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Synthesis of Unnatural Flavonoids and Stilbenes by Exploiting the Plant Biosynthetic Pathway in *Escherichia coli*

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SUMMARY

Flavonoids and stilbenes have attracted much attention as potential targets for nutraceuticals, cosmetics, and pharmaceuticals. We have developed a system for producing "unnatural" flavonoids and stilbenes in *Escherichia coli*. The artificial biosynthetic pathway included three steps. These included a substrate synthesis step for CoA esters synthesis from carboxylic acids by 4-coumarate:CoA ligase, a polyketide synthesis step for conversion of the CoA esters into flavanones by chalcone synthase and chalcone isomerase, and into stilbenes by stilbene synthase, and a modification step for modification of the flavanones by flavone synthase, flavanone 3β-hydroxylase and flavonol synthase. Incubation of the recombinant *E. coli* with exogenously supplied carboxylic acids led to production of 87 polyketides, including 36 unnatural flavonoids and stilbenes. This system is promising for construction of a larger library by employing other polyketide synthases and modification enzymes.

INTRODUCTION

Flavonoids are polyketide derivatives synthesized exclusively in plants, many of which possess various functions, such as acting as floral pigments, signal molecules, and antimicrobial compounds [1]. In addition, several beneficial effects on human health because of their antioxidant [2], anti-inflammatory [3], antitumor [3], and estrogenic [4] activities have been reported. Flavonoids are, therefore, potential targets for nutraceuticals, cosmetics, and pharmaceuticals. However, although more than 5000 flavonoids have been identified, their application has been hampered because they usually exist as a mixture of multiple compounds and are difficult to isolate, or their activities are not satisfactory. Stilbenes, represented by resveratrol, are plant-derived polyketides that have also been shown to prevent or slow the progression of a wide variety

of diseases, including cancer, cardiovascular disease, and ischemic injuries [5].

The biosynthesis of flavonoids starts with the naringenin chalcone synthesis by chalcone synthase (CHS). Naringenin chalcone is synthesized through the stepwise condensation of three malonyl-CoAs onto *p*-coumaroyl-CoA by CHS (Figure 1A). *p*-Coumaroyl-CoA as the substrate of CHS is synthesized by thioesterification of *p*-coumaric acid, which is generated from the phenylpropanoid pathway with CoASH by 4-coumarate:CoA ligase (4CL). Naringenin chalcone is converted to (2*S*)-naringenin by intramolecular cyclization by chalcone isomerase (CHI), which accomplishes the synthesis of a flavanone scaffold. All flavonoids are produced from a common precursor, (2*S*)-naringenin, through modifications by various tailoring enzymes [6]. For instance, flavone synthase I (FNS I) synthesizes apigenin from (2*S*)-naringenin and flavanone 3β-hydroxylase (F3H) and flavonol synthase (FLS) sequentially convert (2*S*)-naringenin into dihydrokaempferol and kaempferol. FNS I, F3H, and FLS belong to the 2-oxoglutarate-dependent oxygenases that are nonheme iron dioxygenases utilizing 2-oxoglutarate as a cofactor [7]. 2-Oxoglutarate-dependent oxygenases are soluble and can therefore be functionally expressed in *E. coli*. We have recently succeeded in microbial production of flavanone [8], flavone, and flavonol [9] by reconstituting the flavonoid biosynthetic pathway in *E. coli*.

Polyketides, including flavonoids and stilbenes, show a great structural diversity. This structural diversity of polyketide scaffolds is governed mainly by the selectivity of starter and extender substrates, the number of condensation reactions, and the mode of ring closure of the resultant polyketide chains [10]. Polyketides are synthesized by polyketide synthases (PKSs) that fall into three groups, types I through III [11]. Type I and type II PKSs are ketosynthases with accessory enzymes that control the sizes and shapes of the polyketide scaffolds [11]. In contrast, type III PKSs, composed of only a ketosynthase domain, are structurally the simplest PKSs that synthesize aromatic polyketides by iterative condensation of malonyl-CoA derivatives with acyl-CoA esters [12]. CHS, belonging to the type III PKS superfamily, catalyzes condensation of *p*-coumaroyl-CoA with three malonyl-CoA-derived acetate units, intramolecular cyclization, and aromatization to produce naringenin chalcone (Figure 1A). Stilbene synthase
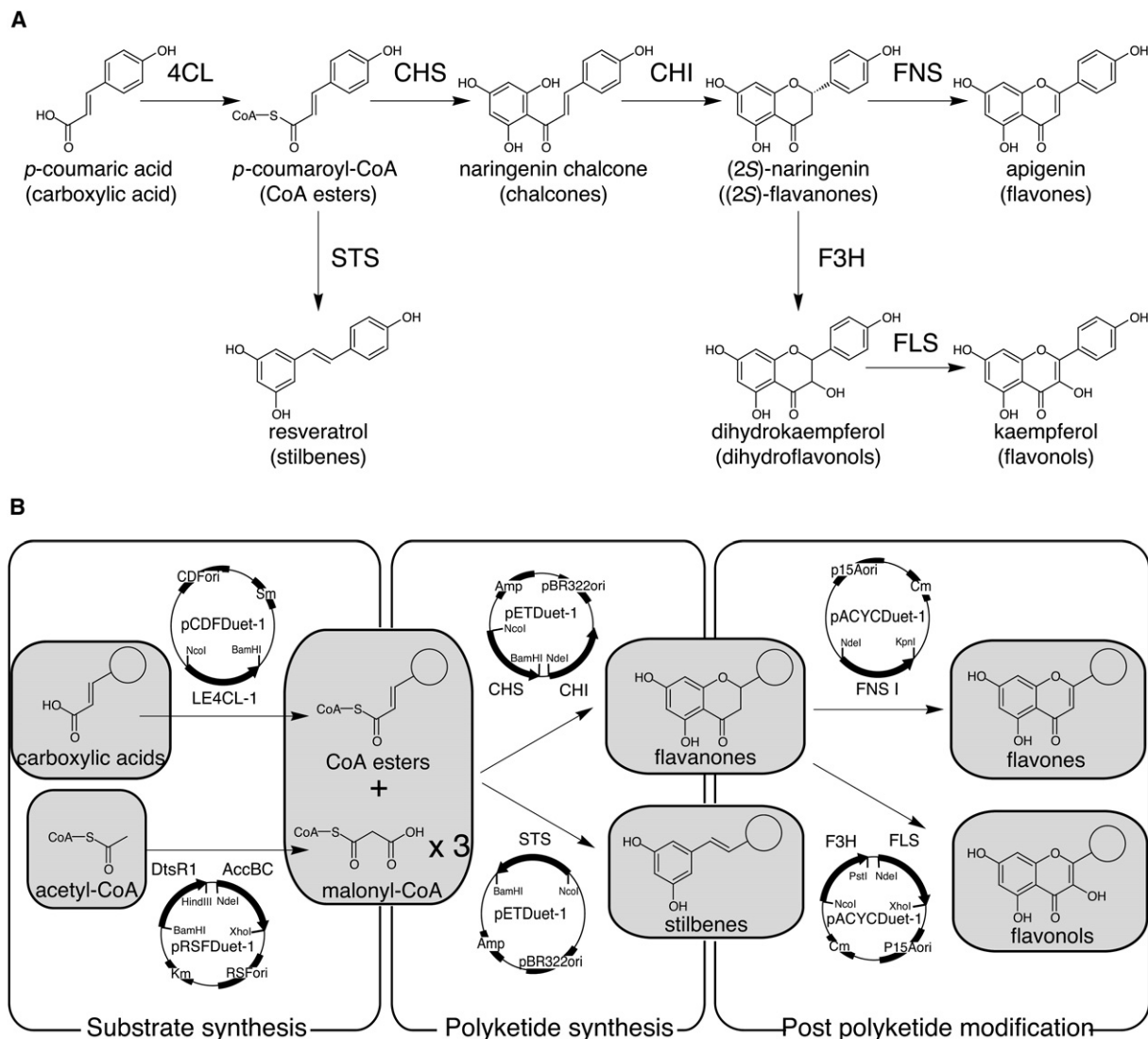
Figure 1. Synthesis Routes of Plant Polyketides in Plants and Recombinant *E. coli*

