Journal of Molecular Catalysis B: Enzymatic 66 (2010) 257–263

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Synthesis of non-natural flavanones and dihydrochalcones in metabolically engineered yeast

Sean R. Werner, Hao Chen, Hanxiao Jiang, John A. Morgan*

School of Chemical Engineering, Purdue University, West Lafayette, IN 47907, United States

ARTICLE INFO

Article history:
Received 1 December 2009
Received in revised form 26 May 2010
Accepted 31 May 2010
Available online 8 June 2010

Keywords:
Phloretin
Polyketides
Galactose inducible promoter
Saccharomyces cerevisiae

ABSTRACT

Flavonoids are plant phenolic compounds that have many interesting medicinal properties. Therefore, there is interest in the synthesis of non-natural flavonoids as they may possess new or enhanced biological activities. In this study, metabolically engineered *Saccharomyces cerevisiae* expressing 4-coumaroyl:CoA-ligase (4CL) and chalcone synthase (CHS) was explored as a platform for producing non-natural flavanones and dihydrochalcones. By precursor addition of cinnamic acid analogues to the engineered yeast, numerous non-natural flavanones and dihydrochalcones were formed *in vivo*. Also, several CHS derailment products were formed. Of the isolated compounds, one flavanone and three derailment products were found to be novel compounds.

1. Introduction

Flavonoids are a large class of secondary metabolites derived from the phenylpropanoid pathway. In plants, flavonoids have several functions including act as pigments, as well as protection against UV radiation, microbial invasion, insects, and animals [1–3]. It has been shown that many flavonoids benefit human health, as they possess antioxidant, anti-inflammatory, and anti-carcinogenic activity [4–9] In the first step of flavonoid biosynthesis, phenylalanine and/or tyrosine are deaminated by phenylalanine ammonia-lyase (PAL) to give cinnamic acid and *p*-coumaric acid, respectively. Cinnamate-4-hydroxylase (C4H) hydroxylates cinnamic acid at the para position, giving *p*-coumaric acid. Phenylpropanoic acids are ligated with coenzyme A (CoA) by 4-coumaroyl:CoA-ligase (4CL), creating CoA thioesters. Chalcone synthase (CHS) then catalyzes the condensation of phenylpropanoid CoA thioesters with three acetate units from malonyl-CoA to form chalcones. Chalcones can either undergo ring closure, to give a racemic mixture of flavanones, or a single enantiomer by chalcone isomerase (CHI). Flavanones are the precursors to the further biosynthesis of other flavonoids, such as flavones, flavonols, isoflavones, and anthocyanins [10–12].

Since flavonoids have significant medicinal properties, non-natural flavonoids (flavonoids with non-natural substituents incorporated in their structures) may also possess interesting biological activity, and hence have potential pharmaceutical uses. Therefore, there is an interest in synthesizing non-natural flavonoids. Chemical-enzymatic synthesis of non-natural flavonoids and polyketides have been pursued, but these methods require enzymatic or chemical synthesis of CoA thioesters which are then used as substrates to the polyketide synthase [13–15]. The enzymatic methods require the use of expensive cofactors (e.g. ATP, CoA) limiting the ability to scale up the synthesis. There has been considerable recent interest in the area of metabolic engineering of microorganisms for the production of flavonoids [12,16,17]. Flavonoids can be synthesized by expressing enzymes involved in flavonoid biosynthesis in recombinant hosts such as *Saccharomyces cerevisiae* and *E. coli*, where the cofactors required by the enzymes are biosynthesized by the host. These metabolically engineered microorganisms can be used to synthesize non-natural flavonoids by a precursor directed biosynthesis approach [18]. First, a non-natural substrate is added to an organism expressing a biosynthetic pathway, and if the substrate and the corresponding intermediates are accepted by the enzymes in the pathway, this non-natural substrate is incorporated into the final products. This method has shown promise for production of non-natural flavonoids such as flavanones, flavonols, and stilbenes [19,20].

This study demonstrates the ability of metabolically engineered *S. cerevisiae*, expressing 4CL and CHS from the flavonoid biosynthesis pathway, to synthesize novel non-natural flavanones from cinnamic acid analogues and dihydrochalcones from phenylpropanoic acids by the precursor directed biosynthesis approach.

* Corresponding author at: FRNY Hall of Chemical Engineering, West Lafayette, IN 47907-2050, United States. Tel.: +1 765 494 4088; fax: +1 765 494 0805.
E-mail address: jamorgan@purdue.edu (J.A. Morgan).

1381-1177/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2010.05.017

2. Materials and methods

2.1. Chemicals

2-Aminocinnamic acid and 3-aminocinnamic acid was purchased from City Chemical LLC (West Haven, CT). 2-Chlorocinnamic acid was purchased from ACROS Organics (Geel, Belgium). 3-Methoxycinnamic acid, 2-methylcinnamic acid, 2-methoxycinnamic acid, was purchased from Alfa Aesar (Ward Hill, MA). 5-Bromo-2-methoxy cinnamic acid, 2-chloro-6-fluoro cinnamic acid, 3-chlorocinnamic acid, *trans*-2,3-dimethoxycinnamic acid, 2-ethoxycinnamic acid, 2-fluorocinnamic acid, 3-fluorocinnamic acid, hydrocinnamic acid (phenylpropanoic acid), 2-hydroxycinnamic acid, 3-hydroxycinnamic acid, 3-methylcinnamic acid, 4-methoxycinnamic acid, 2-nitrocinnamic acid, 3-nitrocinnamic acid, phloretic acid (dihydro-p-coumaric acid), urocanic acid (3-(4-Imidazolyl)acrylic acid), were of the highest available purity and purchased from Sigma–Aldrich Co. (St. Louis, MO). Phloretin was purchased from MP Biomedical (Solon, OH).

