SYNTHESIS OF ALLENIC KETONES AND THE ROLE OF YEAST IN THEIR CONVERSION TO DAMASCENONE

Natoiya Dee Rayette Lloyd

A Thesis Submitted for the

Degree of Doctor of Philosophy

University of Adelaide

School of Agriculture, Food and Wine

Faculty of Science

October 2013

Dedication

This Thesis is dedicated to my dad, Robert Lloyd who passed away (03/10/1941 – 16/11/2009) and my wonderful mom, Maxine and brother, Ryan who are always there to support me. I am so grateful to have both of you in my life.

Table of Contents

Abstract

The thesis describes the formation of damascenone during fermentation conditions from potential ketone precursors. An introduction to the thesis and aims of the study are described in Chapter 1, the synthesis of the precursors is detailed in Chapter 2, identification of the precursors as natural products and hydrolysis studies are included in Chapter 3, fermentation studies are detailed in Chapters 4 and the damascenone stability studies are in Chapter 5.

Chapter 2 describes the synthesis of megastigma-4,6,7-triene-3,9-dione (**26**) and 3-hydroxymegastigma-4,6,7-trien-9-one (**27**) from the common starting material, 4-oxoisophorone as well as the synthesis of 9-hydroxymegastigma-4,6,7-trien-3-one (**28**) from diketone **26**. The allene 3-*tert*-butyldimethylsilyloxy-9-hydroxymegastigma-4,6,7-triene (**29**) was synthesised first and this was then used to produce ketones **26** and **27**. The synthesis of **26** occurred in two steps from the silylated allene diol **29** which involved deprotection, followed by a Dess-Martin oxidation. The synthesis of ketone **27** was achieved by a Dess-Martin oxidation of the silylated allene diol **29** followed by deprotection using TBAF. The ketone **28** was synthesised by a selective reduction of **26**.

Chapter 3 covers the identification of the ketones **26** and **28** as natural products. It describes the hydrolysis studies performed with ketone **27** in model wine and includes a discussion as to why **27** was not seen in grape juice or honey extracts, in contrast to the analogues **26** and **28**.

The fermentation study involving the synthesised ketones **26**, **27** and **28** is discussed in Chapter 4. The fermentation studies demonstrate that damascenone can be formed by the action of yeast during fermentation, from precursors **26**, **27** and **28**. The results emphasise that the presence of yeast is essential for the conversion. The concentration of damascenone at the end of fermentation was also shown to be dependent on the yeast strain. Yeast strain AWRI 796 showed to be more efficient in the formation of damascenone compared to yeast strain AWRI 1537.

The final section of the thesis (*Chapter 5*) details the stability of damascenone during fermentation. A substantial loss of damascenone was observed during fermentation and the factors involved were further explored. The possible contributing factors included the loss of damascenone through the action of yeast metabolism, loss from reaction with components in the model or real juice and loss via evaporation of damascenone via the ferment flask during fermentation.

Declaration

I, Natoiya Dee Rayette Lloyd certify that this work contains no material which as 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.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australian Digital Thesis Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

…….....

Natoiya Dee Rayette Lloyd

Acknowledgments

There are many individuals who have helped me throughout my PhD, some who have been there since the beginning and some who came on board mid way through. Over the years my supervisors included Prof. Dennis Taylor, Dr. Mark Sefton, Dr. Chris Curtin, Dr. Gordon Elsey, Dr. Maurizio Ugliano and Dr Martin Johnston and I can't express how appreciative I am for all of the help and guidance you all gave me.

I want to particularly thank the Grape and Wine Research and Development Corporation for providing the funding for my PhD project and also enabling me to attend the 9th Wartburg Flavour Symposium 2010, in Eisenach, Germany. Thank you to Flinders University, The University of Adelaide and The Australian Wine Research Institute for providing me the opportunity to work on such a great project with access to outstanding facilities and a friendly and supportive working environment.

I particularly want to thank Dennis for all his enthusiasm and positive encouragement in regard to every aspect of my project giving me the tools to continue forward and to always strive to achieve great results. Thanks so much for all of your guidance and support.

I want to especially thank Mark Sefton for your supervision from the very beginning of the project. You helped me in many ways and you were always keen to chat with me about my research providing me with many ideas that would strengthen my work. I learnt a lot from you and always appreciated your feedback especially when the results were sometimes overwhelming. The time you gave towards my work will always be remembered and I am forever grateful.

I want to especially thank Dr. Dimitra Capone. Your help throughout my PhD was endless even at your busiest moments. You have been great to work with over the years and I have learnt so much from you. I can't thank you enough for your help, guidance and encouragement to finish.

Gordon, you were there at the beginning of my PhD project and I will never forget all of the things I have learnt from you over the years. I always looked up to you and I will take what you have taught me and use it throughout my chemistry career. I want to thank Kevin for your endless help in the lab, you taught me a lot and it was always very enjoyable to work with you.

Thank you to Maurizio for all his assistance with the biochemistry aspects of my project. Your direction and support throughout my PhD will always be valued. I want to thank Dr. Chris Curtin for becoming involved in my PhD work as a supervisor and always being interested in the work and results I obtained. I really appreciate the contributions you made and the time you gave to help me finish. I want to also thank Dr. George Skouroumounis for all of his inspiring ideas towards my work which created some major turning points in my research.

I would have not made it through to the end without the people that surrounded me on a day to day basis. I want to thank my fellow PhD students both at Flinders University, the University of Adelaide and my work colleagues at the AWRI. I especially want to thank Josh, Jo, Kerry, Anthea, Nicole, Stacey, Pete, Dave, Taryn, Simon, Rachel, Bek, Andrew, Angus, Caroline, Radka, Jane, June, Jenny, Ally, Ruyi, Xin, Mao and Anson.

Lastly, I cannot forget my beautiful family who I love so much, who wish me well in anything I do and always believe in me. My mum, the best mum in the world, I want to thank you for your endless support. My brother Ryan I thank you for all your words of encouragement to get through and finish and a big thank you for the awesome coffee machine to help me daily particularly near the end of my PhD. My dad, I love you so much. I think of your positive words often and will always cherish our time together.

Matt, you mean the world to me and have been there since I started my PhD. You have always been there for me, respected, and supported me during my research. Thanks for cooking me awesome dinners every night and supporting me in whatever I needed. You truly are one of a kind.

Publications

Refereed Publications

1. Lloyd, N. D. R.; Capone, D. L.; Ugliano, M.; Taylor, D. K.; Skouroumounis, G. K.; Sefton, M. A.; Elsey, G. M., Formation of damascenone under both commercial and model fermentation conditions. *J. Agric. Food. Chem.* **2011**, 59, (4), 1338-1343.

2. Lloyd, N. D. R.; Capone, D. L.; Ugliano, M.; Taylor, D. K.; Skouroumounis, G. K.; Sefton, M. A.; Elsey, G. M. The role of yeast in the generation of the odorant damascenone in wine. *Proceedings of the 9th Wartburg Symposium on Flavor Chemistry and Biology*, Eisenach, Germany,13 – 16th April, **2010**, pp. 447 – 451.

Symposia

1. Lloyd, N. D. R.; Capone, D. L.; Ugliano, M.; Taylor, D. K.; Sefton, M. A.; Elsey, G. M. The role of yeast in the generation of damascenone in wine. Crush Conference, Adelaide, South Australia, **2011**.

2. Lloyd, N. D. R.; Capone, D. L.; Ugliano, M.; Taylor, D. K.; Sefton, M. A.; Elsey, G. M. The role of yeast in the generation of the odorant damascenone in wine. 14th Australian Wine Industry Technical Conference, Adelaide, South Australia, **2010**.

3. Lloyd, N. D. R.; Capone, D. L.; Ugliano, M.; Taylor, D. K.; Elsey, G. M. The formation of damascenone under fermentation conditions. The 23rd RACI Organic Division Conference, 'Organic 08', Hobart, Tasmania, **2008**.

Abbreviations

CDGJ medium	Chemically defined grape juice medium
CDMW	Chemically defined model wine (made from CDGJ medium)
COSY	Correlation spectroscopy
DCM	Methylene chloride
DMAP	4-Dimethylaminopyridine
DMSO	Dimethylsulfoxide
EIC	Extracted ion chromatogram
EtOAc	Ethyl acetate
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
hr	Hour
Hz	Hertz
KI	Kovats Index
LAH	Lithium Aluminium Hydride
MeOH	Methanol
mM	millimolar
NaOMe	Sodium methoxide
ppb	parts per billion, μg/L
ppm	parts per million, mg/L
ppt	parts per trillion, ng/L
RT	Room temperature
sat. aq.	Saturated aqueous
TBAF	Tetrabutylammonium fluoride
TBDMS	*tert*-Butyldimethylsilyl
TBDMSCl	*tert*-Butyldimethylsilyl chloride
THF	Tetrahydrofuran
TIC	Total ion chromatogram
TLC	Thin layer chromatography
μM	micromolar

List of Figures, Schemes and Tables

Chapter 1

Introduction

CHAPTER 1 Introduction

1.1 The Australian wine industry

Historically, the wine industry in Australia began with the arrival of white settlers on the first fleet to this country in 1788. It wasn't until the first fleet arrived, that vines were introduced to Australia as part of the, 'Plants for Settlement'.[1] The grape vines are commonly grouped in the genus *Vitis*, in the family vitaceae.[2] The V*itis vinifera* varieties were introduced from Europe and South Africa. Amongst the 60 varieties in Australia, the most commonly known are Shiraz, Cabernet Sauvignon, Merlot, Pinot Noir, Ruby Cabernet, Chardonnay, Sauvignon Blanc, Semillon, Colombard and Riesling.[3,4]