(A) Biosynthesis of flavanones and stilbenes in plants. These key intermediates are further modified into flavones and flavonols.

(B) The multiplasmid approach for synthesis of unnatural plant polyketides in *E. coli*. The polyketide synthesis is divided into three steps: substrate synthesis from carboxylic acids by LE4CL-1, polyketide biosynthesis by CHS/CHI and STS, and modification by FNS and F3H/FLS.

(STS), another representative of the type III PKS superfamily, uses the same substrates as CHS, but differs in the ring folding, thus producing resveratrol, but not naringenin chalcone (Figure 1A).

Recent development of "combinatorial biosynthesis" has enabled us to manipulate biosynthetic pathways to generate a library of hybrid or unnatural compounds [10]. For example, 50 macrolides were prepared by systematic modification of a modular type I PKS, 6-deoxyerythronolide B synthase [13]. However, this approach cannot be applied to the type III PKS. The diverse reactions of type III PKSs depend on subtle differences in the amino acid residues forming the active site cavity, since the polyketide chain elongation, cyclization, and aromatization catalyzed by type III PKSs occur in a single, multifunctional

active cavity formed by a homodimer of the ketosynthase [12]. This structural simplicity has hampered systematic exchange of functional domains or rational mutagenesis for the synthesis of unnatural polyketides. Until now, the reports concerning the synthesis of unnatural polyketides by type III PKSs have been limited to in vitro synthesis depending on the promiscuity of their substrate specificity [14, 15].

However, plants produce a vast array of flavonoids that are divided into nine major subgroups: chalcones, flavanones, flavones, flavonols, aurones, isoflavonoids, flavanediols, anthocyanins, and tannins [6]. This structural diversity is generated through postpolyketide modifications by various tailoring enzymes [6]. Therefore, in the case of type III PKSs, combinatorial biosynthesis for production

of a wide variety of unnatural flavonoids and stilbenes could be established by means of combining various PKSs and postpolyketide modification enzymes, rather than systematic exchange of functional domains or rational mutagenesis on the basis of their structure-function relationship. In the present study, we have employed a multiplasmid approach for producing unnatural plant polyketides in *E. coli* by the combinatorial use of generous substrate specificity of type III PKSs and a variety of tailoring enzymes. A combination of the multiplasmid approach with precursor-directed biosynthesis has generated a library of natural and unnatural plant polyketide compounds.

RESULTS AND DISCUSSION

Construction of Unnatural Polyketide Production System

We divided the flavonoid biosynthetic pathway into three steps: substrate synthesis, polyketide synthesis, and post-polyketide modification. These three steps were each placed on a plasmid with different replication origins and selectable markers and simultaneously introduced in *E. coli* (Figure 1B). The plasmid for the substrate synthesis step was pCDF-LE4CL-1 carrying the 4-coumarate:CoA ligase gene from *Lithospermum erythrohizon* (gromwell) on the vector pCDFDuet-1. The plasmids for the polyketide synthesis step were pET-CHS/CHI, carrying the chalcone synthase and chalcone isomerase genes from *Glycyrrhiza echinata* (licorice) and *Pueraria lobata* (kudzu), respectively, on pETDuet-1, and pET-STS, carrying the stilbene synthase gene from *Arachis hypogaea* (peanut) on pETDuet-1. The plasmids for the modification step were pACYC-FNS I, carrying the flavone synthase gene from *Petroselinum crispum* (parsley) on pACYCDuet-1, and pACYC-F3H/FLS, carrying the flavanone 3β-hydroxylase and flavonol synthase genes from the *Citrus* plant genus on pACYC-Duet-1. In addition to these plasmids, we also introduced pRSF-ACC, carrying the two subunit genes for acetyl-CoA carboxylase from *Corynebacterium glutamicum* to increase the intracellular pool of malonyl-CoA [8]. This would allow the efficient connection between the primary metabolism of *E. coli* and the secondary metabolisms that are reconstituted by the plant genes.

The above system employed two techniques, a multiplasmid approach [16] and precursor-directed biosynthesis [17]. One of the advantages of the multiplasmid approach was that construction of recombinant biosynthetic pathways by cotransformation of plasmids was rapid and efficient, avoiding the laborious and time-consuming tasks required to construct plasmids for individual biosynthetic steps [16]. Precursor-directed biosynthesis is a promising approach to generating novel analogs of polyketides. This technique was successfully applied to production of novel polyketides by a type I PKS mutant that was blocked in the early stage of the biosynthesis of a natural product [17]. Polyketide synthesis by the mutant enzyme was restored by the introduction of natural precursors. Introduction of analogs of natural precursors, in

turn, gave novel compounds. To apply precursor-directed biosynthesis to type III PKSs, we used *E. coli* as a host where the phenyl propanoid pathway is absent. Consequently, exogenous addition of unnatural carboxylic acids to the recombinant *E. coli* cell would lead to production of the corresponding CoA esters by the action of 4CL without competition by the plant natural carboxylic acid, *p*-coumaric acid. CoA esters of unnatural carboxylic acids would be recognized as a starter substrate of CHS to give the corresponding chalcones, which would be subsequently converted to (2*S*)-flavanones by the intramolecular cyclization activity of CHI (Figure 1B). STS, instead of CHS/CHI, could be readily introduced to this system by the multiplasmid method, leading to the synthesis of unnatural stilbenes. Another approach for the generation of molecular diversity by the present system is the introduction of postpolyketide modification enzymes, FNS I and F3H/FLS, which would convert the flavonoids into flavones and flavonols, respectively (Figure 1B). A key to the success of the precursor-directed biosynthesis by type III PKSs is the recognition and processing of unnatural precursors and intermediates by the enzymes.