2.2. Strain and plasmid

Saccharomyces cerevisiae strain AH22 (MATa *leu2-3 leu2-112 his4-519 can1*) (ATCC 38626) and *S. cerevisiae* strain, WAT11U, a derivative of the W303-B strain (*MAT a*; *ade*2-1; *his*3-11, -15; *leu*2-3, -112; *ura*3-1; *can*^{R}; *cyr*$^{+}$) were used as the host strains. The plasmid pKS2μHyg containing 4CL and CHS open reading frames (pKS2μHyg-4CL-CHS) with individual galactose inducible promoters (GAL10) controlling expression was constructed as previously described [21,22]. The 4CL gene (GenBank accession no. U18675) is from the plant *Arabidopsis thaliana*, and CHS (GenBank accession no. AF315345) is from the plant *Hypericum androsaemum*.

2.3. E. coli and yeast manipulations

The plasmid pKS2μHyg-4CL-CHS was transformed into chemically competent *E. coli* TOP10 F' cells according to the manufacturer's protocol. Recombinant *E. coli* TOP10 F' (Invitrogen) was grown at 37 °C in Luria Bertani broth supplemented with 100 μg/mL of ampicillin for maintenance of plasmid. The plasmid pKS2μHyg-4CL-CHS was transformed into *S. cerevisiae* using the lithium acetate/SS carrier DNA/PEG method [23]. Yeast transformants were selected on YPDH agar plates that were incubated at 30 °C for about 3 days. Yeast strains were grown at 30 °C in YPDH and YPLH media. YPDH media contains 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 200 μg/mL Hygromycin (A.G. Scientific, San Diego, CA). YPLH is similar to YPDH, but contains the same quantity of galactose instead of glucose. Hygromycin was added before inoculation of media.

2.4. Substrate Screening

A single colony of AH22 or WAT11U harboring pKS2μHyg-4CL-CHS from a YPDH plate was inoculated in 10 mL of YPDH and grown for about 24 h at 30 °C with shaking at 300 rpm. Culture tubes containing 10 mL of YPDH were inoculated to achieve an initial OD_{600} of 0.05. After approximately 20 h of growth, the cultures were centrifuged at 1000 × *g* for 5 min. The cells were resuspended in 10 mL of YPLH medium in order to induce gene expression. After 6 h of induction, substrate dissolved in DMSO was added to the culture to give an initial concentration of 500–600 μM. Samples were taken after about 20 h and were quenched with an equal volume of methanol. The samples were then centrifuged at 18,000 × *g* for 1 min and the supernatant was stored at −20 °C prior to HPLC analysis. Product formation was determined by formation of new peaks on the HPLC chromatogram.

2.5. Fermentation and product isolation

A single colony of AH22 harboring pKS2μHyg-4CL-CHS from a YPDH plate was inoculated in 50 mL of YPDH in a 250 mL flask and grown for about 24 h at 30 °C with shaking at 300 rpm. Three 1-L flasks containing 300 mL of YPLH were inoculated with the appropriate volume of cells from overnight culture to achieve an OD_{600} = 0.05. After 5 h the cinnamic acid analogue was added to the culture in powder form to achieve a calculated initial concentration of 1 mM. Cultures were incubated at 30 °C with shaking at 300 rpm for 3 days. After fermentation, the cells were removed by centrifugation at 600 × *g* (2000 rpm using JA-14 rotor) for 10 min. Next, the combined supernatant was extracted twice with equal volumes of ethyl acetate. The ethyl acetate fractions were concentrated by rotary evaporation and the isolated residue was resuspended in methanol and stored at −20 °C.

2.6. Chromatography and product purification

Flavonoids produced by AH22 expressing 4CL and CHS were analyzed by high performance liquid chromatography (HPLC) using an Agilent Zorbax SB-C18 column (4.6 mm × 75 mm) with the column temperature maintained at 30 °C. Solvent A was 1.5% (v/v) acetic acid in water; solvent B was methanol. Solvent B was maintained at 10% for 4 min, then increased to 60% over 42 min, and held for 4 min before increased to 70% in 2 min and maintained for 2 min. Solvent B was returned to 10% over 2 min. Phloretin production was analyzed by HPLC using the following method. Solvent A was 1.5% (v/v) acetic acid in water and solvent B was acetonitrile. Solvent A was then held at 90% for 4 min. Solvent A was decreased from 90% to 40% over 42 min, and then held at 40% for 4 min. Solvent A was decreased further to 30% over 2 min and held for 2 min before returning back to 90%. The flow rate was 0.9 mL/min with an injection volume of 20 μL. Preparative HPLC was performed using an Agilent Zorbax SB-C18 column (9.4 mm × 100 mm) with the column temperature maintained at 30 °C. Solvent A was 1.5% (v/v) acetic acid in water; solvent B was acetonitrile. The flow rate was 2.0 mL/min. Solvent A was held at 95% for 2 min and then decreased from 95% to 35% over 23 min. Solvent A was then increased back to 95% over 2 min. Fractions were collected with a Foxy Jr. fraction collector (Teledyne Isco, Lincoln, NE).