The wine industry in Australia has developed enormously, particularly over the past 50 years and has achieved international recognition for the quality of its wines. The volume of Australian wine exports has increased over the past decade from around 300 million litres to about 700 million litres.[5]

Australia's total volume of wine exported in 2011 was 703 million litres which was valued at A$ 1.89 billion and is 10% lower than the previous year. However, the average value of wine did increase by 1% to A$ 2.69 per litre which is the first increase observed since 2001. This volume is similar to the amount of exports in previous years but it is at the lower end of the scale over the past five years. This is primarily due to the decline in the production of Australian wine which reached a high in 2005 at 1.42 billion litres and decreased to a low of 1.07 billion litres in 2011. A major shift in the market has occurred between the volumes that are shipped in bottles compared to the volume shipped in bulk containers. Wine shipped in bulk containers is at a record high now accounting for 49% of wine exported compared to bottled wine exported which has seen a decrease by 3%, sharing 50%.[5,6]

Australia is the fourth largest exporter of wine by volume behind countries like Italy, France and Spain.[7] Australia has five top markets that account for 80% of the Australian export volume during the year; these are the UK, US, Canada, China and

Germany. The volume of wine exported to these countries was 248 million litres, 179 million litres, 50 million litres, 41 million litres and 41 million litres, respectively.[6]

Australia now has more than 60 designated wine regions with the total number of wine producers in Australia having increased from 1625 to 2299 between 2003 and 2011. Australia has become one of the world leaders in both the quantity and quality of wine.[5,8]

The top five largest wine companies by total wine production for 2009 and 2010 were: Accolade Wines, Treasury Wine Estate, Casella Wines, Pernod Ricard Pacific and Australian Vintage (*Table 1.1*).[9]

Table 1.1 The top wine companies by total wine production in Australia and some of their associated brands.[9]

Wine Company	Wineries
Accolade Wines	Hardy's Houghton Banrock Station Berri Estates
Treasury Wine Estates	Wolf Blass Wynns Coonawarra Estate Penfolds Rosemount Estate Lindeman's
Casella wines	Yellow Tail Yendah
Pernod Ricard Pacific	Orlando Wines Richmond Grove Wines Wyndham Estate
Australian Vinatge	McGuigan Nepenthe Miranda

The initial development of the wine industry in South Australia dates back from the 1830's to the 1860's. South Australia's most famous regions include, the McLaren Vale, Langhorne Creek, Barossa Valley, Clare Valley and Coonawarra. The first vines were planted close to the centre of Adelaide at Reynella, in the McLaren Vale and Langhorne Creek districts. German immigrants began the vineyards in the Barossa Valley around 1847 with John Gramp responsible for the plantation of the vines at

Jacob's Creek. The initial planting of the Sevenhill's vineyard in the Clare Valley region was in 1848 by Austrian Jesuits. The Coonawarra region was discovered by John Riddoch in the 1890s. The Coonawarra has only become famous more recently due to the work of David Wynn who established Coonawarra Estate Winery in 1951.[4]

South Australia is the leading state of wine production in the country producing 546,306 litres for 2009 to 2010. It is responsible for almost 50% of the total wine produced in Australia which was 1,132,102 litres. The production of wine in South Australia has declined in the last 5 years but the general trend from the early 70's shows significant growth and it still remains the top wine producing state as it has been for many years. The total production of wine by state in decreasing order is SA, NSW/ACT, VIC, WA, TAS followed by QLD.[5,10]

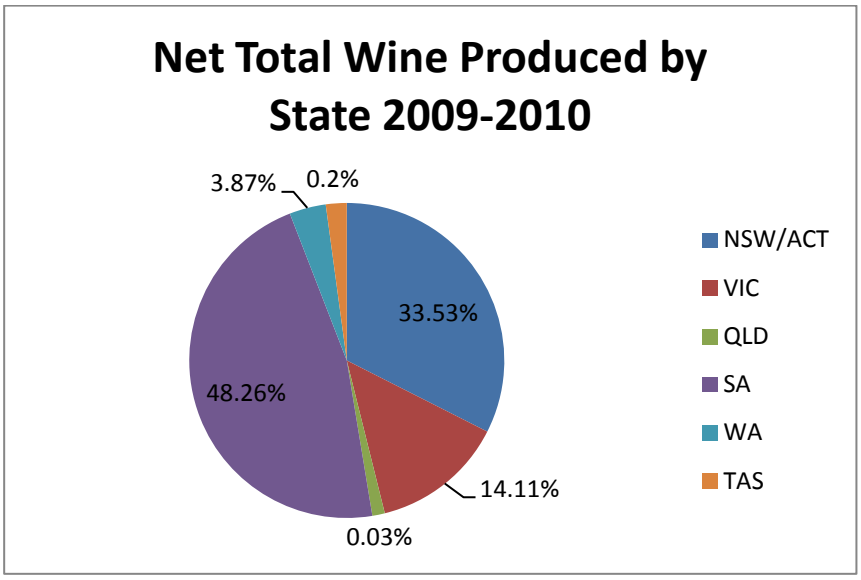


Figure 1.1 2009–2010 Net total wine production (includes wineries that crush more than 400 T/yr).[10]

1.2 Aroma and flavour of wine

Aroma and flavour are perhaps two of the most important factors contributing to the overall sensory experience of wine. Flavour is the result of sensations perceived in the mouth including smell, taste and touch. The perceived flavour of wine is due to volatile compounds contributing to aroma and non-volatile constituents that cause other taste and mouth-feel sensations such as sweetness, saltiness, sourness, bitterness, and umami. A consumer's interest in wine is considered to be predominantly driven by the flavour so the knowledge about the underlying chemical constituents responsible is highly important.[11]

The formation of wine aroma and flavour compounds has long been investigated since there has been a desire to produce more prominent flavours in foods. Understanding the origin, formation and fate of important aroma compounds is imperative to controlling their concentration. This information enables the modulation of essential aroma compounds, thus influencing the overall wine style and improving wine quality. The key flavour and aroma compounds present in a finished wine can act as sensory drivers and govern its overall bouquet.

Wine is a complex matrix with many components contributing to the perceived sensory characteristics. In general, non-volatile compounds that have some sensory impact in wine include sugars, organic acids, polymeric phenols and mineral substances. Importantly the volatile aroma compounds can be detected at very low concentrations in wine from ppm to as low as ppt and they have a great impact on the sensory perception of wine. There are hundreds of volatile compounds present but only a portion of them impact upon the aroma of wine.[3,11-13]

The total volatile aroma content of a wine, excluding ethanol is 0.8 g/L to 1.2 g/L which is predominantly made up of acetic acid, acetaldehyde, ethyl acetate, propanol, isobutanol, 2- and 3-methylbutanol. The remaining portion of volatiles comprises about 600-800 different volatile compounds present in varying amounts. Some of the important classes of these volatile compounds found in wine include acetals, organic acids including hydroxycinnamic acids, alcohols, esters, acetates, phenolic and

heterocyclic compounds, terpenes (monoterpenes and sesquiterpenes), norisoprenoids, sulfur containing compounds, methoxypyrazines and lactones.[14-16] Examples of compounds from some of the main important classes of volatile compounds and their odour description is detailed in the table below (*Table 1.2*).

Table 1.2 Examples of volatile aroma compounds identified in wine and their odour description.[13]

Class	Compound	Odour description
Esters	Ethyl isobutyrate Ethyl decanoate	Sweet, rubber Grape
Acids	Isobutyric acid Isovaleric acid	Rancid, butter, cheese Sweat, acid, rancid
Alcohols	Isoamyl alcohol 1-Hexanol	Whiskey, malt, burnt Resin, flower, green (cut grass)
Acetates	Isoamylacetate	banana
Phenolics	Guaiacol	Smoke, sweet, medicine
Terpenes	Linalool (monoterpene) Rotundone (sesquiterpene)	Flower, lavender Black pepper
Norisoprenoids	β-Damascenone β-Ionone	Apple, rose, honey Seaweed, violet, flower, raspberry
Methoxypyrazines	3-Isobutyl-2-methoxypyrazine	Earth, spice, green pepper
Lactones	*cis*-Oak lactone γ-decalactone	Coconut, flower Peach, fat
Volatile thiols	3-Mercaptohexan-1-ol 4-Mercapto-4-methylpentan-2-one	Passionfruit, grapefruit Boxwood

The odour detection threshold is quite variable between the different volatile compounds. Some compounds are more potent than others and their aroma description can significantly change depending on their concentration and the matrix. The aroma of dimethyl sulfide (DMS) at low concentrations can be described as cooked/canned asparagus, at higher concentrations like blackcurrant and at further increased concentrations its aroma is considered to be more like cooked corn/tomato.[13] 4-Mercapto-4-methyl pentan-2-one (4-MMP) is a potent sulfur compound and at high concentrations it has a distinct box tree/cat urine aroma but at low concentrations it is described to be like passionfruit/tropical fruit.[13]

The quantity and quality of the aroma and flavour composition of wine is determined by various factors including grape variety, the environmental conditions, vinification processes, yeast utilised during fermentation and ageing.[14,17] Volatile compounds can

originate from various sources during the winemaking process. They can be derived directly from the grape berry or arise from non-volatile precursors that release the volatile component during processing and/or storage. Volatile compounds can also come from yeast and bacterial metabolism, oak wood extraction and chemical reactions during the winemaking processes and/or upon storage.[13]

An important portion of the aroma compounds identified in wine are derived from non-volatile aroma precursors. A mature grape berry contains a number of non-volatile compounds including unsaturated lipids, phenolic acids, carotenoids, *S*-cysteine conjugates, glycoconjugates and *S*-methylmethionine.[16,18]

For example, the potent volatile thiol, 4-MMP is predominantly found in the form of non-volatile cysteine or glutathione conjugates.[3,19-21] Tominaga and co-workers showed that 4-MMP, 4-mercapto-4-methylpentan-2-ol (4-MMPOH) and 3-mercaptohexan-1-ol (3-MH) are released by the action of yeast during alcoholic fermentation due to the cleavage, by yeast, of the corresponding *S*-cysteine conjugates.[22] It has been shown that yeast strain selection, fermentation temperature and grape berry transportation can impact on the resulting amount of volatile thiols released.[19,20] Grant-Preece et al.[23] also showed the release of the volatile 3-MH from 3-*S*-glutathionylhexan-1-ol (3-Glut-3-MH) under model wine conditions where the cysteine conjugate is also formed, presumably as an intermediate, in the process.

