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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.6b04074 • Publication Date (Web): 19 Nov 2016

Hydroxycinnamoyl Glucose and Tartrate Esters and Their Role in the Formation of Ethylphenols in Wine

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ABSTRACT

Synthesized *p*-coumaroyl and feruloyl *L*-tartrate esters were submitted to *Brettanomyces bruxellensis* strains AWRI 1499, 1608 and 1613 to assess their role as precursors to ethylphenols in wine. No evolution of ethylphenols was observed. Additionally, *p*-coumaroyl and feruloyl glucose were synthesized and submitted to *B. bruxellensis* AWRI 1499 which yielded both 4-ethylphenol and 4-ethylguaiacol. Unexpected chemical transformations of the hydroxycinnamoyl glucose esters during preparation were investigated to prevent these in subsequent synthetic attempts. Photoisomerization gave an isomeric mixture containing the *trans*-esters and undesired *cis*-esters, and acyl migration resulted in a mixture of the desired 1-*O*-β-ester and two additional migrated forms, the 2-*O*-α- and 6-*O*-α-esters. Theoretical studies indicated that the photoisomerization was facilitated by deprotonation of the phenol, and acyl migration is favored during acidic, non-aqueous handling. Preliminary LC-MS/MS studies observed the migrated hydroxycinnamoyl glucose esters in wine, and allowed for identification of feruloyl glucose in red wine for the first time.

KEYWORDS: ***Brettanomyces,*** **hydroxycinnamate ester, ethylphenol, photoisomerization, acyl migration.**

INTRODUCTION

The formation of 4-ethylphenol (**7**) and 4-ethylguaiacol (**8**) in red wine has previously been linked to the breakdown of free hydroxycinnamic acids (**3** and **4**) by *Brettanomyces* yeast.[1] Recently, we have shown that some commonly isolated strains of *B. bruxellensis* from Australia also possess the ability to convert ethyl hydroxycinnamates (**1** and **2**) to ethylphenols to varying extents (Figure 1),[2] and that these esters can therefore contribute to the undesirable aromas and flavors associated with wines effected by *Brettanomyces*. Presumably, this occurs via esterase mediated release of hydroxycinnamic acids from the ethyl esters and this supposition has lead us to investigate the possibility that other hydroxycinnamoyl esters present during wine maturation could be metabolized by *B. bruxellensis* and contribute to the accumulation of ethylphenols in red wine.

Hydroxycinnamoyl *L*-tartaric acid esters, including *p*-coumaroyl and feruloyl *L*-tartrate (**9** and **10**), have been quantified during the vinification process,[3, 4] and concentration of the tartrates in wine has been shown to be greatly reduced from that of the initial must concentrations with most of this loss seen during vinification, and a smaller amount lost during the ageing process.[4, 5] The decrease in tartrate concentration during winemaking has been linked to fermentation and the yeast *Saccharomyces cerevisiae*,[6] malolactic fermentation and the bacteria *Oenococcus oeni*,[7] as well as wine ageing,[5] and commercial enzyme preparations added to must.[8, 9] One study linked a decrease in tartrate esters to the yeast genus *Brettanomyces* but did not correlate these loses with an increase in volatile phenol production,[10] while another implied that *Brettanomyces* is unable to metabolize the tartrate esters of hydroxycinnamic acids.[11] Model fermentation experiments with synthesized hydroxycinnamoyl tartrates would definitively assess the ability of *Brettanomyces* to produce ethylphenols from the tartrate esters.

In addition to the tartrate esters, the existence of *p*-coumaroyl and feruloyl glucose esters (**11** and **12**) was confirmed in white grapes[12] and Riesling wine,[13] followed by the identification of *p*-coumaroyl glucose esters in red wines.[14] The identification of two separate *p*-coumaroyl glucose esters in several red wines was achieved via liquid-liquid extraction,[15] with the authors presuming

that the two esters differed by the position of attachment to glucose. In subsequent quantification of these two esters over 26 months of ageing in three wines the glucose ester concentration was in the range of 0.1-1.0 mg/L and only fluctuated slightly over time,[14] indicating that the glucose esters are stable over long periods of storage. Another group, using a similar extraction procedure identified two *p*-coumaroyl hexoses with initial concentrations of 1.23 and 1.51 mg/L, increasing throughout malolactic fermentation.[7] Subsequent studies have also identified two *p*-coumaroyl glucose esters in red grapes or wine, without providing further structural information.[16, 17] To date there is no study linking hydroxycinnamoyl glucose esters to metabolism by *Brettanomyces*.

Hydroxycinnamates can undergo photoisomerization via ultra-violet radiation which is dependent on the substrate, solvent, pH, wavelength of incident light and is susceptible to concentration effects.[18-21] The *cis/trans*-isomerization of *p*-coumaric acid, and related derivatives, has been the subject of numerous theoretical studies,[22-27] but the isomerization, and even the different isomers of the hydroxycinnamoyl glucose esters are yet to be studied experimentally or theoretically. The stereochemistry of hydroxycinnamates is of importance in the accumulation of ethylphenols as the decarboxylase of *B. bruxellensis* was recently observed to only be active towards the *trans*-hydroxycinnamic acids.[28]

Additionally, acyl migrations, or intra-molecular transesterifications, within glucose derivatives are widely known. Examples of migrations around glucose rings by small or simple moieties have been observed for decades,[29, 30] and can yield different outcomes under changing conditions,[31] including catalysis by silica.[32] The products and final outcomes of acyl migrations are determined by thermodynamic influences,[33] the speed at which these products can be formed are governed by mechanistic restraints such as the ring-size of the intermediates formed[34] and the electronic characteristics of the esters involved.[35] Although acyl migration has not previously been described for hydroxycinnamoyl glucose esters in these mechanistic studies,[33, 36] they provide a starting point for examining the possibility that multiple glucose esters previously observed in wine extracts could be products of acyl migration.

This paper details the synthesis of hydroxycinnamoyl tartrate and glucose esters, and role of both classes of hydroxycinnamoyl ester as potential precursors to ethylphenols was examined by submitting them to fermentation experiments with *B. bruxellensis*. Computational studies into the observed photoisomerization and acyl migrations during synthesis of the hydroxycinnamoyl glucose esters, and preliminary studies into the presence of migrated forms of the glucose esters in wine were both undertaken to prevent these unwanted transformations in future synthetic attempts.

MATERIALS AND METHODS

Chemicals. Dry organic solvents were purchased and dispensed using a Puresolv™ solvent purification system (*Innovative Technologies, Massachusetts, USA*). General organic solvents were obtained (*Chemsupply, Adelaide, Australia)* and distilled where needed; other chemicals were purchased (*Sigma-Aldrich Chemical Company, Castle Hill, Australia)*.

General. Reverse-phase C18 chromatography was performed using pre-packed cartridges designed for use with automated flash chromatography systems (*Teledyne Isco, Nebraska, USA*). The cartridge was loaded with compound dissolved in a minimal volume of methanol, before being washed with water, eluted with acetonitrile/water/formic acid (30:69:1), flushed with acetonitrile/formic acid (99:1), and re-equilibrated with water. The ^{1}H NMR spectra were acquired with a Bruker Ultrashield Plus 400 MHz or 600 MHz spectrometer as where specified.

Synthesis of substrates and standards. Detailed synthetic preparations and additional characterization data can be found in the supporting information.

*1-O-Chloroacetyl hydroxycinnamates (**13** and **14**).* Preparation of **13** and **14** were achieved using a literature procedure.[37] Characterization data of **13** was as previously described,[37] NMR assignment for **14** was made based on **13**.

Tartrate coupling reactions. Synthesis of di-tert-butyl *L*-tartrate (**18**) from *L*-tartaric acid was achieved via *O,O'*-diacetyl *L*-tartaric anhydride (**15**), *O,O'*-diacetyl *L*-tartaric acid (**16**) and *O,O'*-diacetyl-di-*tert*-butyl *L*-tartrate (**17**) based on literature procedures.[38-42] Characterization data

matched literature reports for **15**,[40] **16**,[40] **17**[42] and **18**.[42] Coupling reactions of chloroacetyl hydroxycinnamate (**13** or **14**) with **18** to give **19** or **21**were achieved in an analogous fashion to previous reports.[43]

*p-Coumaroyl di-tert-butyl L-tartrate (**19**).* ^{1}H NMR: (400 MHz, $CDCl_3$) δ: 7.60 (d, 1H, J = 15.9 Hz, H_7), 7.29 (app. d, 2H, J = 8.6 Hz, $H_{3,5}$), 6.84 (app. d, 2H, J = 8.6 Hz, $H_{2,6}$), 6.18 (d, 1H, J = 15.9 Hz, H_8), 5.51 (d, 1H, J = 2.3 Hz, $H_{2'}$), 4.68 (d, 1H, J = 2.3 Hz, $H_{3'}$), 3.34 (br. s, 1H, OH), 1.52 (s, 9H, *t*-Bu_4), 1.44 (s, 9H, *t*-Bu_1).

