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A Versatile Microbial System for Biosynthesis of Novel Polyphenols with Altered Estrogen Receptor Binding Activity

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SUMMARY

Isoflavonoids possess enormous potential for human health with potential impact on heart disease and cancer, and some display striking affinities for steroid receptors. Synthesized primarily by legumes, isoflavonoids are present in low and variable abundance within complex mixtures, complicating efforts to assess their clinical potential. To satisfy the need for controlled, efficient, and flexible biosynthesis of isoflavonoids, a three-enzyme system has been constructed in yeast that can convert natural and synthetic flavanones into their corresponding isoflavones in practical quantities. Based on the determination of the substrate requirements of isoflavone synthase, a series of natural and nonnatural isoflavones were prepared and their binding affinities for the human estrogen receptors (ERα and ERβ) were determined. Structure activity relationships are suggested based on changes to binding affinities related to small variations on the isoflavone structure.

INTRODUCTION

Among the classes of plant compounds that exhibit potentially important biological activities are the isoflavonoids, three-ringed compounds originating from the condensation of malonyl-CoA and *p*-coumaric acid by a type III polyketide synthase (Chemler et al., 2008). Isoflavones have been demonstrated to have an impressive array of pharmacological activities and potential benefits for human health in multiple areas including cardiovascular disease (Siow et al., 2007; Squadrito et al., 2003), cancer (Yamamoto et al., 2003), and other potential health-promoting activities (Liu et al., 2006; Rasbach and Schnellmann, 2008; Zhao and Brinton, 2007) although some occur at concentrations unlikely to be attained from diet.

One particularly striking group of interactions is that of certain isoflavones with steroid receptors (Ji et al., 2006; McCarty, 2006), most famously, the 100 nM interaction of genistein with human estrogen receptor α (hERα) and the even more striking 10 nM affinity of genistein for human estrogen receptor β (hERβ) (Zhao and Brinton, 2005). Estrogen receptors are involved in numerous developmental and biological interactions and the precise response is determined by the interplay between estrogen, the estrogen receptors, and an array of modifying factors (Harris et al., 2005; Leung et al., 2006). Among the goals of investigators looking for estrogen analogs has been the discovery of molecules, referred to as selective estrogen response modifiers or SERMs (Setchell, 2001), that elicit some of estrogen's effects but not others (Hollmer, 2006). Based on their striking affinities for the estrogen and other steroid receptors, the isoflavonoids are a potential class of drugs for modifying estrogen action and metabolism.

The abundance of isoflavonoids from plants is generally low and is determined by a host of environmental factors that vary from year to year and locale to locale, to say nothing of the additional variability introduced in the supply chain from field to processor to distributor to consumer (Mortensen et al., 2009). In addition, the "active" isoflavonoids are present in undefined mixtures and, in some natural extracts, components have mutually off-setting effects (Liew et al., 2003; Liu et al., 2007; Meezan et al., 2005).

Chemical synthesis is certainly the preferred method for making most isoflavonoids. However, for compounds with multiple similar substitution sites like polyphenols or saccharides, partial biosynthesis with natural or mutated enzymes provides a means for the production of more defined compounds in improved yield. The incorporation of natural and hybrid biosynthetic pathways into microorganisms has created the potential for not only the defined biosynthesis of a multitude of natural products but also the opportunity to generate nonnatural derivatives with potentially novel or refined pharmaceutical activities (Minami et al., 2008; Willits et al., 2004). The ability to do what amounts to combinatorial chemistry in microorganisms by using a variety of natural or nonnatural precursors and different combinations of modifying enzymes has opened the possibility of creating new compounds and at the same time inventing the method for their synthesis (Challis and Hopwood, 2007; Chemler et al., 2007; Katsuyama et al., 2007a; Straathof et al., 2002).

Presented here is an approach that combines organic synthesis and metabolic engineering for the synthesis of natural and nonnatural isoflavones in practical quantities in *Saccharomyces cerevisiae*. Based on the essential determination of the substrate requirements of the key enzyme, isoflavone synthase (IFS), this approach is the initial step in the construction of a system for the potential synthesis of thousands of natural

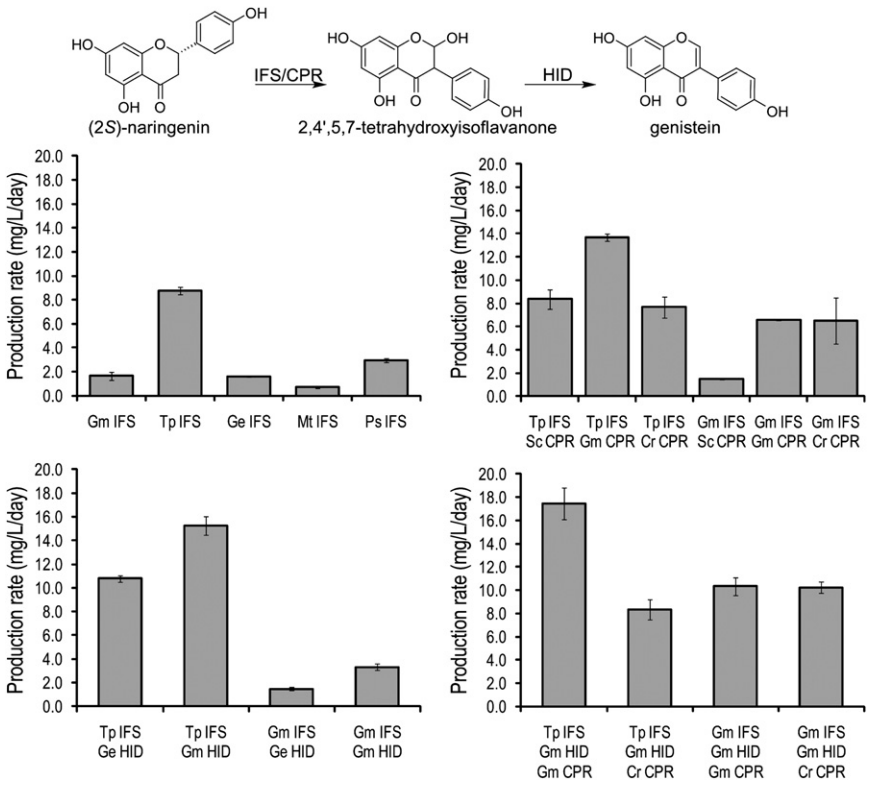

Figure 1. Genistein Production Rates from Naringenin in *S. cerevisiae* Shake Flask Cultures