Apocarotenoids are generally found in grapes as non-volatile glycoconjugates and have been extensively studied due to their structural diversity and influential aromas. Important aroma compounds such as β-damascenone and 1,1,6-trimethyl-1,2-dihydronapthalene (TDN), form by a complex multistep process. They are derived from norisoprenoid glycosides that undergo chemical rearrangements and biotransformations to produce the aromatic aglycone during winemaking or ageing processes. The pathways involved in the formation of these aglycone norisoprenoids are better understood than the biogenesis of their corresponding glycosidic precursors in plants.[18,24]

1.3 C_{13} norisoprenoids

C_{13} norisoprenoids are the most common and widespread group of volatile compounds in many grape varieties and include several powerful odorants. They are derived from the oxidative degradation of carotenoids by regiospecific oxygenases. The breakdown of carotenoids in grapes leads to the formation of C_9, C_{10}, C_{11} and mainly C_{13} norisoprenoids (*Figure 1.2*). The cleavage at the C_9-C_{10} position is governed by enzyme specificity, producing the most common family of C_{13} norisoprenoid derivatives.[16,18,25] Norisoprenoids such as β-damascenone (**1**) (*Figure 1.3*) and β-ionone have been found in the exocarp tissue of a grape berry.[26]

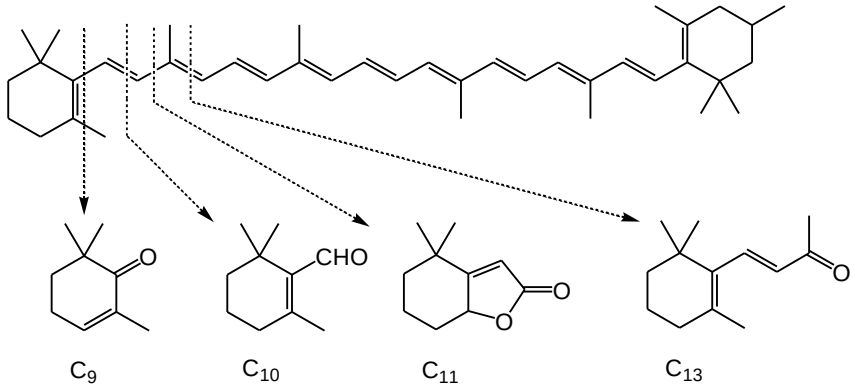
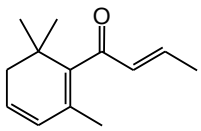


Figure 1.2 Formation of the cleavage products from β-carotene.[12,25,27]



1

Figure 1.3 Structure of damascenone 1.

There are two main types of norisoprenoids, those with a basic megastigmane skeleton (*Figure 1.4* and *1.5*) and the non-megastigmane compounds (*Figure 1.6*). The C_{13} megastigmane skeleton consists of a cyclohexyl ring substituted at the C_1, C_5 and C_6 positions with an unsaturated four carbon chain attached to C_6. The various classes of C_{13} norisoprenoid derivatives include the the rose ketones (**2**) where the skeleton is oxygenated at the C_7 position and the ionone series (**3**) where the skeleton is

oxygenated at the C_9 position (*Figure 1.5*). The non-megastigmane C_{13} norisoprenoid forms include the vitispiranes (**4**) and the theaspiranes (**5**) (*Figure 1.6*) and are known or presumed to be formed by rearrangement of megastigmane precursors.[12,28]

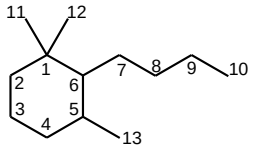


Figure 1.4 Basic C_{13} megastigmane skeleton.

2 3

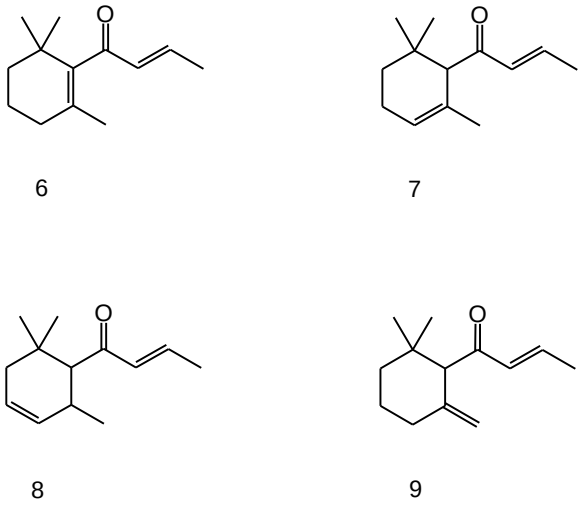
Figure 1.5 Megastigmane forms.[28]

NB: ≡ C=C / C–C

4 5

Figure 1.6 Non-megastigmane forms.

The rose ketone series are noted for their "rose like" aroma. These include damascenone (**1**), β-damascone (**6**), α-damascone (**7**), δ-damascone (**8**) and γ-damascone (**9**) (*Figure 1.7*).[29]



6 7

8 9

Figure 1.7 Some of the rose ketones.

1.4 Damascenone

β-Damascenone (**1**) (*Figure 1.3*), commonly referred to as damascenone is an important flavour and aroma compound found ubiquitously, from tobacco and honey, to tomatoes, cheese and both red and white wine.[30] Damascenone was first isolated from *Rosa damascena Mill.* (Bulgarian rose) oil in 1967[29] and reported in 1970 by Demole and co-workers.[31] The name "Damascenone" originates from the botanical name of the rose (*Rosa damascena Mill.*), giving the *root* "damasc" and the suffix "enone" which represents the chemical functionality of the compound.[29]

Damascenone **1** has a large array of aroma descriptors such as fruity, floral, rose, honey, plum, grape, raspberry, sugar and stewed apple.[29] These characters are considered desirable attributes in both red and white wine.[12] Damascenone was first identified in wine in 1974 in the extracts of Riesling, Rulander, Traminer and Scheurebe varieties.[32] It is a potent odorant with a low aroma detection threshold of 2 ppt[33] in water and 50 ppt in model wine.[34]

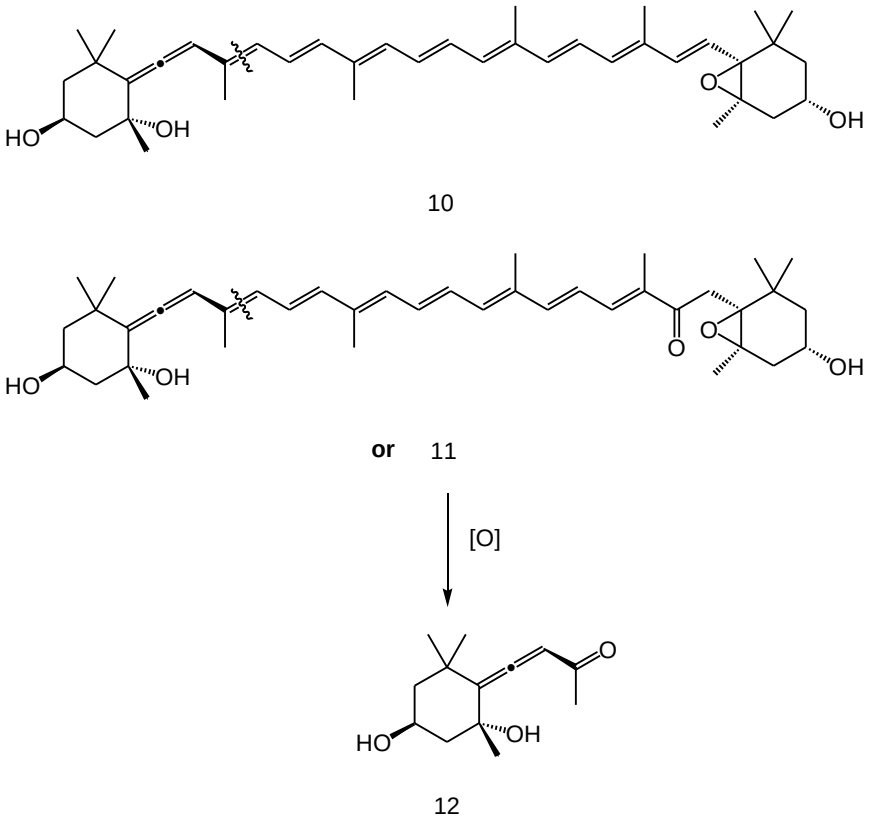
The first sensory description of damascenone was recorded as "magnificent"[29] and studies carried out by Guth[34] and Ferreira et al.[35] show that it enhances the positive bouquet of a wine rather than adding one specific character. Guth conducted a study focused on the blend of the most important odorants with high odour activity values of Gewürztraminer and Scheurebe white wines. The research revealed that the

removal of damascenone from the mixture decreased odour intensity but did not change odour quality.[34]

