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Combinatorial Mutasynthesis of Flavonoid Analogues from Acrylic Acids in Microorganisms

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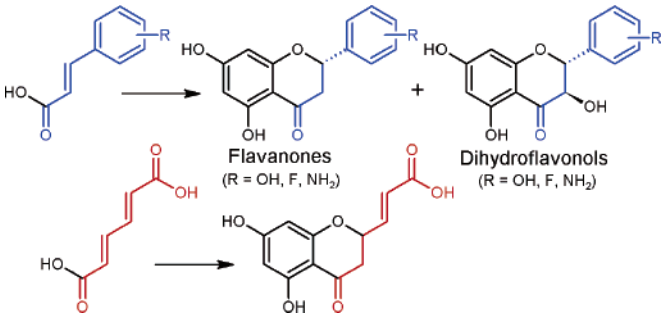
ABSTRACT



Flavonoids are plant secondary metabolites often used as nutraceutical supplements, but a growing number of unnatural flavonoids are being investigated as therapeutic agents. Cultures of *Saccharomyces cerevisiae* expressing recombinant flavonoid enzymes, including 4-coumaroyl: CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3β-hydroxylase (FHT), produced novel flavanones and dihydroflavonols when fed with a number of aromatic acrylic acids. The flavonoid network also exhibited broad substrate specificity by converting muconic acid into a unique polypropanoid.

Flavonoids are a diverse group of plant secondary metabolites that contain a 15-carbon phenylpropanoid core, which is extensively modified by rearrangement, alkylation, oxidation, and glycosylation.[1] These fascinating compounds possess extraordinary antioxidant activity with recent evidence pointing to their potential as digestive enzyme inhibitors.[2−4] Many flavonoids are used as nutraceutical supplements, but a growing number of unnatural flavonoids are being used as therapeutic agents in clinical trials including Phenoxodiol, flavopiridol, and ipriflavone. This is not surprising because about 50−60% of new drugs introduced into the market during the past 20 years are natural products or they are structurally related to them.[5,6] Because chemical synthesis of natural compounds is often complex and expensive, mutasynthesis and metabolic engineering are attractive alternatives for creating flavonoid analogues from inexpensive starting materials and endogenous molecules derived from sugars through large-scale fermentation processes. For example, unnatural polypropanoids have been made using a flavonoid enzyme, chalcone synthase from *Scutellaria baicalensis*, which had a broad substrate specificity toward synthesized *p*-coumaroyl-CoA analogues.[7,8]

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To mutasynthesize unnatural flavonoids, we employed the same strategy used to produce a range of natural flavonoids that relies on recombinant microbes as production platforms.[9−14] According to this strategy, *p*-coumaric acid (**1a**) first enters into the recombinant cells where it is ligated to coenzyme A by 4-coumaroyl:CoA ligase (4CL). The CoA ester then locks into the binding pocket of chalcone synthase (CHS), and the enzyme-bound thioester serves as an initiation unit for chain elongation and aromatization reactions. Upon being released from the enzyme, the newly formed chalcone undergoes a second cyclization but in a stereospecific manner by chalcone isomerase (CHI) to produce the first stable flavonoid, (2*S*)-naringenin (**4a**).

In this paper, we report the viability of a flavonoid network to utilize acrylic acid analogues and describe the combinatorial mutasynthesis of novel unnatural flavonoids using recombinant *Saccharomyces cerevisiae*. A two-stage screening protocol was first developed to screen a number of cinnamic acid analogues for their potential to be substrates of the flavonoid enzymes. The analogues differed from *p*-coumaric acid in that the *p*-hydroxyl group was replaced by a single or multiple halogen, amine, *O*-methoxy, or translocated hydroxyl groups (**1b**−**n**) (see Supporting Information). We also tested acrylic acids containing five-membered heteroaromatic rings, furan, and thiophene, as well as *trans*,*trans*-muconic acid (**1g**).

The first stage of the screening protocol employed a modified in vitro assay[15] where the acrylic acids were incubated with malonyl-CoA, adenosine triphosphate, and the soluble protein fraction of *Escherichia coli* containing recombinant 4CL from parsley.[16] The ultraviolet spectrum of the reactions was monitored between 250 and 400 nm for a bathochromatic shift in the maximum absorption indicating the formation of the respective CoA-ligated product (Figure 1). When the acrylic acids were incubated with the 4CL enzyme, the λ_{max} shifted from 309 to 338 nm for *p*-coumaric acid (**1a** into **2a**) (reported value of 333 nm[17]), from 278 to 326 nm for *m*-coumaric acid (**1b** into **2b**), from 324 to 348 nm for *o*-coumaric acid (**1c** into **2c**), from 275 to 340 nm for *p*-fluorocinnamic acid (**1d** into **2d**), from 269 to 310 nm for *o*-fluorocinnamic acid (**1e** into **2e**), and from 333 to 366 nm for *p*-aminocinnamic acid (**1f** into **2f**). The UV maximum of the muconic acid CoA analogue (**2g**) could not be definitively determined due to low activity (**1g** into **2g**). No shifts were seen for compounds containing chlorines, multiple halogens, *O*-methoxy groups, or five-membered rings.