*Feruloyl di-tert-butyl L-tartrate (**21**).* ^{1}H NMR: (600 MHz, $CDCl_3$) δ: 7.68 (d, 1H, J = 15.9 Hz, H_7), 7.07 (dd, 1H, J = 8.1 and 1.8 Hz, H_5), 7.04 (d, 1H, J = 1.8 Hz, H_3), 6.92 (d, 1H, J = 8.1 Hz, H_6), 6.35 (d, 1H, J = 15.9 Hz, H_8), 5.50 (d, 1H, J = 2.3 Hz, $H_{2'}$), 4.67 (dd, 1H, J = 6.9 and 2.3 Hz, $H_{3'}$), 3.93 (s, 3H, OCH_3), 3.20 (d, 1H, J = 6.9 Hz, OH), 1.51 (s, 9H, *t*-Bu_4), 1.44 (s, 9H, *t*-Bu_1).

*p-Coumaroyl L-tartrate (**9**).* *p*-Coumaroyl *tert*-butyl *L*-tartrate (**19**) (46.2 mg, 0.11 mmol) was dissolved in dry dichloromethane (5 mL) followed by the addition of trifluoroacetic acid (0.18 mL, 2.29 mmol) and the mixture was stirred at room temperature under a nitrogen atmosphere for 24 hours before the solvent was removed in vacuo. Purification by reversed-phase chromatography on C18 gave **9** (27.5 mg, 82%) as an amorphous solid. R_f (20% MeOH/CH_2Cl_2): 0.00. ^{1}H NMR: (400 MHz, CD_3OD) δ: 7.74 (d, 1H, J = 15.9 Hz, H_7), 7.48 (app. d, 2H, J = 8.7 Hz, $H_{3,5}$), 6.81 (app. d, 2H, J = 8.7 Hz, $H_{2,6}$), 6.38 (d, 1H, J = 15.9 Hz, H_8), 5.55 (d, 1H, J = 2.3 Hz, $H_{2'}$), 4.77 (d, 1H, J = 2.3 Hz, $H_{3'}$). ^{13}C NMR spectral properties were as previously described.[44]

*Feruloyl L-tartrate (**10**).* Feruloyl *tert*-butyl *L*-tartrate (**21**) (35.9 mg, 0.082 mmol) was submitted to the same reaction conditions as described for **19** (above). This gave **10** (11.0 mg, 41%) as an off-white amorphous solid. R_f (20% MeOH/CH_2Cl_2): 0.00. ^{1}H NMR: (400 MHz, CD_3OD) δ: 7.73 (d, 1H, J = 16.0 Hz, H_7), 7.20 (d, 1H, J = 1.9 Hz, H_3), 7.10 (dd, 1H, J = 8.2 and 1.9 Hz, H_5), 6.82 (d, 1H, J = 8.2 Hz, H_6), 6.41 (d, 1H, J = 16.0 Hz, H_8), 5.57 (d, 1H, J = 2.4 Hz, $H_{2'}$), 4.78 (d, 1H, J = 2.4 Hz, $H_{3'}$), 3.89 (s, 3H, OCH_3). ^{13}C NMR spectral properties were as previously reported.[44, 45]

Glycosylation reactions. The preparation of 2,3,4,6-tetra-*O*-chloroacetyl-α-D-glucopyranosyltrichloroacetimidate (**25**) from *D*-glucose and the method of glycosylation were as previously reported.[46] The characterization data for the product and intermediates (**23**-**25**) were analogous to previous reports although anomeric ratios tended to vary.[37, 46, 47]

*2,3,4,6-Tetra-O-chloroacetyl-β-D-glucopyranosyl 1-O-chloroacetyl coumarate (**26**).* From **13** (210.6 mg, 0.88 mmol), gave 0.30 g (48%) of **26** as a white honeycomb. ^{1}H NMR: (400 MHz, $CDCl_3$) δ: 7.73 (d, 1H, J = 16.0 Hz, H_7), 7.58 (app. d, 2H, J = 8.7 Hz, $H_{3,5}$), 7.20 (app. d, 2H, J = 8.7 Hz, $H_{2,6}$), 6.37 (d, 1H, J = 16.0 Hz, H_8), 5.90 (d, 1H, J = 8.2 Hz, $H_{1'}$), 5.44 (dd, 1H, J = 9.6 and 9.5 Hz, $H_{3'}$), 5.33 (dd, 1H, J = 9.5 and 8.2 Hz, $H_{2'}$), 5.26 (dd, 1H, J = 9.7 and 9.6 Hz, $H_{4'}$), 4.42 (dd, 1H, J = 12.5 and 4.3 Hz, $H_{6a'}$), 4.33 (m, 3H, $ArOCOCH_2Cl$ and $H_{6b'}$), 4.12 (app. s, 2H, OCH_2Cl), 4.04-4.01 (m, 7H, 3 x OCH_2Cl and $H_{5'}$).

*2,3,4,6-Tetra-O-chloroacetyl-β-D-glucopyranosyl 1-O-chloroacetyl ferulate (**27**).* From **14** (224.0 mg, 0.83 mmol), afforded 0.38 g (64%) of **27** as a pale-yellow honeycomb. NMR and mass spectral properties were as previously reported.[46]

General procedure for de-chloroacetylation (causing cis/trans-isomerization). 2,3,4,6-Tetra-*O*-chloroacetyl-*β-D*-glucopyranosyl hydroxycinnamate (**26** or **27**) (100.0 mg) was dissolved in pyridine/water (1:1, 10 mL) and stirred at room temperature for 6 hours. The reaction mixture was concentrated and the crude mixture purified using XAD-8 resin (eluted with 60% MeOH/H_2O) to give a mixture of *cis*- and *trans-β-D*-glucopyranosyl hydroxycinnamate (**11** or **12**) as a colorless residue. The spectrum of the *cis*-isomer was extracted from the mixture. See below for characterization data for the *trans*-isomers.

cis-p-Coumaroyl glucose. ^{1}H NMR: (400 MHz, CD_3OD) δ: 7.73 (m, 2H, $H_{3,5}$), 6.94 (d, 1H, J = 12.9 Hz, H_7), 6.82 (app. d, 2H, J = 8.8 Hz, $H_{2,6}$), 5.82 (d, 1H, J = 12.9 Hz, H_8), 5.55 (d, 1H, J = 8.0 Hz, $H_{1'}$), 3.85 (m, 1H, $H_{6a'}$), 3.68 (m, 1H, $H_{6b'}$), 3.47-3.32 (m, 4H, $H_{2', 3', 4', 5'}$). Assignment and identification of the *cis*-isomer was performed using the known *trans*-isomer (*trans*-**11**) and the data for the *cis*-aglycone (*cis*-**3**).[48, 49]

cis-Feruloyl glucose. ^{1}H NMR: (400 MHz, CD_3OD) δ: 7.87 (d, 1H, J = 1.9 Hz, H_3), 7.17 (dd, 1H, J = 8.3 and 1.9 Hz, H_5), 6.94 (d, 1H, J = 13.0 Hz, H_7), 6.77 (d, 1H, J = 8.3 Hz, H_6), 5.83 (d, 1H, J = 13.0 Hz, H_8), 5.56 (d, 1H, J = 7.8 Hz, $H_{1'}$), 3.88 (s, 3H, OCH_3), 3.88-3.84 (m, 1H, $H_{6a'}$), 3.72-3.66 (m, 1H, $H_{6b'}$), 3.49-3.35 (m, 4H, $H_{2',3',4',5'}$). Assignment and identification of the *cis*-isomer was performed using the known *trans*-isomer (*trans*-**12**) and the data for the *cis*-aglycone (*cis*-**4**).[48, 50]

General procedure for de-chloroacetylation (causing migration). 2,3,4,6-Tetra-*O*-chloroacetyl-*β*-*D*-glucopyranosyl hydroxycinnamate (**26** and **27**) (300.0 mg) was dissolved in pyridine/water (1:1, 20 mL) and stirred at room temperature in the dark for 6 hours. Only being exposed to red light, the reaction mixture was concentrated and the crude mixture purified using column chromatography (10% MeOH/CH_2Cl_2) to give migrated mixtures of the *trans*-*β*-*D*-glucopyranosyl hydroxycinnamate, but largely consisting of the 1-*O*-*β*-ester (approx. 80% for **11** and 90% for **12**). The migrated mixture was found to revert back to the 1-*O*-*β*-ester after standing in buffered water at wine pH (3.5).