1.4.1 Formation of damascenone

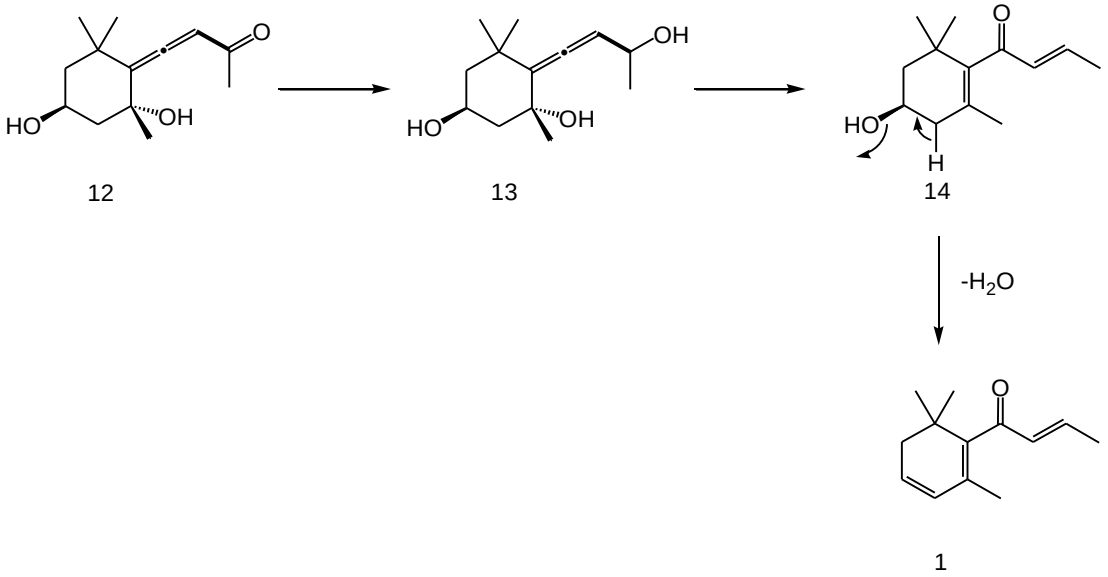
The formation of damascenone **1** in wine has been investigated for approximately the last 30 years, with most of the work focused on its formation via chemical mechanisms as reviewed by Sefton et al.[30] It is generally accepted that damascenone **1** is formed in nature by the hydrolytic breakdown of complex secondary metabolites derived from carotenoids with an allene functional group such as neoxanthin **10** or fucoxanthin **11**.[36,37] In particular, the allene group has been identified in 43 carotenoids out of approximately 700 naturally occurring carotenoids.[38] These carotenoids are natural pigments and are commonly found in a wide range of fruit and vegetables including lettuce, broccoli, grapes, mango, carrot, sweet potato, tomato and many more.[39] Sunlight is required for their biosynthesis and they will start forming in the grape at the first stage of fruit development and continue through until the onset of berry ripening (veraison). During berry development from veraison to maturity, the level of carotenoids decreases due to factors such as climate, agriculture practices and/or grape cultivar.[16]

The formation of damascenone **1** from various precursors was first proposed in 1973. It was initially suggested that the C_{13} norisoprenoid could be produced via various intermediates from grasshopper ketone **12**.[36,40] Grasshopper ketone **12** was first isolated in 1968 by Meinwald and co-workers and the structure and stereochemistry of this compound was confirmed by Russell and Weedon.[37,41] Meinwald et al.[37] proposed that the structures of the carotenoid pigments neoxanthin **10** or fucoxanthin **11** were closely related to the structure of **12** and thus could directly lead to the formation of **12** as a result of oxidative fission of **10** and/or **11** (*Scheme 1.1*). Grasshopper ketone has been identified in grape and wine extracts that were subjected to hydrolysis using a glycosidase enzyme indicating its presence as a glycoside. [42-45]



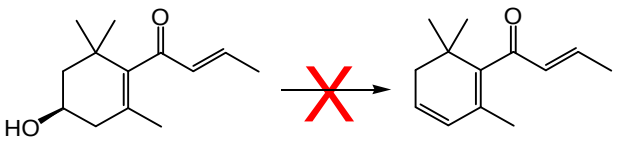
Scheme 1.1 Oxidative fission of neoxanthin 10 and/or fucoxanthin 11.

Isoe et al.[36] and Ohloff et al.[36,40] studied the metabolism of carotenoids and the possible biogenesis of damascenone. Isoe et al.[36] proposed that damascenone is derived from allenic carotenoids with grasshopper ketone **12**, the allenic triol **13** and 3-hydroxy-β-damascone **14** as intermediates (*Scheme 1.2*).



Scheme 1.2 Proposed formation of damascenone 1.

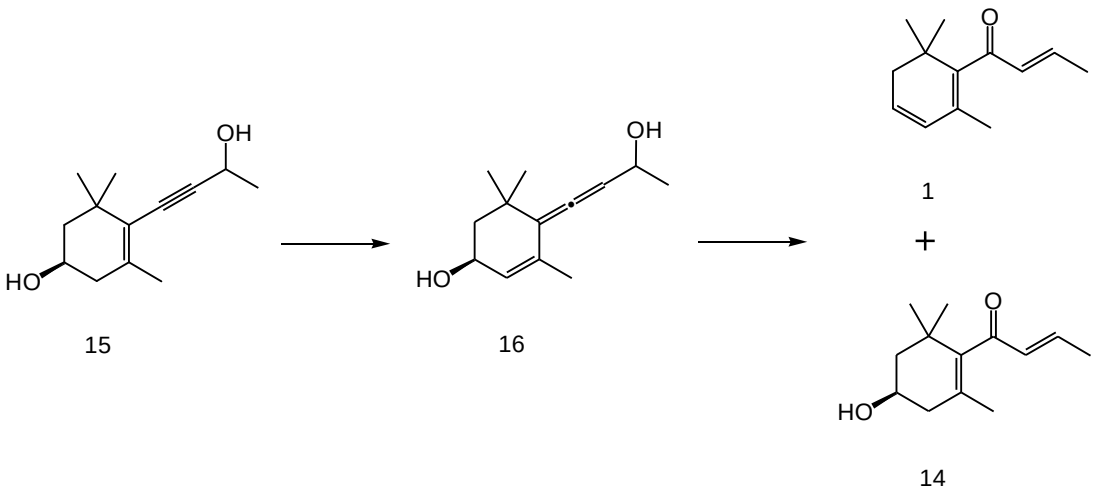
Ohloff showed that under acidic conditions (30% sulfuric acid) damascenone was not produced from 3-hydroxy-β-damascone **14** (*Scheme 1.3*) indicating that **14** was not an intermediate and damascenone was produced via an alternative pathway from the triol **13**.[40]



14 1

Scheme 1.3 Reaction of 3-hydroxy-β-damascone 14 under acidic conditions.

Ohloff also suggested that the acetylenic diol **15** and allenic diol **16** were intermediates in the formation of damascenone from the triol **13**. When compound **15** was treated with 30% sulfuric acid, the products obtained were **1** and **14** with **16** proposed as a potential intermediate (*Scheme 1.4*).[46]

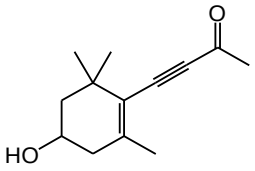


15 16 14

Scheme 1.4 Conversion of acetylenic diol 15 to ketones 1 and 14 under acidic conditions.

Thorough hydrolytic studies have been conducted on various possible precursors to damascenone over the years. Sefton et al.[47] confirmed the formation of **1** and **14** from **15** at room temperature and pH 3 but the conversion was extremely slow and they also verified that damascenone does not form from 3-hydroxy-β-damascone (**14**).

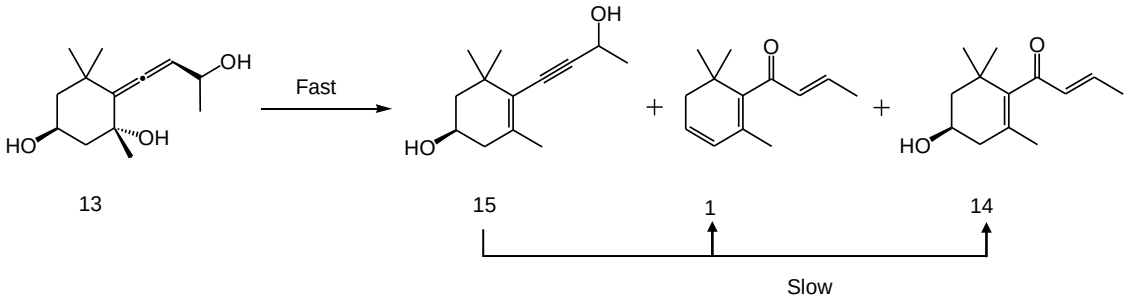
Winterhalter et al.[48,49] suggested **1** is derived from the carotenoids containing an allenic functional group such as neoxanthin **10** and/or fucoxanthin **11**. Winterhalter et al.[48] identified the oxidised form of **15**, the acetylenic ketone **17** (*Figure 1.8*). It was suggested that acetylenic ketone **17** could produce **15** via enzymatic reduction. It was thought that acetylene **17** could be produced from acetylenic carotenoids by the cleavage of the C_9, C_{10}-bond of a corresponding carotenoid. This would be an alternative pathway for the formation of **1** to the one previously proposed by Ohloff et al.[40] and Isoe et al.[36] However, it is possible that **17** could also form from the oxidation of **15** or by the dehydration and rearrangement of **12**.