Rates are from the second day of fermentation. Gm, *G. max* (soy bean); Tp, *T. pratense* (red clover); Ge, *G. enchinata* (licorice); Mt, *M. truncatula* (alfalfa); Ps, *P. sativum* (pea); Sc, *S. cerevisiae* (yeast); Cr, *C. roseus* (Madagascar periwinkle). Standard deviations are from triplicates.

and nonnatural isoflavonoids. The pathway for the biosynthesis of the isoflavonoids begins with flavanones, which are converted to isoflavones by the combined action of three enzymes: IFS (Jung et al., 2000; Kim et al., 2003; Veitch, 2007), 2-hydroxyisoflavanone dehydratase (HID) (Akashi et al., 2005), and the cytochrome P450 reductase (CPR) (Kim et al., 2005; Seeger et al., 2003). The expression and interaction of these three plant enzymes in *S. cerevisiae* enabled the biosynthesis of natural and nonnatural isoflavones that displayed a spectrum of interactions with ERα and ERβ.

RESULTS AND DISCUSSION

Among Five IFS Genes Tested, *Trifolium pratense* IFS Had the Highest Observed Activity When Expressed in *S. cerevisiae*

A primary objective was the construction of a yeast strain that would efficiently convert a diverse array of flavanone precursors into natural and nonnatural isoflavones. The central enzyme in this process is IFS and only limited data is available on the two key selection criteria: relative conversion rates and substrate flexibility. In order to find the best natural enzyme, sequences from the approximately 25 known legume-derived IFS proteins were compared. The IFS proteins were found to be highly conserved; overall sequence identity among these proteins exceeded 80%. Consequently, the search for the best IFS was directed toward those that had in some way been distinguished in the literature: *Glycine max* (soy bean) because of the high levels of isoflavones in soy beans and because of prior experience with chimeric soy IFS (Leonard and Koffas, 2007; Tian and Dixon, 2006), *Trifolium pratense* (red clover) because of reported substrate flexibility and interaction with coexpressed CPR (Kim et al., 2005), *Glycyrrhiza echinata* (licorice) because of its reported productivity in yeast (Katsuyama et al., 2007b), and *Pisum sativum* (pea) and *Medicago truncatula* (alfalfa) because of modest sequence differences near the putative catalytic site.

The five IFS genes were cloned separately into pYES2.1 yeast expression vector under the control of the *GAL1* promoter and transformed in yeast strain INVSc1, where the necessary NADPH-derived reducing equivalents would be provided by the endogenous CPR. Each strain was then grown in selective minimal medium with galactose as an inducer and carbon source and supplemented with naringenin (Figure 1A). Conversion of naringenin to genistein was monitored over 7 days with peak conversion rate observed on the second day. Presented in Figure 1B, maximum observed production of genistein by the cloned and expressed IFS genes was ranked as follows: *T. pratense* > *P. sativum* > *G. max* ≥ *G. echinata* > *M. truncatula*. The IFS from *T. pratense* was selected as the best candidate to continue work toward improving isoflavone yields.

Conversion of Naringenin to Genistein by IFS Is Dependent on the Source of the Supporting CPR

NADPH-derived reducing equivalents are transferred from CPR to a recipient P450 enzyme like IFS by transient, direct contact between the two membrane-bound enzymes. Interestingly, multiple eukaryotic microsomal P450 enzymes are supported by a single endogenous CPR, which suggests a universal electron transfer mechanism conserved among higher order species allowing the possibility of a CPR to support heterogeneous P450 enzymes. However, the source of the CPR can influence the efficiency of the coupled redox reaction as seen with P450s of plant origin (Jennewein et al., 2005; Pompon et al., 1996; Ponnamperuma and Croteau, 1996) and even the stereochemistry of the reaction (Kraus and Kutchan, 1995). Using microsomes prepared from yeast coexpressing *T. pratense* IFS and rice CPR, genistein production from naringenin increased 4.3-fold (Kim et al., 2005). Based on these examples, it was of interest to clone NADPH-dependent CPRs from different sources and compare the production rates of genistein when overexpressed with the different IFSs.

To examine the ability of particular CPRs to enhance IFS activity in vivo, the CPR genes from *Catharanthus roseus*

(Madagascar periwinkle), *G. max*, and *S. cerevisiae* were coexpressed in *S. cerevisiae* cells with either IFS from *G. max* or *T. pratense* (the sequence for the CPR from *T. pratense* was not available). The CPRs from *G. max* and *C. roseus* have 66.2% protein identity with each other but only about 30% protein identity with the CPR from yeast. CPR activity, the transfer of electrons from NADPH to the P450 monooxygenase, was determined indirectly by measuring the concentration of genistein produced by the recombinant yeast fed with naringenin and cultured over 7 days.

The maximum production rates were then compared to yeast strains expressing only IFS supported by the endogenous yeast CPR (Figure 1C). Coexpression of IFS and plant CPR in yeast cells led to a significant increase in the production rate. For example, the rate of genistein production increased by over 50% to 15 mg/liter/day, in yeast coexpressing *T. pratense* IFS and *G. max* CPR. Coexpression of *T. pratense* IFS with the CPR from *C. roseus* or *S. cerevisiae* had no significant effect on production rates compared to control. When *G. max* IFS was coexpressed with the CPR from *G. max* or *C. roseus*, maximum product formation rates increased to 6.5 mg/liter/day, a 4-fold rate increase over expression of *G. max* IFS alone. In contrast, overexpression of yeast CPR with *G. max* IFS did not improve the genistein production rate. These results suggest optimal pairing of proteins is crucial for an efficient redox reaction.

HID from *G. max* Enhances Genistein Production Rate

The conversion of 2-hydroxyisoflavanones to isoflavones can proceed spontaneously (Hashim et al., 1990) but in plants it is facilitated by the enzyme HID. Only two HID enzymes have been characterized and share 60.1% protein identity. The HID from *G. max* displays a kinetic bias toward 2-hydroxyisoflavanones with a hydroxyl group at the C4′ position while HID from *G. echinata* has a higher activity toward substrates that bear a C4′ methoxy substituent (Akashi et al., 1999, 2005).

Demonstrations of the requirement for HID have been equivocal. In one attempt to reconstruct the isoflavone pathway in hairy root cultures of the legume *Lotus japonicus*, the heterologous expression of *G. echinata* HID was determined to be a critical determinant of the accumulation of intracellular isoflavones even though the concentration was modest (less than 0.5 mg/liter) (Shimamura et al., 2007). In contrast, the expression of an artificial chimera of IFS and CPR in *E. coli* yielded 4 mg/liter of genistein without an HID (Leonard and Koffas, 2007). In construction of an isoflavone-producing system, it is essential to know if the coexpression of HID will increase productivity or yield.

