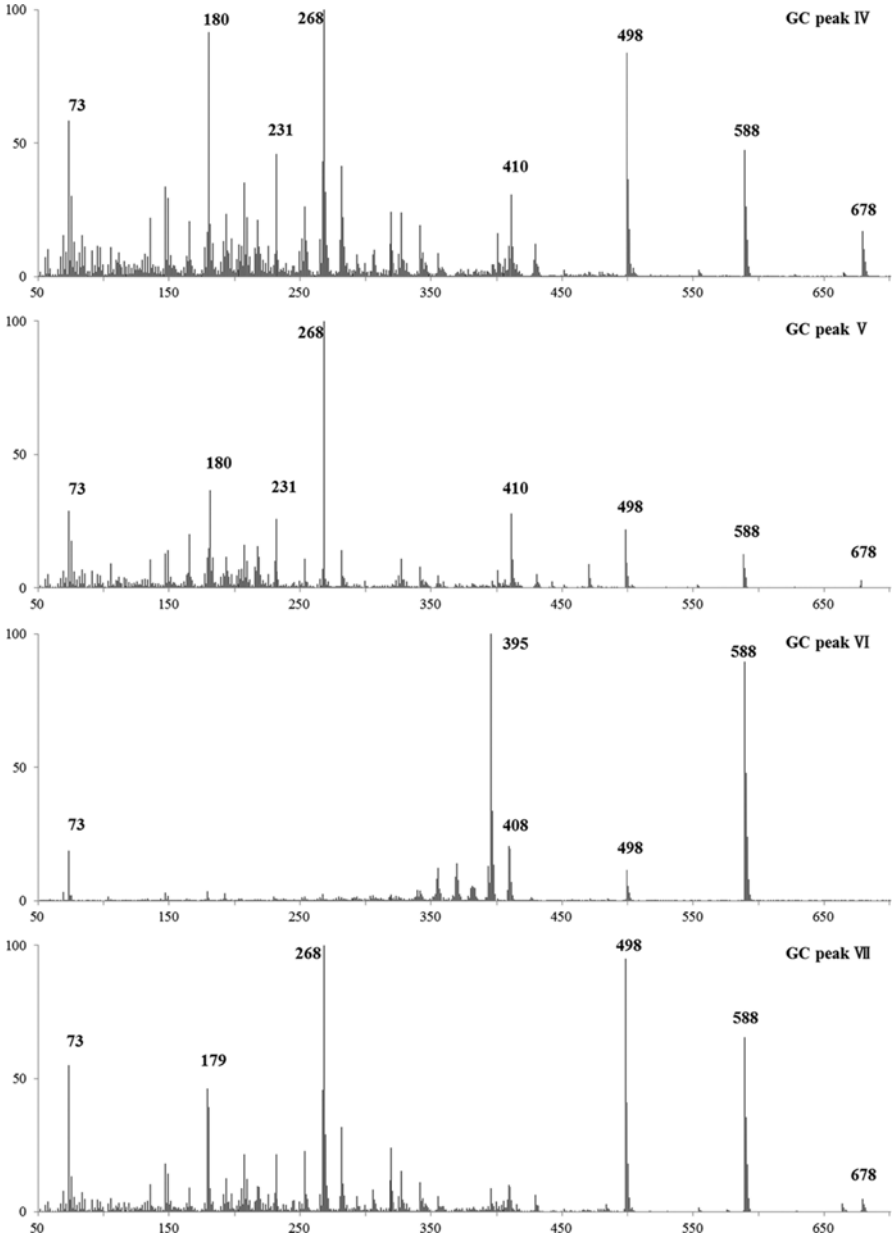

Fig. 2. Continued.