17

Figure 1.8 Acetylenic ketone 17.

Skouroumounis and co-workers aimed to test the hypothesis that the triol **13** produces damascenone at wine pH. Hydrolysis of the triol at pH 3.0 gave damascenone **1**, 3-hydroxy-β-damascone **14** and the acetylenic diol **15** (*Scheme 1.5*). Damascenone was very much the minor product (< 1%), although it was concluded that the rate of formation of damascenone from the hydrolysis of the allene triol, was enough to account for the damascenone found in grapes and other fruits.[50]

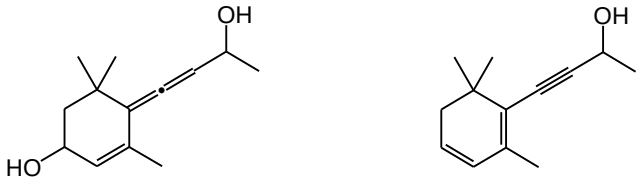


13 15 1 14

Scheme 1.5 Formation of damascenone 1, acetylenic diol 15 and 3-hydroxy-β-damascone 14.

Skouroumounis and co-workers reported two intermediates observed during the hydrolysis of the allene triol. They were tentatively assigned using mass spectral data

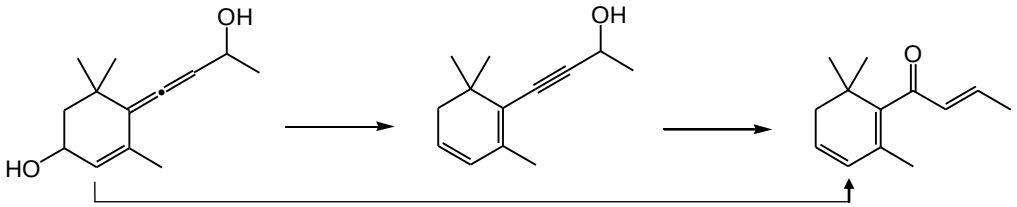
as megastigma-4,6,7-triene-3,9-diol (**16**) and megastigma-3,5-dien-7-yn-9-ol (**18**). The dienyne alcohol **18** had previously been reported as a constituent of rum, which is also known to contain damascenone. Skouroumounis and co-workers proposed this dienyne alcohol as a possible immediate precursor to damascenone (*Figure 1.9*).[50]



16 18

Figure 1.9 Allene diol 16 and dienyne alcohol 18.

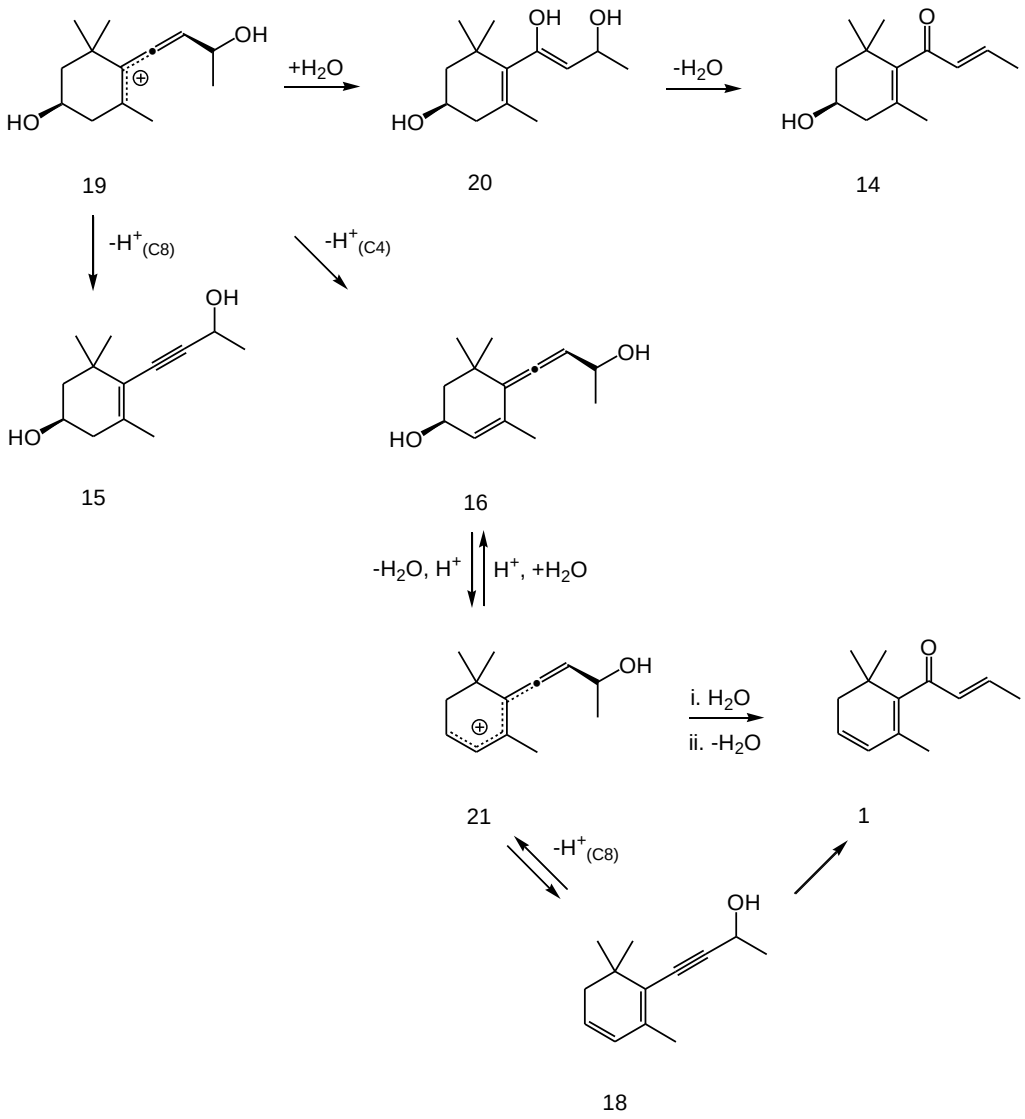
Puglisi and co-workers further investigated the two intermediates, the allene diol **16** and the dienyne alcohol **18**, confirming their identities by synthesis. In 2001 they found that hydrolysis of **18** in model wine at room temperature produced damascenone **1** in > 90% yield, with no trace of 3-hydroxy-β-damascone **14**. This indicated that the dienyne alcohol was an immediate precursor to natural damascenone (*Scheme 1.6*). Acid hydrolysis of the allene diol **16** also yielded damascenone as the main product. The dienyne alcohol **18** and some epimerised starting material were observed as intermediates. This work showed that the three main products from the triol **13**, 3-hydroxy-β-damascone **14**, the allene diol **16** and the enyne diol **15** all form by competing pathways (*Scheme 1.7*).[51,52] Additionally, hydrolytic data showed the conversion of the acetylenic alcohol to damascenone occurred at a slower rate than the conversion of the allenic diol **16** to damascenone. This indicated that there are two pathways from the allenic diol **16** to damascenone **1**, directly from the diol or via intermediate **18**.[49,50]



16 18 1

Scheme 1.6 Conversion of allenic diol 16 to damascenone 1.

It is evident from these findings that the presence of the $C_{4,5}$ double bond is an absolute prerequisite for the formation of damascenone. This activates the C_3 hydroxyl, which is then lost via dehydration to produce damascenone.[53]



Scheme 1.7 Summary of the chemical pathways involved in the formation of damascenone 1.

Based upon the vast amount of hydrolytic data, Daniel et al.[54] proposed a chemical mechanism for the formation of damascenone **1** from the allenic diol **16**. The cation **19** is expected to form from the loss of the C_5 hydroxyl from the allene triol **13** under acidic conditions. The subsequent loss of the C_8 proton leads to the formation of the acetylenic diol **15** or hydration at the C_7 position forms 3-hydroxy-β-damascone (**14**). The loss of a proton at the C_4 position from the cation **19** would lead to the formation of the allenic diol **16**. The loss of the C_3 hydroxyl from the allenic diol **16** affords

damascenone as the main product or alternatively the formation of the acetylenic alcohol **18** occurs through a C_3 carbocation formed by the loss of the C_8 proton leading indirectly to the formation of damascenone (*Scheme 1.7*).[54]

1.5 Yeast and fermentation

Much development has occurred in the area of flavour and aroma optimisation in wine by the selection of yeast strains for fermentation.[55] The impact of yeast strain on wine composition has proved to be a major contributor to the resulting flavours after fermentation. In particular the characterisation of *Saccharomyces cerevisiae (S. cerevisiae)* has revealed that it produces many secondary metabolites that are key sensory drivers.[56] The knowledge of how yeast contributes to the processes involved in the formation of the aroma compound damascenone **1** is one of the main focuses of this thesis. Being able to modulate the formation of this aroma active and desirable character by the selection of yeast might provide winemakers a better guide to achieving their targeted aroma profile.