To determine whether HID could improve genistein production activity in vitro, the soluble protein from yeast cells solely expressing *G. max* HID was incubated with microsomes prepared from yeast expressing only *T. pratense* IFS. The rate of consumption of naringenin was monitored by HPLC and compared to a control with soluble protein prepared from yeast harboring an empty vector. The reaction containing HID increased IFS rate of conversion of naringenin by 45% over that of microsomes with IFS coincubated with the yeast soluble protein devoid of HID.

The ability of HID to improve genistein production rate by IFS was then examined in vivo. IFSs from *T. pratense* or *G. max* were coexpressed with either *G. max* or *G. echinata* HID in yeast cultures and the concentration of genistein was measured for up to 7 days. For yeast cultures expressing *T. pratense* IFS, the coexpression of *G. max* HID led to a 75% increase in the maximum genistein production rate; a 25% increase was observed when *G. echinata* HID was expressed. For yeast cultures coexpressing *G. max* IFS and *G. max* HID, a 2-fold increase in the maximum production rate was seen while coexpression of *G. max* IFS with *G. echinata* HID had no effect on genistein production rate (Figure 1D). These results demonstrate that the *G. max* HID augments IFS activity and hence was selected for construction of the isoflavone production pathway.

Reconstructing the Entire Isoflavone Biosynthesis System Is the Key for Maximum Production

In order to reconstitute the optimal functional isoflavone synthesis system within yeast cell cultures, the *G. max* and *T. pratense* IFS genes were coexpressed with the available CPR and HID genes. The hypothesis was that the complete system would have a synergistic effect on the production of genistein in yeast. HID and CPR genes under separate *GAL1* promoters were cloned onto yEPlac112; IFS was present on a separate pYES2.1 vector. The yeast strains were constructed by transforming both vectors into INVSc1 yeast cells sequentially and the vectors were maintained on selective minimal SC medium.

Coexpression of the best single and double enzyme combinations led to the construction of the yeast strain coexpressing *T. pratense* IFS, *G. max* CPR, and *G. max* HID. This construct improved genistein production rate by 15% compared to the coexpression of *G. max* IFS with *G. max* HID, by 25% compared to *T. pratense* IFS with *G. max* CPR, and by 200% compared to expression of *T. pratense* IFS alone (Figure 1E). The combination of *T. pratense* IFS, *G. max* HID, and *C. roseus* CPR provided no improvement and was equivalent to the yeast strain coexpressing only *T. pratense* IFS and *C. roseus* CPR.

The cognate combination of *G. max* IFS, CPR, and HID yielded significant improvements in genistein production rate over any other combinations with the *G. max* IFS. The three-enzyme combination was 50% more active compared to just IFS and CPR, three times higher than IFS with only HID, and over ten times faster than IFS alone. Despite different initial rates of the strains, the final concentrations of genistein all maximized to approximately the same level of about 35 mg/liter (Figure 2).

IFS Activity Is Substantially Inhibited by Genistein and Biochanin A

One phenomenon observed during fermentations was the consistent decrease in genistein production rate over time and an apparent concentration "ceiling" noted above. The fall in productivity cannot be accounted for by genistein's low solubility or by consumption of the precursor naringenin. To determine whether isoflavones like genistein act as product inhibitors of IFS, yeast microsomes containing *T. pratense* IFS were incubated with a fixed concentration of naringenin and different concentrations of either genistein, biochanin A, or genistin. Similar studies were performed on microsomes prepared containing the other IFSs but the consumption rates were too slow to measure accurately. The consumption rate of naringenin was monitored over 30 min and the rates were calculated as

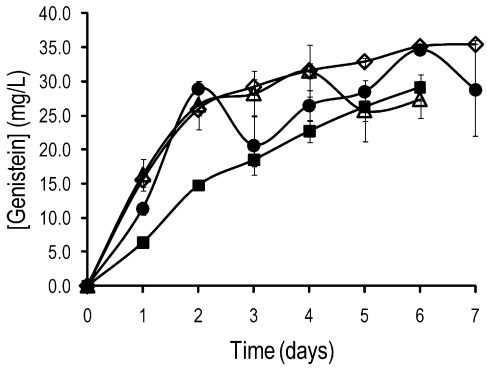

Figure 2. Genistein Production Curves from *S. cerevisiae* Coexpressing IFS, CPR, and HID

●: *T. pratense* IFS, *G. max* HID, *G. max* CPR. ■: *T. pratense* IFS, *G. max* HID, *C. roseus* CPR. ◇: *G. max* IFS, *G. max* HID, *G. max* CPR. △: *G. max* IFS, *G. max* HID, *C. roseus* CPR. Standard deviations are from triplicates.

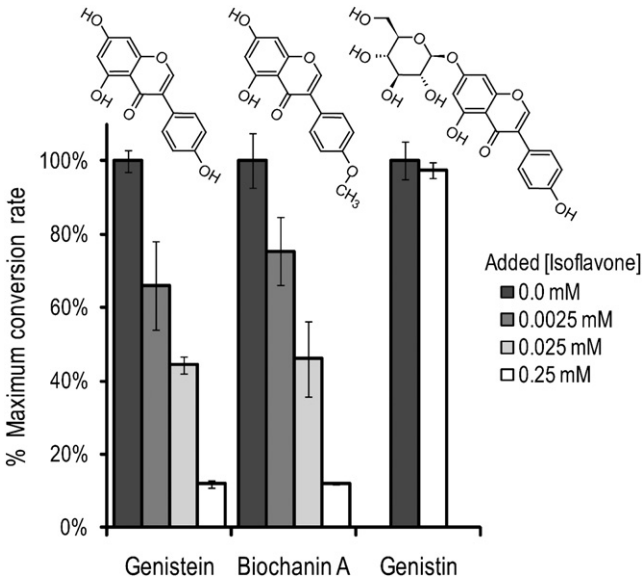

Figure 3. Isoflavone Inhibition of the Conversion of 0.25 mM Naringenin by IFS

0.0025, 0.025, and 0.25 mM genistein are equivalent to 0.675, 6.75, and 67.5 mg/liter, respectively. Standard deviations are from duplicates.

a percentage of the maximum consumption rate when no inhibitor was present. When genistein was initially added, significant inhibition was observed across three orders of magnitude including a 34% decrease in IFS activity in the presence of 2.5 μM of genistein (Figure 3). An almost identical inhibition profile was observed when the inhibitor was biochanin A (4′-methoxygenistein). In contrast, 0.25 mM genistin (genistein 7-*O*-glucoside) exhibited no inhibition of the conversion of naringenin. Based on these results it appears that binding to IFS is dependent on the presence of an unmodified hydroxyl group at the C7 position. The majority of isoflavonoids are accumulated in the form of their glyco- and malonylconjugates in soybean seeds. Acylation and glycosylation increases isoflavone solubility and stability while enabling compartmentalization in soy seeds (Heller and Forkmann, 1994). Isoflavone-modifying enzymes such as glycosyltransferases and methyltransferases (Dhaubhadel et al., 2008) may also alleviate product inhibition of IFS by genistein.