*p-Coumaroyl glucose (**11**).* From **26** (261.7 mg, 0.37 mmol), gave 51.5 mg (43%) of **11** as a white residue. R_f (20 % MeOH/CH_2Cl_2): 0.29. MS (-EI) *m/z* (*%*): 325.7 (M^-, *100*), 265.5 (*7*), 187.7 (*8*), 163.4 (*21*), 145.2 (*44*). ^{1}H NMR: (400 MHz, CD_3OD) δ: 7.73 (d, 1H, J = 15.9 Hz, H_7), 7.48 (app. d, 2H, J = 8.5 Hz, $H_{3,5}$), 6.82 (app. d, 2H, J = 8.5 Hz, $H_{2,6}$), 6.37 (d, 1H, J = 15.9 Hz, H_8), 5.57 (d, 1H, J = 7.9 Hz, $H_{1'}$), 3.85 (dd, 1H, J = 12.1 and 1.8 Hz, $H_{6a'}$), 3.69 (dd, 1H, J = 12.1 and 4.6 Hz, $H_{6b'}$), 3.45-3.38 (m, 4H, $H_{2',3',4',5'}$). ^{13}C NMR spectral properties for the 1-*O*-*β*-ester were as previously reported.[13]

*Feruloyl glucose (**12**).* From **27** (501.8 mg, 0.68 mmol), 47.5 mg (20%) of **12** as an off-white residue. R_f (20% MeOH/CH_2Cl_2): 0.32. MS (-EI) *m/z* (*%*): 355.3 (M^-, *100*), 295.5 (*8*), 217.2 (*20*), 193.6 (*25*), 175.4 (*32*). ^{1}H NMR: (400 MHz, CD_3OD) δ: 7.73 (d, 1H, J = 15.9 Hz, H_7), 7.21 (d, 1H, J = 1.9 Hz, H_3), 7.10 (dd, 1H, J = 8.2 and 1.9 Hz, H_5), 6.82 (d, 1H, J = 8.2 Hz, H_6), 6.41 (d, 1H, J = 15.9 Hz, H_8), 5.58 (d, 1H, J = 7.5 Hz, $H_{1'}$), 3.90 (s, 3H, OCH_3), 3.86 (dd, 1H, J = 12.1 Hz,

174 $H_{6a'}$), 3.70 (dd, 1H, J = 12.1 and 4.5 Hz, $H_{6b'}$), 3.49-3.35 (m, 4H, $H_{2', 3', 4', 5'}$). ^{13}C NMR spectral
175 properties for the 1-*O*-*β*-ester were as previously reported.[13]

176 *Migrated glucose esters.* ^{1}H NMR shifts of migrated glucose esters were partially
177 determined for glucose proton shifts that were significantly different from those of the 1-*O*-*β*-ester,
178 with assignments made using coupling patterns and changes in chemical shifts analogous to
179 literature data.[51]

180 **Computational studies.** Theoretical calculations were performed using the Spartan '08
181 package (*Wavefunction Inc., California, USA*) with geometry optimizations and final energies
182 determined using the DFT B3LYP 6-31G* basis set. Full experimental conditions for the
183 computational studies can be found in the supporting information.

184 **Wine samples for analysis.** One white wine (Stanley Classic Dry White) and one red wine
185 (Yalumba 1997 Shiraz) were used throughout the study. Concentrated wine samples were prepared
186 from 50 mL of wine at 30 ^{o}C under reduced pressure until the volume had reduced to 5 mL.
187 Unconcentrated and concentrated wine samples were passed through a 45 μm syringe filter and
188 analyzed directly. Standards of *p*-coumaroyl glucose (**11**) and feruloyl glucose (**12**) were prepared
189 (in methanol, 10 mg/L) to determine retention times and obtain reference mass spectral data.

190 **HPLC-MS analysis of wine samples.** HPLC-MS or MS/MS analysis was carried out using
191 a 4000 Q TRAP hybrid tandem mass spectrometer interfaced with a Turbo V ion source for
192 elecrospray ionization (AB Sciex AB Sciex, Foster City, CA), combined with an Agilent 1200
193 HPLC system equipped with a binary pump, degasser, autosampler, column oven, and photodiode
194 array (PDA) detector.

195 **HPLC conditions.** A 10 μL aliquot of the samples was injected and chromatographed using
196 a 250 x 4.6 mm, 3 μm, 100 Å Luna C18 column, operated at 25 ºC and protected by a C18 guard
197 column (4 x 2 mm) (Phenomenex, Lane Cove, NSW, Australia). The eluents were formic
198 acid/water (0.5:99.5 v/v, Eluent A) and formic acid/acetonitrile/water (0.5:25.0:74.5 v/v, Eluent B)
199 with a flow rate of 1 mL/min. A gradient was applied as follows: 20% to 30% B linear from 0 to 20

minutes; 30% to 50% B linear from 20 to 50 minutes; 50% B to 100% B linear from 50 to 60 minutes; 100% B to 20% B from 60 to 65 minutes. The column was equilibrated with 20% B for 10 minutes prior to an injection. The eluent from the HPLC was split by use of a splitter (a tee) and delivered at a follow rate of 0.45 mL/min to the mass spectrometer and at 0.55 mL/min to the PDA detector with monitoring wavelengths at 290, 320 and 370 nm with a slit width of 4 and a bandwidth of 16 nm.

Electrospray and mass spectrometric conditions. All mass spectrometric data were obtained in negative ion mode. Nitrogen gas was used for the curtain, nebulizer, turbo and collision gases. The Turbo V ion source parameter were set at -3500 V for the ion spray potential, -60 V for the declustering potential, -10 V for the entrance potential, 50 psi for gas 1 (nebulizer) and gas 2 (turbo), 15 psi for the curtain gas, and 500 °C for the turbo gas (gas 2) temperature.

For tandem mass spectrometry, the collision potential was set in an appropriate range from -15 to -25 V and the collision gas pressure was set at high. Product ion spectra of *m/z* 325 for *p*-coumaroyl glucose and *m/z* 355 for feruloyl glucose were recorded in a mass range from *m/z* 50 to 400 with a scan time of 1 s and a step mass of 0.1. For multiple reaction monitoring (HPLC-MRM), the following mass transitions were monitored with a dwell time of 50 ms; *m/z* 325→119, 145, 163 and 187 for *p*-coumaroyl glucose, and *m/z* 355→119, 175, 193 and 217 for feruloyl glucose.

Yeast and growth/Fermentation experiments/Ethylphenol analyses. These were as previously described.[2] Briefly, starter cultures of *B. bruxellensis* were prepared in YPD (Yeast Peptone Dextrose). Model fermentation experiments were performed in triplicate in YNB (Yeast Nitrogen Base) media (supplemented with 20 g/L glucose, pH 3.5). Ferments were spiked with either **9** and **10** or **11** and **12** at 10 mg/L, inoculated with a single strain of *B. bruxellensis* and incubated at 28 ^{o}C. Control experiments were run concurrently, performed under analogous conditions without yeast inoculation. Samples (5 mL) were collected every second day, centrifuged (4000 r.p.m. for 5 mins) and the supernatant decanted from the yeast pellet and stored at -20 ^{o}C until required for analysis. Ethylphenol analysis was performed as previously described.[52]

RESULTS AND DISCUSSION

Synthesis of hydroxycinnamoyl tartrate esters (Figure 2). Preparation of di-*tert*-butyl tartrate (**18**) from *L*-tartaric acid was achieved as shown. The acid chlorides of **13** and **14** were formed, concentrated and used directly, being added drop-wise in dry hexane to **18** in dry pyridine,[43] which for short reaction times gave the chloroacetylated products (**20** or **22**) with minor dechloroacetylation. However, longer exposure to pyridine gave further deprotection to give mainly the desired products (**19** or **21**), but always with some intermediate **20** or **22**, that could be isolated and deprotected further. Hydrolysis of the *tert*-butyl esters and purification with reverse-phase chromatography yielded pure hydroxycinnamoyl tartrate esters (**9** or **10**).

Synthesis of hydroxycinnamoyl glucose esters (Figure 3). The synthesis of *p*-coumaroyl and feruloyl glucose was based on the glucosyl donor (**25**) used previously to prepare hydroxycinnamoyl glucose esters,[37, 47] and the synthetic pathway has since been validated in preparation of feruloyl and sinapoyl glucose.[46]

Glycosylation between the glycosyl donor, **25**, and acids (**13** or **14**) gave penta-chloroacetyl protected esters **26** or **27**. Removal of the chloroacetyl groups gave a mixture of the desired products (**11** or **12**) and 6-*O*-chloroacetyl glucopyranosyl hydroxycinnamates (**28** or **29**). Under ambient light conditions an initial attempt to separate the product resulted in rapid photoisomerization to give *cis*-**11** or **12**, which could be controlled by handling in the dark, or under red light. Using a modified purification method, flash chromatography to remove the mono-protected species, **28** and **29**, using 10% methanol in dichloromethane, and indeed multiple solvent combinations, resulted in acyl migration. This gave a combination of esters mostly consisting of the desired 1-*O*-β-ester as well as significant amounts of 2-*O*-α- and 6-*O*-α-esters and trace amounts of the 2-*O*-β-, 6-*O*-β– and 3-*O*-α/β-esters, which could be partially characterized. In these minor esters the chemical shifts for hydroxycinnamate alkene protons (H_7 and H_8) were moved upfield a negligible amount, and for each positional isomer the glucose proton shifts were identical between

feruloyl and *p*-coumaroyl derivatives. In the mixtures a greater extent of migration for the *p*-coumaroyl ester was observed. It was found that when migrated mixtures were stored under aqueous acidic conditions glucose esters reverted back to the 1-*O*-*β*-esters, which will be detailed below.