The concentration of damascenone has been shown to change during various stages of the wine making and storage processes, as reviewed by Sefton et al.[30] In general it has been observed that, damascenone increases in concentration post-fermentation by significant amounts, degrades over the first few months of maturation but has also been seen to increase during the maturation of wine depending on the environmental conditions.

The formation of damascenone during fermentation has yet to be fully understood. Many studies have shown an increase in damascenone concentration during and at the end of fermentation but the precursors responsible for this increase are unknown. This thesis is focused on identifying potential precursors to damascenone that not only undergo chemical transformation but also biotransformation by the action of yeast during fermentation to give damascenone.

1.5.1 *Saccharomyces cerevisiae*

Wine fermentation is generally carried out by the yeast *S. cerevisiae* which converts grape sugars into ethanol and carbon dioxide and a range of secondary products.[3,4,57,58] It wasn't until the second half of the 19th century that the role of yeasts in the transformation of grape juice into wine was well understood. Traditionally, wine fermentation occured spontaneously due to yeast present on the surface of the grapes and the results were often unpredictable.[1] There is a range of microflora that exists on the grape including moulds, bacteria and yeasts. Only a small portion of the large variety of yeast present of the grape plays a role in alcoholic fermentation.[56]

The growth of non-*Saccharomyces* yeasts dominates the beginning stages of grape must fermentation. They are characterised by their low fermentative power but contribute to the flavour of wine by complex processes involving different yeast genera and species. The non-Saccharomyces yeasts utilised in spontaneous and inoculated fermentation include *Hanseniaspora* (*Kloeckera*) and *Candida*.[56]

Due to the rising ethanol concentration during fermentation, the growth of the non-*Saccharomyces* yeasts are limited to the first 2-3 days of fermentation and the strains of *S. cerevisiae* then become more dominant and complete the fermentation.[56] The most important yeast species involved in grape must fermentation is *S. cerevisiae* which comprises a very large number of strains with varied technological properties. In its various forms it can act as wine yeast, brewer's yeast, distiller's yeast and baker's yeast.[15]

1.5.2 Yeast and the organoleptic properties of wine

Microorganisms have a major role in determining the organoleptic properties of a wine with yeast having a significant influence due to their role in alcoholic fermentation. The microflora present on the surface of grapes can contribute to a number of biochemical reactions. These reactions occur during ripening of the grapes and wine production and thus can influence the overall composition of wine.[17,56]

The non-*Saccaromyces* and *S. cerevisiae* yeast strains that are involved in alcoholic fermentation influence the fermentation speed, the nature and quantity of secondary products and the aroma characters of wine.[58] The non-*Saccharomyces* yeasts compete with the *Saccharomyces* for nutrients, contribute to the nature of secondary products present in the final wine and as such affect the overall bouquet.[56]

The volatile profile of a wine can change depending on the yeast strain used during fermentation. Loscos et al.[24] reported the significant effect that yeast strains of *S. cerevisiae* can have on the concentration of various important aroma compounds. The study involved three commercial strains with a strong influence for compounds, *E*-2-hexenol, *E*-whiskylactone, δ-octalactone, γ-nonalactone, *o*- and *m*-cresols, 4-ethyl-2-methoxyphenol, 4-allyl-2-methoxyphenol, 4-vinylphenol, zingerone, actinidiols, 3,7-dimethyl-1,5-octadien-3,7-diol and farnesol. The majority of the compounds reported are grape or oak derived and the change in their concentrations is still not fully understood. The greatest difference between yeast strains of *S. cerevisiae* was for the compound 4-vinylphenol which showed a 10 fold difference between yeast strains.[24] 4-Vinylphenol can be formed as a result of decarboxylase activity by a range of microorganisms[59,60] but generally in white wines it is produced by *S. cerevisiae*.

The amount of 4-MMP formed as a result of the fermentation has been shown by Dubourdieu and coworkers to be dependent upon the type of yeast strain utilised to conduct the fermentation. Notably, fermentations with S*accharomyces bayanus* strains have been shown to release more 4-MMP than with *S.cerevisiae* strains.[61-63] Howell and co-workers confirmed these observations by showing that the amount of 4-MMP that can be obtained from the Cys-4-MMP precursor is dependent on the commercial wine strain utilised with the yeasts all varying their ability to release this important volatile thiol.[20,62]

Yeasts of different genera have been reported to be distinguished by their contribution to the varietal aroma produced from non-volatile grape precursors. Fermentations with *Saccharomyces* yeasts resulted in significant amounts of the important aroma compounds β-damascenone, β-ionone and linalool whereas those

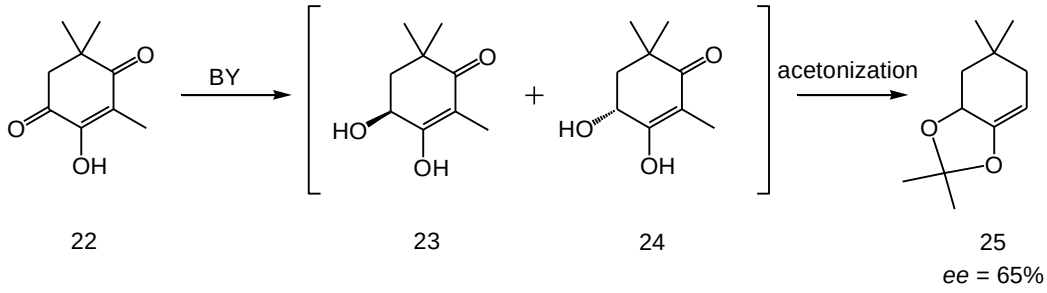
with *Brettanomyces* had higher amounts of lactones, Riesling acetal, ethyl vanillate and ethyl dihydrocinnamate.[64]

1.5.3 Biotransformation

Microbial transformations including yeast-mediated transformations have been utilised for thousands of years to make bread, dairy products and alcoholic beverages. These microorganisms not only have a role in human nutrition but are significantly useful in organic chemistry. A significant property of the fermenting yeast *S. cerevisiae* is its reducing action. *S. cerevisiae* has been used as a reducing agent during chemical synthesis.[65]

The reducing action of fermenting yeast, *S. cerevisiae* was first observed by Dumas in 1874.[65] He reported the formation of hydrogen sulfide from the addition of powdered sulfur to a suspension of fresh yeast in a sugar solution.

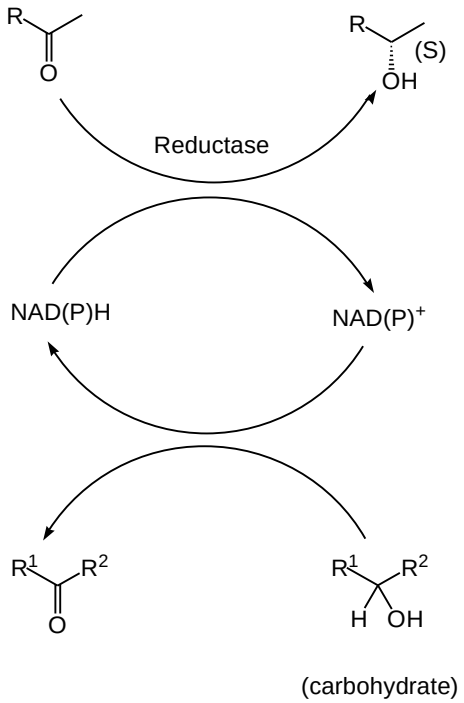
It has been shown that using fermenting *S. cerevisiae* to reduce diketone compounds is an effective method to produce the corresponding alcohols **23** and **24** in an enantioselective manner. Reduction of 4-hydroxyisophorone (**22**) using *S. cerevisiae* followed by acetonization gave 25-30% of **25** with an ee of 65% (*Scheme 1.8*).[65]



Scheme 1.8 Biotransformation using S. cerevisiae.[65]

The most common application of this organism to organic synthesis is the asymmetric reduction of ketones. The *S. cerevisiae* mediated reductions of ketones can be enantioselective or diastereoselective. Development in the area of engineered yeast cells has been aimed at improving the stereoselectivity of these reactions where the

main problem is the issue of multiple yeast reductase enzymes all with different stereoselectivities.[66]



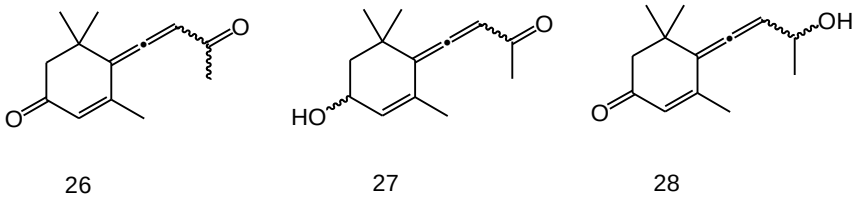
Scheme 1.9 Bioreduction of ketone to alcohol by S. cerevisiae.
R, R^1, R^2 = H, alkyl, aryl etc.

