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UNIVERSITÀ DEGLI STUDI DI TORINO

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Production of novel antioxidative phenolic amides through heterologous expression of the plant's chlorogenic acid biosynthesis genes in yeast

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Running title: **Expression of the chlorogenic acid pathway in yeast**

ABSTRACT

Phenolic esters like chlorogenic acid play an important role in therapeutic properties of many plant extracts. We aimed to produce phenolic esters in baker's yeast, by expressing tobacco *4CL* and globe artichoke *HCT*. Indeed yeast produced phenolic esters. However, the primary product was identified as N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid by NMR. This compound is an amide condensation product of *p*-coumaric acid, which was supplied to the yeast, with 3-hydroxyanthranilic acid, which was unexpectedly recruited from the yeast metabolism by the HCT enzyme. N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid has not yet been described before, and shows structural similarity to avenanthramides, a group of inflammation-inhibiting compounds present in oat. When applied to mouse fibroblasts, N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid induced a reduction of intracellular reactive oxygen species, indicating a potential therapeutic value for this novel compound.

Key words: chlorogenic acid; yeast; avenanthramide; artichoke

1. Introduction

The expression of plant metabolic pathways in microbial organisms such as brewers' yeast (*Saccharomyces cerevisiae*) is an attractive strategy for the production of valuable secondary metabolites, and may even provide a means of synthesizing compounds not present in nature. Microbial expression systems offer several advantages over chemical synthesis or direct extraction from plant tissue, e.g. reduced requirements of toxic chemicals, constant quality, simple extraction and potential for a higher synthesis efficiency (Limem et al., 2008). For these reasons, yeast has been engineered to produce isoprenoids (such as carotenoids, sterols, polyprenyl alcohols, ubiquinone) (Engels et al., 2008), long chain polyunsaturated fatty acids, and early intermediates of the plant phenylpropanoid pathway (Chemler et al., 2006). In particular, the health-promoting properties of flavonoids and other phenolic compounds have stimulated the development of heterologous expression systems based on *S. cerevisiae* (Forkmann and Martens, 2001; Katsuyama, et al. 2007; Trantas et al., 2009). Examples include flavanones (Jiang et al., 2005; Yan et al., 2007), 5-deoxyflavones (Yan et al., 2007), flavones (Leonard et al., 2005), isoflavones (Kim et al., 2005; Ralston et al., 2005), resveratrol (Becker et al., 2003; Beekwilder et al., 2006; Zhang et al., 2006), phenolic acids (Ro and Douglas, 2004; Vannelli et al., 2007) and raspberry ketone (Beekwilder et al., 2007). Production of phenolic esters in microbial systems has not been reported.

The human diet includes several plants which are rich in phenolic esters, e.g. coffee, apples, lettuce and globe artichoke (Clifford, 1999; Moglia et al., 2008). Among the phenolic esters, chlorogenic acid, an ester of caffeic acid and quinic acid, is the most well known compound (Fig. 1). In plants they are involved in defense against pathogens, protection from UV radiation, or signalling of stress (Dixon and Paiva, 1995; Treutter, 2005). Most relevantly, being part of the human diet, many phenolic esters have properties beneficial to human health (Clifford, 1999). Typically they act as strong antioxidants, preventing the oxidative damage and lipid peroxidation mediated by harmful free radicals (RiceEvans et al., 1997). Plant phenolic compounds have been shown to be

prophylactic against arteriosclerosis, cardiovascular diseases, inflammatory processes and certain forms of cancer (Jang et al., 1997; Boots et al., 2008).

Biosynthesis of phenolic esters starts from the phenylpropanoid pathway, which catalyses the conversion of phenylalanine to secondary products, such as lignin, phenolic esters and flavonoids (Liu et al., 2007). Via a series of hydroxylation, methylation and dehydration reactions, phenylalanine is converted into a range of hydroxycinnamic acid compounds, which may be incorporated into either polyphenols like lignin or flavonoids, or phenolic amides, or phenolic esters. At present, a number of enzymes involved in the biosynthesis of phenolic esters in plants have been identified (Hoffmann et al., 2003; Niggeweg et al., 2004; Comino et al., 2007; Mahesh et al., 2007; Comino et al., 2009; Moglia et al., 2009). Key enzymes are 4CL (4-coumarate:CoA-ligase), which forms phenolic acid – CoA esters (Lee and Douglas, 1996), and HCT (hydroxycinnamoyl-CoA:shikimate/quinic acid hydroxycinnamoyl transferase) (Hoffmann et al., 2003; Comino et al., 2007), which couples the CoA esters to intermediates of the phenylalanine biosynthetic pathway, such as quinic acid or shikimic acid, to form phenolic esters (Fig. 1).

Here we describe a production system of conjugated hydroxycinnamates, based on a brewers' yeast which co-expresses tobacco 4CL and globe artichoke HCT.

2. Materials and methods

2.1 Materials

Avenanthramide standards were kindly provided by Dr. Meydani (JM USDA Human Nutrition Research Center on Aging at Tufts University, Boston) and Dr. Collins (Eastern Cereals and Oilseeds Research Center (ECORC), Ottawa). The standard *p*-coumaroyl quinate was kindly provided by Dr. Ullmann (Université Louis Pasteur, Strasbourg). All chemicals used were from Sigma (St. Louis, unless indicated otherwise).

2.2 The pUC4CL-HCT plasmid

A globe artichoke HCT gene (accession DQ104740) was PCR amplified from the plasmid pGEM-HCT using primers 5'-TCTCCATGGGTAAGATCGAGGTGAGAGAATCAACGATG and 5'-TCTCTCGAGTTAGATATCATATAGGAACTTGCTG, and the resulting amplicon was digested with *NcoI* and *XhoI* and ligated into linearized pUC-4CL, which harbours the tobacco 4CL-2 gene (Beekwilder et al., 2006). The resulting pUC4CL-HCT plasmid was introduced into *S. cerevisiae* CEN.PK113-3b (*ura3 his3*), so that 4CL and HCT were under the control, respectively, of the galactose GAL 10 and GAL 1 promoters. The construct was integrated within the yeast LEU2 locus, along with ILV2-SMR, which confers resistance to the herbicide chlorosulfometuron methyl.

2.3 Yeast transformation and cultivation

The transformation of *S. cerevisiae* strain CEN.PK113-3b (*ura3 his3*) was achieved using the lithium acetate / single strand carrier DNA / polyethylene glycol method (Gietz and Woods, 2002). Transformants were selected on 2xYT plates (16 g/L tryptone, 10 g/L yeast extract, 10 g/L sodium chloride, 20 mg/L uracil, 20 mg/L histidine) containing 25 mg/L chlorosulfuron methyl. The success of the transformation was confirmed by PCR analysis. Single colonies of the transformed yeast strain were transferred to 5 mL 2 x YT medium at 30 °C for 20 hr with shaking (250 rpm).