2.7. LC/MS

Electrospray ionization analyses were carried out on a Waters Micromass Q-TOF micro mass spectrometer system (Waters, Milford, MA). Samples were analyzed by HPLC on a C18 column (2.1 mm × 150 mm, Waters) by the following method. A was 1.5% (v/v) acetic acid in water; solvent B was acetonitrile. The flow rate was 0.3 mL/min and the column temperature maintained at 30 °C. After 20 μL of sample was injected, solvent B was 5% for 4 min, then was increased to 50% over 56 min, and held for 5 min, before it was returned to 5% in 5 min. UV absorbance at 290 nm was monitored for detection of novel flavonoids. Negative ion mode was applied with the mass spectrometer scan range from 50 to 600 *m*/*z*.

2.8. MS analysis

Isolated residues samples were dissolved in methanol in preparation for mass spectrometry analysis. Electrospray ionization MS and Tandem MS (MS/MS) were carried out on a FinniganMAT LCQ Classic (ThermoFinnigan Corp., San Jose, CA) mass spectrometer system in either the negative or positive ion mode. Tandem MS

results were obtained by selecting the ion of interest. The precursor ion was then subjected to collision-induced dissociation, resulting in the formation of product ions. The collision energy was set to 40% of the maximum available from the 5-V tickle voltage, with a 2-mass-unit isolation window.

2.9. ^{1}H NMR analysis

In preparation for proton NMR analysis, the isolated residues were dried then resuspended in deuterated methanol. The ^{1}H NMR spectra were acquired on a Bruker DRX 500 MHz spectrometer. Correlation spectroscopy (COSY) was used to find which protons are spin–spin coupled to each other to determine molecular structure. Chemical shifts are referenced to the methanol solvent peak at 3.3 ppm.

3. Results and discussion

3.1. Substrate screening for production of non-natural flavonones

In a previous study, Jiang et al. [22] constructed a metabolically engineered *S. cerevisiae* expressing PAL, 4CL and CHS from the flavonoid biosynthetic pathway capable of producing the flavanones naringenin and pinocembrin from the amino acids phenylalanine and tyrosine. The dihydrochalcones, phloretin and 2′,4′,6′-trihydroxydihydrochalcone as well as CHS derailment products were also produced. Jiang et al. [21] also reported formation of flavanones and dihydrochalcones by addition of cinnamic acid and *p*-coumaric acid to *S. cerevisiae* coexpressing 4CL and CHS. Therefore, we examined the ability of this yeast stain expressing 4CL and CHS as a platform for production of non-natural flavonoids by addition of cinnamic acid analogues to the yeast cultures.

As a screen for cinnamic acids that could be accepted through the engineered pathway, 22 cinnamic acid analogues were individually added to recombinant yeast cultures expressing 4CL and CHS, and the products formed were compared against a control where no substrate was added. Formation of new peaks on HPLC chromatograms by monitoring 290 nm compared to the control indicated the possible formation of non-natural flavonoids and novel CHS derailment products. The majority of the substrates tested showed formation of new products by HPLC (Table 1). This could indicate 4CL and CHS have broad substrate specificity and capable of metabolizing many non-natural cinnamic acids. To confirm the new products being formed in the substrate screening assay were the corresponding non-natural flavanones or derailment products from CHS, LC/MS analysis was performed on a select number samples. For some of the screened analogues, only one or two of the proposed products were detected (Table 2). Two types of possible products, analogues of pinocembrin (**1b**–**10b**) and tetraketide lactones (**1c**–**10c**), have the same molecular weight and could not be differentiated by LC/MS. Due to lack of authentic standards, it is not possible to definitely conclude which of these products have been produced. Products from 10 of the 22 cinnamate analogues added to the AH22 host coexpressing 4CL and CHS were analyzed by LC/MS (Table 2).

The results showed that all ten of the tested compounds could be metabolized by 4CL and CHS. Based on the structure of products from CHS and the molecular weight of metabolites detected by negative ion mode LC/MS, the structures of major products were proposed. The effects of functional group substitution on the cinnamic acid aromatic ring gave interesting trends in product distributions. Among the tested analogues, **1a** produced only tetraketide products but **2a** produced only dihydrochalcone and triketide lactone products. **3a** produced only tetraketide products but **4a** produced both tetraketide products and triketide lactone.

Table 1
Screening of cinnamic acid analogues for metabolism by 4CL and CHS in recombinant *S. cerevisiae*.