All Five IFS Enzymes Have the Same Substrate Flexibility

The second key selection criterion for IFS enzymes was substrate flexibility. In principle, system substrate flexibility can be maximized by combining IFS enzymes that accept different subsets of flavanones as precursors. To determine whether IFSs from different species had different substrate flexibility, the five IFSs cloned into yeast were challenged against the library of natural and nonnatural flavanones (Table 1 and Figure 4).

Flavanones were purchased when possible but additional flavanones were synthesized by reacting an appropriate acetaphenone and benzaldehyde to form a chalcone that was subsequently cyclized to yield the corresponding flavanone (see Supplemental Information available online) (Huang et al., 1999; Urgaonkar et al., 2005). Flavanones were designed to mimic plant-derived flavanones and isoflavones. For example, all natural flavonoids are hydroxylated at the C7 position of the A ring while the C5 position may or may not be hydroxylated. The resulting library of flavanones had many 7-monohydroxylated and 5,7-dihydroxylated members. Natural flavanones and isoflavones are further differentiated by single or multiple substitutions on the B ring so the library was designed to consist of flavanones with a variety of hydroxyl, fluoro, chloro, bromo, methyl, methoxy, and ethoxy substituents at various positions on the B ring. In total, 7 natural and 19 nonnatural flavanones were available for assessment of IFS substrate flexibility.

Microsomes bearing the membrane-bound IFS along with the endogenous *S. cerevisiae* CPR were prepared and incubated with each flavanone for 24 hr and the reactions were analyzed by reverse-phase HPLC. New products were identified in the elution profiles by comparison with reactions performed with yeast microsomes that lacked any IFS. Each IFS displayed the same convertible substrate profile and no diversity in substrate flexibility was observed. While this might be expected from the high degree of sequence conservation, the practical consequence is that the range of convertible substrates cannot be expanded by exploiting natural variations among this ensemble of IFSs. However, distinct criteria were observed that dictated whether a flavanone was accepted as a substrate.

Substrate Requirements for IFS Enzymes

While the panel of potential flavanone substrates is not comprehensive, a few suggestions about IFS substrate requirements can be made from a survey of those flavanones that were acceptable substrates. First, a hydroxyl group at the C7 position is required to bind to the IFS as suggested by the conversion of 7-hydroxyflavanone but not flavanone itself. Second, hydroxylation at the C5 position is not necessary as the synthesis of both genistein and daidzein proceeds without the need for an intervening genistein dehydratase. Third, substitutions at the C2′ or C6′ position (OH and Cl) were not tolerated, possibly due to interference of the hydroxylation reaction at the C2 position. Fourth, substitutions at the C3′ and/or C5′ position (OH, F,

Table 1. Biotransformations of Flavanones by the Isoflavone Pathway

	R_1		R_2				
Flavanone	5	7	2′	3′	4′	5′	Primary biotransformation product
Flavanone (1)	H	H	H	H	H	H	NR
7-Hydroxyflavanone (2)	H	OH	H	H	H	H	3,7-Dihydroxydihydroflavonol (D1)
Pinocembrin (3)	OH	OH	H	H	H	H	3,5,7-Trihydroxydihydroflavonol (D2)
Naringenin (4)	OH	OH	H	H	OH	H	Genistein (28)
Liquiritigenin (5)	H	OH	H	H	OH	H	Diadzein (29)
Eriodictyol (6)	OH	OH	H	OH	OH	H	Orobol (30)
Butin[a] (7)	H	OH	H	OH	OH	H	3′,4′,7-Trihydroxyisoflavone (31)
2′,4′,5,7-Tetrahydroxyflavanone[a] (8)	OH	OH	OH	H	OH	H	NR
Hesperitin (9)	OH	OH	H	OH	OCH_3	H	NR
Homoeriodictyol (10)	OH	OH	H	OCH_3	OH	H	3′-Methoxy-4′,5,7-trihydroxyisoflavone (32)
4′,7-Dihydroxy-3′-methoxyflavanone[a] (11)	H	OH	H	OCH_3	OH	H	4′,7-Dihydroxy-3′-methoxyisoflavone (33)
3′,5′-Dimethoxy-4′,5,7-trihydroxyflavanone[a] (12)	OH	OH	H	OCH_3	OH	OCH_3	3′,5′-Dimethoxy-4′,5,7-trihydroxyisoflavone (34)
4′,7-Dihydroxy-3′,5′-dimethoxyflavanone[a] (13)	H	OH	H	OCH_3	OH	OCH_3	4′,7-Dihydroxy-3′,5′-dimethoxyisoflavone (35)
3′-Ethoxy-4′,5,7-trihydroxyflavanone[a] (14)	OH	OH	H	OCH_2CH_3	OH	H	3′-Ethoxy-4′,5,7-trihydroxyflavanone (36)
4′,7-Dihydroxy-3′-ethoxyflavanone[a] (15)	H	OH	H	OCH_2CH_3	OH	H	4′,7-Dihydroxy-3′-ethoxyflavanone (37)
3′-Methyl-4′,5,7-trihydroxyflavanone[a] (16)	OH	OH	H	CH_3	OH	H	3′-Methyl-4′,5,7-trihydroxyisoflavone (38)
4′,7-Dihydroxy-3′-methylflavanone[a] (17)	H	OH	H	CH_3	OH	H	4′,7-Dihydroxy-3′-methylisoflavone (39)
3′,5′-Dimethyl-4′,5,7-trihydroxyflavanone[a] (18)	OH	OH	H	CH_3	OH	CH_3	3′,5′-Dimethyl-4′,5,7-trihydroxyisoflavone (40)
4′,7-Dihydroxy-3′,5′-dimethylflavanone[a] (19)	H	OH	H	CH_3	OH	CH_3	4′,7-Dihydroxy-3′,5′-dimethylisoflavone (41)
5,7-Dihydroxy-4′-fluoroflavanone[a] (20)	OH	OH	H	H	F	H	NR
5,7-Dihydroxy-3′-fluoroflavanone[a] (21)	OH	OH	H	F	H	H	NR
5,7-Dihydroxy-2′-fluoroflavanone[a] (22)	OH	OH	F	H	H	H	NR
4′-Chloro-5,7-dihydroxyflavanone[a] (23)	OH	OH	H	H	Cl	H	NR
3′-Chloro-4′,5,7-trihydroxyflavanone[a] (24)	OH	OH	H	Cl	OH	H	3′-Chloro-4′,5,7-trihydroxyisoflavone (42)
3′-Chloro-4′,7-dihydroxyflavanone[a] (25)	H	OH	H	Cl	OH	H	3′-Chloro-4′,7-dihydroxyisoflavone (43)
3′-Bromo-4′,5,7-trihydroxyflavanone[a] (26)	OH	OH	H	Br	OH	H	3′-Bromo-4′,5,7-trihydroxyisoflavone (44)
3′-Bromo-4′,7-dihydroxyflavanone[a] (27)	OH	OH	H	Br	OH	H	3′-Bromo-4′,7-dihydroxyisoflavone (45)