The overnight culture was diluted 1:25 in 50 mL of fresh 2 x YT medium and cultured at 28 °C in the presence of galactose to induce transgene expression. When the OD₆₀₀ reached 0.4, *p*-coumaric acid (5 mM), caffeic acid (5 mM), phenylpropionic acid (5 mM), ferulic acid (5 mM) or cinnamic acid (5 mM) were added. Expression was monitored over the following 96 hr, by taking 5mL samples every ~12 hr for HPLC analysis. Each 5 mL aliquot mixed in a glass tube with 4 mL of ethyl acetate, vortexed for 10 s and centrifuged for 5 min at 4000 rpm. The upper phase was transferred to a new tube, placed under nitrogen flow to reduce the volume to about 1 mL, and finally transferred to a glass vial for HPLC-PDA and HPLC QTOF-MS analysis.

2.4 HPLC-PDA analysis

The HPLC system comprised a Waters 600 controller, a Waters 996 photodiode array detector (PDA) and a column incubator held at 40 °C. For the chromatographic separation an analytical column Luna C18 (2) (2 x 150 mm, 100 Å, particle size 3 µM) with a pre-column (2 x 4 mm) from Phenomenex was used. The mobile phase consisted of ultrapure water acidified with 0.1% trifluoroacetic acid (eluate A), and acetonitrile acidified with 0.1% trifluoroacetic acid (eluate B). The system used a linear gradient from 5% B to 35% B in 45 minutes. The flow rate was 1 mL/min, the injection volume was 10 µL, and the range of detection wavelength was 240-600 nm.

2.5 HPLC QTOF-MS

To identify the polyphenolic metabolites in the extracts, accurate mass LC-MS and MS/MS on a high-resolution quadrupole time-of-flight (Q-TOF) mass spectrometer with lockmass correction was used, in conjunction with spectral analysis by a PDA detector (Moco et al. 2006). HPLC was performed using a Waters Alliance 2795 HT HPLC system, providing a linear gradient from 5 to 35% acetonitrile (acidified with 0.5% formic acid) in 45 minutes at a flow rate of 1 mL/min. The chromatographic separation employed an analytical column Luna C18 (2) (2 x 150 mm, 100 Å, particle size 3 µM) with a pre-column (2 x 4 mm) from Phenomenex. Eluting compounds were first

detected on line at 240-600 nm using a Waters 2996 PDA, before entering a QTOF Ultima API mass spectrometer equipped with an electrospray ionization source and a separate LockSpray. The eluate flow was split after PDA detection to obtain a flow of 0.2 mL/min into the mass spectrometer. Before each series of analyses, the mass spectrometer was calibrated using phosphoric acid:acetonitrile:water (1:1000:1000, v/v). During sample analysis, the capillary voltage was 2.75 kV and the cone voltage 35 V. Source and desolvation temperatures were 120 °C and 250 °C, respectively.

The negative detection mode was adopted, because during mass spectrometry analysis, polyphenolic acids ionize better in this mode (Moco et al., 2006). The collision energy was 10 eV. Ions in the m/z range of 100-1500 were detected using a scan time of 0.9 s and an interscan delay of 0.1 s. Tandem mass spectrometry was performed on-line using three different collision energies (5, 10, and 50 eV) on up to eight masses per survey scan. Leucine encephalin (Sigma), dissolved in 50% acetonitrile with 20 μ M ammonium acetate, was used as a lock mass and was measured every 10 s. Masslynx software v4.1 was used for the visualization and processing of data.

2.6 Preparative HPLC

One liter of yeast culture was incubated with *p*-coumaric acid as described above. After centrifugation, the supernatant of the culture was extracted twice with 100 mL of ethyl acetate. Ethyl acetate phases were pooled and ethyl acetate was removed by evaporation under a nitrogen flow. Dry material was dissolved in 8 mL of methanol. Material was loaded in three injections on a Luna C18 (2) column with dimensions 21.2 x 150 mm, 100 Å, particle size 5 μ m (Phenomenex), operated on a Shimadzu preparative HPLC LC-8A with a SIL-10AP autosampler and a SPD-M10Avp photodiode array detector. After loading the sample, the column was first washed with 160 mL of 29% acetonitrile with 0.1% TFA at 15 mL/min, and subsequently the compound was eluted with 34% acetonitrile with 0.1% TFA. Eluting material with absorbance at 312 nm was collected and evaporated to dryness by vacuum film evaporation.

2.7 Identification by spectral analysis

UV spectra were recorded in methanol on a Varian Cary 50 Scan UV-Visible spectrophotometer. IR spectra were recorded in KBr on a Bruker Vector 22 IR spectrophotometer. NMR spectra were recorded in methanol-d₄ or DMSO-d₆ on a Bruker Avance 500 at 500 MHz at room temperature.

Spectral data N-(E)-p-coumaroyl-3-hydroxyanthranilic acid

UV (MeOH): λ_{\max} 337, 304 (sh) and 295 (sh) nm.

IR (KBr): 3416, 1683, 1650, 1603 (s), 1561, 1515 (s), 1468, 1384, 1279, 1240, 1203, 1171, 1014, 978, 829, 764 cm^{-1} .

ESI-TOF-MS: m/z 298.0705 $[\text{M}-\text{H}]^-$ ($\text{C}_{16}\text{H}_{13}\text{NO}_5$ corresponds with 298.0710 Da). MS/MS: 254 $[\text{M}-\text{CO}_2-\text{H}]^-$, 145.0291 $[\text{M}-\text{H}-\text{C}_7\text{H}_7\text{NO}_3]^-$ ($\text{C}_9\text{H}_7\text{O}_2$ corresponds with 145.0284 Da).

^{13}C -NMR (125 MHz, CD_3OD): δ (ppm) 171.5 (C7), 168.5 (C9'), 161.2 (C4'), 151.7 (C3), 144.8 (C7'), 131.2 (C2'/6'), 128.9 (C1), 127.3 (C1'), 127.0 (C5), 127.0 (C2), 124.5 (C6), 124.0 (C4), 117.4 (C8'), 116.9 (C3'/5'). ^{13}C -NMR (125 MHz, DMSO-d₆ + 0.2% TFA): δ (ppm) 168.3 (C7), 165.0 (C9'), 159.3 (C4'), 151.2 (C3), 140.9 (C7'), 129.7 (C2'/6'), 127.7 (C1), 125.7 (C1'), 125.7 (C5), 125.1 (C2), 120.7 (C6), 120.1 (C4), 118.1 (C8'), 115.9 (C3'/5').