Substrate Name	New products formed by 4CL-CHS[a]	# of new products detected by HPLC.
Cinnamic acid analogues		
2-Aminocinnamic acid[b]	Yes	1
3-Aminocinnamic acid[b]	Yes	2
5-Bromo-2-methoxy cinnamic acid	Yes	3
2-Chlorocinnamic acid [b]	Yes	1
3-Chlorocinnamic acid [b]	Yes	1
2-Chloro-6-fluoro cinnamic acid	Yes	4
2,3-Dimethoxycinnamic acid	No	-
2-Ethoxycinnamic acid	No	-
2-Fluorocinnamic acid [b]	Yes	2
3-Fluorocinnamic acid [b]	Yes	2
2-Hydroxycinnamic acid[b]	Yes	3
3-Hydroxycinnamic acid [b]	Yes	3
2-Methylcinnamic acid [b]	Yes	3
3-Methylcinnamic acid [b]	Yes	2
2-Methoxycinnamic acid	No	-
3-Methoxycinnamic acid	Yes	2
4-Methoxycinnamic acid	No	-
2-Nitrocinnamic acid	No	-
3-Nitrocinnamic acid	Yes	2
Urocanic acid	Yes	1
Phenylpropanoic acids		
Phenylpropanoic acid	Yes	1
Phloretic acid	Yes	2

[a] As determined by new peaks on the HPLC chromatogram compared to control by monitoring 290 nm.
[b] Samples that were selected for LC/MS analysis to confirm formation of products from CHS.

5a and **6a** gave products with a reduced carbon-carbon double bond, dihydrochalcone and the triketide derailment products. **7a** produced a tetraketide product but **8a** produced only a triketide lactone. **9a** and **10a** both produced tetraketide products. In general, all substrates with R1 substitution regardless of size or polarity produced tetraketide products except for **5a**. Substrates with R2 substitutions tend to produce the reduced dihydrochalcone and triketide lactone products.

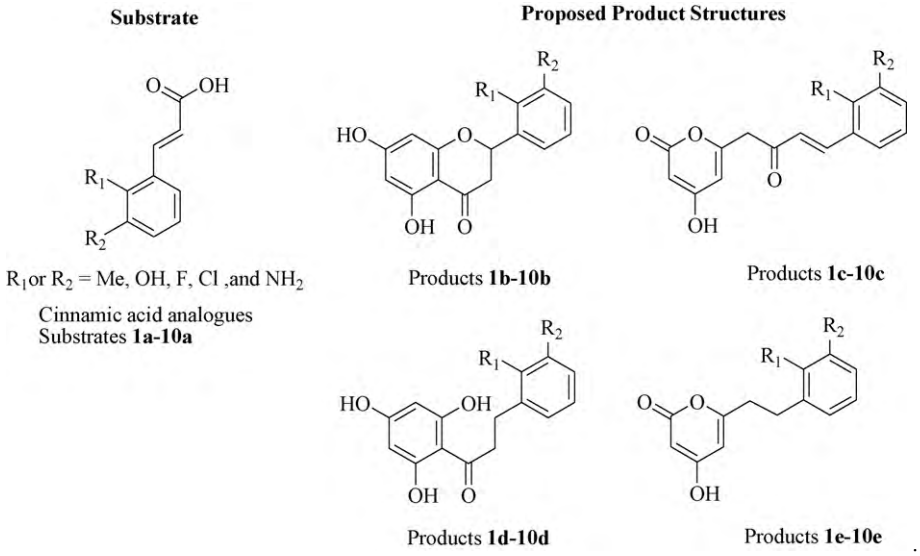
3.2. Production of non-natural flavanones and polyketides

Precursor addition of cinnamic acid analogues to the *S. cerevesiae* strain AH22 expressing 4CL and CHS was scaled up to 3× 300 mL scale fermentations (900 mL combined) to isolate a significant quantity of products for further characterization. The non-natural flavanones and polyketides that were isolated in significant quantity are shown in Fig. 1.

The product isolated from addition of 2-methylcinnamic acid (**1a**, Fig. 1) to the engineered yeast occurred at a retention time of 25.5 min and UV spectrum had λ_{max} = 289 nm. ESI-MS spectrum gave a parent ion peak $[M+H]^+$ at *m*/*z* 271 suggesting the condensation of three malonyl-CoA by CHS. Analysis of ^{1}H NMR and COSY spectra revealed two aromatic protons of ring-A (δ 5.9), four coupled aromatic protons of ring-B (δ 7.53 and 7.25), three methyl protons (δ 2.38), and two α-carbonyl protons (δ 3.1 and 2.72) coupled to one proton (δ 5.67), confirming ring closure and the structure of 2′-methyl-5,7-dihydroxyflavanone (**1b**, Fig. 1). A quantity of 3 mg of **1b** was isolated from the fermentation. A chemical synthesis of this flavanone has been reported in the literature, but this is the first report using precursor directed biosynthesis [24,25].

The product isolated from addition of 2-aminocinnamic acid (**9a**, Fig. 1) to the engineered yeast occurred at a retention time of 19.8 min and the UV spectrum had λ_{max} = 289 nm. ESI-MS spectrum gave a parent ion peak $[M-H]^-$ at *m*/*z* 270 suggesting the condensation of three malonyl-CoA by CHS. Tandem MS on the parent ion

Table 2
Products from incubation of cinnamic acid analogue substrates (**1a–10a**) with AH22 coexpressing 4CL and CHS. R1 and R2 are substituted functional group on the aromatic ring of cinnamate; **1b–10b** are pinocembrin analogues; **1c–10c** are coumaroyltriacetic acid lactones (CTAL); **1d–10d** are 2′,4′,6′-trihydroxydihydrochalcone products; **1e–10e** are triketidelactones.