***cis/trans*-Isomerization of glucose esters of *p*-coumaric and ferulic acids.** The ease of *cis/trans*-isomerization that occurred in the glucose esters, and comparative lack thereof in other hydroxycinnamates prepared synthetically in this work, led to the investigation of the nature of the isomerization and the factors that influenced this phenomenon in the glucose esters. Initial investigations into excitation to the first excited triplet state (T_1), calculated in a vacuum, gave energy profiles for *p*-coumaric acid and *p*-coumaroyl glucose that explained the propensity for photoisomerization, with the lowest energy conformations possessing a C_6-C_7-C_8-C_9 dihedral angle of approximately 90^o (Figures 4a and 4b). However, the energy differences between the singlet ground state (S_0) and T_1 for the *trans*-isomers (180^o dihedral) for *p*-coumaric acid and *p*-coumaroyl glucose were found to be 229.4 and 226.2 kJ/mol, respectively, and not considered large enough to explain the difference in isomerization away from the *trans*-isomer based on substrate alone.

During the preparation of numerous hydroxycinnamate derivatives, changes in solubility required different compounds to be handled in various solvents, with the glucose esters handled in more polar solvents than the free hydroxycinnamic acid were. To understand the role that solvents may play in hydroxycinnamate isomerization, solvents of differing polarities (expressed as ET_{30})[53] were introduced into the calculations. The vertical excitation energy of the *trans*-substrate, or the energy required to achieve an excited state and facilitate isomerization away from the *trans*-isomers, showed reduction with increasing polarity (Figure 4c). Additionally, the vertical excitation energies determined for *p*-coumaroyl glucose were lower than those for *p*-coumaric acid for all solvents calculated. This trend was also mirrored in the HOMO-LUMO gap for each S_0 species (Figure 4d). The minor changes in vertical excitation energy and HOMO-LUMO gap with solvents of differing polarities, along with minor changes also due to substrate, start to provide an

explanation as to the ease of isomerization observed for the glucose esters, assisting in lowering the energy required to achieve an excited state and hence, isomerization.

The mirrored trends in S_0-T_1 vertical excitation energy and S_0 HOMO-LUMO gap removed the need to calculate the energy of both the S_0 and T_1 state and allowed for rapid determination of energy barriers simply from the HOMO-LUMO gap of the S_0 state. Hence a representation of ease of isomerization for multiple hydroxycinnamates could be rapidly studied by examining the relationship between the HOMO-LUMO gap and structural features. With phenolic deprotonation shown to lower excitation energy in *p*-coumaric acid,[25] as well as pH effecting isomeric ratios in neutral hydroxycinnamates,[18] the structures studied were chosen because of their differing electronic configurations with respect to their ability to become resonance stabilized by movement of electrons from the phenol through the alkene and towards the carboxyl group. This included structures possessing carboxyl anions and phenolic protection that are expected to have very little resonance contribution resulting in an alkene with increased double bond character (Table 1).

Across the 11 structures, the HOMO-LUMO gap correlated with both the charge ratio between oxygens 1 and 3 (r-value = -0.89, $P = 0.0002$) and the alkene double bond length (r-value = -0.9677, $P < 0.0001$), and adequately explained what was observed experimentally. By altering the electron density at the extremities of the molecules (oxygens 1 and 3), the double bond character of the C_7=C_8 alkene was being affected (as shown by double bond length) as was the energy required to excite an electron to the first excited state (HOMO-LUMO gap) and facilitate isomerization. As such, hydroxycinnamate esters that are handled under basic conditions, producing a phenolate anion, will possess a lower HOMO-LUMO gap and a higher propensity to isomerize. Substrate, solvent, pH, and electronic environment influence the energy required for isomerization between hydroxycinnamates, and these results support what was observed experimentally. Environments that contribute to increasing the electron density on the phenolic oxygen, or even diminish electron density at the carboxyl oxygen should be avoided under most lighting conditions.

Migration of glucose esters. To better understand the acyl migration of the glucose esters, to explain the ratios of products observed and to investigate the possibility of this occurring in wine or organic solvent wine extracts, theoretical studies were first performed. Water and dichloromethane were used to study the positional isomers, the most common solvents that the glucose esters experienced, along with ethanol and toluene which were included as examples of polar protic and apolar aprotic solvents. The relative energies of the esters in water (Figure 5) justified the predominance of the 1-*O*-β-esters in aqueous environments, being the thermodynamically most stable, though this is not the case for any of the other solvents studied (Figure 5). In dichloromethane, ethanol and toluene a thermodynamic preference for migration to the 6-*O*-α-ester was observed. The 2-*O*-α-ester was also lower in energy than the 1-*O*-β-ester in dichloromethane and ethanol. For NMR characterization of the glucose esters, analysis in d_4-methanol may have influenced the ratio of esters present.

Experimentally, the extent of migration differed between the two glucose esters with greater amounts of 2-*O*-α- and 6-*O*-α-esters observed in the ^{1}H NMR spectrum of *p*-coumaroyl glucose than for feruloyl glucose, which is not evident based on the thermodynamic calculations. While the exact route to migration is not understood, we have mechanistically investigated the plausible migrations away from the 1-*O*-β-esters to explain the relative extents of migrations observed between the two esters. During synthesis, the migration was thought to be facilitated by silica gel, and it was also reversed in wine-like conditions. Therefore, the mechanism applied here (as described by Horrobin[33]) is that which would occur in aqueous acidic environments, and reflect the possibilities in wine. The key intermediates employed in calculation of the 1-*O*-β- to 2-*O*-β-*p*-coumaroyl glucose migration are shown in Figure 6 (with analogous intermediates used for subsequent migrations) along with relative energies. Although the migrated mixtures contained α-anomers, 6-*O*-α- and 2-*O*-α-esters, migration away from the 1-*O*-β-ester was proposed to have occurred before anomerization due to the mechanistic challenges faced with direct anomerization of the 1-*O*-β- to 1- *O*-α-esters, with previous evidence supporting this.[51]

For both the 1-*O*- to 2-*O*- and 1-*O*- to 6-*O*-migrations were less favored, as energy barriers were increased, in an aqueous environment for both glucose esters when compared with dichloromethane. This supports the rapid migration seen in 10 % methanol/dichloromethane on silica gel (Figure 6). Not only are the 2-*O*-α- and 6-*O*-α-esters thermodynamically favored in dichloromethane (Figure 5), but migration directly to these positions is likely to happen more rapidly also. For a 1-*O*- to 2-*O*-migration, the difference in energy of intermediate 2 in dichloromethane between feruloyl and *p*-coumaroyl (with an energy barrier between intermediates 1 and 2 of 30 kJ/mol for the *p*-coumaroyl and 65 kJ/mol for the feruloyl) describes a kinetically more favorable migration for the *p*-coumaroyl glucose. If the mixtures had not yet reached equilibrium and were not yet representing the thermodynamic products, the energy barriers for migration directly to the 2-*O*-position helps to explain why a greater extent of migration was observed for *p*-coumaroyl glucose (**11**) than in feruloyl glucose (**12**) at the time of characterization.

In studying direct migrations to both the 6-*O*- and 3-*O*-positions a ring-flipped glucose was produced, giving 1-OH, 3-OH and 6-OH on the same side of the ring. As such, the bicyclic intermediate 2 in the 1-*O*- to 3-*O*-migration is stabilized by the ring-flip and has a similar, or lower energy than intermediate 1 for both esters in all conditions investigated (Figure 6). Additionally, the 1-*O*- to 3-*O*-migrations involve a favorable 6-membered cyclic intermediate.[34, 54] Although the ring-flipped glucose allows for facile migration to the 3-*O*-position, subsequent migration to the thermodynamically favored 6-*O*-position is also facilitated,[55] and most likely why the 3-*O*-esters were not prevalent in the migrated ester mixtures.

However, these results do indicate that in largely aqueous conditions that the 1-*O*-β-esters will be thermodynamically favored, and that most migrations will occur more slowly due to higher energy barriers to migration, with the only exception being the migration to the 3-*O*-position which requires a ring flip of the glucose ring before the migration can take place.