2.8 *In vitro* enzyme-assays

The HCT open reading frame was amplified using primers with additional recognition sites of *NdeI* and *BamHI*. The amplicon was *NdeI* and *BamHI* restricted, and the digestion product ligated into the pET3a plasmid linearized using the same restriction enzymes. The resulting recombinant pET3a-HCT plasmid was transformed into *E.coli* BL21(DE)pLysE and grown on a selective medium (LB, broth agar, 34 mg/L chloramphenicol and 50 mg/L ampicillin). Transformants and control colonies were inoculated in 10 mL of LB medium, with 1% w/v glucose and 30 mg/L chloramphenicol and 50 mg/L ampicillin. The overnight cultures were diluted 1:25 in 50 mL LB

medium in the presence of chloramphenicol and ampicillin, and incubated at 37 °C, until the OD₆₀₀ reached 0.6. At this point, IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to a final concentration of 1 mM and the cultures were grown overnight at 28 °C on a shaker (250 rpm). Cultures were centrifuged (3,500 rpm, 5 min) and cells were re-suspended in 1 mL of phosphate-buffered saline 7.5 and lysed by three cycles of freezing (in liquid nitrogen) and thawing (at 37°C). Following sonication (5 cycles, 30 s), the solution was clarified by centrifugation, and the supernatant assayed for HCT activity. Each 30 μL assay reaction contained 100 mM sodium phosphate buffer (pH 7.0), 1 mM dithiothreitol, bacterial supernatant, 300 μM caffeoyl-CoA or *p*-coumaroyl-CoA (both provided by Transmit) and 1 mM 3-hydroxyanthranilic acid (Sigma). The same reaction was also performed replacing 3-hydroxyanthranilic acid with anthranilic acid and 5-hydroxyanthranilic (Sigma). Reactions were incubated for 30 min at 30°C, extracted with 30 μL of acetic acid:acetonitrile (1:99) and analysed by HPLC.

The amount of HCT recombinant protein in the supernatant fraction was quantified by densitometry of the bands on polyacrylamide gels stained with Coomassie Brilliant-Blue R-250 (Fluka). For Km determination, varying substrate-dependent concentrations were used. For Km with 3-hydroxyanthranilic acid 0.75 μg enzyme, 300 μM *p*-coumaroyl-CoA and 0.5-40 mM 3-hydroxyanthranilic acid were used. For Km with shikimic acid 0.75 μg enzyme, 300 μM *p*-coumaroyl-CoA and 0.5-40 mM shikimic acid acid were used. Reactions were incubated at 30°C for 30 min. An HPLC calibration curve was established for each molecule for quantification purposes. Km and Vmax values were calculated in duplicate by Lineweaver-Burk plots.

2.9 HPLC antioxidant detection system

Antioxidant activity in yeast extracts and of avenanthramide A and B standards was determined by HPLC coupled to an on-line post-column antioxidant system (Beekwilder et al., 2005). Compounds eluting from the analytical column first passed through a PDA detector (absorbance set at 240-600 nm) and reacted during 30 s with a buffered solution of 2,2-azinobis (3-ethylbenzothiazoline-6-

sulfonic acid, ABTS^{•+}; Roche) radical cations in a post-column reactor before passing through a second detector [(dual wavelength UV-vis detector (Waters)] which monitors the ABTS^{•+} radicals. The ABTS^{•+} radical solution was prepared by dissolving 55 g ABTS in 50 mL water, followed by the addition of potassium permanganate. After 16 h incubation in the dark, the solution was diluted in three volumes of 0.2 M sodium phosphate buffer, pH 8.0. The post-column reaction loop was a 3 m stainless steel tube (internal diameter 0.508 mm) held at 40°C, and the column eluate remained for exactly 30 s in this tube before detection. The decreased absorption of ABTS^{•+} following its reaction with antioxidants was monitored at 412 nm.

2.10 FACS analysis of intracellular ROS levels

Mouse embryonic fibroblasts (MEF) cells characterized by high steady-state levels of intracellular ROS were used as a cellular model to test the antioxidant activity of YAv (N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid). Intracellular ROS levels were measured using the cell-permeable redox-sensitive fluorogenic probe 2',7'-dichlorofluorescein diacetate (H₂DCFDA). YAv standard was prepared by dissolving it in dimethyl sulfoxide (DMSO). Cells were incubated over-night in complete medium containing four different concentrations of YAv (40, 120, 200, 300 μM) or DMSO as control. Cells were then washed twice in PBS, incubated for 20 min with 5 μM DCFH-DA (Sigma) in PBS, collected in PBS by trypsin treatment, and analyzed by a Becton Dickinson FACScalibur using a 488 nm excitation laser and FL1 channel – 530/30 nm band pass emission filter. Mean of fluorescence was determined using CellQuest software (Becton Dickinson). Autofluorescence of unlabeled cells was used for background fluorescence measurement.

3. Results

3.1 Production of phenolic compounds in HCT-expressing yeast

With the aim to establish a food-grade production platform for phenolic esters, the *4cl-2* gene from tobacco and the *hct* gene from globe artichoke were introduced into the yeast *S. cerevisiae*. To this end, a DNA plasmid containing the tobacco *4cl-2* gene under control of the GAL10 promoter, the globe artichoke *hct* gene under control of the GAL1 promoter, and the selectable marker ILV2-SMR was constructed. This construct was integrated into the LEU2 locus of *S. cerevisiae*, leading to the strain 4CL-HCT. Subsequently this strain was compared to untransformed (control) yeast for metabolizing phenolic acids added to the culture medium. When the strains were fed with *p*-coumaric acid (5 mM), the 4CL-HCT yeast readily fully consumed the substrate, and at 72h the highest concentration of new compounds was observed (data not shown). In contrast, in the control yeast culture, hardly any change of concentration of *p*-coumaric acid was detected. The HCT cultures showed formation of a small amount (1.6 mg/L) of a compound which was identified as *p*-coumaroyl shikimate (Fig. 2A; $R_t = 22.55$ min; see below). Notably, also formation of a compound that eluted late in the chromatographic gradient (Fig. 2A; $R_t = 45.32$ min) was observed. To assess the cellular localization of these products, yeast cells were separated from the medium by centrifugation, and both fractions were assayed for the presence of these compounds. The culture medium contained about 10-fold more of these compounds than the pellet, indicating that products were secreted into the medium. When the 4CL-HCT recombinant yeast was incubated with other phenolic acids, such as caffeic acid, phenylpropionic acid, ferulic acid or cinnamic acid, only in the case of caffeic acid strong substrate consumption and synthesis of a new compound ($R_t = 39.72$ min, Fig. 2B) was observed.

3.2 Identification of products on HPLC-PDA and LC-MS

Chromatographically separated compounds were preliminary identified on the basis of their

absorbance spectrum, the exact mass of the pseudomolecular ion and its MS/MS fragments. In the incubation of 4CL-HCT yeast with *p*-coumaric acid, the peak eluting at $R_t = 22.55$ min showed an $[M-H]^-$ at m/z 319.0802 (Fig. 2A). This mass corresponds within 5 ppm to the predicted molecular mass of *p*-coumaroyl shikimate, and the compound has the same R_t and absorbance spectrum (λ_{max} at 217 and 311 nm) as the *p*-coumaroyl shikimate standard.