Combinatorial Biosynthesis of Unnatural Plant Flavonoids

We first tried to produce natural and unnatural flavonoids and stilbenes in a small scale in which 500 μl of the cell suspension containing 40 g/l of glucose and 1 mM each of the substrate carboxylic acids was incubated at 26°C for 60 hr. Since the cells were incubated in minimal medium, and no cell mass increased during incubation [8, 9], detection and purification of the polyketides produced were easily accomplished. In other words, the *E. coli* cell was an enzyme bag that converted the carboxylic acids into the corresponding flavonoids and stilbenes. For the purpose of production of natural and unnatural flavanones, pCDF-LE4CL-1, pRSF-ACC, and pET-CHS/CHI were simultaneously introduced in *E. coli*. As expected, exogenous supply of natural carboxylic acids, such as cinnamic acid (**1a**), *p*-coumaric acid (**2a**), *m*-coumaric acid (**3a**), *o*-coumaric acid (**4a**), and caffeic acid (**5a**), to the recombinant *E. coli* cells led to production of the corresponding flavanones (**1g–5g**), as revealed by liquid chromatography electrospray ionization mass spectrometry (LC-ESI/MS) and LC-ESI/MS/MS analysis (Figure 2; see also Figure S1 in the Supplemental Data available with this article online). In contrast, ferulic (**6a**) and sinapic (**7a**) acids, which are natural carboxylic acids, were not converted to flavanones, suggesting that the culture conditions optimized for naringenin production from **2a** were not suitable for some carboxylic acids. It is known that the optimal pH for flavanone formation is different among the starter substrates [18].

We next added unnatural carboxylic acids to the recombinant *E. coli* cells. A variety of unnatural carboxylic acids, such as fluorocinnamic acids (**8a–11a**), furyl (**15a** and **17a**), thienyl (**16a** and **18a**), and naphthyl (**20a**) acrylic acids, gave the corresponding unnatural flavanones (**8g–11g**, **15g–18g**, and **20g**), which demonstrated the
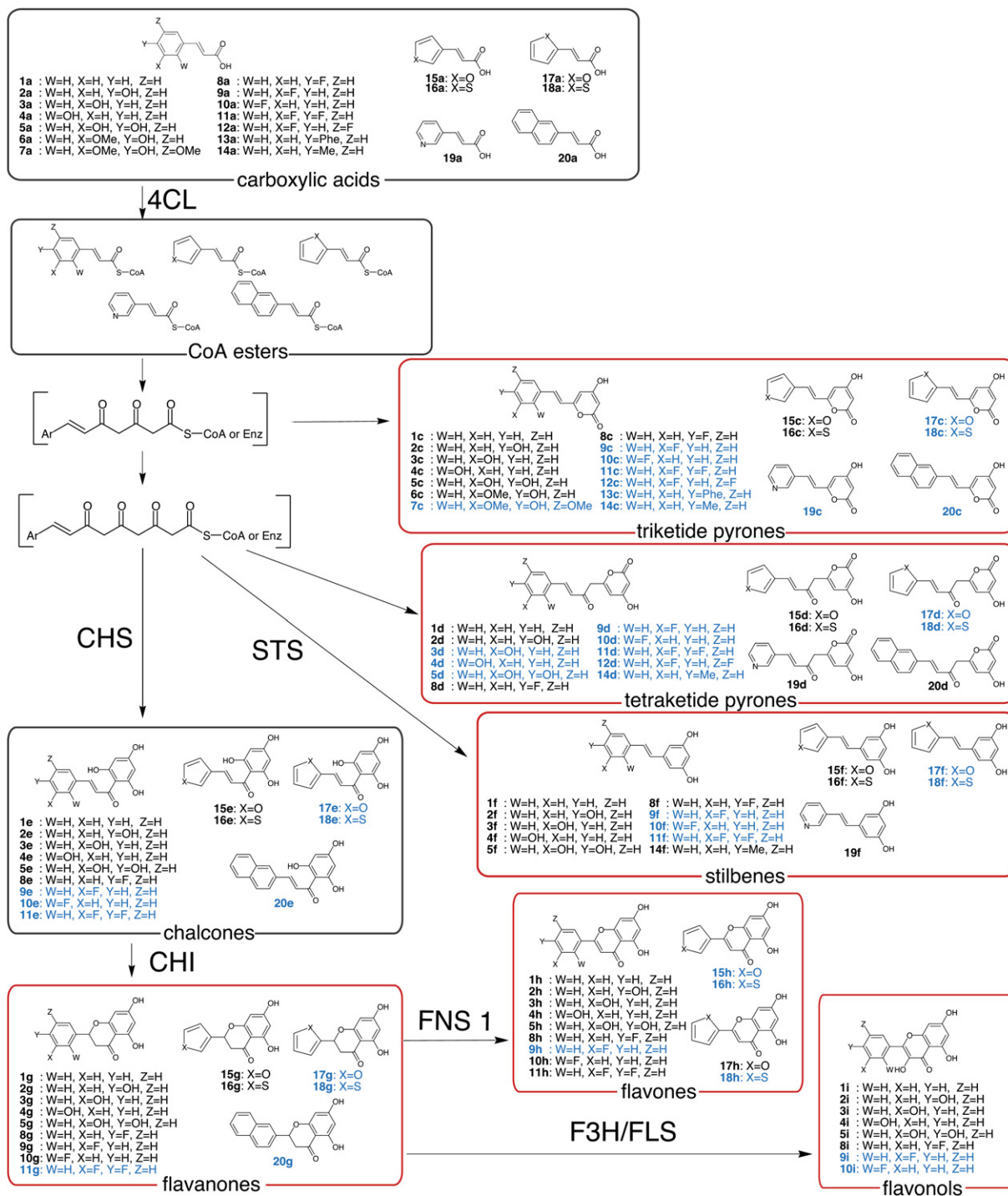
Figure 2. Summary of the Structures of Plant Polyketides Produced in This Study

The polyketides synthesized are surrounded by red squares. Novel compounds, which have not been reported in the literature, are indicated in blue.