et al., 1999), the mass values of the monohydroxylated ENDs at the aromatic or aliphatic moiety were reported. Table 1 shows the major ions in the mass spectra of the TMS derivative of END and its monohydroxylated products. The GC separation showed different patterns of hydroxylated ENDs (Fig. 1). Among the seven products from the reaction with *S. avermitilis*, four Al-OH-ENDs and three Ar-OH-ENDs were observed (Fig. 1(a) and Fig. 2). The *m/z* values of three highest ions at GC peaks I, II, III and VI in Fig. 1(a) were 395, 408 and 588 corresponding to GC/MS fragment patterns of Al-OH-END. However, any reference compounds of Al-OH-END were not observed here. So the specific hydroxylation position was not identified. The *m/z* value of major ions of fragmented Ar-OH-END was equivalent to the *m/z* value of three highest ions at GC peaks IV, V and VII in Fig. 1(a) as 268, 498 and 588. The position of the additional hydroxyl group was identified using GC chromatography of three synthetic reference compounds of the Ar-OH-END [6]. As a result, GC peak IV was 6-hydroxy-END (6-OH-END), that is, hydroxylation in para position, GC peak V was 2-OH-END, that is, hydroxylation in ortho position and GC peak VII was 4-OH-END, that is, hydroxylation in another ortho position (Fig. 3). The mass fragmentation pattern is proposed in Fig. 4. From the whole cell reaction by *N. farcinica* IFM10152, two hydroxylated products were analyzed, and those corresponded to ortho-hydroxylation END showing the identical retention time in Fig. 1(b) and mass spectra compared to the results from *S. avermitilis* MA-4680 (data are not shown). From 15 mg/L of END, 900 μg/L of Al-OH-END and 210 μg/L of 4-OH-END were produced by *S. avermitilis* and 300 μg/L of 2-OH-END and 480 μg/L of 4-OH-END were biotransformed by *N. farcinica* IFM10152 (Table 2).

2. Identification of P450 Genes Responsible for the END Monohydroxylation Reaction

To identify P450s which are responsible for the END hydroxylation activity, the P450 library in *E. coli* was constructed. First, 33

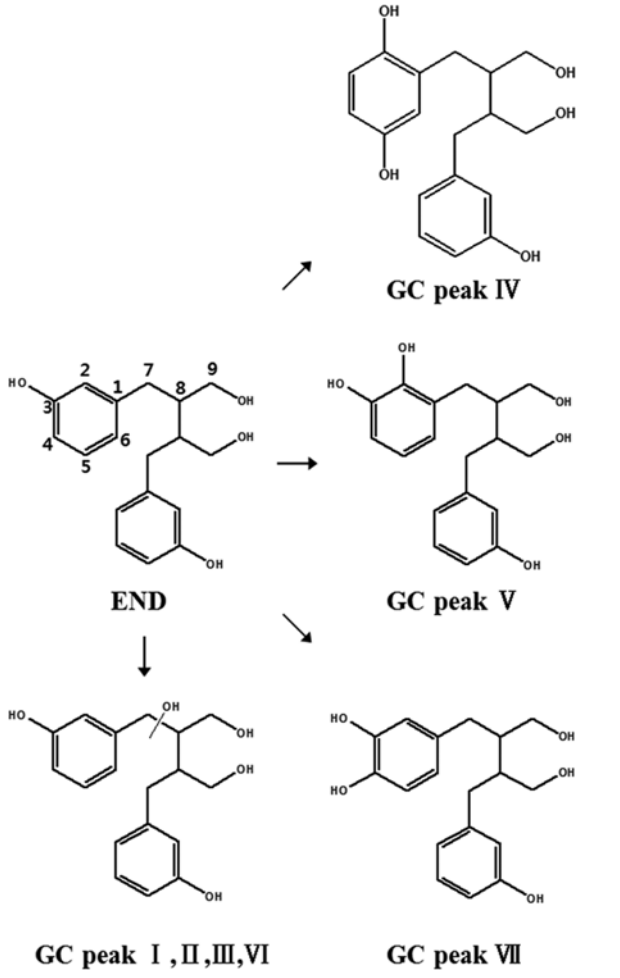

Fig. 3. Structures of END and hydroxylation products. The GC peak numbers refer to Fig. 1 and Fig. 2.