The main product peak ($R_t = 45.32$ min, Fig. 2A) was subjected to further characterization. Accurate mass measurement of this peak showed a pseudomolecular ion $[M-H]^-$ at m/z 298.0707 (Fig. 2A), which corresponds within 5 ppm to a molecule with composition $C_{16}H_{13}NO_5$ ($[M-H]^- = 298.0721$ m/z). As *p*-coumaric acid was used as precursor (MW = 164, $C_9H_8O_3$), and the absorbance spectrum of this precursor shared some properties with the novel compound, we hypothesized that the novel compound resulted from a coupling of coumaric acid with a hydroxy-aminobenzoic acid (e.g. hydroxyanthranilic acid or aminosalicic acid, $C_7H_7NO_3$) via an ester or amide linkage. MS/MS showed the neutral loss of 153, i.e. a hydroxy-aminobenzoic acid, yielding a fragment at m/z 145. The UV spectrum showed a broad maximum around 337 nm with shoulders at 304 and 295 nm which is similar to the UV of avenanthramide A (amide of 5-hydroxyanthranilic acid and *p*-coumaric acid) (Collins and Wiliam, 1989).

Likewise, incubation of the 4CL-HCT yeast with caffeic acid resulted in the accumulation of an unknown compound. This compound eluted at $R_t = 39.72$ min, i.e. slightly more hydrophylic compared to the *p*-coumaric acid product, showed λ_{max} at 217 and 339 nm (Fig. 2B), its accurate mass was 314.0645 ($[M-H]^-$) and upon MS/MS a fragment at m/z 161 was observed. Again, the neutral loss of 153 corresponds to a loss of hydroxyaminobenzoic acid, and all data together suggests that this caffeic-acid derived compound is structurally similar to the coumaric acid-derived compound.

3.3 Identification by NMR

The main product of the 4CL-HCT yeast fed with *p*-coumaric acid ($R_t = 45.32$ min) was produced

in a 1 L culture, purified by preparative HPLC, and about 20 mg of a yellow amorphous solid was yielded for further identification. The ^1H -NMR spectrum showed the characteristic pattern of an (E)-*p*-coumaric unit [(two ^2H doublets ($J=8.5$ Hz) at 6.83 and 7.51 ppm and two ^1H doublets ($J=15.2$ Hz) at 6.69 and 7.68 ppm)] (Table 1). The presence of the 3-hydroxyanthranilic unit was deduced by the observation of signals at 7.61 (dd, $J=7.2$ and 1.9 Hz), 7.18 (dd, $J=8.1$ and 7.2 Hz) and 7.15 ppm (dd, 8.1 and 1.9 Hz). The ^1H - and ^{13}C -NMR chemical shifts excluded the presence of a 3- or 6-aminosalicylic acid moiety. What remained to be deduced was the coupling between the two halves, both an ester or amide linkage is plausible. The IR spectrum showed bands characteristic of an amide, while the lack of any bands above 1680 cm^{-1} excluded the possibility of a phenolic ester moiety. The combined data suggested the structure depicted in Fig. 3A, N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid. The ^1H - and ^{13}C -NMR data of the *p*-coumaroyl part are superimposable with those reported for N-(E)-*p*-coumaroyl-3,5-dihydroxyanthranilic acid (Blaakmeer et al., 1994).

This structure was finally confirmed by the ^{13}C -NMR data, a Heteronuclear Multiple Bond Correlation (HMBC) spectrum and a ^1H -NMR spectrum in DMSO- d_6 . The latter spectrum showed four signals at 9.96, 10.12, 10.77 and 12.75 ppm corresponding to the 4'-OH, the 3'-OH, the amide NH and carboxylic acid respectively. The ^{13}C -NMR chemical shifts of this compound fitted perfectly with the proposed structure of N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid and were incompatible with an ester linkage. In that case, one would expect two shifts of around 140 ppm for C2 and C3 instead of the observed 25 ppm difference. An HMBC spectrum showing cross peaks between the amide NH and carbon signals in both halves of the molecule was obtained only by recording the spectrum in ultra dry DMSO- d_6 with 0.2% trifluoroacetic acid (TFA). Without TFA the N-H signal is broadened due to exchange revealing no crosspeaks in the HMBC. The OH and COOH proton signals are present in the spectrum recorded in DMSO with TFA as a very broad line between 11 and 14 ppm. The HMBC cross peaks are given in Fig. 3B. N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid (trivially called yeast avenanthramide, YAv) has not been described before

as a natural product (Fig. 3A).

3.4 *In vitro* enzyme activity

The globe artichoke HCT enzyme is known to accept quinic acid and shikimic acid as acceptors for caffeoyl-CoA and *p*-coumaroyl-CoA (Comino et al., 2007). To confirm the ability of HCT to use 3-hydroxyanthranilic acid as an acceptor *in vitro*, HCT was produced in *E. coli*, and the recombinant enzyme was incubated with 3-hydroxyanthranilic acid and either caffeoyl-CoA or *p*-coumaroyl-CoA. The products of reaction were analysed by HPLC coupled to a photodiode-array detector. In the presence of active HCT, incubation with both phenolic acids lead to products which were absent in the control reactions (inactive enzyme). The chromatographic and spectral properties of these compounds ($R_t = 39.7$ min, λ_{max} 217 and 339 nm for the caffeoyl CoA product; $R_t = 45.3$ min and λ_{max} 337 nm for the coumaroyl CoA product) exactly matched those of the compounds produced *in vivo* by the recombinant yeast after incubation with caffeic acid and *p*-coumaric acid, respectively (Fig. 2). Thus, the HCT enzyme displayed the surprising ability to couple phenolic acids to the amino-moiety of 3-hydroxyanthranilic acid, and catalyze amide-bond formation. When anthranilic acid and 5-hydroxyanthranilic acid were tested as potential acceptors in the enzymatic reaction with caffeoyl CoA, no enzymatic activity was observed (data not shown), indicating that the position of the amino-group on the anthranilic acid is important for recognition by the enzyme. In order to explain the different yields of produced phenolic ester (*p*-coumaroyl shikimate) and phenolic amide (N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid) in the culture medium of recombinant yeast, we evaluated the kinetic parameters of the HCT enzyme with shikimic acid and 3-hydroxyanthranilic acid as acceptors in the presence of *p*-coumaroyl-CoA as acyl donor.

While the affinity of the enzyme for shikimic acid as acceptor was similar to the one observed with 3-hydroxyanthranilic acid ($K_m = 1.44 \pm 0.3$ vs 1.64 ± 0.9 ; Table 2), the reaction velocity was higher for 3-hydroxyanthranilic than for shikimic acid (Table 2). Thus, the catalytic efficiency was four times higher when HCT was provided with 3-hydroxyanthranilic as compared to shikimic acid

(V_{max}/K_m 1.18 and 0.30, respectively; Table 2).

3.5 Evaluation of antioxidant activity

We investigated the antioxidant properties of the new yeast compounds by HPLC coupled to an on-line post-column antioxidant detection system (Beekwilder et al., 2005; Niederländer et al., 2008). An example is illustrated in Fig. 4A. The chromatogram shows the ABTS^{•+} radical quenching activity of yeast-produced compounds N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid (YAv) and its caffeoyl variant (YAvII), and compares them with avenanthramide A and B, which have a similar amide structure (Fig. 5). Avenanthramides have been described in literature as being strong antioxidants (Chen et al., 2007). For N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid and its caffeoyl variant, similar levels of antioxidant activity were observed (Fig. 4A).