reliability and validity of the culture conditions (Figure 2, Figure S1). Extracted ion LC-ESI/MS chromatograms and LC-ESI/MS/MS fragmentation spectra of **8g** are shown in Figures 3B and 3E as representatives. Of the unnatural flavanones produced, novel compounds that have not been reported in the literature are shown in blue in Fig-

ure 2. Roughly estimated, 50% of the product was present in the culture broth, suggesting that the flavanones could be readily transported through the cell membrane of *E. coli*. We also detected negligible amounts of triketide pyrones (**1c–12c** and **15c–18c**), a typical derailment product of type III PKSs, by the ion chromatograph analysis of
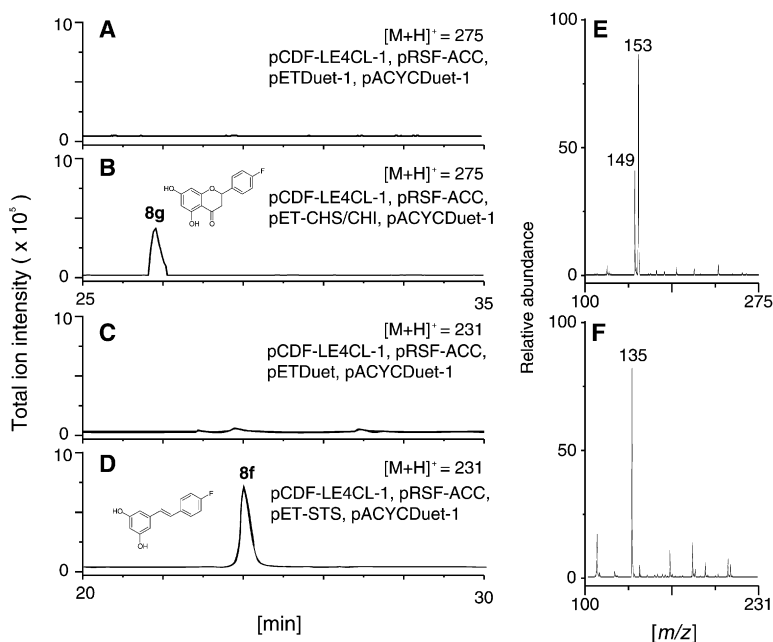
Figure 3. Extracted Ion LC-ESI/MS Chromatograms and LC-ESI/MS/MS Fragmentation Spectra of 8g and 8f

(A) *E. coli* harboring pCDF-LE4CL-1, pRSF-ACC, pETDuet-1, and pACYCDuet-1, as a negative control, produced no flavanones. The target ions of the extracted ion chromatograms are shown in the upper right of the chromatograms.

(B) Supplementation of **8a** to *E. coli* harboring pCDF-LE4CL-1, pRSF-ACC, pET-CHS/CHI, and pACYCDuet-1 led to production of **8g**.

(C) *E. coli* harboring pCDF-LE4CL-1, pRSF-ACC, pETDuet-1, and pACYCDuet-1, as a negative control, produced no stilbenes. The target ions of the extracted ion chromatograms are shown in the upper right of the chromatograms.

(D) Supplementation of **8a** to *E. coli* harboring pCDF-LE4CL-1, pRSF-ACC, pET-STS, and pACYCDuet-1 led to production of **8f**.

(E) The LC-ESI/MS/MS fragmentation spectrum of **8g**.

(F) The LC-ESI/MS/MS fragmentation spectrum of **8f**.

LC-ESI/MS (data not shown). In all cases, no tetraketide pyrones were detected.

We further introduced the plasmids for the modification step into the *E. coli* cell expressing LE4CL-1, ACC, CHS, and CHI. When pACYC-FNS I was introduced, 13 flavones (**1h–5h**, **8h–11h**, and **15h–18h**) were synthesized from the corresponding flavanones (Figure 2). An exception was the flavanone (**20g**) derived from 3-(2-naphthyl) acrylic acid, which was not modified by the FNS I. These findings suggested that FNS I modified a broad spectrum of unnatural flavanones (Figure 2, Figure S2). Similarly, the F3H/FLS on pET-CHS/CHI also showed rather broad substrate specificity, giving eight flavonols derived from cinnamic, hydroxycinnamic, and monofluorocinnamic acids (**1i–5i** and **8i–10i**), albeit in small amounts (Figure 2, Figure S3).

We scaled up the reactions starting from cinnamic acid (**1a**), *p*-coumaric acid (**2a**), fluorocinnamic acid (**8a**), furyl- (**15a**), and thienyl- (**16a**) acrylic acids to evaluate the productivity of the system. The yields of the natural flavanones derived from **1a** and **2a** reached roughly 70–90 mg/l and those of the unnatural flavanones derived from **8a**, **15a** and **16a** reached ca. 50 to 100 mg/l (Table 1). In contrast, the yields of natural (**1c**) and unnatural (**16c**) pyrones were 3.6 ± 1.1 and 2.7 ± 0.8 mg/l, respectively. These observations were consistent with the above-described in vitro reaction of CHS that produced byproduct pyrones in small amounts. Furthermore, the unnatural flavones derived from **8a**, **15a**, and **16a** were produced in yields of 30 to 50 mg/l, which were comparable to the yields of the natural flavones derived from **1a** and **2a**. These results showed that the system was useful for production of novel polyketides. However, the unnatural flavonols derived from **8a**, **15a**, and **16a** were produced in trace amounts, and considerable amounts of the flavanones remained intact (data not shown).

We assume that no efficient conversion of the flavanones to flavonols was due to the substrate preference of F3H, because no dihydroflavonols were detected in the reaction.