NR, no reaction.
[a] Chemically synthesized for this study.

Cl, CH_3, OCH_3, and OCH_2CH_3) were tolerated if the group was relatively small in size. Fifth, the presence of a C4′ hydroxyl group was an absolute requirement for 2-hydroxyisoflavanone synthesis provided that all other criteria were met. Compounds lacking any substitution on the B ring, as is the case with 7-hydroxyflavanone and pinocembrin (5,7-dihydroxyflavanone), were converted by IFS but gave rise to corresponding dihydroxyflavonols (Table 1 and Figure 4B). Identical HPLC profiles were obtained when the same two substrates were incubated with flavanone 3β-hydroxylase from *Malus domestica*, which

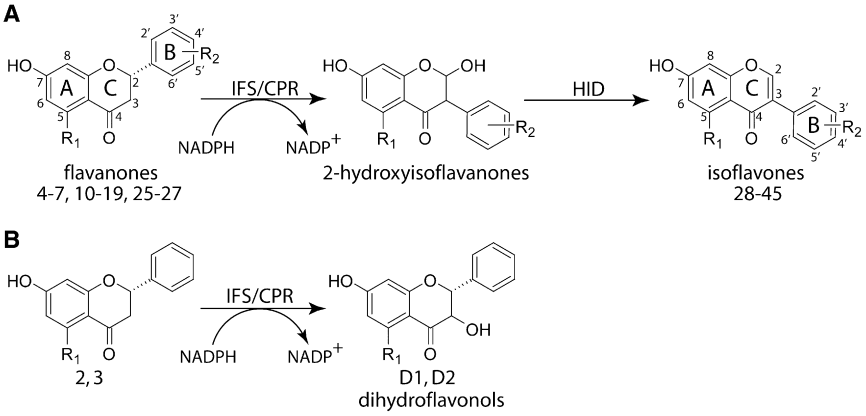

Figure 4. Biotransformations of Flavanones by the Isoflavone Pathway
(A) Conversion of 4′-hydroxyflavanones into isoflavones.
(B) Conversion of 7-hydroxyflavanone and pinocembrin into dihydroflavonols.

converts flavanones to dihydroxyflavonols (Chemler et al., 2007) (data not shown). The identification of dihydroflavonols corrects a prior misinterpretation of substrate flexibility (Kim et al., 2003). Therefore, the C4′ hydroxyl group appears to be a strict requirement for ring migration. All of these observations are consistent with a previous model of the mechanism of IFS (Hashim et al., 1990).

The screening of a substrate library against IFS opened up the ability to reexamine the role of key flavanone features required for catalytic activity and how they might interact with residues within the active site of the enzyme. No crystal structure of IFS exists but previous docking experiments using homology modeling of CYP93C2 (*G. enchinata* IFS) derived from P450BM3 were performed to help identify key residues in the catalytic site (Sawada et al., 2002). Using site-directed mutagenesis, residues Ser 310 and Lys 375 were identified to play roles in the unique aryl migration from the C2 to the C3 position (Sawada et al., 2002). Modification of Lys 375 led to complete abolition of 2-hydroxyisoflavanone synthesis and resulted in the exclusive formation of a dihydroflavonol and/or flavone depending on additional mutations. A follow up study identified Leu 371 to play an important role in not only catalytic activity but protein stability (Sawada and Ayabe, 2005). Both these studies proposed that Lys 375 is an absolute requirement for aryl ring migration and put forth a model that this residue interacts with the C7 hydroxyl group on the flavanone substrate (Sawada and Ayabe, 2005). The screening library used in this study identified the necessity of a C4′ hydroxyl group in order to obtain an isoflavone, and with substrates lacking B ring substituents (7-hydroxyflavanone and pinocembrin) only the respective dihydroflavonols were produced. With these two observations coupled with the previous site mutagenesis results, we propose an alternative role of Lys 375 as the residue directly involved in aryl ring migration. That is, Lys 375 binds to a flavanone's C4′ hydroxyl group and possibly contributes to the shift reaction of the B ring from the C2 to the C3 position. In addition, the hydroxyl group at the C7 position appears to be necessary for binding to the catalytic site since no product was observed when flavanone was the substrate. A more likely residue for the C7 hydroxyl group to bind to would then be the Ser 310 as the S310T CYP93C2 mutant lowered but did not abolish 2-hydroxyisoflavanone production (Sawada et al., 2002). All of these observations, taken together, may guide helpful modifications to a computer-based model of IFS: substrate interactions (Sawada and Ayabe, 2005).

New Guidelines Identified for the Design of Isoflavone as Potent Estrogen Receptor Agonists

Among the attributes of the soy isoflavones and their derivatives, such as equol, is their remarkably high affinity for the human estrogen receptors (Choi et al., 2008; Davis et al., 2008; Pinto et al., 2008; Zhao and Brinton, 2005). Many of the most promising benefits of isoflavones in general and genistein in particular appear to be mediated through interaction with ERβ while certain of the less encouraging effects take place through ERα (McCarty, 2006). Further, the observations that estrogen-replacement therapy promotes both cancer and chronic heart fatigue have led to an intensive search for compounds that promote the benefits of estrogen replacement therapy while reducing some of its worrisome side effects (Hollmer, 2006).