Various enzymes responsible for the reduction that occurs have been suggested to be alcohol dehydrogenase, aldose reductase and NADPH reductase. *S. cerevisiae* is the most popular whole-cell biocatalyst rather than the utilisation of isolated enzymes because cofactor regeneration is not required due to the presence of nicotinamide cofactors (usually NADPH). Cofactor regeneration occurs because yeast contains the required enzymes to carry out this process. The $NAD(P)^+$ captures a hydride from glucose or other carbohydrates present during fermentation and this hydride is transferred to a substrate. This enables the reduction of a substrate such as ketones to the corresponding alcohol (*Scheme 1.9*).[67,68] Monoterpenoid ketones have also been shown to undergo reduction by the action of alcohol dehydrogenase enzymes.[69,70]

1.6 Aims

This thesis will focus predominantly on the role of yeast in the formation of damascenone **1** from the ketone precursors **26**, **27** and **28**, during fermentation. The formation of damascenone under hydrolytic conditions has been well documented but the full extent of the role of yeast contributing to its formation has not been completely elucidated. The concentration of damascenone increases post fermentation, but the cause of this increase deserves further attention. Understanding the means by which this important aroma compound is generated is the first step towards the goal of being able to control its formation in finished wines.

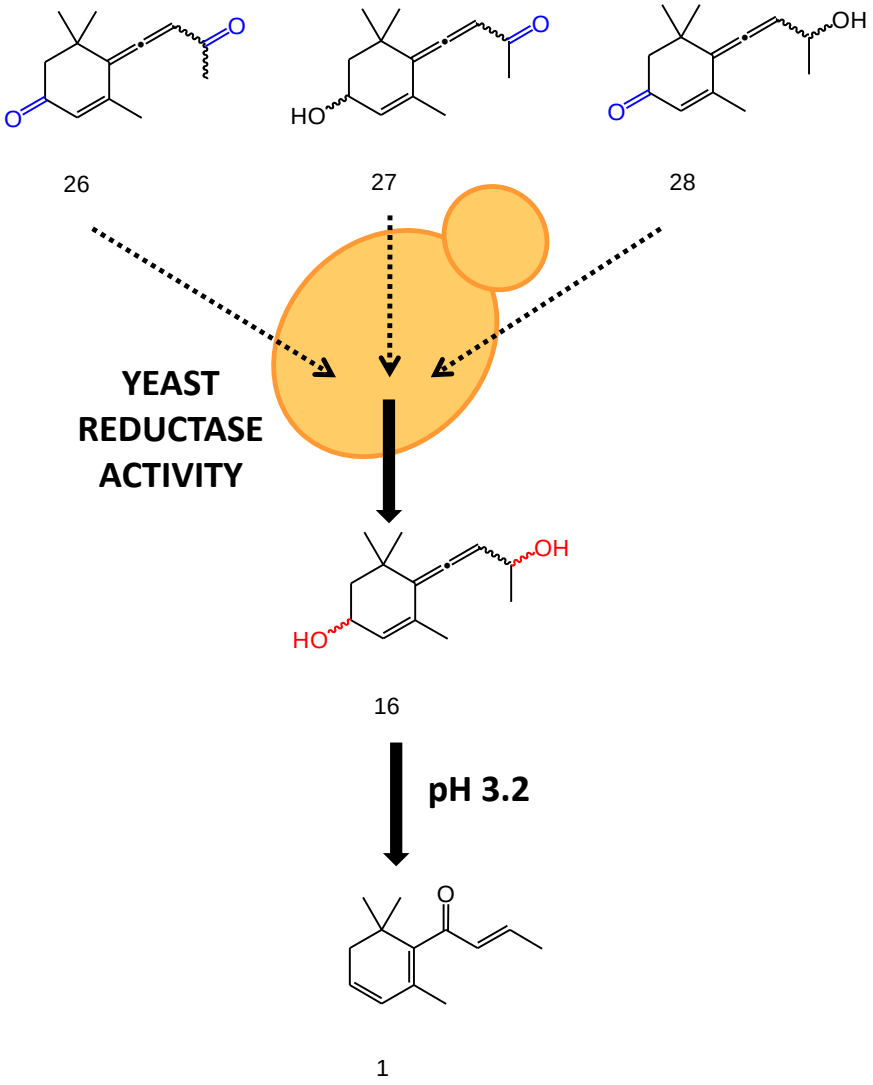
Potential precursors **26**, **27** and **28** were selected for synthesis, namely ketones which could then be used in fermentation studies to investigate the formation and concentration of damascenone obtained from these compounds (*Figure 1.10*).



26 27 28

Figure 1.10 Allenic ketones 26, 27 and 28.

It was proposed that these potential ketone precursors might undergo transformations that are both fermentation induced and acid catalysed. Reduction to their corresponding diols **16** by the action of the yeast could occur, followed by hydrolysis under wine acidic conditions to form damascenone as depicted in *Scheme 1.10*.



Scheme 1.10 Proposed biotransformation of the ketones 26, 27 and 28.

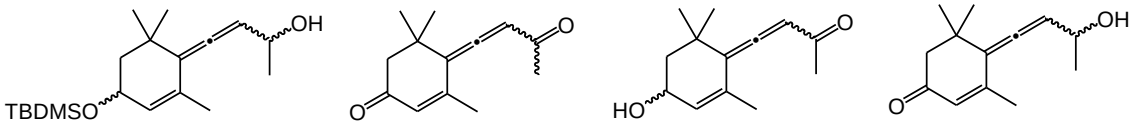
Different yeast strains were utilised in this study to investigate if there are differences between strains in their ability to produce damascenone **1**.

Chapter 2

Synthesis of allenic ketones

CHAPTER 2 Synthesis of allenic ketones

The allenic ketones **26**, **27** and **28** (*Figure 2.1*) were synthesised and characterised in this study. The various methods trialled to synthesise the ketone **28** are also discussed.



29 26 27 28

Figure 2.1 Protected allene alcohol, 29 and allenic ketones 26, 27 and 28.

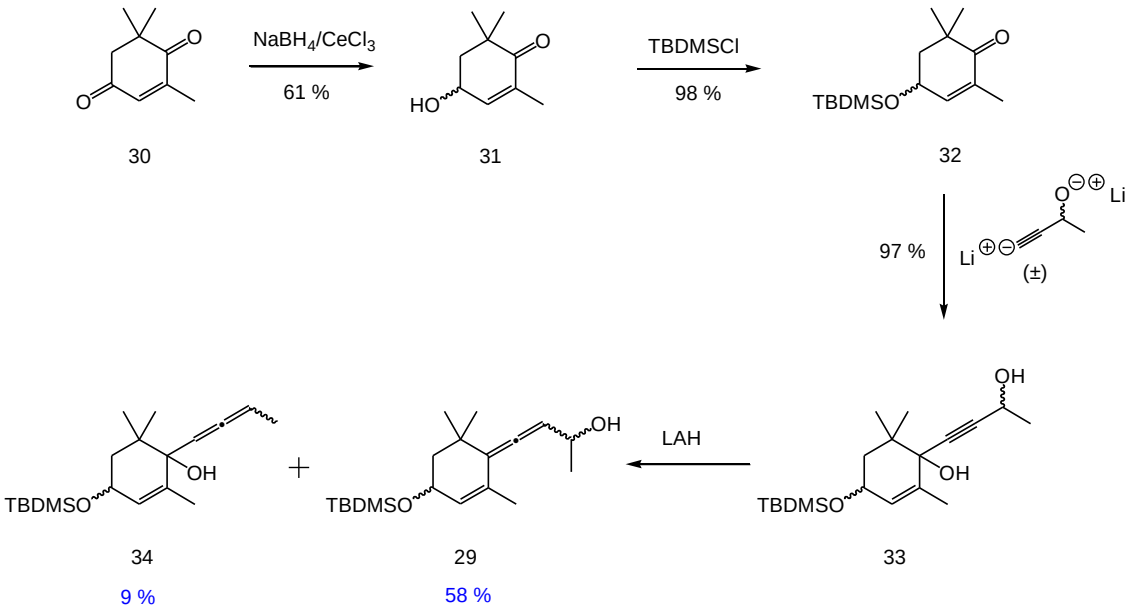
The allenic ketones were selected based on previous literature by Skouroumounis et al.,[50] Puglisi et al.[51,52] and Daniel et al.[54] on the allene diol **16** which was converted to damascenone as the main product under acid hydrolysis conditions. Daniel et al.[54] observed damascenone to account for more than 60% of the products from the allene diol and no other product was observed by GCMS.

Oxidised forms of the allene diol **16** were selected as potential precursors to damascenone. It was anticipated that these precursors could be reduced by the yeast during fermentation to produce the allene diol **16** and then, under the acidic conditions of wine, they would furnish damascenone.

2.1 Synthesis of C_3 protected allenic alcohol (29)

The synthesis of the protected allene alcohol **29** was performed over 4 steps using a modified form of the procedure as described by Puglisi[52] (*Scheme 2.1*). The synthesis of 2,6,6-trimethyl-4-hydroxycyclohex-2-en-1-one (**31**) was achieved by a Luche reduction with 4-oxoisophorone (**30**), $NaBH_4$ and $CeCl_3$. The alcohol **31** was the major product and was accompanied 3,5,5-trimethyl-4-hydroxycyclohex-2-en-1-one. The absence of the $CeCl_3$ in this reaction produced the latter isomer only, indicating the importance of coordination of the caesium ion to the less hindered carbonyl to promote the required reduction.[71] The ketone **31** was subsequently protected at the C_4 position as its silyl ether using TBDMS chloride, giving **32**. This product was then treated with a di-lithio derivative of (±)-but-3-yn-2-ol to produce the C_3 protected

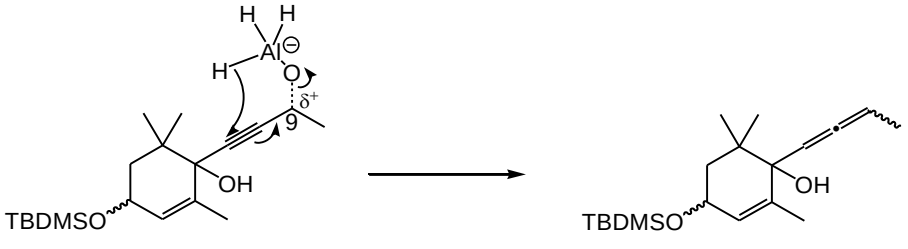
dienyne diol **33**. The final step in the synthesis of the C_3 protected allene diol **29** (*Scheme 2.1*) involved treating the dienyne diol with LAH under reflux conditions to furnish a mixture of two diastereomeric regioisomers (**29** and **34**).