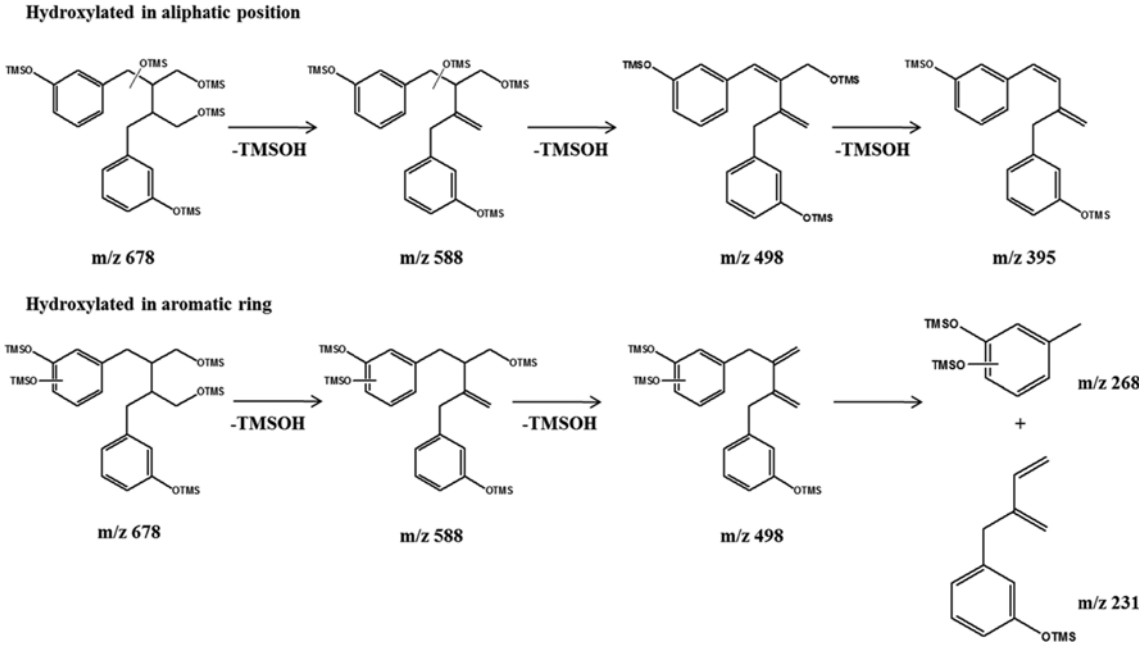

Fig. 4. Proposed mass fragmentation scheme for Al-OH-ENDs and Ar-OH-ENDs.

Table 2. The yield of major three hydroxylated products by *S. avermitilis* MA-4680 and *N. facinica* IFM10152

Strains	Yield (μg/L)		
	Al-OH-END	2-OH-END	4-OH-END
S.avermitilis MA-4680	900	90	210
Nocardia facinica IFM10152	n.d.[a]	300	480

[a]Not detected

P450s from *S. avermitilis* and 26 P450s from *N. farcinica* were cloned and expressed in *E. coli*. 59 P450s belong to 29 CYP families, which have sequence identity above 40% compared to P450s in same families (Table S2). Using cell extract, CO binding spectra were measured to confirm the protein soluble expression. As a result, 14 P450s from *S. avermitilis* MA-4680 and 26 P450s from *N. farcinica* IFM10152 showed UV absorption CO binding spectra, suggesting that those were expressed with functionality. For their electron transfer proteins, putidaredoxin reductase (CamA) and putidaredoxin (CamB) from *P. putida* were chosen and coexpressed. Finally, 40 recombinants were constructed and screened for END hydroxylation activity. As a result, three CYPs from *S. avermitilis* MA-4680 and four CYPs from *N. farcinica* IFM10152 showed the activity for hydroxylation of END (Table S2). Especially, one CYP encoded by *nfa45180* showed the highest activity. This CYP enzyme belongs to CYP154 family, which is well known for its activity toward aromatic compounds [12]. By GC/MS analysis, the structure of product was revealed as 4-hydroxylation END due to the identical retention time and mass spectra compared to the results from *N. farcinica* (Fig. 5).

3. Docking Simulation of Nfa45180 with Substrates and Identification of Key Residues

Computer modeling of Nfa45180 and its complexes with END was performed to obtain more insight about the structural basis

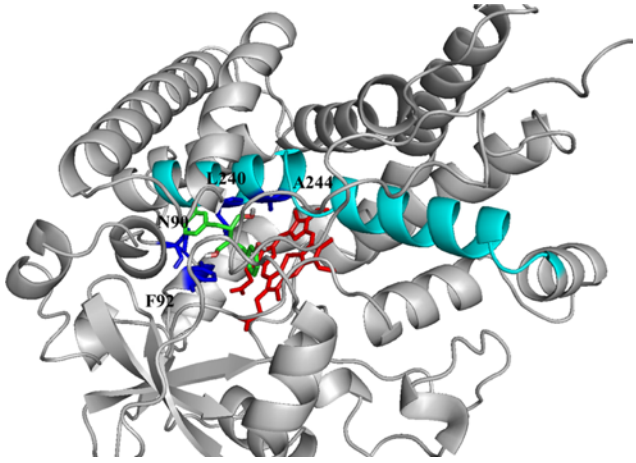

Fig. 6. Docking simulation in the homology model of Nfa45180. I-helix which is the most important secondary structure around heme is shown in cyan and key residues surrounding substrate are shown in blue.