Along with six commercially available natural isoflavones and one flavanone, 12 compounds of the series produced as described above were evaluated for their direct interactions for the human estrogen receptors ERα and ERβ using commercially available in vitro competitive binding assays and a time-resolved fluorescence resonance energy transfer-capable microplate reader (Figure 5). Only 13 out of 20 flavonoids showed binding toward ERα and only 3′-bromo-4′,5,7-trihydroxyisoflavone was equivalent to genistein for affinity for ERα (35 nM) (Table 2). Only 16 out of 20 flavonoids showed binding toward ERβ and none had superior affinity than genistein for ERβ (5 nM) (Table 2). All of the tested isoflavonoids had a higher selectivity (ERα IC_{50}/ERβ IC_{50}) for ERβ, with the single exception of 3′-chloro-4′,7-dihydroxyisoflavone with an α/β value of 0.4. The selectivity for ERβ was greatest for daidzein, genistein, and orobol with α/β values greater than 5. Compounds such as 3′-chloro-4′,5,7-trihydroxyisoflavone, 3′-methyl-4′,5,7-trihydroxyisoflavone, 4′,7-dihydroxy-3′-methylisoflavone, 3′,4′,7-trihydroxyisoflavone, and biochanin A also exhibited slight preference for ERβ with α/β values just over 2.

The presence of a hydroxyl group at the C5 position significantly enhanced binding affinity for both ERα and ERβ. Eight isoflavone pairs differing by only the presence or lack of the C5 hydroxyl group were compared. The genistein analogs (with a C5 hydroxyl group) had at least a 4-fold lower IC_{50} value for ERβ than their daidzein counterparts, which lacked a C5 hydroxyl group. The same pattern was observed with ERα but to an even greater degree. On average, the C5 hydroxyl group reduced IC_{50} values by at least one order of magnitude. Another trend observed was that substituents in the C3′ position generally reduced affinity for both ERα and ERβ as an inverse to their relative bulk. For genistein analogs, the substitution groups with decreasing affinity toward ERα went as follows: Br > H > OH = CH_3 > Cl > OCH_3 > OCH_2CH_3. For daidzein analogs, it went as follows: Cl > H > CH_3 > OH > Br > OCH_3 > OCH_2CH_3. For genistein analogs, the substitution groups with decreasing affinity toward ERβ went as follows: H > OH = Br > CH_3 = Cl > OCH_3 > OCH_2CH_3. For daidzein analogs, it went as follows: H > CH_3 > OH = Cl > OCH_3 = Br > OCH_2CH_3. The next generation of isoflavonoids designed to interact with the estrogen receptors should have small substituents at the C3′ position.

Modifications of hydroxyl groups give rise to the numerous variations of isoflavones found in plant extracts. For instance, the bulk of soy isoflavones are glycosylated (e.g., genistin) and red clover isoflavones are primarily methylated (e.g., biochanin A, prunetin, and formononetin). In either case, modifications of the hydroxyl group at either the C7 or C4′ position greatly reduced their affinity to either estrogen receptor and increased the IC_{50} values by at least a factor of ten compared to genistein.

Our understanding of the structure relationship between isoflavones and estrogen receptors has benefited from the extensive research on genistein and daidzein. Most notable is that genistein is more estrogenic than daidzein, indicating the importance of the C5 hydroxyl group to enhance binding (Fang et al., 2001; Miksicek, 1994). After screening a number of natural flavonoids, two QSAR models were proposed to describe the ERα and ERβ individually (Choi et al., 2008). The expanded substrate library tested here included novel isoflavones, thereby

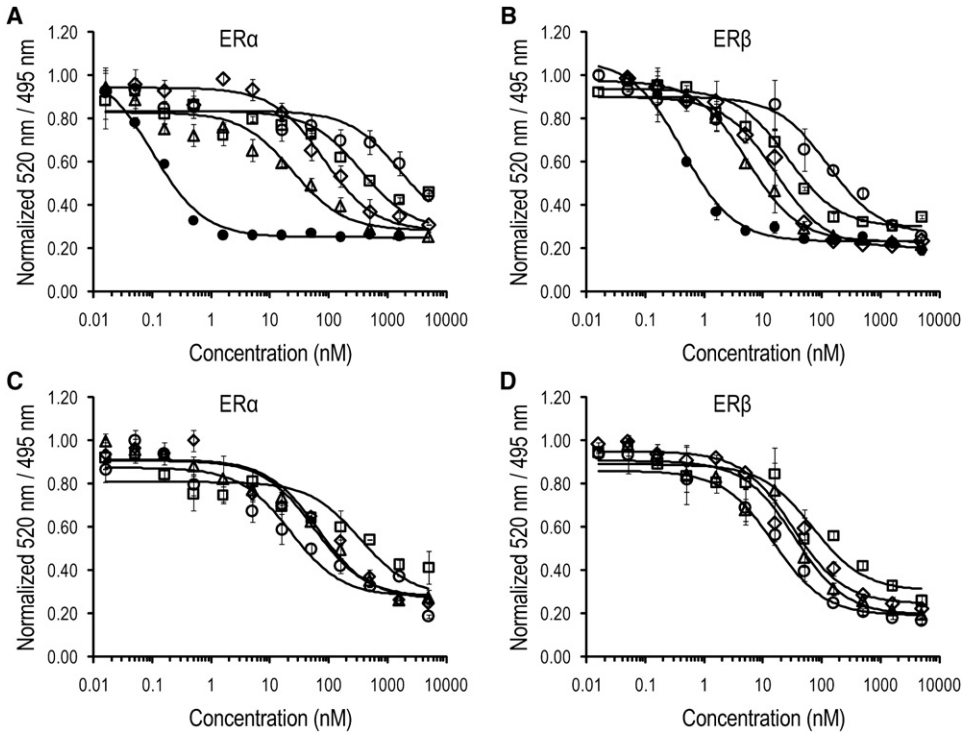

Figure 5. Binding Affinities for ERα and ERβ of Isoflavones
(A and B) ●: estradiol, △: 28, □: 29, ○: 30, ◇: 31.
(C and D) △: 32, □: 33, ○: 42, ◇: 44. Standard deviations are from duplicates.

broadening the base of knowledge of the interactions between phytoestrogens and estrogen receptors.