Combinatorial Biosynthesis of Unnatural Plant Stilbenes

We examined the feasibility of this approach by using STS instead of CHS for the polyketide synthesis step. Exogenous supply of carboxylic acids to the recombinant *E. coli* cell containing 4CL, ACC, and STS resulted in the production of 15 stilbenes (**1f–5f**, **8f–11f**, **14f–19f**) (Figure 2, Figure S4), which demonstrated that a type III PKS other than CHS was applicable to this system. Extracted ion LC-ESI/MS chromatograms and LC-ESI/MS/MS fragmentation spectra of **8f** are shown in Figures 3D

Table 1. Yields of Plant Polyketides Produced by *E. coli*

Substrate	Flavanone (mg/l)	Flavone (mg/l)	Flavonol (mg/l)	Stilbene (mg/l)
1a	65 ± 7	33 ± 6	0.47 ± 0.08	155 ± 17
2a	87 ± 15	84 ± 14	33 ± 3	171 ± 17
8a	102 ± 16	30 ± 4	Trace	132 ± 26
15a	53 ± 30	26 ± 5	—	85 ± 44
16a	45 ± 25	46 ± 2	—	54 ± 3

Flavanones were isolated from *E. coli* harboring pCDF-LE4CL-1, pRSF-ACC, pET-CHS/CHI, and pACYCDuet-1. Flavones were isolated from *E. coli* harboring pCDF-LE4CL-1, pRSF-ACC, pET-CHS/CHI, and pACYC-FNS. Flavonols were isolated from *E. coli* harboring pCDF-LE4CL-1, pRSF-ACC, pET-CHS/CHI, and pACYC-F3H/FLS. Stilbenes were isolated from *E. coli* harboring pCDF-LE4CL-1, pRSF-ACC, pET-STS, and pACYCDuet-1. Results are mean ± SE (n = 3).

and 3F as representatives. In contrast with the CHS, the STS converted the tetraketide intermediates derived from 4-methylcinnamic acid (**14a**) and 3-(3-pyridyl) acrylic acid (**19a**) into the corresponding stilbenes. On the other hand, the tetraketide intermediate derived from naphthyl acrylic acid (**20a**) was correctly ring-folded only by the CHS. Besides stilbenes, negligible amounts of triketide pyrones (**1c**–**20c**) and tetraketide pyrones (**1d**–**5d**, **8d**–**12d**, and **14d**–**20d**) were observed by selected ion chromatography in LC-ESI/MS analysis (data not shown), showing the occurrence of derailment reactions by the STS. The yields of **1c** and **16c** were 2.8 ± 0.4 and 6.1 ± 2.2 mg/l, showing that STS produced byproduct pyrones in trace amounts, as observed for CHS. The yields of stilbenes derived from the unnatural carboxylic acids **8a**, **15a**, and **16a** were 55–130 mg/l, which is comparable to those of 130–170 mg/l, derived from the natural carboxylic acids, **1a** and **2a** (Table 1).

Bioactivity of Unnatural Plant Polyketides

We measured CYP1B1 inhibitory activity of the plant polyketides generated. CYP1B1, which catalyzes hydroxylation of 17β-estradiol, is a major enzyme for carcinogenic estrogen metabolism and is thus involved in the metabolic activation of procarcinogens [19]. Therefore, the inhibition of CYP1B1 is an attractive oncological therapeutic strategy [19]. The ethoxyresorufin-*O*-deethylase (EROD) activity is commonly used to test the activity of CYP1B1. We first measured the inhibitory activity of ethylacetate extracts of the *E. coli* cells producing polyketides (flavonoids and stilbenes) against the EROD activity of CYP1B1 and compared them with that of a negative control, an ethylacetate extract of the *E. coli* cells harboring pCDF-LE4CL-1, pRSF-ACC, pETDuet-1, and pACYC-Duet-1. Note that this primary test was just to screen for candidates having CYP1B1-inhibitory activity, because the amounts of the polyketides and probable byproducts were not taken into consideration. As a result, besides the natural polyketides produced from **1a** to **5a**, the polyketides from **8a** to **11a** and **16a** to **19a** appeared to show CYP1B1 inhibitory activity (data not shown). We chose and purified five stilbenes (**1f**, **2f**, **8f**, **15f**, and **16f**) as representatives to confirm that the CYP1B1-inhibitory activity detected in the ethylacetate extracts was actually due to the stilbenes produced and for further analysis of the inhibition kinetics (Table 2). The results obtained with purified stilbenes showed that the unnatural stilbenes (**8f**, **15f**, and **16f**) had distinct CYP1B1 inhibition kinetics similar to those of the natural stilbenes (**1f** and **2f**). This result suggested that the bioactivity of unnatural polyketides was comparable to that of natural products, and that a library of unnatural products would be promising as a source for screening of bioactive compounds of various functions.

Further Insight into Combinatorial Biosynthesis

Although several attempts to develop a system for production of plant polyketides have been reported [8, 9, 20–24], these studies were concentrated on the produc-

Table 2. Inhibition of the EROD activity of CYP1B1 by Stilbenes

Inhibitor[a]	V_{max} (pmol/mg/min)[b]	K_M (μM)[c]	K_i (μM)[d]
—	885	0.315	—
1f	377	0.158	0.0743
2f	565	0.232	0.177
8f	676	0.276	0.323
15f	610	0.270	1.11
16f	621	0.262	1.18

K_i, inhibitor constant; K_M, Michaelis constant; V_{max}, maximum velocity.
[a] The concentrations of inhibitors are described in Experimental Procedures.
[b] Maximum velocity of the EROD activity by CYP1B1.
[c] Michaelis constant of ethoxyresorufin.
[d] K_i was calculated from the equation, $1/V_{max}' = (1/V_{max0})(1 + [I]/K_i)$. V_{max0} indicates V_{max} without an inhibitor, V_{max}' indicates V_{max} with an inhibitor, and [I] indicates the concentration of an inhibitor.

tion of natural products. In this study, we have developed a system for producing variants of plant polyketides by recombinant *E. coli*. This newly developed system allowed the synthesis of 87 polyketides (14 flavanones, 13 flavones, 8 flavonols, 15 stilbenes, and 37 pyrones), including 36 novel compounds. In addition, our findings suggest that the relaxed substrate specificity of FNS I enables us to synthesize unnatural flavones, whereas the substrate preference of F3H was a bottleneck for the synthesis of unnatural flavonols. It may be possible to overcome this bottleneck by random or systematic mutagenesis of F3H. Furthermore, the positive clones that contain mutated F3H with broader substrate specificity can be easily selected by substituting the part of F3H in pACYC-F3H/FLS in *E. coli* harboring pCDF-LE4CL-1, pRSF-ACC, and pET-CHS/CHI, in addition to the substituted plasmid, and examining production of unnatural flavonols. A similar procedure would be possible for screening of novel enzymes, such as type III PKSs and flavonoid-tailoring enzymes with broader substrate specificity, from plant and microbial origins.

The yields of the unnatural plant polyketides were comparable to those of the natural polyketides. The yields of flavanones and stilbenes were approximately 50–150 mg/l, and those of flavones were 30–80 mg/l. This means that the flavanones are efficiently converted to the corresponding flavones, indicating rather broad substrate specificity of the FNS. On the other hand, most flavonols, except for **2i** and **5i** (Figure S2), were produced in small amounts. We assume that the low yields of the flavonols were due to strict substrate specificity of the F3H, because no accumulation of dihydroflavonols was observed. Notwithstanding the low yields of flavonols, the system in this study is useful in the high-yield production of natural and unnatural flavanones, stilbenes, and flavones. The present system is convenient in the purification of the products, because they are produced in minimal medium,

containing few contaminants. Furthermore, because the phenylpropanoid-flavonoid pathway is absent in *E. coli*, the present system is free from background reactions that would result in the formation of undesirable byproducts. In plants, polyketides are usually produced as a mixture of more than 20 derivatives [25–27], making the purification step very laborious.