Cinnamic acid analog substrates	Substitution		Products		
	R1	R2	b or c	d	e
1a	Me	–	Yes	No	No
2a	–	Me	No	Yes	Yes
3a	OH	–	Yes	No	No
4a	–	OH	Yes	Yes	Yes
5a	F	–	No	Yes	Yes
6a	–	F	No	Yes	Yes
7a	Cl	–	Yes	No	No
8a	–	Cl	No	No	Yes
9a	NH_2	–	Yes	No	No
10a	–	NH_2	Yes	Yes	No

gave a major fragment at *m*/*z* 252 corresponding to $[M–H–H_2O]^-$. Analysis of ^{1}H NMR and COSY spectra revealed two aromatic protons of ring-A (δ 5.9), four coupled aromatic protons of ring-B (δ 7.23, 7.1, 6.78 and 6.72), and two α-carbonyl protons (δ 3.3 and 2.75) coupled to one proton (δ 5.55), confirming ring closure and the structure of 2′-amino-5,7-dihydroxyflavanone (**9b**, Fig. 1). A quantity of 1.2 mg of **9b** was isolated from the fermentation. This flavanone has not been previously reported.

When 3-aminocinnamic acid (**10a**, Fig. 1) was incubated with the engineered yeast, two products were isolated by HPLC. The first product occurred at a retention time of 14.5 min and UV spectrum had λ_{max} = 290 nm. ESI-MS spectrum gave a parent ion peak $[M–H]^-$ at *m*/*z* 270 suggesting the condensation of three malonyl-CoA by CHS. Tandem MS on the parent ion gave a major fragment at *m*/*z* 226 corresponding to $[M–H–CO_2]^-$, suggesting the presence of a pyrone ring. In addition, positive ion mode ESI-MS spectrum gave a parent ion peak $[M+H]^+$ at *m*/*z* 272 consistent with the condensation of three malonyl-CoA by CHS. Tandem MS on the parent ion in positive ion mode gave the fragments at *m*/*z* 153 and *m*/*z* 146 corresponding to fragmentation around the central ketone of a CHS derailment product. Based on the above evidence, we hypothesize the residue is a derailment product from CHS that could be identified as the structure of **10c** (Fig. 1). A quantity of <1 mg of the product was isolated from the fermentation. The second product occurred at a retention time of 12.9 min and UV spectrum had λ_{max} = 287 nm. ESI-MS spectrum gave a parent ion peak $[M–H]^-$ at *m*/*z* 272 suggesting the condensation of three malonyl-CoA by CHS. Tandem MS on the parent ion gave a fragment at *m*/*z* 228 corresponding to $[M–H–CO_2]^-$, suggesting the presence of a pyrone ring. In addition, positive ion mode ESI-MS spectrum gave a parent ion peak $[M+H]^+$ at *m*/*z* 274 consistent with the condensation of three malonyl-CoA by CHS. Tandem MS on the parent ion in positive ion mode gave a fragment at *m*/*z* 148 corresponding fragmentation around the central ketone of a CHS derailment product. Based on the above evidence, we hypothesize the residue is a derailment product from CHS that could be identified as the structure **10f**, the dihydro form of **10c** (Fig. 1). A quantity of <1 mg of product was isolated from the fermentation. These derailment products have not been reported in the literature.

The product isolated from addition of urocanic acid (**11a**, Fig. 1) to the engineered yeast occurred at a retention time of 8.2 min and UV spectrum had λ_{max} = 284 nm. ESI-MS spectrum gave a parent ions peak $[M–H]^-$ at *m*/*z* 245 and $[2M–H]^-$ *m*/*z* 491 (dimer) suggesting the condensation of three malonyl-CoA by CHS. Tandem MS of the parent ion gave a major fragment at *m*/*z* 201 corresponding to $[M–H–CO_2]^-$, indicating the presence of a pyrone ring. A quantity of 2.2 mg of product was isolated from the fermentation. Based on the above evidence, we hypothesize the residue is a derailment product from CHS that could be identified as the structure of **11c** (Fig. 1). This derailment product has not been reported in the literature.

3.3. Production of dihydrochalcones

Jiang et al. [22] hypothesized the dihydrochalcones formed in addition to the flavanones naringenin and pinocembrin from the metabolically engineered yeast is due to endogenous reductases capable of reducing the carbon–carbon double bonds of cinnamic acid and *p*-coumaric acid. This suggested, in addition