This study confirms the phytoestrogenic character of isoflavones and their remarkable interaction with estrogen receptors at nanomolar levels. In particular, the rare natural compound orobol and the unnatural 3′-bromo-4′,5,7-trihydroxyflavone exhibited binding capabilities equivalent to that of genistein. The results obtained from the screening library demonstrate that estrogen receptor binding is strongly increased by hydroxyl groups at positions C4′, C5, and C7 and that C3′ substituents generally reduce the affinity. With these guidelines, a new series of natural and nonnatural isoflavones can be prepared with the aim of making compounds more specific than genistein for ERα and ERβ.

SIGNIFICANCE

Isoflavones have been demonstrated to have an impressive array of pharmacological activities and potential benefits for human health in multiple areas including cardiovascular disease and cancer. Therefore, the establishment of an efficient production platform is desired. This paper describes the construction of an optimized three-enzyme system in *S. cerevisiae* for the production of isoflavones. A number of genes from various plant sources were screened for the most active enzyme and enzyme combinations resulting in a final construct with synergistic properties capable of high isoflavone yields. Additionally, further insight into the IFS mechanism was gained after screening the enzyme against a library of synthetic flavanone precursors. The IFS system was able to produce 4 natural isoflavones and 14 unnatural isoflavone analogs. Several isoflavones produced by this system showed significant binding affinities to human estrogen receptors, particularly the β isoform. Structure-activity relationships between the isoflavone structure and the estrogen receptors were determined. The present study furthers the development of mutasynthesis of natural product analogs, particularly for systems involving cytochrome P450 enzymes and their tailoring enzymes. Various enzymes of plant and microbial origin can be readily incorporated together but identification of the most active enzymes can lead to improved production rates and yields. The biosynthesis of libraries of natural products and analogs then allows the ability to screen for relevant biological activities.

EXPERIMENTAL PROCEDURES

Materials

Estradiol, glucose-6-phosphate, NADPH, and all materials for the chemical synthesis of flavanones were purchased from Sigma-Aldrich (see Supplemental Experimental Procedures for flavanone reaction details). Genistein, daidzein, biochanin A, prunetin, 3′,4′,7-trihydroxyisoflavone, genistin, flavanone, 7-hydroxyflavanone, pinocembrin, liquiritigenin, naringenin, eriodictyol, butin, homoeriodictyol, and hesperitin were purchased from Indofine. Materials for the LanthaScreen estrogen receptor assays were obtained directly from Invitrogen.

Table 2. Estrogen Receptor IC_{50} Values of Natural and Unnatural Isoflavones

Ligand	ERα IC_{50} (nM)	ERβ IC_{50} (nM)	α/β
Estradiol	0.1 ± 0.0	0.2 ± 0.1	0.4
Genistein (28)	34.8 ± 12.0	5.4 ± 0.6	6.4
Daidzein (29)	485.4 ± 145.4	26.3 ± 6.7	18.5
Orobol (30)	70.8 ± 10.5	16.5 ± 5.6	4.3
3′,4′,7-Trihydroxyisoflavone (31)	>1000	187 ± 96.4	NA
3′-Methoxy-4′,5,7-trihydroxyisoflavone (32)	880.3 ± 287.0	449.8 ± 74.0	2.0
4′,7-Dihydroxy-3′-methoxyisoflavone (33)	>1000	706.4 ± 153.9	NA
3′-Ethoxy-4′,5,7-trihydroxyflavanone (36)	>1000	>1000	NA
4′,7-Dihydroxy-3′-ethoxyflavanone (37)	>1000	>1000	NA
3′-Methyl-4′,5,7-trihydroxyisoflavone (38)	69.3 ± 12.4	30.8 ± 9.5	2.2
4′,7-Dihydroxy-3′-methylisoflavone (39)	540.9 ± 159.5	70.8 ± 20.9	7.6
3′,5′-Dimethyl-4′,5,7-trihydroxyisoflavone (40)	>1000	155.4 ± 55.5	NA
4′,7-Dihydroxy-3′,5′-dimethylisoflavone (41)	>1000	>1000	NA
3′-Chloro-4′,5,7-trihydroxyisoflavone (42)	101.4 ± 32.3	39.5 ± 6.0	2.6
3′-Chloro-4′,7-dihydroxyisoflavone (43)	104.4 ± 41.7	208.7 ± 49.8	0.5
3′-Bromo-4′,5,7-trihydroxyisoflavone (44)	28.4 ± 10.5	17.1 ± 4.2	1.7
3′-Bromo-4′,7-dihydroxyisoflavone (45)	>1000	841.5 ± 183.7	NA
Genistin (genistein-7-*O*-glucoside)	>1000	>1000	NA
Prunetin (4′,5-dihydroxy-7-methoxyisoflavone)	413.3 ± 91.5	299.4 ± 51.7	1.4
Biochantin A (5,7-dihydroxy-4′-methoxyisoflavone)	465.4 ± 115.2	157.1 ± 24.1	3.0
Naringenin (4)	328.8 ± 127.2	186.4 ± 32.6	1.8

Standard deviation from duplicates. NA, not applicable.

Plasmids and Strains

IFS from *Glycine max* (soy), *Glycyrrhiza echinata* (licorice), *Medicago truncatula* (alfalfa), *Pisum sativum* (pea), and *Trifolium pratense* (red clover), HID from *Glycine max* and *Glycyrrhiza echinata*, and CPR from *Catharanthus roseus* (Madagascar periwinkle) and *Glycine max* were isolated from their respective plants. First, the seeds were germinated in the dark at 30°C in a growth chamber to obtain etiolated seedlings. Seedlings were wounded either by cutting small slits (~1 mm sections) or exposed to UV for 5 min. Incubation in the dark continued for 6 hr before harvesting. Harvested plant tissues were flash frozen using liquid nitrogen and stored at −70°C until further use. RNAs from plant tissue were isolated using the RNeasy Plant Mini Kit (QIAGEN). cDNAs were obtained from the RNAs first by reverse transcription and then by PCR using the SuperScript One-Step RT-PCR with Platinum Taq System (Invitrogen). Primers included in the reaction were designed based on their respective sequence in GenBank. CPR from *S. cerevisiae* was obtained directly using PCR of DNA isolated from yeast strain INVSc1 using the DNeasy Blood and Tissue Kit (QIAGEN) accordingly. Once each gene was obtained, they were cloned into the pYES2.1-TOPO vector (Invitrogen), downstream of the *GAL1* promoter. *E. coli* strain TOP10F′ (Invitrogen) was used for plasmid maintenance and manipulation. All genes were sequenced and errors found were corrected using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). Each HID and each CPR under a *GAL1* promoter was then cloned separately into YEplac112 (ATCC). The YEplac112 plasmid harboring HID from *G. max* was then used to clone separately CPR from *G. max* and *C. roseus* with their own *GAL1* promoter. pYES2.1 plasmids harboring an IFS transformed into *S. cerevisiae* strain INVSc1 (Invitrogen) or in combination with a YEplac112 plasmid harboring HID and/or CPR. Positive transformants were selected by growth on uracil- and/or tryptophan-deficient SC yeast medium agar plates. A complete list of plasmids can be found in Table S1.