The ABTS^{•+} radical quenching assay, like most popular antioxidant activity assays, is performed in a cell-free system and does not account for cellular mechanisms influencing the response to a substance. To examine the biological relevance of the chemically tested antioxidant activity of N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid (YAv), we performed a cellular antioxidant activity (CAA) assay (Wolfe and Liu, 2007) using mouse embryonic fibroblasts (MEF) cells. MEF cells are characterized by high steady-state levels of intracellular ROS. *In vivo*, fibroblasts are known to play a role in the initiation of inflammation (Smith et al., 1997). Cells were incubated with different concentrations of YAv, and intracellular ROS levels were monitored and quantified by Fluorescence Activated Cell Sorting (FACS) analysis using a well-established method based on the cell-permeable, ROS-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA). This compound is deacetylated by cellular esterases to form the non-fluorescent 2',7'-dichlorodihydrofluorescein (DCFH) which is trapped within cells and stoichiometrically oxidized to the highly fluorescent dichlorofluorescein (DCF) through its reaction with intracellular ROS (Wolfe and Liu, 2007). As shown in Fig. 4 (panels B and C), cell treatment with 200 and 300 μ M of YAv caused a significant reduction of intracellular ROS levels (around 40% as compared with vehicle

(DMSO)-treated cells), demonstrating that N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid can exert a biologically relevant antioxidant activity.

4. Discussion

In this study, *S. cerevisiae* was deployed as cell factory for the biosynthesis of phenolic esters. To establish a microbial production system, two plant genes, which encode an essential part of the plant biochemical pathway leading to phenolic esters, were engineered into yeast. It was demonstrated that yeast can produce *p*-coumaroyl shikimate, a phenolic ester present in nature. Surprisingly, also a novel amide between *p*-coumaric acid (added to the culture medium) and endogenous 3-hydroxyanthranilic acid was formed. This novel compound, N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid, was demonstrated to exert antioxidant activity *in vitro* and to lower the intracellular reactive oxygen species (ROS) in mouse embryonic fibroblast cells, and thus appeared to be a bio-available anti-oxidant.

Oxidative stress is thought to be an important contributing factor in the development of cancer and cardiovascular diseases (Berlett and Stadtman, 1997; Lee and Blair, 2001). It results from an imbalance between the production of ROS and the efficiency of the antioxidant defence mechanisms to cope with ROS, and may lead to irreversible damage of fundamental cellular components, including DNA, protein and lipids. In addition to the endogenously produced antioxidants and enzymes that prevent oxidative damage, exogenously obtained antioxidants, including phenolic compounds, are able to scavenge ROS and may help to prevent oxidative damage.

4.1 Phenolic compounds produced by globe artichoke HCT

With the production of *p*-coumaroyl shikimate, a new category of plant phenolic compounds, i.e. phenolic esters, has been added to the production repertoire of yeast. Chlorogenic acid, which belongs to the same category, has been implicated in a wide range of antimicrobial, UV-protective and antioxidant properties (Wang et al., 2003; Kukic et al., 2008) . In addition to known compounds such as *p*-coumaroyl shikimate, the 4CL-HCT yeast produced a novel compound, N-(E)-*p*-

coumaroyl-3-hydroxyanthranilic acid (YAv), which to date has not been described (Fig. 3A). The properties of this novel compound relate to those of avenanthramides. Avenanthramides are a group of 30 hydroxycinnamoylanthranilates, which, so far, have only been found in oat (*Avena sativa*), and are the main soluble phenolic compounds in oat kernels (Collins and Wiliam, 1989; Okazaki et al., 2004; Chen et al., 2007). In addition, they have been reported as host-marking pheromones in the eggs of the butterfly *Pieris brassicae* (Blaakmeer et al., 1994). Avenanthramides are amide conjugates of anthranilic acid (or its hydroxylated derivatives) and hydroxycinnamic acids. As an example, avenanthramides A and C are shown in Fig. 5. Avenanthramides from oat behave as phytoalexins in presence of pathogen infection. They have been described to possess an anti-oxidant activity *in vitro* (Bratt et al., 2003; Fagerlund et al., 2009) and in animal models (Ji et al., 2003; Chen et al., 2004; Chen et al., 2007), and have shown to exert an anti-inflammatory activity, by reducing release of interleukin-8 (Liu et al., 2004). As such, they have been incorporated in topical anti-inflammatory skin-care products, aimed to reduce inflammations that accompany allergic reactions (Meydani, 2006). The structures of the newly compounds formed in the 4CL-HCT yeast differ from avenanthramides in the position of the hydroxyl group in the anthranilic part (Fig. 5). We compared the antioxidant activity of the new compounds to avenanthramide standards from oat, by means of an HPLC system with online antioxidant analysis. The outcome of this *in vitro* assay showed that the antioxidant activity of the new compounds was quite similar to what observed for avenanthramides (Fig. 4A). In addition, by performing a cellular antioxidant activity (CAA) assay, we demonstrated that N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid can function as a ROS scavenger in biological systems (Fig. 4B, C), suggesting that it might constitute an additional, easily produced compound for biomedical applications. Future studies will be aimed at evaluating anti-oxidant properties of the new compounds in animal models and their ability to act as anti-inflammatory agents.

4.2 Amide bond formation by globe artichoke HCT

The HCT enzyme is part of a well characterized group of the BAHD-family of acyltransferases (D'Auria, 2006). Members of this group have been implicated in the formation of ester bonds between hydroxycinnamic acids and shikimic acid or quinic acid, leading to accumulation of chlorogenic acid. The HCT from globe artichoke, used in this study for expression in yeast, demonstrated to produce these esters *in vitro* (Comino et al., 2007). The HCT gene from tobacco has been tested with a range of alternative acceptors (e.g. anthranilate, glucose, malate, tyramine, spermidine, spermine, agmatine), but appeared to be specific for quinate and shikimate (Hoffmann et al., 2003). However, the possibility of using 3-hydroxyanthranilic acid as an acceptor has not been previously tested. In this study we show that the globe artichoke HCT enzyme is active on 3-hydroxyanthranilic acid (Table 2) and, surprisingly, uses the amino-group of this substrate for amide bond formation. It should be noted that the only amino-group accepted as a substrate is the one from 3-hydroxyanthranilic acid, while amino groups of anthranilic acid or 5-hydroxyanthranilic acid are not accepted. Still, it is a surprising observation that HCT can transfer hydroxycinnamic acids to both amino and alcohol groups. Some subfamilies of the BAHD family of acyltransferases are known to catalyze formation of amide bonds, but not ester formation. These comprise the oat AsHHT enzyme, involved in synthesis of avenanthramides (Yang et al., 2004), potato tyramine hydroxycinnamoyltransferase (THT) (Hohlfeld et al., 1995; Negrel and Javelle, 1997), pepper serotonin N-hydroxycinnamoyltransferase (Kang et al., 2006; Kang and Back, 2009), putrescine N-hydroxycinnamoyl transferase (Negrel et al., 1992) and the barley ACT enzyme, involved in synthesis of agmatine (Burhenne et al., 2003). None of these enzymes is known to catalyze both ester and amide-bond formation. Notably, the oat AsHHT enzyme is relatively closely related to the *Arabidopsis* and tobacco HCT genes (D'Auria, 2006), which would be in keeping with a tendency of HCT genes to facilitate amide bond formation, alongside with regular ester formation.