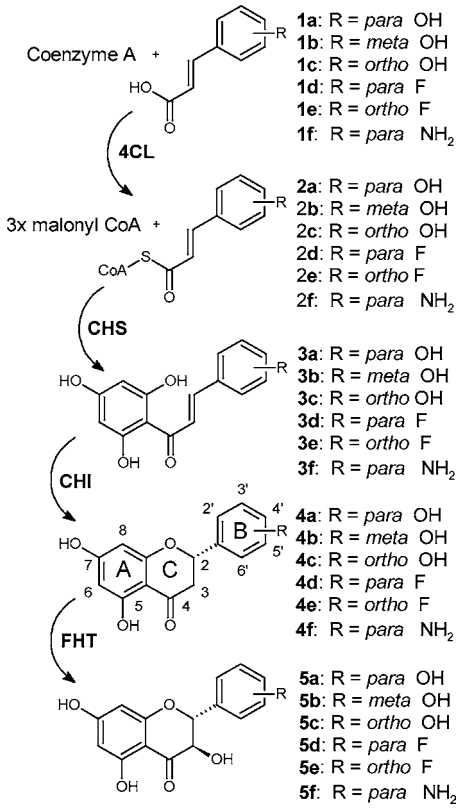

Figure 1. Formation of flavanones (**4a**−**f**) and dihydroflavonols (**5a**−**f**) by 4CL, CHS, CHI, and FHT.

The second stage of the screening protocol employed an in vitro assay where the acrylic acids were incubated with a mixture of crude *E. coli* cell extracts containing 4CL along with CHS and CHI cloned from petunia. Additionally, malonate and coenzyme A along with crude *E. coli* cell extracts containing malonyl-CoA synthetase (MATB) from cloned *Rhizobium trifolii*[18] were added to create a regenerative malonyl-CoA pool. The conversion of **1b**−**f** into flavanone analogues was monitored by high-performance liquid chromatography (HPLC), and the spectra were compared to control assays deficient in CHS protein to identify new peaks. The UV profiles of the products of **1b**−**f** had UV spectra (λ_{max} 287, 287, 289, 288, and 289 nm, respectively) that shared a striking similarity with that of **4a** (λ_{max} 288 nm), suggesting flavanone structures belonging to (2*S*)-3′,5,7-trihydroxyflavanone (**4b**), (2*S*)-2′,5,7-trihydroxyflavanone (**4c**), (2*S*)-5,7-dihydroxy-4′-fluoroflavanone (**4d**), (2*S*)-dihydroxy-2′-fluoroflavanone (**4e**), and (2*S*)-4′-amino-5,7-dihydroxyflavanone (**4f**). A previous study[7] reported creating novel flavonoids along with several CHS derailment products when converting chemically synthesized CoA

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analogues using only CHS from *S. baicalensis*. In contrast, in our study, only a single peak was observed for each reaction.

The enzymatic products were further characterized by electron spray ionization MS and ^{1}H NMR. To generate enough material, *S. cerevisiae* harboring a leucine-deficient selective plasmid expressing 4CL, CHS, and CHI was used as a production platform to convert the prescreened acrylic acids (**1b**−**f**) into unnatural flavanones (**4b**−**f**) (Figure 1). Yeast fermentations using SC minimal media were carried out in shake flasks for 2−3 days, and the consumption of acrylic acids and production of corresponding products was monitored daily by HPLC. Products were recovered from cell-free cultures by liquid extraction and purified by HPLC. The relative activities of each substrate (Table 1) were calculated on the basis of the maximum concentrations reached over 72 h and compared to the conversion of **1a** into **4a**. The ESIMS spectra of **4b** and **4c** gave negative ion peaks $[M - H]^-$ at *m*/*z* 271; both **4d** and **4e** had negative ion peaks $[M - H]^-$ at *m*/*z* 273; and **4f** had a negative ion peak $[M - H]^-$ at *m*/*z* 270 and a positive ion peak $[M + H]^+$ at *m*/*z* 272.

