Chemistry of Hydroxycinnamate Esters and their Role as Precursors to Dekkera Produced Off-flavour in Wine

A thesis presented in fulfilment of the requirements for the degree of

Doctor of Philosophy

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Abstract

The potential for malodour in wine caused by the accumulation of ethylphenols has been widely studied with respect to the breakdown of the hydroxycinnamic acids, *p*-coumaric and ferulic acid, by *D. bruxellensis*. The presence of esterified hydroxycinnamate conjugates in grapes and wine is well established and they account for a large proportion of the hydroxycinnamate content. There exists the possibility that these conjugates could also provide the potential for spoilage, though they have never been linked to the direct formation of ethylphenols. The research highlighted within this thesis examines the potential role of a number of esterified conjugates in the production of ethylphenols by *D. bruxellensis*. Two classes of berry derived esters, the tartaric acid and glucose bound hydroxycinnamates, as well as the vinification formed ethyl esters, were synthesised and used for model fermentation experiments.

Chapter 2 describes the preparation of a number of protected hydroxycinnamic acid derivatives that were used in the synthesis of the hydroxycinnamoyl tartrate esters (**7** and **8**) for the first time. Coupling 1-*O*-chloroacetyl protected *p*-coumaric and ferulic acids (**21** and **22**) with di-*tert*-butyl-*L*-tartrate (**34**) followed by selective hydrolysis of the *tert*-butyl esters yielded *p*-coumaroyl tartrate (**7**) and feruloyl tartrate (**8**). Hydroxycinnamoyl glucose esters (**9** and **10**) were prepared using the same hydroxycinnamates (**21** and **22**), esterifying with a prepared trichloroacetimidate glucosyl donor sequence, though purification of the glucose esters resulted in undesired chemical transformations. It was found that photoisomerisation of the glucose esters could be prevented via synthesis under red light, which gave *trans*-**9** and **10**, however migration of the hydroxycinnamoyl moiety around the glucose ring, which yielded mainly the 2-*O*-α- and 6-*O*-α-esters, was a product of submitting the esters to non-aqueous solvents and could not be avoided.

The acyl migration of the glucose esters that was observed in Chapter 2 has been researched at a DFT B3LYP 6-31G* theoretical level in Chapter 3 with respect to both the thermodynamics and kinetics of the transformations. The desired 1-*O*-β-esters were thermodynamically favoured only in water, while in any other solvent studied the 2-*O*-α- and 6-*O*-α-esters would prevail. Kinetically, migration to the 3-*O*-position involved lower energy barriers which can be equated to a more rapid process, although the ring-flipped

conformation needed to achieve the migration would promote subsequent migration to the 6-*O*-position. Step-wise migration, from the 1-*O*- to the 2-*O*-position, was found to be thermodynamically less favoured than other migrations investigated. This effect can be rationalised by the formation of a 5-membered cyclic intermediate in comparison to the 6-membered intermediate produced during 1-*O*- to 3-*O*-migration. However, the energy barriers involved in 1-*O*-β- to 2-*O*-β-migration better explain the comparative extent of migration observed between the *p*-coumaroyl and feruloyl glucose esters. The possibility of multiple glucose esters existing in wine was the focus of a brief study, finding two separate *p*-coumaroyl glucose esters in red and white wine, while a lesser extent of migration in feruloyl glucose limited observation to concentrated wine alone. However, due to co-elution of feruloyl glucose (**10**) with suspected *p*-coumaroyl anthocyanin derivatives in red wine, HPLC-MRM was required to detect it, which is the first report of this compound in red wine.

Theoretical studies into observed photoisomerisations and the synthesis of *cis*-hydroxycinnamates are described in Chapter 4. The *cis*-ethyl hydroxycinnamates were isolated and hydrolysed to give a mixture of *cis/trans*-hydroxycinnamic acids (**3** and **4**), which could be separated by flash chromatography, though the pure *cis*-isomers isomerised rapidly under ambient conditions and slowly under red light back to the *trans*-isomers. Stable isomeric mixtures were achieved by irradiation with ultra-violet light giving mixtures of 40-50% of the *cis*-isomer which could be used further in fermentation studies. Computational evidence suggested that isomerisation of the hydroxycinnamic acids was favoured with greater resonance throughout the molecule. Those with deprotonated phenolic moieties possessed the most intramolecular electron movement, decreasing the HOMO-LUMO gap and promoting photoisomerisation. Smaller solvent and substrate effects were also noted, though the nature of the phenol and carboxyl clearly played the most important role in determining stability of each isomer.

Fermentation in the presence of the synthesised *trans*-hydroxycinnamoyl esters (**7**-**12**) and investigation into the stereospecificity of *D. bruxellensis* enzyme activities was performed as detailed in Chapter 5. In Australia, three genetic groups of *D. bruxellensis* account for 98% of isolates, with the largest of these groups making up 85%. AWRI 1499 is a representative of the largest genetic group, with AWRI 1608 and AWRI 1613 belonging to the two remaining significant genetic groups. In the presence of AWRI 1499, the *trans*-

ethyl esters (**11** and **12**) were metabolised to varying extents with the preference for breakdown of ethyl coumarate (**11**) over ethyl ferulate (**12**). This selectivity was investigated further and found to be common for both AWRI 1499 and AWRI 1608, while AWRI 1613 was unable to breakdown either ester. The preference for formation of 4-ethylphenol (**1**) over 4-ethylguaiacol (**2**) from the ethyl esters could accentuate the ratio of these compounds as seen in wine, initially thought to be brought about by the relative concentration of the precursor acids.