4.3 Substrates for phenolic compounds in yeast

This work indicates that yeast is a suitable non-plant system for the production of phenolic esters.

Advantages of *Saccharomyces cerevisiae* over other microbial hosts include its food-grade status, the extensive knowledge on its large scale production and genomic structure (Giaever et al., 2002; Forster et al., 2003), and its suitability to express plant genes such as cytochrome P450 monooxygenases (Pompon et al., 1996; Jiang and Morgan, 2004; Leonard et al., 2006), many of which are involved in biosynthesis of plant phenolic compounds.

The production of phenolic esters and amides by yeast has not been described before. It involves the coupling of exogenously provided phenolic esters to precursors of phenylalanine, which are recruited from the yeast metabolism. Also biosynthesis of flavonoids and stilbenes in yeast involves recruitment of an intermediate from the yeast metabolism, malonyl CoA (Beekwilder et al., 2006). However, so far this has not lead to significant synthesis of unexpected side products, likely because no molecules closely related to malonyl CoA are both available in yeast and acceptable to the recombinant enzymes. In the case of HCT there appear to be two endogenous yeast substrates, shikimic acid and 3-hydroxyanthranilic acid. The ratio of the two different metabolites formed (about 10 fold less *p*-coumaroyl shikimate than N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid, Fig. 2) could partly be explained by the turn-over rate of the HCT enzyme, as V_{max}/K_m values were four times higher for hydroxyanthranilic acid than for shikimic acid (Table 2). However, this difference could also relate to the metabolic environment of the HCT enzyme in yeast. Firstly, shikimic acid occurs in yeast as part of the pathway leading to aromatic aminoacids, such as phenylalanine, tyrosine and tryptophan (Duncan et al., 1987). Its synthesis is mediated by the *arom* protein, which is a multifunctional enzyme catalysing five consecutive steps in the shikimate pathway. Expression of the *arom* enzyme is regulated by the well characterised *S. cerevisiae* 'general control' mechanism, in response to amino-acid limitation (Duncan et al., 1987). Secondly, hydroxyanthranilic acid is an intermediate in the formation nicotinamide adenine dinucleotide (NAD) from tryptophan. In *S. cerevisiae* it is secreted into the culture medium when it has been supplied with an excess of tryptophane (Shetty and Gaertner, 1973). Apparently hydroxyanthranilic acid functions as a sink for tryptophan, when sufficient NAD is available in the cells. Therefore,

differences in availability of shikimic acid and hydroxyanthranilic acid for esterification with hydroxycinnamic acid-CoA esters could be explained by the presence of excess of aromatic amino-acids in the (rich) culture medium. As a consequence, the yield of *p*-coumaroyl shikimate and N-(*E*)-*p*-coumaroyl-3-hydroxyanthranilic acid may be influenced by the concentration of aromatic amino acids in the medium. This would offer the opportunity to tune production of either compounds. Our future efforts will be aimed at testing fermentation in a poor yeast medium, without aromatic amino-acids, to detect any change in the ratio *p*-coumaroyl shikimate / YAv change and to evaluate if the production route of new compounds is more economically feasible.

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Table 1: ¹H-NMR parameters of N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid¹H-NMR (400 MHz, CD₃OD; 500 MHz, DMSO-d₆ + TFA)

Solvent Position	CD ₃ OD		DMSO-d ₆ + TFA	
	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)
<i>p</i> -Coumaric acid part				
H2', H6'	7.51	8.5	7.48	8.6
H3', H5'	6.83	8.5	6.82	8.6
H7'	7.68	15.2	7.46	15.6
H8'	6.69	15.2	6.78	15.6
anthranilic acid part				
H4	7.15	8.1, 1.9	7.09	8.1, 1.7
H5	7.18	8.1, 7.2	7.13	8.1, 7.6
H6	7.61	7.2, 1.9	7.26	7.6, 1.7
NH	-	-	9.91	

Table 2: Kinetic properties of recombinant HCT

Kinetic parameters of recombinant HCT with 3-hydroxyanthranilic acid and shikimic acid as acceptors in presence of *p*-coumaroyl-CoA as acyl donor. The standard deviation for K_m and V_{max} values indicate was calculated from three different replicates.

Varying substrate	Saturating substrate	K_m (mM)	V_{max} (pKat/mg)	V_{max}/K_m (pKat/mg/mM)
Shikimic acid	<i>p</i> -coumaroyl-CoA	1.44 ± 0.3	0.44 ± 0.004	0.3
3-Hydroxyanthranilic acid	<i>p</i> -coumaroyl-CoA	1.64 ± 0.9	1.95 ± 0.15	1.18

FIGURE LEGENDS

Fig. 1. Biosynthetic pathway of phenolic acids in plant.

Enzymes involved in the biosynthesis are 4CL (4-coumarate:CoA-ligase) and HCT (hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase). The pathway starts from either *p*-coumaric acid or caffeic acid.

Fig. 2. Production of phenolic compounds by recombinant yeast

An aliquot of yeast culture expressing 4CL and HCT genes was analyzed after 72 hr growth with *p*-coumaric acid (A) or caffeic acid (B) by means of HPLC-PDA analysis. The chromatograms were recorded at 312 nm. Compounds that are novel with respect to the control are indicated, with their retention time and mass spectrum.

Fig. 3. Molecular structure of N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid

(A) N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid (YAv) produced by *S. cerevisiae* 4CL/HCT. Numbers of the carbon atoms are indicated. (B) Observed HMBC interactions. For the 2'/6' and 3'/5' H's and C's only one of two identical interactions is indicated.

Fig. 4. Antioxidant activity of N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid

(A) HPLC analysis of antioxidant power of new compounds (YAv and YAvII) produced in CEN.PK 4CL-HCT, compared to avenanthramide A (AvA) and B (AvB). The chromatograms were recorded at 412 nm, after reaction with ABTS^{•+}. Yeast avenanthramide (YAv) corresponds to N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid observed in the incubation with *p*-coumaric acid, while Yeast avenanthramide II (YAvII) is the compound produced in the incubation with caffeic acid.

(B-C) MEF cells were treated over-night with different concentrations of yeast avenanthramide (YAv) or DMSO (CTR) in complete culture medium. After incubation with DCFH-DA, cells were analyzed by FACS to quantify the steady-state levels of intracellular ROS. A quantitative graph of the mean fluorescence intensity values (expressed as percents unit of the control fluorescence, ± SD) (panel B) and a FACS profile (panel C) representative of three independent experiments are shown. Notice that both 200 and 300 μM yeast avenanthramide (YAv) treatment caused a significant reduction of intracellular ROS levels (*P<0.01).