Production Curves of Genistein

Recombinant yeast cells were grown to saturation in 3 ml cultures for 2 days at 30°C on SC dropout media containing 2% glucose and lacking uracil and/or tryptophan. Yeast cells were then quantified by spectrophotometry at 600 nm. Yeast cultures at final OD_{600} of 0.8 were used to inoculate 5 ml of SC dropout media containing 2% galactose and supplemented with 1.0 mM of the flavanone substrate naringenin [equivalent to 0.5 mM (2*S*)-naringenin]. Cultures were incubated at 30°C to produce genistein. For isoflavone production analysis, 150 μl of culture was harvested and frozen for later analysis. The thawed cultures were centrifuged to remove cell debris and the supernatant was analyzed by HPLC. Naringenin and genistein concentrations were quantified from 50 μl of sample directly injected into an HPLC system (1100 series; Agilent) equipped with a ZORBAX SB-18 column (5 μm, 4.6 × 150 mm) held at 25°C and a diode array detector. The isocratic mobile phase at a flow rate of 1 ml/min consisted of 50% water, 35% methanol, and 15% acetonitrile. The elution time of naringenin was 7.08 min and of genistein was 8.20 min. Concentrations were determined using authentic standards; naringenin was quantified at 290 nm while genistein was quantified at 260 nm.

Measurement of Inhibition of IFS by Isoflavones

Yeast microsomes from yeast harboring pYES2.1-TpIFS were prepared according to the procedure previously described (Pompon et al., 1996), and microsomal proteins were quantified colorimetrically (Protein Assay Dye Reagent; Bio-Rad). Enzyme assays for determining the inhibition of the rate at which IFS consumed naringenin by various isoflavones were performed at 25°C in 2 ml vials at a final volume of 250 μl. Yeast microsomes (250 μg microsomal protein/reaction) were incubated with 0.0025, 0.025, and 0.25 mM isoflavone (genistein, genistin, or biochanin A), 1 mM EDTA, and 0.25 mM naringenin in 50 mM Tris-HCl (pH 7.4). Nicotinamide cofactor recycle was performed using 0.25 U/reaction of glucose-6-phophate dehydrogenase from *S. cerevisiae* (Sigma-Aldrich) and 2.5 mM glucose-6-phosphate. Reactions were initiated by adding 1 mM NADPH. Samples of 25 μl were taken every 10 min for 1 hr and injected directly for HPLC analysis. Percent inhibition of the rate of consumption of naringenin was determined for three different isoflavone concentrations and compared to when no isoflavone was present initially.

Measurement of In Vitro Rate of Naringenin Consumption of IFS with HID

Soluble yeast protein from yeast harboring pYES2.1-GmHID was prepared according to the procedure previously described (Pompon et al., 1996) but the supernatant obtained following cell lysis was saved and the insoluble proteins and cell debris discarded. The total soluble protein was quantified colorimetrically (Protein Assay Dye Reagent). In vitro assays using yeast microsomes containing IFS from *T. pratense* were performed as previously described but 125 μg of soluble yeast protein containing HID from *G. max* was added. Percent increase of the rate of consumption of naringenin was determined and compared to when no HID was added.

General Procedure for Bioconversion of Flavanone into Isoflavones (Compounds 28–45)

Cultures of *S. cerevisiae* strain INVSc1 harboring the plasmids pYES2.1-GmIFS and YEplac121-GmHID-CrCPR where grown in 3 ml of yeast SC minimal knockout medium (without uracil and tryptophan) supplemented with 2% glucose at 30°C overnight. The next day, the preinoculum was added to 100 ml of yeast SC minimal knockout medium supplemented with 2% galactose so that the initial OD_{600} was 0.4. The appropriate flavanone dissolved in DMSO was added to the culture to a final concentration of 0.1 mM. The cultures were incubated in a horizontal shaker at 30°C for 72 hr. Cells were then removed by centrifugation and the aqueous medium was extracted three times with ethyl acetate. The organic fractions were pooled and dried by rotary evaporation and crude extracts were resuspended in 1:1 acetonitrile and water. Purification was performed using an Agilent 1100 series HPLC with a diode array detector using an Agilent semi-preparative C18 column (9.1 × 250 mm) held at 25°C in line with a fraction collector. The isocratic mobile phase used was 35% methanol and 15% acetonitrile in water.

Characterization of Unnatural Isoflavones

Purified unnatural isoflavones were characterized by their UV profile in 10:7:3 acetonitrile/methanol/water and by high resolution mass spectroscopy in positive ionization as summarized in Table S2. Compounds 31, 32, 43, and 44 were chosen as representatives for ^{1}H nuclear magnetic resonance spectroscopic studies and were prepared from 200 ml cultures in SC dropout medium described above (see Supplemental Experimental Procedures).

Estrogen Receptor Assays

Estrogen receptor binding studies were conducted using Invitrogen's LanthaScreen ER alpha and beta competitive binding assay and performed according to the manufacturer's protocol. Time-resolved fluorescence resonance energy transfer was measured using Bio-Tek's Synergy 2 microplate reader for excitation at 340 nm and detection of emission signals of terbium at 495 nm and the tracer, Fluormone ES2 Green, at 520 nm. Purified unnatural isoflavones and flavonoid standards were dissolved in DMSO and finally diluted to 2× concentration in Invitrogen's complete ES2 screening buffer so that the final concentration of DMSO did not exceed 2% v/v. Each sample was performed in at least duplicates. Final concentrations of substrates, chosen to be evenly spaced on a logarithmic scale (incremented by $10^{0.5}$), were 5000, 1581, 500.0, 158.1, 50.00, 15.89, 5.000, 1.589, 0.5000, 0.1581, 0.0500, and 0.01581 nmol/liter. The IC_{50} values were determined as the concentration of the competitor that produces 50% displacement of the tracer used in the assay (3 nM). The IC_{50} values were calculated by a logistic three-parameter curve fit with a Hill coefficient of −1.0 using Prism 5.0 (GraphPad) and IC_{50} values with $R^2 > 0.7$ were reported else they were considered non-binders.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two tables and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2010.03.010.

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