In synthesis or isolation of the hydroxycinnamoyl glucose esters, the use of solvents other than water should be limited, especially under acidic conditions conducive to migration. If organic

355 solvents are employed, they should be done so under neutral conditions, or in the presence of a
356 buffer. In the event of migration away from the desired 1-*O*-*β*-esters, it was found that for storage
357 under aqueous acidic conditions, migrated mixtures will revert to the 1-*O*-*β*-esters, with the spectral
358 data of each ester corresponding to literature data, as reported in the experimental section.[15, 56]
359 (extracted ion chromatograms of glucose esters stored in pH = 3.5 model wine and mass spectra of
360 identified peaks can be found in supporting information).

361 **HPLC-MS analysis of glucose esters in wine.** The ease of migration of the glucose esters
362 in non-aqueous solvents brings into question whether multiple esters observed in previous
363 quantifications[7, 14, 15] are an artefact of extraction using non-aqueous solvents promoting migration
364 and multiple positional isomers, rather than being present in grape or wine products.

365 When the synthetically prepared glucose esters were stored in model wine, subsequent
366 analysis by HPLC-MS gave extracted ion chromatograms that showed largely single esters. This
367 analysis, without a solvent extraction or purification step, suggests that migration will not be greatly
368 facilitated by the method of analysis solely.

369 Unconcentrated red and white wine along with concentrated samples (5 times concentrated
370 under reduced pressure) were submitted to analysis by HPLC-MS through direct injection rather
371 than extraction by organic solvent, to avoid solvent induced migration. While *p*-coumaroyl glucose
372 and feruloyl glucose could be identified in concentrated white wine (extracted ion chromatograms
373 and mass spectra in supporting information), the HPLC-MS analysis of red wine failed to identify
374 feruloyl glucose due to insensitivity caused by co-eluting species (tentatively identified as *p*-
375 coumaroyl anthocyanin derivatives). This led to decreased resolution of the *m/z* 355 extracted ion
376 chromatogram and no fragmentations were found matching that of the reference sample, which may
377 explain why feruloyl glucose has yet to be identified in red wine. Increased sensitivity was achieved
378 using HPLC-MRM with the predominant fragmentations, those from the precursor ions (*m/z* 325 or
379 355) to the aglycone (*m/z* 163 or 193) and to the aglycone minus water (*m/z* 145 or 175), displayed
380 in Figure 7.

Multiple *p*-coumaroyl glucose esters were observed in unconcentrated white wine, concentrated white wine and concentrated red wine (29.7 and 30.6 mins, Figures 7b, c and d, left side). The presence of a second feruloyl glucose ester was not obvious in unconcentrated white wine (Figure 7b), but slightly better resolution in concentrated white wine facilitated identification of a second feruloyl glucose peak (37.7 mins, Figure 7c). As near identical chromatograms were observed for unconcentrated and concentrated white wine, the concentration step was not considered to alter the compounds present, only providing improved resolution. As such, unconcentrated red wine was not analyzed, instead only concentrated red wine was used to obtain that improved sensitivity while providing information that was representative of an unconcentrated sample. Although the concentration of feruloyl glucose in concentrated red wine appears to be somewhat lower than in white wine, evidence of a second peak in the concentrated red wine sample was seen (37.6 mins, Figure 7d). These data confirm the previous findings that there are multiple glucose esters in wine,[3, 14, 16] but the theoretical studies show that the extraction method can contribute to the extent of migration. These results also suggest why the feruloyl glucose ester was not observed in previous studies, as the presence of what are likely to be *p*-coumaroyl anthocyanin derivatives in red wine co-elute, and prevent identification unless HPLC-MRM is used.

Metabolism of *p*-coumaroyl and feruloyl glucose by *Brettanomyces* yeast. The synthetic samples of the glucose esters were spiked as migrated mixtures containing mainly the 1-*O*-*β*-esters, with the knowledge that in wine-like environments the 1-*O*-*β*-esters will prevail, or will at least reside in a ratio that is representative of what occurs in wine. As acyl migration is a dynamic process, isolating single positional isomers and submitting to fermentation experiments would simply result in a mixture governed by the stability of each positional isomer in a given medium. Regardless, during fermentation, to release the free hydroxycinnamic acid the esterase of *Brettanomyces* must be active towards a hydroxycinnamoyl glucose ester.

The evolution of ethylphenols during fermentation (Figure 8) with AWRI 1499 gave a similar percentage conversion for **11** and **12** of approximately 36%. The uninoculated control

ferments showed no evolution of ethylphenols, and the stability of the glucose esters has been shown by Monagas[14] and was proven by storage in fermentation-like conditions for the length of the experiment, so it can be confirmed that the production of ethylphenols was caused by enzymatic cleavage of the glucose esters. The intermediate hydroxycinnamic acids (**3** and **4**) were not quantified in this work as, in our experience, once the acid has been liberated the conversion to the ethylphenol is good,[28] and recovery of both the ester and ethylphenol account for a significant proportion of the spiked material.[2]

This experiment shows for the first time that the hydroxycinnamoyl glucose esters can be metabolized by *B. bruxellensis* and can contribute to the formation of ethylphenols in wine. With moderate conversions from the glucose esters, the spoilage potential during barrel ageing will largely be determined by the concentration present in the wine, which as described previously can range from trace to several mg/L.[3, 14, 16] Assuming a pre-malolactic fermentation concentration of *p*-coumaroyl glucose, as observed by Hernandez et al., of 2.74 mg/L,[3] a 36% molar conversion would result in a 4-ethylphenol concentration of around 370 μg/L. This is more than half the detection threshold,[1] suggesting hydroxycinnamoyl glucose esters should be considered when assessing the potential for ethylphenol accumulation.

Metabolism of *p*-coumaroyl and feruloyl tartrate by *Brettanomyces* yeast. The hydroxycinnamoyl tartrate esters (**9** and **10**), when fermented with AWRI 1499, AWRI 1608 and AWRI 1613 (the three strains tested belonging to the three main genetic groups and being representative of 98% of Australian *Brettanomyces* isolates[57]) gave no conversion to 4-ethylphenol or 4-ethylguaiacol, and the result was confirmed in repeat experiments. This is consistent with that detailed by Schopp et al. in red wine ferments with *B. bruxellensis*.[11]

The potential for the tartrate esters to contribute to spoilage during barrel ageing is limited, with *B. bruxellensis* lacking the enzymatic ability to release hydroxycinnamic acids and subsequently form ethylphenols. However, previous studies showed that the hydroxycinnamoyl tartrates could be hydrolyzed using commercial enzyme preparations.[8, 9] The resulting

hydroxycinnamic acids would be metabolized to yield ethylphenols in the presence of *B. bruxellensis*, and so the tartrate esters might act as indirect precursors to ethylphenols, if first exposed to specific enological conditions, regardless of the ability of *B. bruxellensis* to directly metabolize them.

While the hydroxycinnamoyl tartrates were not metabolized, this work shows that the glucose esters can contribute to the accumulation of ethylphenols, adding to that already known for the free acids and the ethyl esters,[1, 2] and expanding the pool of ethylphenol precursors.

ABBREVIATIONS USED

HPLC, high performance liquid chromatography; MS, mass spectrometry; AWRI, Australian Wine Research Institute; NMR, nuclear magnetic resonance; FTIR, Fourier transform infra-red; HRMS. High resolution mass spectrometry; DFT, density functional theory; MMFF, Merck molecular force field; HOMO, highest occupied molecular orbital; LUMO. Lowest unoccupied molecular orbital; PDA, photodiode array; S_0, singlet ground state; T_1, first excited triplet state; MRM, multiple reaction monitoring.

ACKNOWLEDGEMENT

The School of Agriculture, Food and Wine, the University of Adelaide and The Australian Wine Research Institute, members of the Wine Innovation Cluster in Adelaide, are supported by Australian grape growers and winemakers through their investment body, Wine Australia, with matching funds from the Australian Government.

ASSOCIATED CONTENT

Supporting information. synthetic methodologies and characterization data for preparation of hydroxycinnamoyl esters; computational methodologies and absolute values generated in computational studies of hydroxycinnamoyl isomerization and glucose ester migration; chromatographic data from HPLC-MS studies of glucose esters; absolute values of ethylphenol

evolution from hydroxycinnamoyl glucose esters. This material is available free of charge via the internet at http://pubs.acs.org.

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617

FIGURE CAPTIONS

Figure 1. Presumed pathway for the breakdown of ethyl hydroxycinnamates by *B. bruxellensis.*

Figure 2. Preparation of hydroxycinnamoyl tartrate esters. *i*) acetyl chloride, reflux *ii*) water, acetone *iii*) $MgSO_4$, H_2SO_4, *tert*-BuOH *iv*) KOH, MeOH *v*) $SOCl_2$, reflux *vi*) **18**, pyridine *vii*) trifluoroacetic acid, C18-RP chromatography.