Table 1. Flavanone Production in Cultures with *S. cerevisiae* Expressing 4CL, CHS, and CHI

substrate	flavanone (mg/L)[a]		relative activity
1a	**4a**	60.04 ± 4.11	100.0%
1b	**4b**	6.54 ± 0.42	11.1%
1c	**4c**	6.36 ± 0.40	10.6%
1d	**4d**	2.81 ± 0.35	4.6%
1e	**4e**	6.54 ± 0.69	10.8%
1f	**4f**	15.82 ± 4.23	26.4%
1g	**4g**	nd	nd

[a] $n = 3$.

Interestingly, the flavanone cluster was also capable of converting an acrylic acid analogue (**1g**), in which the benzene ring was replaced by a propanoic acid, producing a unique polypropanoid (Figure 2). Upon performing the second in vitro assay with **1g**, a single product peak was evident (λ_{max} at 285 nm). The product was isolated from fermentations, and a ESIMS ion peak $[M - H]^-$ at *m*/*z* 249 was observed. On the basis of the molecular weight, the structure was presumed to be (5,7-dihydroxy-4-chromanone)-propenoic acid (**4g**), but further chemical characterization could not be performed due to the low quantities capable of being produced and purified.

To further expand the diversity of the novel flavonoids, a second strain, capable of producing dihydroflavonols (Figure 1), was developed from the previous strain by adding a uracil-deficient selective plasmid to express a gene encoding flavanone 3β-hydroxylase (FHT) cloned from *Malus domestica* (apple).[13] In plants, dihydroflavonols are flavanone derivatives that serve as precursors for a variety of compounds including flavonols, catechins, and anthocyanins.

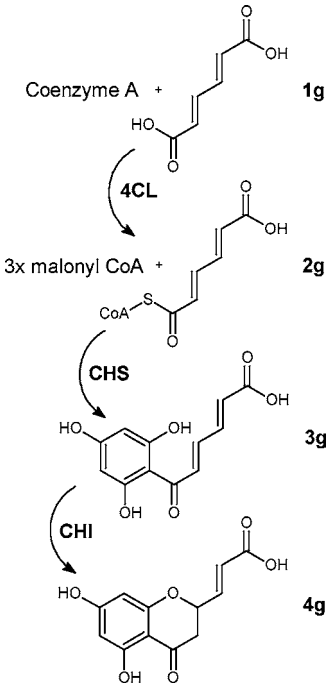

Figure 2. Proposed mechanism for the formation of **4g** by 4CL, CHS, and CHI.

Originating from their corresponding acrylic acids, compounds **5b**−**f** were purified from yeast fermentations and overall yields (Table 2) were compared to the conversion of **1a** into dihydrokaempferol (**5a**). The ESIMS $[M - H]^-$ spectra obtained contained peaks at *m*/*z* 287 for **5b** and **5c**, at *m*/*z* 289 for **5d** and **5e**, and at the corresponding *m*/*z* 286 for **5f**. The stereochemistry was assigned to be identical to (2*R*,3*R*)-dihydrokaempferol (**5a**) which occurs as a single stereoisomer in nature.[19]

Table 2. Dihydroflavonol Production in Cultures with *S. cerevisiae* Expressing 4CL, CHS, CHI, and FHT

substrate	dihydroflavonol (mg/L)[a]		relative activity
1a	**5a**	62.79 ±12.84	100.0%
1b	**5b**	2.98 ± 0.42	4.7%
1c	**5c**	3.85 ± 0.82	6.1%
1d	**5d**	0.75 ± 0.06	1.2%
1e	**5e**	4.50 ± 0.92	7.1%
1f	**5f**	10.44 ± 1.12	16.7%

[a] $n = 3$.

This is the first demonstration of the mutasynthesis of novel flavanones and dihydroflavonols from simple acrylic acids. It is noteworthy that the recombinant metabolic pathway also accepted a substrate containing a propanoic

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acid instead of a heteroaromatic ring moiety. However, cinnamic acids containing aromatic chlorines, multiple halogens, *O*-methoxy groups, or acrylic acids with five-membered rings were not accepted as viable substrates of 4CL suggesting that steric size and electronegative charge may play a role in the exclusion of these molecules. In conclusion, we have presented scalable means to mutasynthesize novel polypropanoids using synthetic analogues to create a library of potentially important medicinal compounds. Further expansion of the library will include novel stilbenes, catechins, flavones, flavonols, and even anthocyanidins with new colors.

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Supporting Information Available: Experimental procedures and spectroscopic data of fermentation products (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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