for the regio-specific hydroxylation of END. Nfa45180 from *N. farcinica* has the highest amino acid identity (64.6%) with CYP154A1 from *Streptomyces coelicolor* among the known crystal structures of bacterial cytochrome P450s deposited in the PDB. The corresponding crystallographic structure (PDB entry 1ODO) was chosen as structural template. Docking simulation of Nfa45180 with enterodiol as a substrate was performed to identify the key residues involved in interaction between the enzyme and the substrate. Fig. 6 shows the heme active site with enterodiol and there are four residues (Asn90, Phe92, Leu240, and Ala244) which interacts with enterodiol. The distances toward the iron atom are 3.92 Å for END. As Asn90/Phe92 and aromatic moiety of END forms hydrophobic interaction, another aromatic moiety of END is located near heme geo-

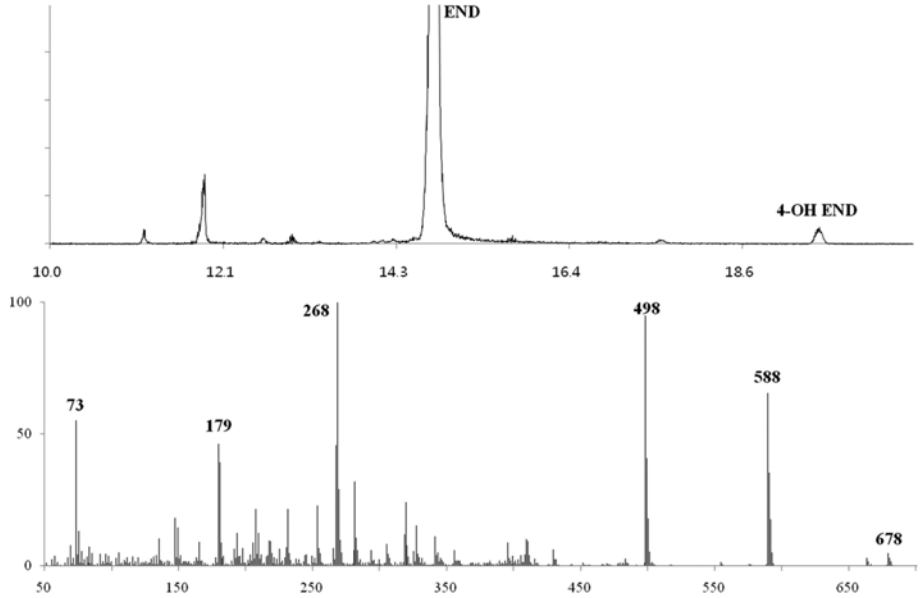

Fig. 5. (a) GC/MS total ion current (TIC) of END conversion in the presence of Nfa45180 in *E. coli*. The product peak (t_R=19.52) was identified as 4-OH-END. (b) Mass spectra of 4-OH-END.

metrically. Also, Ala244, which is the nearest residue from heme, provides room for the aromatic moiety of END. This indicates the reason for the regioselective hydroxylation of END in structural aspect.

CONCLUSION

We investigated the hydroxylation of mammalian lignin END using whole cell reaction of *S. avermitilis* and *N. farcinica*. Seven and two hydroxylated products were separated from *S. avermitilis* and *N. farcinica,* respectively, and their structures were identified by mass fragmentation (GC/MS/MS) study. In the GC spectra, seven hydroxylated products from *S. avermitilis* were identified as four ENDs with aliphatic hydroxylation and three ENDs with aromatic hydroxylation, whereas the two hydroxylated END products from *N. farcinica* IFM10152 were all hydroxylated at ortho-position of an aromatic ring. All the P450s of *S. avermitilis* and *N. farcinica* were examined to determine the P450s responsible for hydroxylation reaction. Among them, Nfa45180 was screened to have hydroxylation activity at C4 position of END. To elucidate the structural understanding of regio-selectivity of the Nfa45180 for END, docking studies were performed using a homology model of Nfa45180. Thus, the model with the shortest distance between heme and C4 position in enterodiol had the lowest energy. This model thoroughly explains the high regio-specificity of CYP154 toward enterodiol.