Fig. 5. Comparison between the novel compounds and avenanthramide A and C.

The novel avenanthramide-like compound in 4CL-HCT yeast (YAv) corresponds to N-(E)-*p*-coumaroyl-3-hydroxyanthranilic acid observed upon incubation with *p*-coumaric acid, while Yeast avenanthramide (YAvII) is the hypothetical structure of the compound produced upon incubation with caffeic acid. The structures of the newly formed compounds differ from avenanthramides in the position of the hydroxyl group, relative to the amide bond (as indicated by arrows).

Figure 1

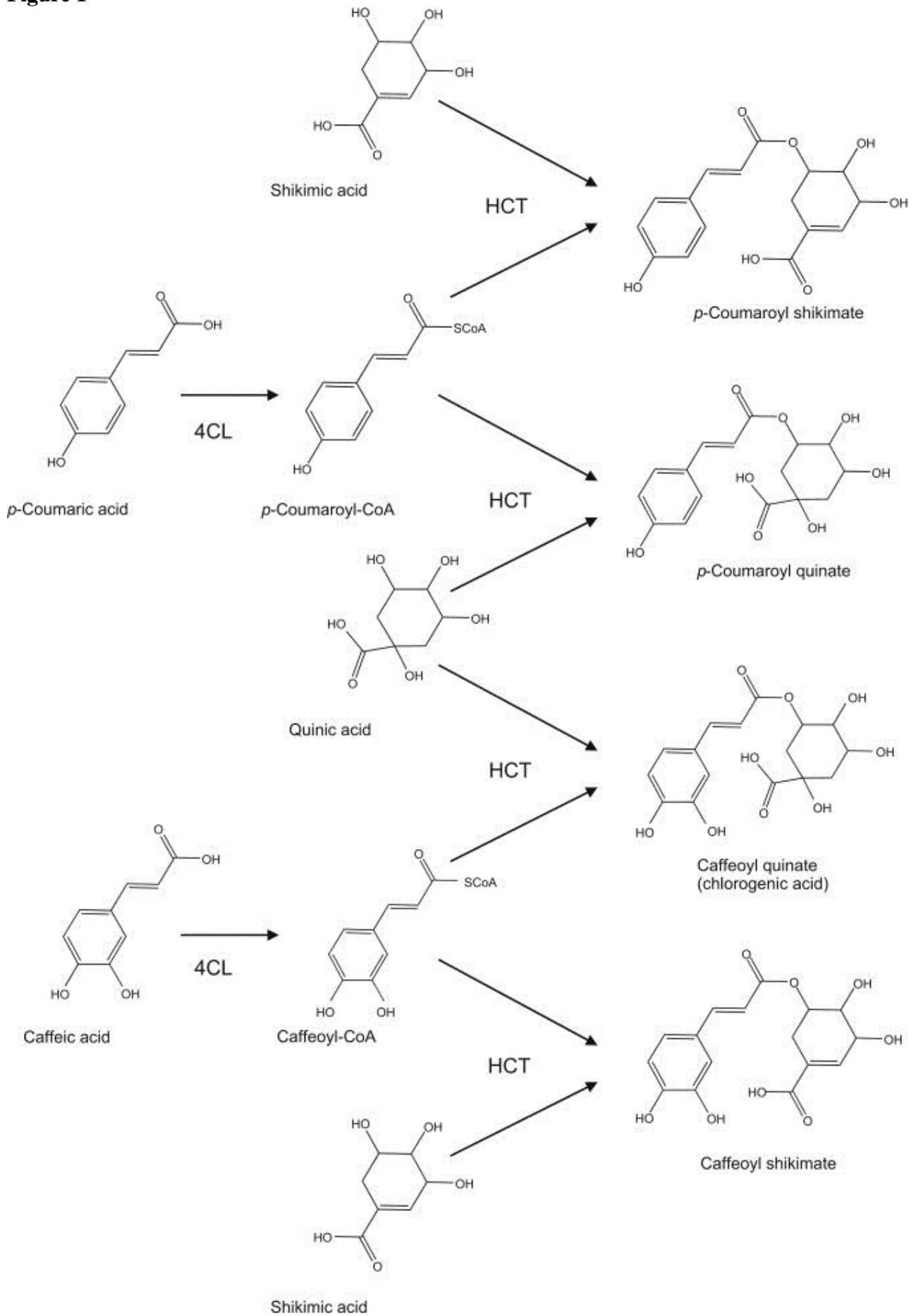


Figure 2

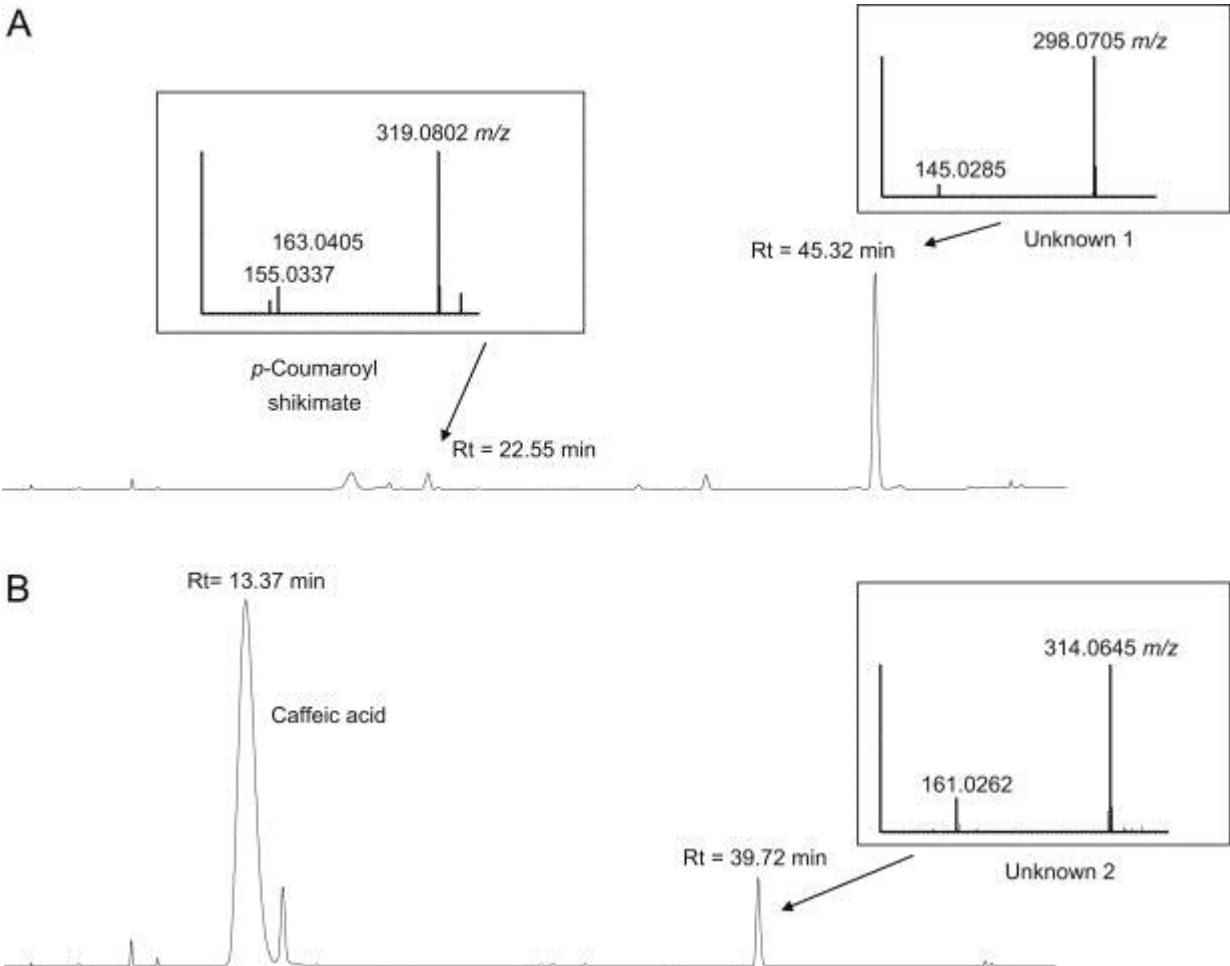
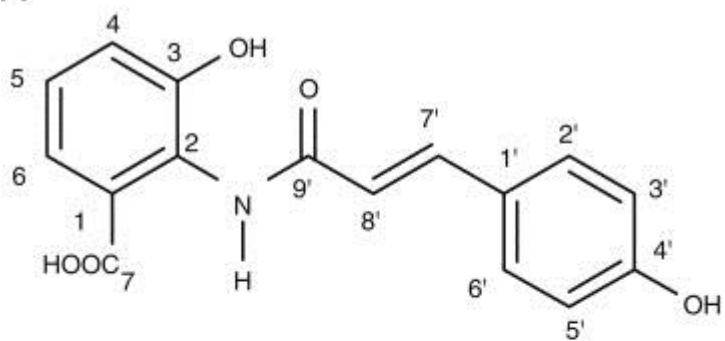