Of the berry derived esters, the tartrate esters (**7** and **8**) were not metabolised by AWRI 1499, and subsequent fermentations with AWRI 1608 and 1613 yielded the same result. This confirmed that the tartrate esters cannot contribute directly to the formation of ethylphenols during exposure to *D. bruxellensis*. The glucose esters were metabolised by AWRI 1499 to a moderate extent (35% conversion), providing information that these can contribute to the accumulation of ethylphenols during barrel ageing. Furthermore, the isomerisation of the glucose esters lead to studies into the stereoselectivity of *D. bruxellensis* enzyme activities, whereby the decarboxylase as well as the ethyl esterase showed selectivity for the *trans*-isomers and that the *cis*-hydroxycinnamate content of grapes and wine are not important in the accumulation of ethylphenols. The experimental procedures employed throughout Chapters 2-5 are outlined in Chapter 6.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Publications and Symposia

Publications:

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Hixson, J. L.; Curtin, C. D.; Sefton, M. A.; Taylor, D. K. Stereospecificity of *D. bruxellensis* in the production of ethylphenol off-flavour in wine. *Proceedings of the 13th Weurman Flavour Research Symposium. In press.*

Hixson, J. L.; Taylor, D. K.; Ng, S. W.; Tiekink, E. R. T. Di-*tert*-butyl (2*R*,3*R*)-2-({(2*E*)-3-[4-(acetyloxy)-3-methoxyphenyl]prop-2-enoyl}oxy)-3-hydroxybutanedioate. *Acta Crystallographica, Section E* **2012**, 68 (3), o509-o510.

Hixson, J. L.; Taylor, D. K.; Ng, S. W.; Tiekink, E. R. T. Di-*tert*-butyl (2*R*,3*R*)-2-({(*2E*)-3-[4-(acetyloxy)phenyl]prop-2-enoyloxy)-3-hydroxybutanedioate. *Acta Crystallographica, Section E* **2012**, 68 (2), o568-o569.

Hixson, J. L.; Elsey, G. M.; Curtin, C. D.; Sefton, M. A.; Taylor, D.K. Hydroxycinnamoyl glucose and tartrate esters and their role in the formation of ethylphenols in wine. *J. Agric. Food Chem. In draft.*

Symposia:

Hixson, J. L.; Curtin, C. D.; Taylor, D. K.; Elsey, G. M. Mapping the Metabolic Inputs of 'Brett' Taint. **Poster** presented at the *2009 YPD conference (Meeting of the Australasian Yeast Group).*

Hixson, J. L.; Curtin, C. D.; Taylor, D. K. Stereospecificity of the Decarboxylase Enzyme of *D. bruxellensis.* **Poster** presented at the *14th Wine Industry Technical Conference, 2010.*

Hixson, J. L.; Elsey, G. M.; Curtin, C. D.; Taylor, D. K. Isomerisation of the Hydroxycinnamic Acids and their Role in the Production of Wine Off-aroma. **Seminar** presented at the *2010 Adelaide Synthetic Chemistry Symposium.*

Hixson, J. L.; Curtin, C. D.; Sefton, M. A.; Taylor, D. K. Determination of Alternative Precursors to *Brettanomyces/Dekkera* Produced Off-flavour. **Seminar** presented at the *13th Weurman Flavour Research Symposium, 2011.*

Abbreviations

4-EG	4-Ethylguaiacol
4-EP	4-Ethylphenol
Å	Angstroms
Ac	Acetyl
AcCl	Chloroacetyl
AcCN	Acetonitrile
app. d	Apparent doublet
Ar	Aromatic
AWRI	Australian Wine Research Institute
Bn	Benzyl
br	Broad
COSY	Correlation spectroscopy
d	Doublet
DAD	Diode array detector
DCM	Dichloromethane
dd	Doublet of doublets
ddd	Doublet of doublet of doublets
DFT	Density functional theory
EIC	Extracted ion chromatogram
ESI	Electrospray ionization
Et	Ethyl
Et_2O	Diethyl ether
EtOAc	Ethyl acetate
g	Grams
GC	Gas chromatography
Glc	Glucose
HCA	Hydroxycinnamic acid
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HOMO	Highest occupied molecular orbital
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectroscopy

Hz	Hertz
hν	Light
J	Coupling constant
kJ	Kilojoules
L	Litre
LC	Liquid chromatography
Lit.	Literature
LUMO	Lowest unoccupied molecular orbital
m	Multiplet
M	Molar (moles/litre)
m/z	Mass to charge ratio
mg	Milligrams
$MgSO_4$	Magnesium sulphate
MHz	Megahertz
ML	Megalitre
mL	Millilitre
MMFF	Merck Molecular Force Field
mmol	Millimoles
mol	Moles
m.p.	Melting point
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MYPG	Malt, yeast extract, peptone, glucose
nm	Nanometres
NMR	Nuclear magnetic resonance
p	Para
Ph	Phenyl
ppb	Parts per billion
ppm	Parts per million
q	Quartet
R_f	Retension factor
rpm	Revolutions per minute
s	Singlet
S_0	Singlet ground state

S_1	Singlet first excited state
t	Triplet
T_1	Triplet first excited state
tert	Tertiary
THF	Tetrahydrofuran
TIC	Total ion chromatogram
TLC	Thin layer chromatography
TMS	Tetramethyl silane
UV	Ultra-violet
Vis	Visible
VNBC	Viable but non-culturable
X4	Hexane fraction
YNB	Yeast extract, nitrogen, base
YPD	Yeast extract, peptone, dextrose
δ	Chemical shift
μ	Micro

Figures, Schemes and Tables

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“Success is the ability to go from one failure to another with no loss of enthusiasm”

Sir Winston Churchill

“I’m a great believer in luck, and I find the harder I work the more I have of it”

Thomas Jefferson