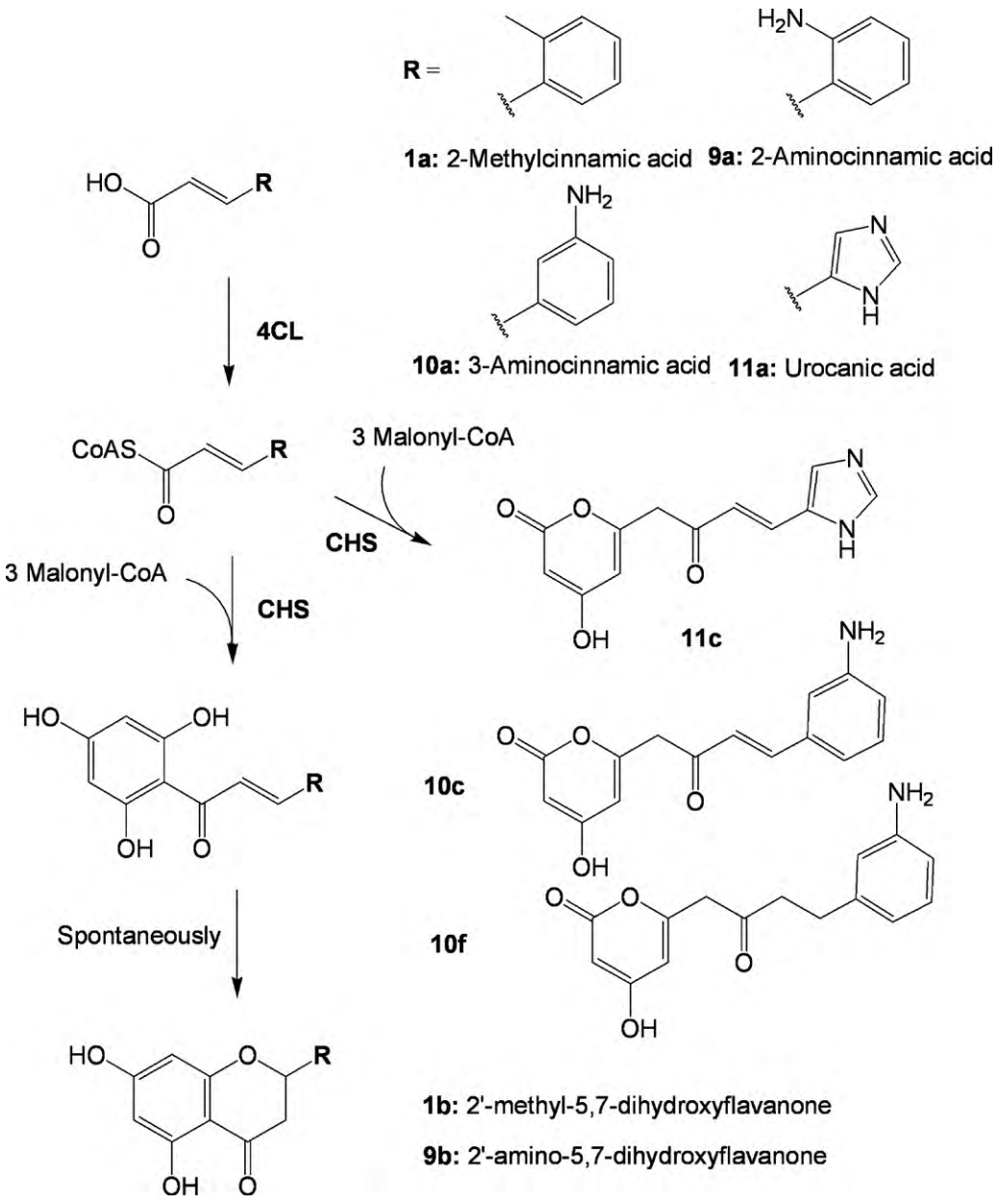


Fig. 1. Biosynthesis of non-natural flavanones and CHS derailment products by precursor addition of cinnamic acid analogues to *S. cerevisiae* AH22 expressing 4CL and CHS.

to cinnamic acids being metabolized through 4CL and CHS, that phenylpropanoic acids are used as precursors in the formation of dihydrochalcones.

Precursor addition of phloretic acid (**12a**, Fig. 3) to *S. cerevisiae* expressing 4CL and CHS resulted in the formation of phloretin (**12d**, Fig. 3). The quantity of phloretin in the supernatant increased to 36 ± 7 mg/L after 17 h. There was no further increase in phloretin concentration after longer fermentation times (Fig. 2). When phloretic acid (**12a**, Fig. 3) was incubated with the engineered yeast on a larger scale, two products were isolated by HPLC. One product occurred at the same retention time as phloretin (22.17 min) and showed the same UV spectrum ($\lambda_{max} = 286$ nm). ESI-MS spectrum gave a parent ion peak $[M-H]^-$ at m/z 273. Analysis of ^{1}H NMR and COSY spectra revealed two aromatic protons of ring-A (δ 5.80), four coupled aromatic protons of ring-B (δ 7.03 and 6.68), and four coupled alkyl protons (δ 3.25 and 2.84), confirming the structure of phloretin (**12d**, Fig. 3). A quantity of 3.8 mg of phloretin was purified from the large scale fermentation. Phloretin was synthesized in a similar manner using metabolically engineered *E. coli* [26]. Small

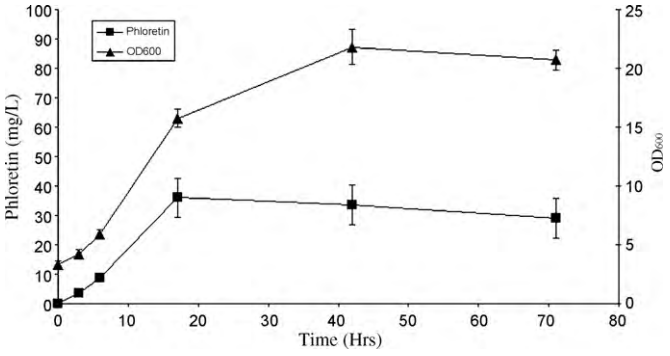


Fig. 2. Addition of the precursor phloretic acid to *S. cerevisiae* AH22 expressing 4CL and CHS for the phloretin production. After the cells were grown in YPDH for 20 h reaching an OD_{600} of 3.5, the cells were resuspended in YPLH and induced for 6 h before addition of substrate. Time zero corresponds with addition of phloretic acid to the induced culture. The data points and error bars correspond to the mean ± stdev ($n = 3$).