There are various polyketide biosynthesis enzymes that could readily be applied to the present system employing the multiplasmid approach; for instance, propionyl-CoA carboxylase giving methylmalony-CoA [28] would provide different extender substrates for polyketide synthesis. Recently discovered type III PKSs that possess novel catalytic activities, such as benzalacetone synthase [29], could provide different polyketide scaffolds. In addition, several flavonoid-modifying enzymes, including methylase [30] and prenyl transferase [31], were identified. Applying not only these enzyme genes, but also many enzyme genes of plant and microbial origin, to this system would enable us to produce a wide variety of natural and unnatural polyketides.

SIGNIFICANCE

Flavonoids show various bioactivities beneficial to human health, such as antioxidant [2], anti-inflammatory [3], antitumor [3], and estrogenic [4] activities. Stilbenes, represented by resveratrol, are reported to prevent cancer, cardiovascular disease, and ischemic injuries [5]. Therefore, establishment of a system for rapid, efficient production of flavonoids and stilbenes has been desired. The bacterial system that we have constructed by employing type III polyketide synthases (PKSs) and a variety of tailoring enzymes is a rapid and efficient one, and is convenient for purification of the products. This system has also proven useful in production of unnatural flavonoids and stilbenes. Of the natural and unnatural polyketides produced by this system, some may show potent and novel bioactivities.

This report describes a convenient and efficient system for production of unnatural flavonoids and stilbenes by microorganisms. A combination of combinatorial biosynthesis and precursor-directed biosynthesis resulted in production of 14 flavanones, 13 flavones, 8 flavonols, and 15 stilbenes. Simultaneous production of 20 triketide pyrones and 17 tetraketide pyrones were also observed. Of the 70 plant polyketides produced in this way, 36 were novel products. This production system allowed the high-yield production of both natural and unnatural plant polyketides. Several stilbenes produced by this system showed inhibition against the ethoxyresorufin-O-deethylase activity of CYP1B1, suggesting that novel, unnatural polyketides become potential bioactive compounds. Thus, the present study opens up the possibility of producing novel compounds by combinatorial biosynthesis employing type III PKSs and a variety of tailoring enzymes. Various enzymes of plant and microbial origin that can be readily incorporated as members in the

artificial biosynthetic pathway would contribute to construction of a larger library of natural and unnatural polyketides.

EXPERIMENTAL PROCEDURES

Materials

E. coli strains JM109 and BLR (DE3), pUC19, restriction enzymes, T4 DNA ligase, *Taq* DNA polymerase, and other DNA-modifying enzymes were purchased from Takara Biochemicals. pETDuet-1, pRSFDuet-1, pACYCDuet-1, and pCDFDuet-1 were purchased from Novagen. *p*-Coumaric acid (**2a**), sinapic acid (**7a**), 4-fluorocinnamic acid (**8a**), 3,4-difluorocinnamic acid (**11a**), 3,5-difluorocinnamic acid (**12a**), 4-methylcinnamic acid (**14a**), 3-(3-furyl)-acrylic acid (**15a**), 3-(3-thienyl)-acrylic acid (**16a**), 3-(3-pyridyl)-acrylic acid (**19a**), chrysin (**1h**), and galangin (**1i**) were purchased from Aldrich. 3-Fluorocinnamic acid (**9a**) was purchased from Alfa Aesar. 3-(2-Naphthyl)-acrylic acid (**20a**) was purchased from Oakwood products. Caffeic acid (**5a**), ferulic acid (**6a**), and naringenin (**2g**) were purchased from Sigma. Cinnamic acid (**1a**), *m*-coumaric acid (**3a**), *o*-coumaric acid (**4a**), 2-fluorocinnamic acid (**10a**), 3-(2-furyl)-acrylic acid (**17a**), 3-(2-thienyl)-acrylic acid (**18a**), and apigenin (**2h**) were purchased from Wako. Resveratrol (**2f**) and kaempferol (**2i**) were purchased from Extrasynthesis. Pinocembrin (**1g**) was purchased from Indofine Chemical Company. 3-(4-Biphenylyl)-acrylic acid (**13a**) was purchased from Avocado Research Chemicals. Human CYP1B1 + P450 Reductase SUPERSOMES were purchased from BD Bioscience.

Construction of Plasmids

pACYC-F3H/FLS [9], pACYC-FNS I [9], and pRSF-ACC [8] have been described previously. To construct pET-CHS/CHI carrying the *CHS* and *CHI* genes under the control of the T7 promoter, we first constructed pET-CHI. With the pET-P$_{T7}$-4GS [8] as a template, the 1.1 kb DNA fragment containing the *CHI*-coding region was amplified by PCR with primer I: 5′-G**GAATTC**<u>CAT*ATG*</u>GCGGCAGCAGCAGCAGT-3′ (with an EcoRI site shown in bold, an NdeI site shown by underlining, and the start codon of *CHI* shown in italics) and primer II: 5′-CG<u>GGATCC</u>*TCA*GACTATAATGCCGTGGC-3′ (with a BamHI site shown by underlining and the stop codon of *CHI* shown in italics). The amplified fragment was cloned between the EcoRI and BamHI sites of pUC19, resulting in pUC19-CHI. The NdeI-BamHI fragment containing the *CHI* sequence was excised from pUC19-CHI and cloned between the NdeI and BglII sites of pETDuet-1, resulting in pET-CHI. With the pET-P$_{T7}$-4GS as a template, the 1.1 kb DNA fragment containing the *CHS*-coding region was amplified by PCR with primer III: 5′-CG**G AATTC**<u>CC*ATGG*</u>TGAGCGTAGCTGAGAT-3′ (with an EcoRI site shown in bold, an NcoI site shown by underlining, and the start codon of *CHS* shown in italics) and primer IV: 5′-CG**GGATCC***TCA*GATGGCCACACT ACGCA-3′ (with a BamHI site shown in bold and the stop codon of *CHS* shown in italics). The amplified fragment was cloned between the EcoRI and BamHI sites of pUC19, resulting in pUC19-CHS. The NcoI-BamHI fragment containing the *CHS* sequence was excised from pUC19-CHS and cloned between the NcoI and BamHI sites of pET-CHI, resulting in pET-CHS/CHI. For construction of pET-STS containing a cDNA of *STS* (AB027606) from *A. hypogaea*, the NcoI-BamHI fragment containing the *STS* sequence on pET-3d-STS [32] was excised and cloned between the NcoI-BamHI sites of pETDuet-1, resulting in pET-STS. For construction of pCDF-LE4CL-1, carrying the *4CL* gene from *L. erythrorhizon*, the 1.1 kb DNA fragment containing the *LE4CL-1*-coding region was amplified by PCR with primer V: 5′-ACAT**GCATGC**<u>CC ATGG</u>GG*ATG*GACACTCAAACCAAAAC-3′ (with an SphI site shown in bold, an NcoI site shown by underlining, and the start codon of *LE4CL-1* shown in italics) and primer VI: 5′-CGC<u>GGATCC</u>*CTA*ATTGTG ATCACCATTTGCAA-3′ (with a BamHI site shown by underlining and the stop codon of *4CL* shown in italics). The amplified fragment was cloned between the SphI and BamHI sites of pUC19, resulting in pUC19-LE4CL-1. The NcoI-BamHI fragment containing the *LE4CL-1*