Figure 3. Preparation and transformations of hydroxycinnamoyl glucose esters. *i*) chloroacetyl chloride, pyridine, reflux *ii*) hydrazine acetate *iii*) trichloroacetonitrile, DBU *iv*) **25**, TMSOTf, molecular sieves *v*) pyridine/water *vi*) XAD-8, MeOH/water *vii*) silica gel, 10% MeOH/CH_2Cl_2.

Figure 4. Energy profiles for isomerization of hydroxycinmmates a) *p*-coumaric acid, and b) *p*-coumaroyl glucose, calculated using DFT B3LYP 6-31G* basis set in a vacuum. Excitation energies for *p*-coumaric acid and *p*-coumaroyl glucose in solvents of differing polarities, c) vertical excitation energy of optimized S_0 configuration to T_1 state, and d) HOMO-LUMO gap energy of S_0 state. Absolute values can be found in the supporting information.

Figure 5. Energies of hydroxycinnamoyl glucose esters, relative to 1-*O*-*β*-ester, calculated using DFT B3LYP 6-31G* basis set in water, dichloromethane, ethanol and toluene. Absolute values can be found in the supporting information.

Figure 6. Key intermediates in acid-catalyzed 1-*O*-*β*- to 2-*O*-*β* acyl migration for *p*-coumaroyl glucose, and energies of key intermediates in acyl migrations, relative to Intermediate 1 determined using the DFT B3LYP 6-31G* basis set. Absolute values can be found in the supporting information.

Figure 7. HPLC-MRM chromatograms (aglycone - blue, aglycone minus water - red) of hydroxycinnamoyl glucose esters. a) Pure glucose esters. b) Unconcentrated white wine. c) Concentrated white wine. d) Concentrated red wine.

Figure 8. Percentage molar conversion of glucose esters to volatile phenols in model ferments, data expressed as average of triplicates ± standard deviation. Absolute values can be found in the supporting information.

TABLES

Table 1. Numbering of key atoms in hydroxycinnamate skeleton and the structural features obtained from B3LYP 6-31G* optimized equilibrium geometries.

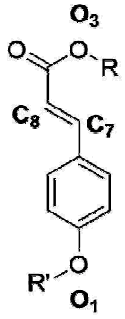
Hydroxycinnamate skeleton	R	R'	C_7=C_8 length (Å)	O_1 charge	O_3 charge	O_1/O_3 charge ratio	HOMO-LUMO gap (kJ/mol)
	H	H	1.351	-0.573	-0.597	0.960	400.95
	anion	H	1.346	-0.589	-0.764	0.771	437.46
	anion	anion	1.354	-0.774	-0.777	0.996	372.07
	CH_2CH_3	H	1.354	-0.58	-0.365	1.589	400.47
	CH_2CH_3	anion	1.369	-0.739	-0.369	2.003	329.87
	Glucose	H	1.355	-0.573	-0.315	1.819	385.99
	Glucose	anion	1.373	-0.728	-0.317	2.297	318.8
	H	$C(O)CH_3$	1.349	-0.476	-0.562	0.847	434.23
	anion	$C(O)CH_3$	1.345	-0.483	-0.754	0.641	449.43
	H	$C(O)CH_2Cl$	1.348	-0.469	-0.561	0.836	437.19
	anion	$C(O)CH_2Cl$	1.344	-0.473	-0.755	0.626	445.27

FIGURE GRAPHICS

Figure 1

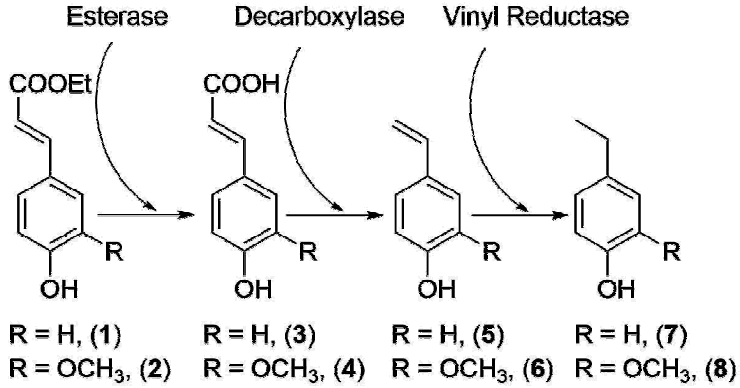


Figure 2

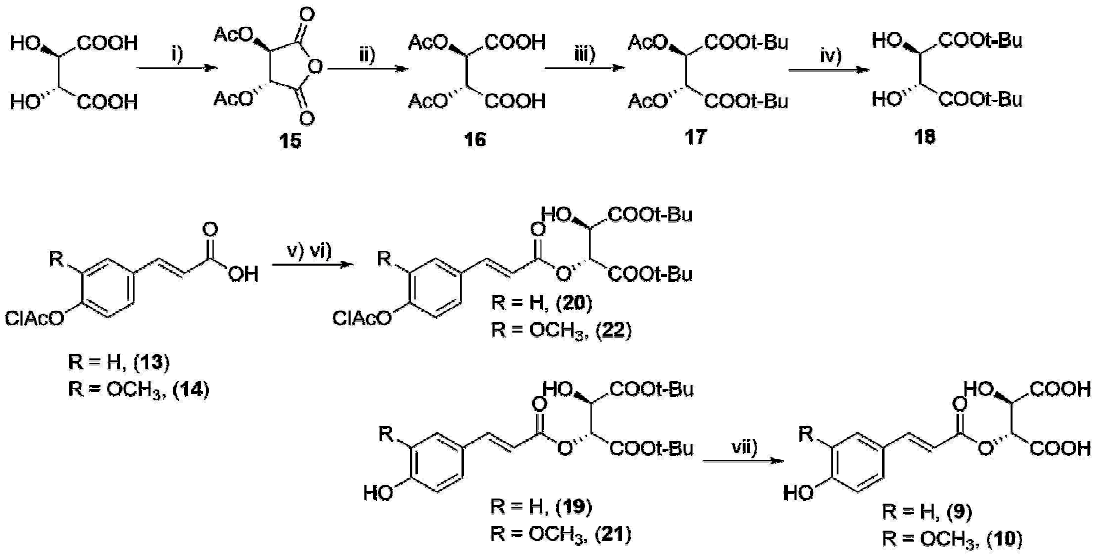

HO COOH
HO COOH
i)
AcO
AcO
O
O
O
15
ii)
AcO COOH
AcO COOH
16
iii)
AcO COOt-Bu
AcO COOt-Bu
17
iv)
HO COOt-Bu
HO COOt-Bu
18
R
ClAcO
O
OH
R = H, (13)
R = OCH3, (14)
v) vi)
HO COOt-Bu
O
O
COOt-Bu
R = H, (20)
R = OCH3, (22)
HO COOt-Bu
O
O
COOt-Bu
HO
R = H, (19)
R = OCH3, (21)
vii)
HO COOH
O
O
COOH
HO
R = H, (9)
R = OCH3, (10)

Figure 3

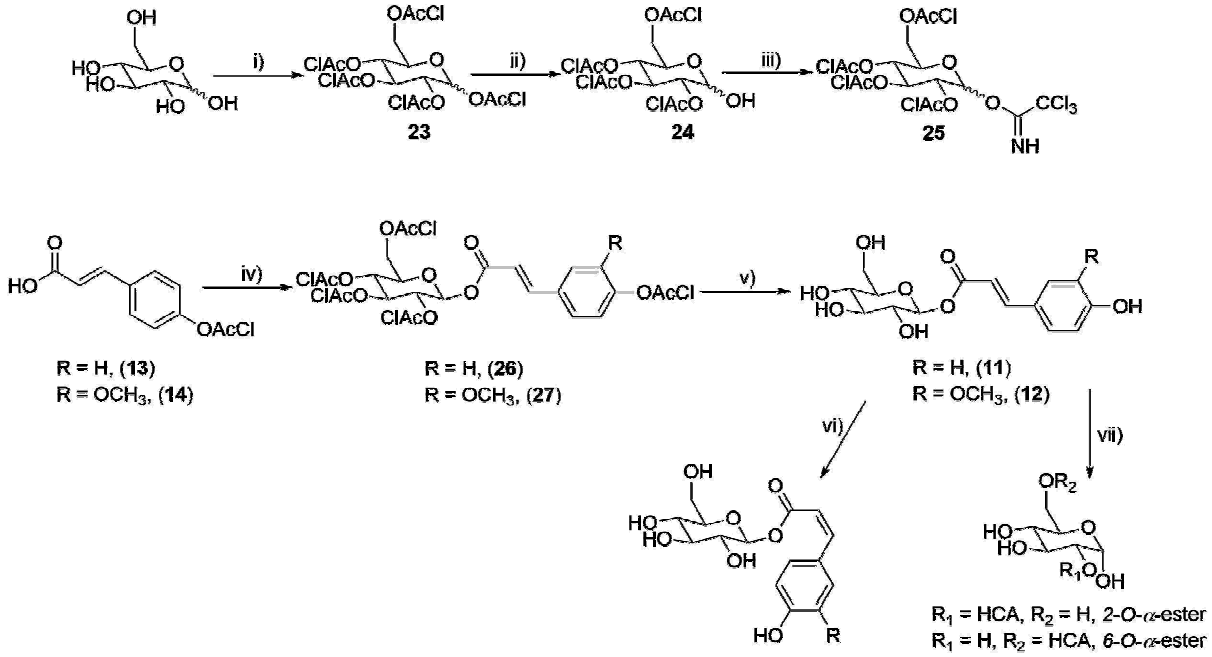

i)
ii)
iii)
23
24
25
iv)
v)
R = H, (13)
R = OCH3, (14)
R = H, (26)
R = OCH3, (27)
R = H, (11)
R = OCH3, (12)
vi)
vii)
R1 = HCA, R2 = H, 2-O-α-ester
R1 = H, R2 = HCA, 6-O-α-ester