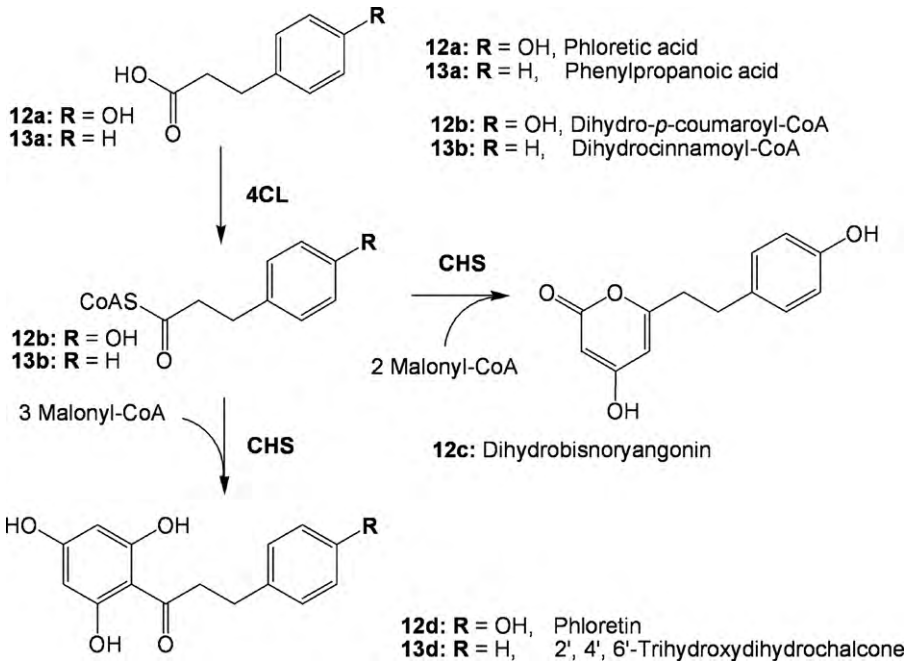


Fig. 3. Biosynthesis of dihydrochalcones by precursor addition of phenylpropanoic acids to *S. cerevisiae* AH22 expressing 4CL and CHS.

amounts of phloretin were detected after 24 h of cultivation, but an absolute quantity was not report.

Besides phloretin, a second product eluted at a retention time of 17.6 min and UV spectrum had λ_{max} = 282 nm. ESI-MS gave a parent ion peak $[M–H]^-$ at *m*/*z* 231 and $[2M–H]^-$ at *m*/*z* 463 (dimer), suggesting the condensation two malonyl-CoA by CHS. Tandem MS of the parent ion gave the major fragment at *m*/*z* 187 corresponding to $[M–H–CO_2]^-$, indicating the presence of a pyrone ring. Analysis of ^{1}H NMR and COSY spectra revealed two protons consistent with a pyrone ring (δ 5.80 and 4.80), four coupled aromatic protons of ring-B (δ 6.99 and 6.67), and four coupled alkyl protons (δ 2.85 and 2.70), confirming the structure of dihydrobisnoryangonin (**12c**, Fig. 3). A quantity of 2.6 mg of product **12c** was isolated from the fermentation. Product **12c** is a derailment product from CHS and has been previously synthesized enzymatically with type III polyketide synthase from *Wachendorfia thyrsiflor* [27].

The product isolated from addition of phenylpropanoic acid (**13a**, Fig. 3) to the engineered yeast occurred at a retention time of 22.6 min and UV spectrum had λ_{max} = 281 nm. ESI-MS gave a parent ion peak $[M–H]^-$ at *m*/*z* 257, and tandem MS of the parent ion resulted in no fragment. Analysis of ^{1}H NMR and COSY spectra revealed two aromatic protons of ring-A (δ 5.79), five coupled aromatic protons of ring-B (δ 7.22 and 7.15), and four coupled alkyl protons (δ 3.31 and 2.94), confirming the structure of 2′,4′,6′-Trihydroxydihydrochalcone (**13d**, Fig. 3). A quantity of 2.1 mg of **13d** was isolated from the fermentation. These studies confirm phenylpropanoic acid can be metabolized by the phenylpropanoid pathway for the production of dihydrochalcones (Fig. 3). This is the first time trihydroxydihydrochalcone has been synthesized using precursor directed biosynthesis starting from phenylpropanoic acid.

There are several explanations for the low yields of product achieved from this system and there are possibilities for improvement. First, the pathway most likely does not accept the non-natural substrates as well as the natural substrates. Use of enzymes with broader substrate specificity could be used or engineered. Second, the non-natural substrates could be inhibiting growth of the yeast cells, impacting product yield. Third, there could be loss of product due to the low efficiency of the extraction and isolation steps. To improve the yield, further metabolic engineering of the yeast strain could be used to increase malonyl-CoA available for CHS as used for a polyketide synthase for production of a fungal polyketide [28].