sequence was excised from pUC19-LE4CL-1 and cloned between the NcoI and BamHI sites of pCDFDuet-1, resulting in pCDF-LE4CL-1.

Production of Plant Polyketides by *E. coli*

Ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (34 μg/ml), and streptomycin (50 μg/ml) were used, when necessary. Recombinant *E. coli* BLR (DE3) cells were precultured overnight at 37°C in 2 ml of Luria-Bertani (LB) medium. The preculture was transferred into 100 ml of LB medium and cultured at 26°C until the optical density at 600 nm reached 0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) was then added at a final concentration of 1 mM, and the culture was further continued for 5 hr. The cells were harvested by centrifugation and the cells (0.5 g wet weight) were suspended in 20 ml of M9 medium. To a portion (500 μl) of the cell suspension in M9 (25 g/l wet weight) was added 1 mM each of substrates, 40 g/l glucose, 25 g/l CaCO$_3$, 0.5 mM FeSO$_4$·2H$_2$O, antibiotics, and 1 mM IPTG, and the culture was incubated at 26°C for 60 hr. The culture broth was adjusted to pH 3.0 with 6 N HCl. After extraction with an equal volume of ethyl acetate, materials were concentrated by evaporation and dissolved in 100 μl dimethyl sulfoxide for LC-ESI/MS/MS analysis. LC-ESI/MS/MS was performed in a positive mode on an esquire HCT plus (Bruker Daltonics) equipped with a DOCOSIL-B column (C$_{22}$, 2 × 200 mm; Senshu Scientific Co.), and the sample was eluted with a gradient of acetonitrile in water (both containing 0.1% acetic acid) at a flow rate of 0.2 ml/min. The conditions of the gradient were: 20% CH$_3$CN for 5 min and 20%–100% CH$_3$CN for 40 min. UV spectra were detected on an Agilent 1100 series UV detector.

For estimation of the amounts of the polyketides produced, the culture was performed with 20 ml medium. The yields were estimated on the basis of the standard curve obtained by integration of the area of the UV peak of the authentic sample on a Waters 600E-996 HPLC system equipped with a DOCOSIL-B column (C$_{22}$, 4.6 × 250 mm; Senshu). The authentic sample was eluted with a gradient of acetonitrile in water (both containing 0.1% trifluoroacetic acid) at a flow rate of 1.0 ml/min. The conditions of the gradient were: 10%–50% CH$_3$CN for 40 min and 50%–100% for 5 min. For the authentic samples, commercially available polyketides and NMR spectroscopically pure flavanones (**8g**, **15g**, and **16g**), flavones (**8h**, **15h**, and **16h**), stilbenes (**1f**, **8f**, **15f**, and **16f**), and triketide pyrones (**1c** and **16c**) were used to prepare the calibration curves.

Characterization of Flavanones

The extracted ion chromatograms of LC-ESI/MS are shown in Figure S1. **8g**, **15g**, and **16g** were chosen as representatives for NMR spectroscopic studies, and were prepared from a 200 ml scale M9 culture, described above. The flavanones were purified by reversed-phase preparative HPLC equipped with a DOCOSIL-B column (C$_{22}$, 20 × 250 mm; Senshu) by elution with 50% of CH$_3$CN in water. 5,7-Dihydroxy-2-(4-fluorophenyl)-chroman-4-one (**8g**) ^{1}H NMR (500 MHz, CDCl$_3$): δ = 7.43 (dd, 2H, *J* = 5.5, 8.5 Hz), 7.12 (t, 2H, *J* = 8.5 Hz), 6.01 (d, 1H, *J* = 2.0 Hz), 5.99 (d, 1H, *J* = 2.0 Hz), 5.41 (dd, 1H, *J* = 3.5, 13.0 Hz), 3.05 (dd, 1H, *J* = 13.0, 17.5 Hz), 2.81 (dd, 1H, *J* = 3.5, 17.5 Hz). 5,7-Dihydroxy-2-(3-furyl)-chroman-4-one (**15g**) ^{1}H NMR (500 MHz, CDCl$_3$): δ = 7.51 (s, 1H), 7.45 (s, 1H), 6.49 (s, 1H), 5.99 (d, 1H, *J* = 2.0 Hz), 5.97 (d, 1H, *J* = 2.0 Hz), 5.43 (dd, 1H, *J* = 3.5, 13.5 Hz), 3.02 (dd, 1H, *J* = 13.5, 17.5 Hz), 2.87 (dd, 1H, *J* = 3.5, 17.5 Hz). 5,7-Dihydroxy-2-(3-thienyl)-chroman-4-one (**16g**) ^{1}H NMR (500 MHz, CDCl$_3$): δ = 7.39 (dd, 1H, *J* = 3.0, 5.0 Hz), 7.36 (br, 1H), 7.68 (dd, 1H, *J* = 1.0, 5.0 Hz), 6.01 (d, 1H, *J* = 2.0 Hz), 6.00 (d, 1H, *J* = 2.0 Hz), 5.51 (dd, 1H, *J* = 3.5, 12.0 Hz), 3.09 (dd, 1H, *J* = 12.0, 17.0 Hz), 3.00 (dd, 1H, *J* = 3.5, 17.0 Hz). We identified the other flavanones by comparing the MS/MS fragmentation patterns with those of authentic naringenin (**2g**). The LC-ESI/MS/MS analysis of flavanones revealed the two major positive fragments, which are characteristic of flavanones (Figure S5).