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Supporting Information

Ortho-hydroxylation of mammalian lignan enterodiol by cytochrome P450s from *Actinomycetes* sp.

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Table S1. The list of strains used in screening for hydroxylation activity toward enterodiol

Strains
Streptomyces avermitilis MA4680
Streptomyces coelicolor A3(2)
Streptomyces carbophilus
Streptomyces venezuelae
Streptomyces peucetius ATCC 27952
Streptomyces lividans
Streptomyces griseolus
Nocardia farcinica IFM10152
Bacillus subtilis subsp. subtilis
Bacillus licheniformis ATCC14580
Bacillus megaterium DSM319
Bacillus cereus KCCM12145
Bacillus amyloliquefaciens ATCC10987

Table S2. The list of CYPs cloned from *Streptomyces avermitilis* and *Nocardia facinica*

Strain	P450	Family	P450	Family	P450	Family
S. avermitilis	Sav109	CYP154A	Sav412	CYP105D	Sav413	CYP105P
	Sav575	CYP102D	Sav584	CYP147B	Sav838	CYP178A
	Sav941	CYP171A	Sav1171	CYP107F	Sav1308	CYP154D
	Sav1611	CYP105Q	Sav1987	CYP107L	Sav2061	CYP179A
	Sav2165	CYP180A	Sav2377	CYP107Y	Sav2385	CYP181A
	Sav2806	CYP182A	Sav2894	CYP107W	Sav2999	CYP183A
	Sav3031	CYP170A	Sav3519	CYP107V	Sav3536	CYP107U
	Sav3704	CYP154B	Sav3881	CYP157A	Sav3882	CYP154C
	Sav4539	CYP107P	Sav5111	CYP184A	Sav5841	CYP125A
	Sav6249	CYP107X	Sav6706	CYP157C	Sav7130	CYP158A
	Sav7186	CYP105R	Sav7426	CYP102B	Sav7469	CYP105D
N. farcinica	Nfa4950	CYP157A	Nfa5180	CYP191A	Nfa11380	CYP136B
	Nfa11960	CYP157A	Nfa12130	CYP193A	Nfa12160	CYP193A
	Nfa21340	CYP157A	Nfa21760	CYP210A	Nfa22290	CYP140A
	Nfa22920	CYP157A	Nfa22930	CYP154B	Nfa24320	CYP125A
	Nfa25810	CYP109A	Nfa25870	CYP125A	Nfa25890	CYP51A
	Nfa30590	CYP104A	Nfa33510	CYP151A	Nfa33880	CYP107E
	Nfa34990	CYP159A	Nfa43600	CYP120A	Nfa45170	CYP157A
	Nfa45180	CYP154A	Nfa46410	CYP107A	Nfa53100	CYP157A
	Nfa53110	CYP154H	Nfa56380	CYP110D		

Table S3. The list of CYPs used in screening for hydroxylation activity toward enterodiol

Strain	P450	Activity	Strain	P450	Activity
S. avermitilis	Sav412	–[a]	*N. farcinica*	Nfa21340	–
	Sav413	+[b]		Nfa21760	–
	Sav838	–		Nfa22290	–
	Sav1171	–		Nfa22920	–
	Sav1987	–		Nfa22930	–
	Sav2377	+		Nfa24320	–
	Sav2894	+		Nfa25810	–
	Sav3519	+		Nfa25870	–
	Sav3882	–		Nfa25890	–
	Sav4539	–		Nfa30590	–
	Sav5111	–		Nfa33510	–
	Sav6249	–		Nfa33880	–
	Sav7186	–		Nfa34990	–
	Sav7469	–		Nfa43600	–
N. farcinica	Nfa4950	–		Nfa45170	–
	Nfa5180	++[c]		Nfa45180	+++[d]
	Nfa11380	–		Nfa46410	–
	Nfa11960	–		Nfa53100	–
	Nfa12130	–		Nfa53110	–
	Nfa12160	+		Nfa56380	–

[a]No activity
[b]Relative hydroxylation activity is within 30%
[c]Relative hydroxylation activity is from 30 to 70%
[d]Relative hydroxylation activity is up to 70%