4. Conclusion

In this study, a metabolically engineered yeast expressing 4CL and CHS was explored as a platform for producing non-natural flavanones and dihydrochalcones. By precursor addition of cinnamic acid analogues to the engineered yeast, several non-natural flavanones and dihydrochalcones were formed. Also, several products from CHS derailment were formed. Of the eight isolated compounds, one flavanone and three derailment products are novel compounds. It is conceivable that introducing non-natural substituents into the flavonoid structure may result in novel compounds with potentially valuable medicinal properties compared. This study indicates metabolically engineered organisms could be used to manufacture such non-natural compounds.

Acknowledgments

The authors thank Kristina Perlas for help with fermentations and product isolation, Dr. John S. Harwood (Purdue Interdepartmental NMR Facility) for help with NMR, and Dr. Karl V. Wood and Deb Miles (Campus-wide Mass Spectrometry Center at Purdue) for help with MS analysis. This work and authors were supported by the School of Chemical Engineering, Purdue University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molcatb.2010.05.017.

References

[1] J.B. Harborne, C.A. Williams, Phytochemistry 55 (2000) 481–504.
[2] B.W. Shirley, Trends Plant Sci. 1 (1996) 377–382.
[3] O. Benavente-Garcia, J. Castillo, F.R. Marin, A. Ortuno, J.A. Del Rio, J. Agr. Food Chem. 45 (1997) 4505–4515.

[4] C.A. Rice-Evans, N.J. Miller, Biochem. Soc. Trans. 24 (1996) 790–795.
[5] G. Di Carlo, N. Mascolo, A.A. Izzo, F. Capasso, Life Sci. 65 (1999) 337–353.
[6] J.A. Manthey, N. Guthrie, K. Grohmann, Curr. Med. Chem. 8 (2001) 135–153.
[7] W.Y. Ren, Z.H. Qiao, H.W. Wang, L. Zhu, L. Zhang, Med. Res. Rev. 23 (2003) 519–534.
[8] L.G. Korkina, Cell. Mol. Biol. 53 (2007) 15–25.
[9] F. Ververidis, E. Trantas, C. Douglas, G. Vollmer, G. Kretzschmar, N. Panopoulos, Biotechnol. J. 2 (2007) 1214–1234.
[10] B. Buchanan, W.G. Russell, L. Jones, B. Buchanan, W. Gruissem, R.L. Jones, Biochemistry and Molecular Biology of Plants, John Wiley and Sons, Somerset, NJ, 2000.
[11] O. Yu, J.M. Jez, Plant J. 54 (2008) 750–762.
[12] Z.L. Fowler, M.A.G. Koffas, Appl. Microbiol. Biotechnol. 83 (2009) 799–808.
[13] H. Morita, Y. Takahashi, H. Noguchi, I. Abe, Biochem. Biophys. Res. Commun. 279 (2000) 190–195.
[14] H. Morita, H. Noguchi, Y. Takahashi, H. Noguchi, I. Abe, Eur. J. Biochem. 268 (2001) 3759–3766.
[15] I. Abe, H. Morita, A. Nomura, H. Noguchi, J. Am. Chem. Soc. 122 (2000) 11242–11243.
[16] I. Limem, E. Guedon, A. Hehn, F. Bourgaud, L.C. Ghedira, J. Engasser, M. Ghoul, Process. Biochem. 43 (2008) 463–479.
[17] F. Ververidis, E. Trantas, C. Douglas, G. Vollmer, G. Kretzschmar, N. Panopoulos, Biotechnol. J. 2 (2007) 1235–1249.
[18] J. Kennedy, Nat. Prod. Rep. 25 (2008) 25–34.
[19] J.A. Chemler, Y.J. Yan, E. Leonard, M.A.G. Koffas, Org. Lett. 9 (2007) 1855–1858.
[20] Y. Katsuyama, N. Funa, I. Miyahisa, S. Horinouchi, Chem. Biol. 14 (2007) 613–621.
[21] H. Jiang, Metabolic engineering of the phenylpropanoid pathway in *Saccharomyces cerevisiae*, PhD Thesis in School of Chemical Engineering, Purdue University, 2005.
[22] H. Jiang, K.V. Wood, J.A. Morgan, Appl. Environ. Microb. 71 (2005) 2962–2969.
[23] R.D. Gietz, R.A. Woods, Transformation of yeast by lithiumacetate/single stranded carrier DNA/polyethylene glycol method, in: C. Guthrie, G. Fink (Eds.), Methods in Enzymology, vol. 350, Academic Press, San Diego, 2002, pp. 87–96.
[24] X. He, F. Yang, X. Lei, J. Chen, Y. Min, Yiyao Gongye 19 (1988) 447–451.
[25] Y. Yao, F. Yang, F. Gao, Zhongguo Yiyao Gongye Zazhi 23 (1992) 211–310.
[26] K.T. Watts, P.C. Lee, C. Schmidt-Dannert, Chembiochemistry 5 (2004) 500–507.
[27] S. Brand, D. Hoelscher, A. Schierhorn, A. Svatos, J. Schroeder, B. Schneider, Planta 224 (2006) 413–428.
[28] S. Wattanachaisaereekul, A.E. Lantz, M.L. Nielsen, J. Nielsen, Metab. Eng. 10 (2008) 246–254.