Figure 4

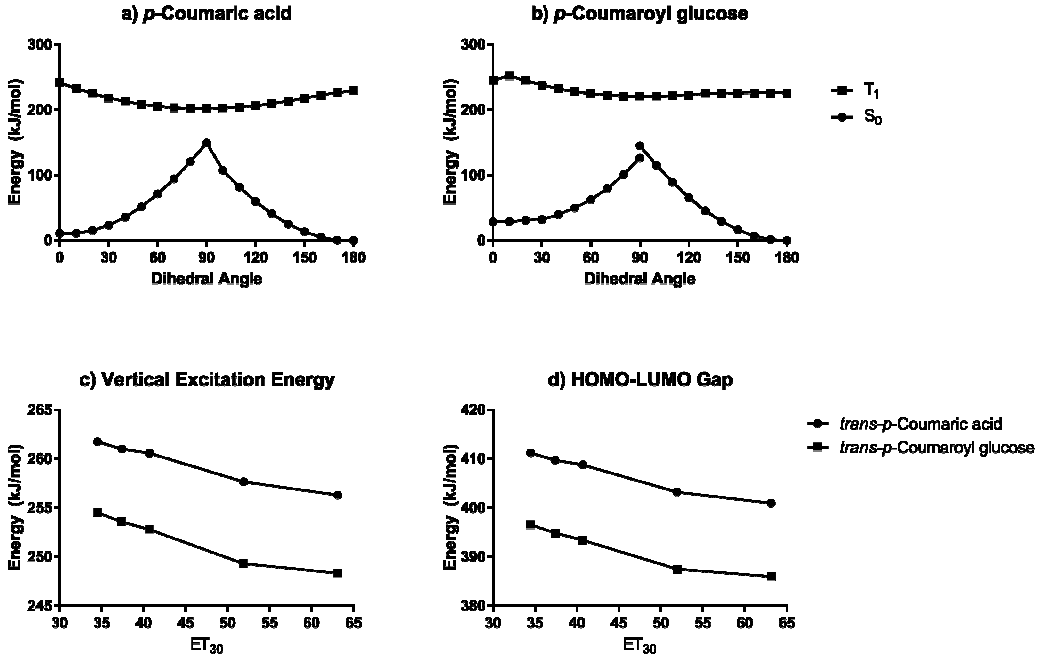

a) p-Coumaric acid
b) p-Coumaroyl glucose
Energy (kJ/mol)
Dihedral Angle
T1
S0
c) Vertical Excitation Energy
d) HOMO-LUMO Gap
ET30
trans-p-Coumaric acid
trans-p-Coumaroyl glucose

Figure 5

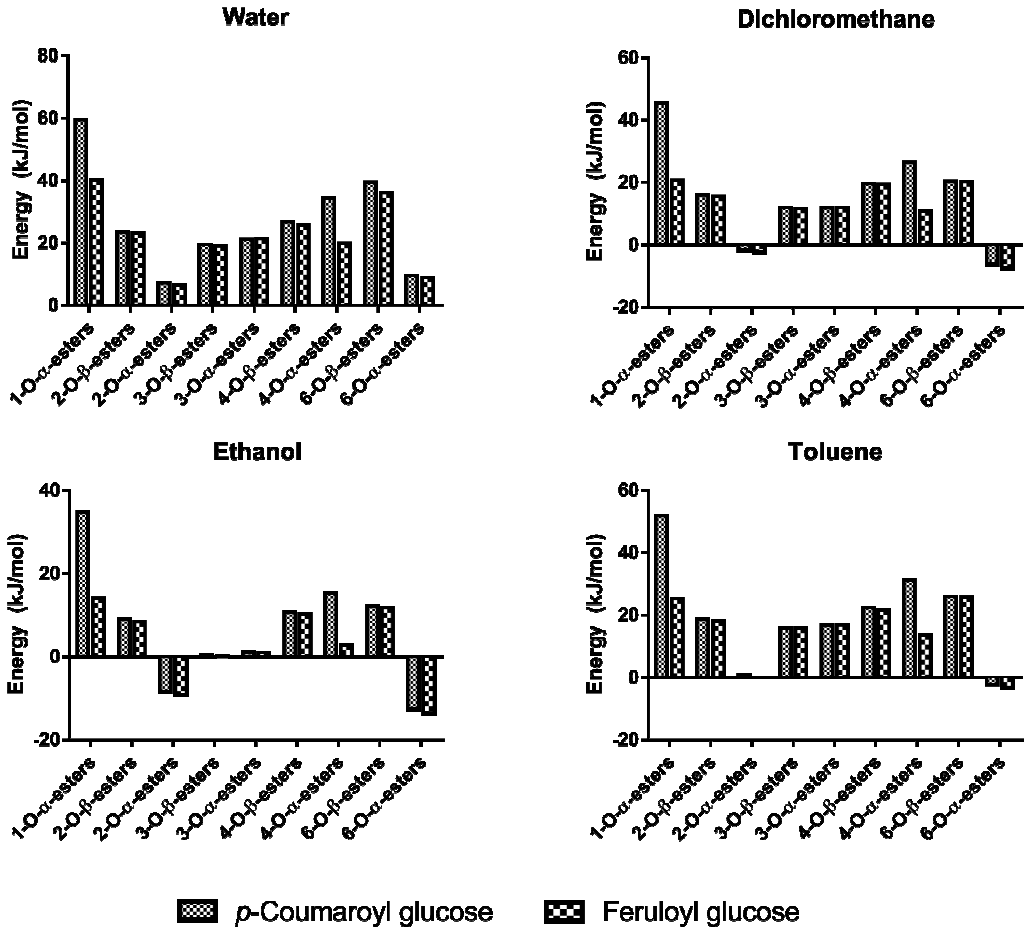

Water
Dichloromethane
Ethanol
Toluene
Energy (kJ/mol)
1-O-α-esters
2-O-β-esters
2-O-α-esters
3-O-β-esters
3-O-α-esters
4-O-β-esters
4-O-α-esters
6-O-β-esters
6-O-α-esters
p-Coumaroyl glucose
Feruloyl glucose