Figure 3

A



B

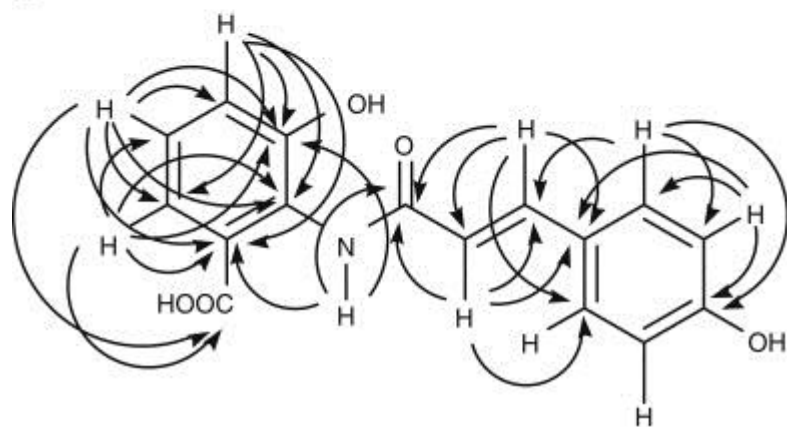


Figure 4

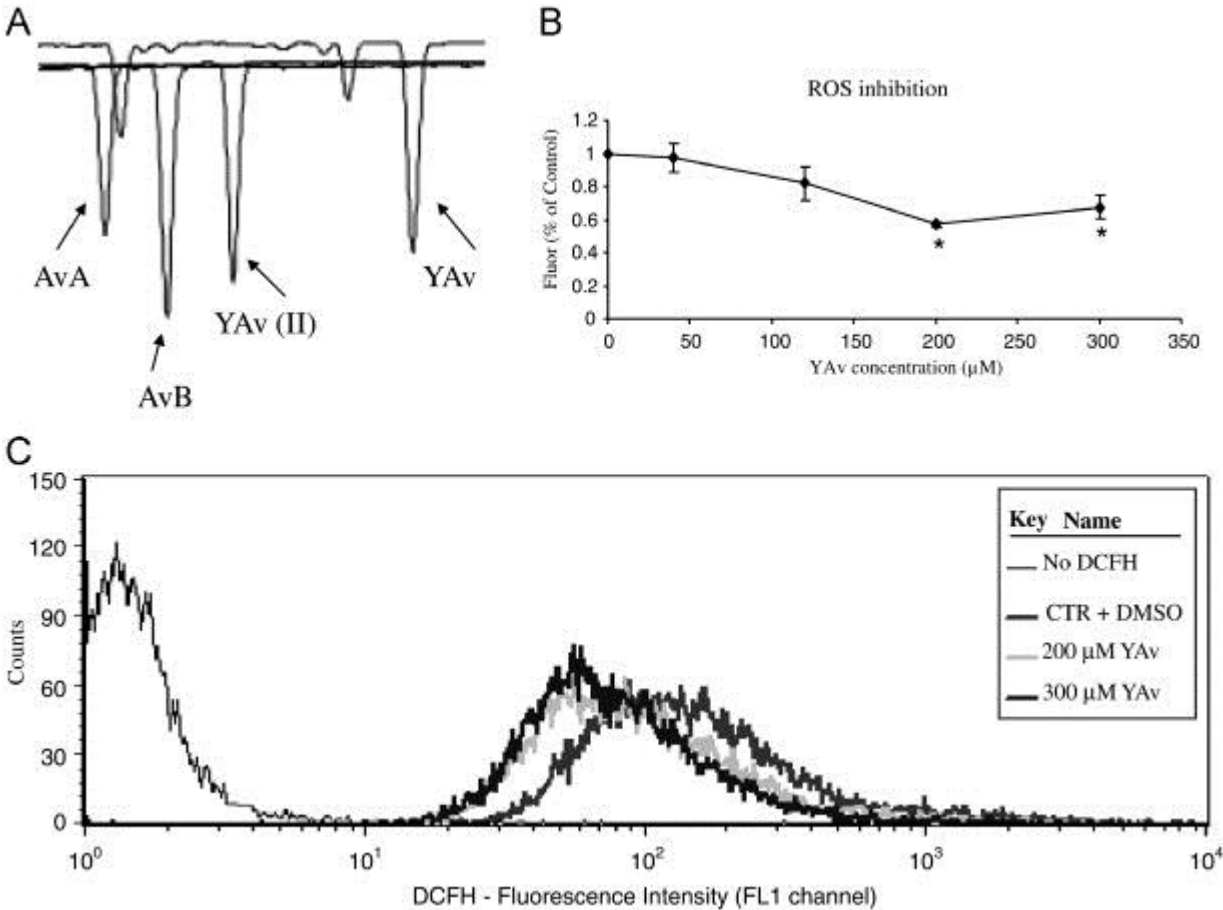


Figure 5