Characterization of Flavones

The extracted ion chromatograms of LC-ESI/MS are shown in Figure S2. The NMR spectra of representatives are: 5,7-dihydroxy-2-(4-fluorophenyl)-chromen-4-one (**8h**) ^{1}H NMR (500 MHz CDCl$_3$): δ = 7.89 (dd, 2H, *J* = 5.5, 9.0 Hz), 7.22 (t, 2H, *J* = 9.0 Hz), 6.65 (s, 1H), 6.46 (d, 1H, *J* = 2.5 Hz), 6.30 (d, 1H, *J* = 2.5 Hz). 5,7-Dihydroxy-2-(3-furyl)-chromen-4-one (**15h**) ^{1}H NMR (500 MHz CD$_3$OD): δ = 8.29 (m, 1H), 7.68 (m, 1H), 6.93 (m, 1H), 6.47 (s, 1H), 6.41 (d, 1H, *J* = 2.0 Hz), 6.18 (d, 1H, *J* = 2.0 Hz). 5,7-Dihydroxy-2-(3-thienyl)-chromen-4-one (**16h**) ^{1}H NMR (500 MHz CD$_3$OD): δ = 8.26 (m, 1H), 7.62 (m, 2H), 6.62 (s, 1H), 6.46 (d, 1H, *J* = 2.0 Hz), 6.21 (d, 1H, *J* = 2.0 Hz). The other flavanones were characterized by comparing the MS/MS fragmentation patterns with those of authentic apigenin (**2h**). The LC-ESI/MS/MS analysis of flavones revealed the two major positive fragments, which are characteristic of flavanones (Figure S5).

Characterization of Flavanols

The extracted ion chromatograms of LC-ESI/MS are shown in Figure S3. The flavonols were characterized by comparing the MS/MS fragmentation patterns with those of authentic galangin (**1i**) and kaempferol (**2i**). The LC-ESI/MS/MS analysis of flavonols revealed the two specific positive fragments, which are characteristic to flavanones (Figure S5).

Characterization of Stilbenes

The extracted ion chromatograms of LC-ESI/MS are shown in Figure S4. The NMR spectra of representatives are: 5-styrylbenzene-1,3-diol (**1f**) ^{1}H NMR (500 MHz, CD$_3$OD): δ = 7.50 (d, 2H, *J* = 7.0 Hz), 7.32 (t, 2H, *J* = 7.0 Hz), 7.22 (t, 1H, *J* = 7.0 Hz), 7.03 (d, 1H, *J* = 16.0 Hz), 6.98 (d, 1H, *J* = 16.0 Hz), 6.48 (d, 2H, *J* = 2.0 Hz), 6.18 (t, 1H, *J* = 2.0 Hz). 5-[2-(4-Fluorophenyl)-vinyl]benzene-1,3-diol (**8f**) ^{1}H NMR (500 MHz, CDCl$_3$): δ = 7.52 (dd, 2H, *J* = 8.5, 5.5 Hz), 7.06 (t, 2H, *J* = 8.5 Hz), 7.02 (d, 1H, *J* = 16 Hz), 6.93 (d, 1H, *J* = 16 Hz), 6.47 (d, 2H, *J* = 2.0 Hz), 6.18 (t, 1H, *J* = 2.0 Hz). 5-[2-(3-Furyl)-vinyl]benzene-1,3-diol (**15f**) ^{1}H NMR (500 MHz, CD$_3$OD): δ = 7.60 (s, 1H), 7.46 (s, 1H), 6.91 (d, 1H, *J* = 16.0 Hz), 6.71 (s, 1H), 6.68 (d, 1H, *J* = 16.0 Hz), 6.41 (d, 2H, *J* = 2.0 Hz), 6.15 (t, 1H, *J* = 2.0 Hz). 5-[2-(3-Thienyl)-vinyl]benzene-1,3-diol (**16f**) ^{1}H NMR (500 MHz, CD$_3$OD): δ = 7.37 (m, 2H), 7.06 (m, 1H), 7.06 (d, 1H, *J* = 16.5 Hz), 6.83 (d, 1H, *J* = 16.5 Hz), 6.44, (d, 2H, *J* = 2.0 Hz), 6.16 (t, 1H, *J* = 2.0 Hz). The other stilbenes were characterized by comparing the MS/MS fragmentation patterns with those of authentic resveratrol (**2f**), except for **19f**. The LC-ESI/MS/MS analysis of flavones revealed a positive fragment, which is characteristic of stilbenes (Figure S5); **19f** was identified only by LC-ESI/MS analysis (Figure S4).

Characterization of Triketide Pyrones

4-Hydroxy-6-styrylpyran-2-one (**1c**) ^{1}H NMR (500 MHz, [CD$_3$]$_2$CO): δ = 7.67 (d, 2H, *J* = 7.5 Hz), 7.42 (t, 2H, *J* = 7.5 Hz), 7.39 (d, 1H, *J* = 16.5 Hz), 7.36 (t, 1H, *J* = 7.5 Hz), 6.94 (d, 1H, *J* = 16.5 Hz), 6.23 (s, 1H), 5.42 (s, 1H). 6-(2-Furan-3-yl-vinyl)-4-hydroxypyran-2-one (**16c**) ^{1}H NMR (500 MHz, [CD$_3$]$_2$CO): δ = 7.77 (d, 1H, *J* = 3.0 Hz), 7.54 (dd, 1H, *J* = 3.0, 5.0 Hz), 7.49 (dd, 1H, *J* = 1.0, 5.0 Hz), 7.43 (d, 1H, *J* = 16 Hz), 6.78 (d, 1H, *J* = 16 Hz), 6.14 (s, 1H), 5.38 (s, 1H).

EROD Assay

The EROD assay was performed as described previously [33]. Briefly, the standard reaction contained 7.5 μg of CYP1B1, 7.5 μg bovine serum albumin, 115 μM NADPH, 110 μM NADH, 5 mM MgSO$_4$, and 0.1 M HEPES (pH 8.0) in a total volume of 100 μl. The stilbenes were added at a final concentration of 0.1 μM (**1f**, **2f**, and **8f**) or 0.5 μM (**15f** and **16f**). The ethylacetate extracts (0.1 μg/ml) of *E. coli* were used. After 2 min of incubation at 37°C, ethoxyresorufin (0.05, 0.1, 0.2, 0.3, 0.4, and 0.6 μM for kinetic analyses and 2 μM for ethylacetate extract samples) was added to initiate the reaction. After 10 or 20 min, 50 μl of methanol was added to stop the reaction. Resorufin was quantified with a fluorescence plate reader (excitation and emission, 530 and 590 nm, respectively).

Supplemental Data

Supplemental Data, including five additional figures, are available online at http://www.chembiol.com/cgi/content/full/14/6/613/DC1/.

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