Figure 6

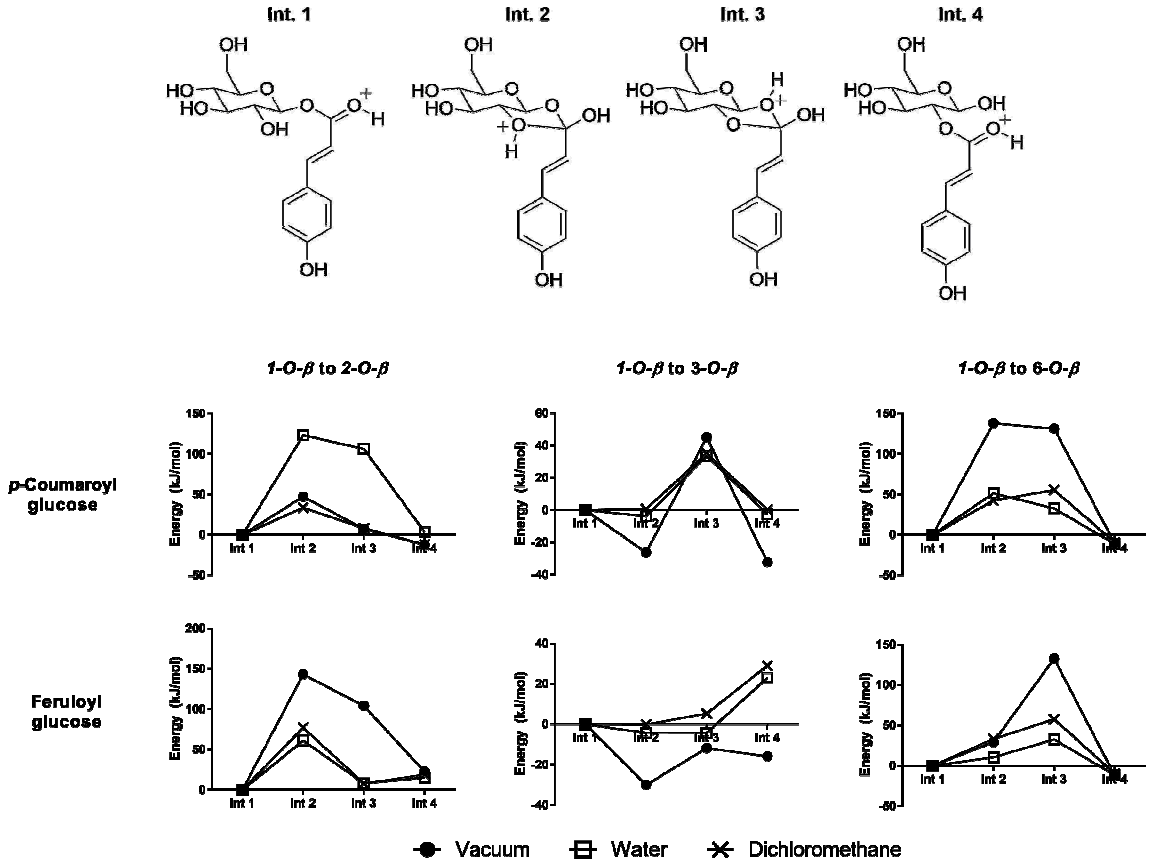

Int. 1
Int. 2
Int. 3
Int. 4
1-O-β to 2-O-β
1-O-β to 3-O-β
1-O-β to 6-O-β
p-Coumaroyl glucose
Feruloyl glucose
Energy (kJ/mol)
Int 1
Int 2
Int 3
Int 4
Vacuum
Water
Dichloromethane

Figure 7

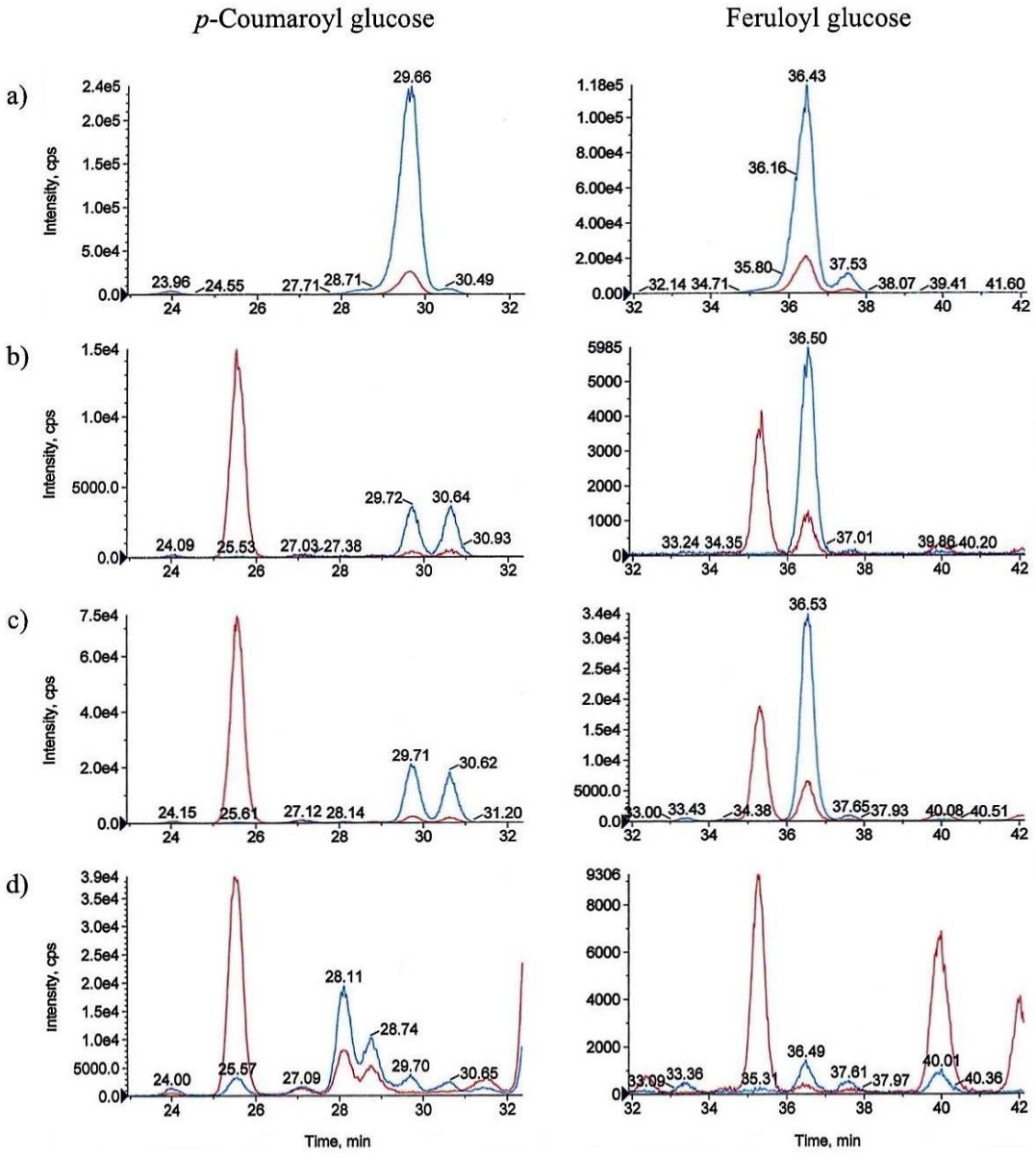
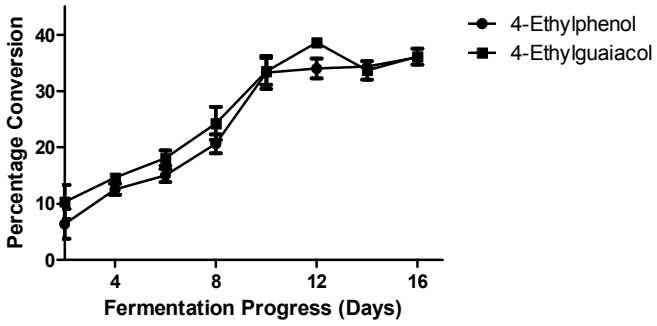
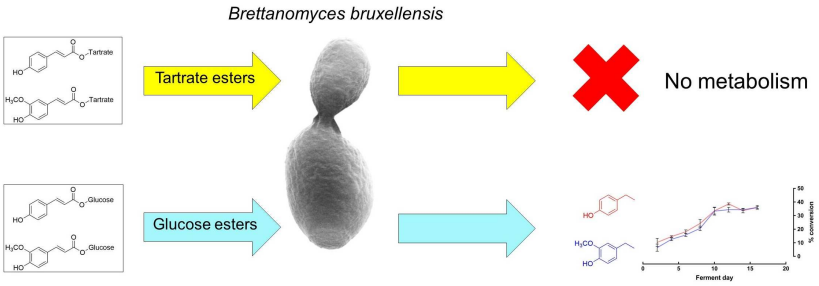

p-Coumaroyl glucose
Feruloyl glucose
a)
b)
c)
d)
Intensity, cps
Time, min
29.66
23.96
24.55
27.71
28.71
30.49
36.43
36.16
35.80
37.53
32.14
34.71
38.07
39.41
41.60
1.5e4
1.0e4
5000.0
0.0
29.72
30.64
30.93
24.09
25.53
27.03
27.38
5985
5000
4000
3000
2000
1000
36.50
33.24
34.35
37.01
39.86
40.20
7.5e4
6.0e4
4.0e4
2.0e4
29.71
30.62
24.15
25.61
27.12
28.14
31.20
3.4e4
3.0e4
2.5e4
2.0e4
1.5e4
1.0e4
36.53
33.00
33.43
34.38
37.65
37.93
40.08
40.51
3.9e4
3.5e4
3.0e4
2.5e4
28.11
28.74
29.70
24.00
25.57
27.09
30.65
9306
8000
6000
4000
2000
36.49
33.09
33.36
35.31
37.61
37.97
40.01
40.36
24
26
28
30
32
34
36
38
40
42

Figure 8


Percentage Conversion
0
10
20
30
40
4
8
12
16
Fermentation Progress (Days)
4-Ethylphenol
4-Ethylguaiacol

TOC GRAPHIC


Brettanomyces bruxellensis
Tartrate esters
Glucose esters
No metabolism
Ferment day
% Conversion