Scheme 2.1 Synthetic pathway towards protected allenic diol 29.

The required major isomer **29** was isolated by column chromatography and used to synthesise the potential precursors to damascenone. Several portions of the allene **29** were synthesised as required. Storing the allene **29** in the fridge or freezer at -10 ^{o}C for about 2 months led to the degradation of this compound and leaving in the sunlight also led to a complex mixture of products within a two week period. Storing this compound at -80 ^{o}C prevented degradation. In general, **29** was synthesised immediately before use to ensure degradation did not occur. The allene **29** was a mixture of four diastereomers but separation was not required for the forthcoming reactions.

The allene **34** was also isolated by column chromatography and fully characterised for the first time. The ratio of the allene **29** to the allene **34** was 5:1, respectively. The allene **34** is presumed to be formed by the complexation of the aluminium to the C_9 hydroxyl (*Scheme 2.2*), in contrast to the complexation to the C_6 hydroxyl giving the allene **29**.



33 34

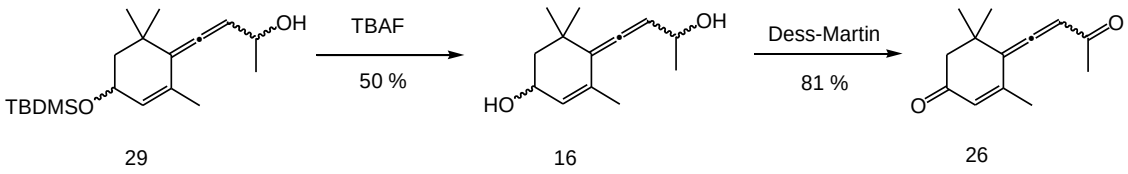
Scheme 2.2 Formation of the allene 34 from the dienyne diol 33.

The proton NMR showed characteristic signals for **34** between 5.45 ppm and 5.40 ppm resonating for the proton at C_9, at 5.36 ppm resulting from the olefinic proton at C_4, between 5.31 ppm and 5.29 ppm resulting from the proton at C_7 and a multiplet between 4.29 ppm and 4.25 ppm resonating from the proton at C_3.

2.2 Synthesis of megastigma-4,6,7-trien-3,9-dione (26)

The diketone **26** was synthesised in two steps from the previously synthesised allene alcohol **29** (*Scheme 2.3*). The first step involved the deprotection of the hydroxyl moiety at the C_3 position to produce the allene diol **16**. This reaction occurred over a few days and produced the allene diol in adequate yield. The second step in the synthesis was to perform a double oxidation on the allene diol to furnish the target diketone **26**.

Previous work conducted on the oxidation of analogous compounds to **16** utilised the reagent manganese dioxide.[72] This reagent however, produced the diketone **26** from the diol **16** in very poor yields. The Swern oxidation which requires oxalyl chloride, triethylamine and dimethylsulfoxide (DMSO) as described in section 2.3.1 was then employed to oxidise the diol **16**. The reaction produced **26** successfully but only gave a yield of 43%. The Dess-Martin periodinane reagent (*Scheme 2.5*) was then trialled for the oxidation of the diol **16** and produced the diketone **26** in a yield of 81%.[73] The Dess-Martin oxidation is discussed in more detail in the next section (*2.3.1 Dess-Martin vs Swern oxidation*).



29 16 26

Scheme 2.3 Synthesis of the diketone 26 precursor from the allene alcohol 29.

The characterisation and purity of the synthesised diketone compound was confirmed by GCMS, ^{1}H and ^{13}C NMR followed by COSY, HMQC and HMBC.

The proton NMR of the diketone **26** revealed the absence of the multiplet between 4.39-4.32 ppm which represented the protons present at C_3 and C_9 in the spectrum for the diol **16**. The signal at 6.17 ppm for the proton resonating at C_8 changed from a broad doublet in the proton NMR for the diol **16**, to a broad singlet. The signal at 5.96 ppm for the proton at C_4 in the diketone changed from a multiplet in the diol **16** to a broad singlet in the diketone **26**. The broad singlets representing the protons resonating at C_4 and C_8 are characteristic for the diketone because their chemical shifts remained downfield at around 6 ppm characteristic for the allenic proton and vinyl proton and the broad singlets indicated the absence of the adjacent protons.

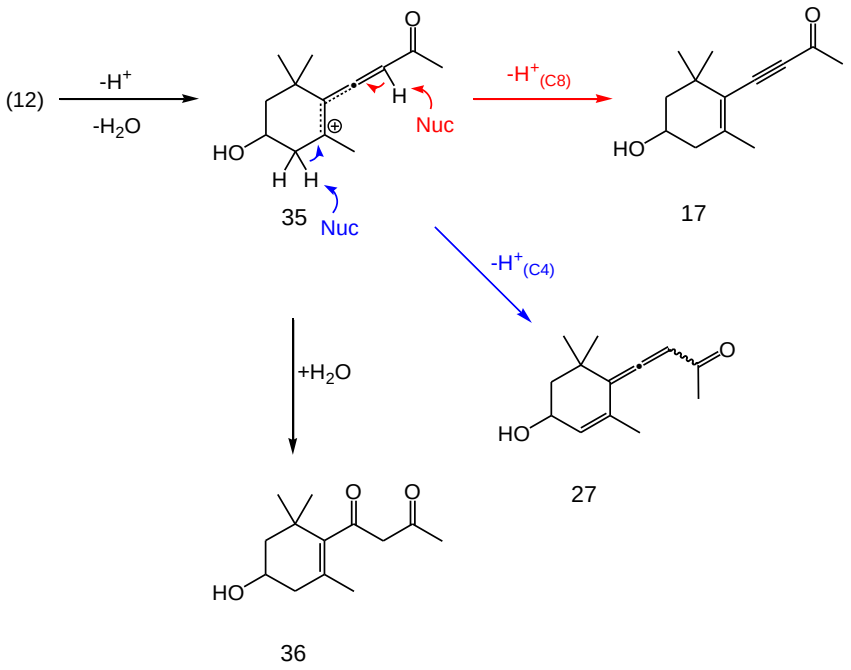
The carbon spectrum showed characteristic carbonyl signals at 197.5 ppm and 197.1 ppm for the C_3 and C_9 carbonyl carbons. The allene carbon was also identified further downfield at 214.3 ppm for the C_7 Carbon.

The mass spectrum showed a small peak at *m/z* 204 for the molecular ion but significant ions at *m/z* 162 and 147 (100%). These peaks were attributed to the loss of the C_9 carbonyl group and the C_{10} methyl group from the molecular ion with proton transfer to the resultant cation to afford the *m/z* 162. A subsequent loss of a methyl group resulted in the fragment $C_{10}H_{11}O^{+\bullet}$ with *m/z* 147.

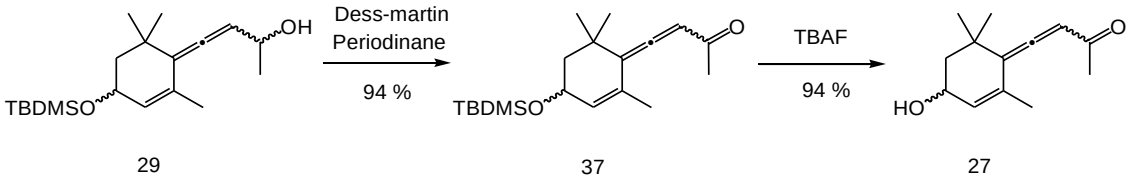
2.3 Synthesis of 3-hydroxymegastigma-4,6,7-trien-9-one (27)

In a similar manner to the diketone **26**, it was hypothesised that biotransformation of ketone **27** would occur under fermentation conditions, producing damascenone **1**. The synthesis of the ketone **27**, a putative *in vivo* dehydration product of grasshopper

ketone[74] (*Scheme 2.4*) was performed in two steps from the protected allene alcohol **29** (*Scheme 2.5*). The Dess-Martin periodinane oxidation of the alcohol moiety at the C_9 position furnished the target compound in a 94% yield. The alcohol at the C_3 position was then deprotected using TBAF in dry THF to produce the ketone **27**. This last step proceeded over 16 hrs and produced the desired product, also in a yield of 94%.



Scheme 2.4 Possible in vivo dehydration products of grasshopper ketone including the ketone 27.



Scheme 2.5 Synthesis of ketone 27.

The characterisation and purity of the synthesised ketone **27** was confirmed by GCMS, ^{1}H and ^{13}C NMR followed by COSY, HMQC and HMBC. The NMR revealed a mixture of the two diastereomers **27** with some of the signals coincident and some of the signals overlapping. For fermentation studies, the ketone **27** was synthesised and isolated as a mixture of diastereomers. The diastereomers of the ketone **27**, were separated by

column chromatography with the first eluted diastereomer being also the first eluted by GCMS. The first eluted diastereomer was analysed by proton NMR (*Figure 2.2*).

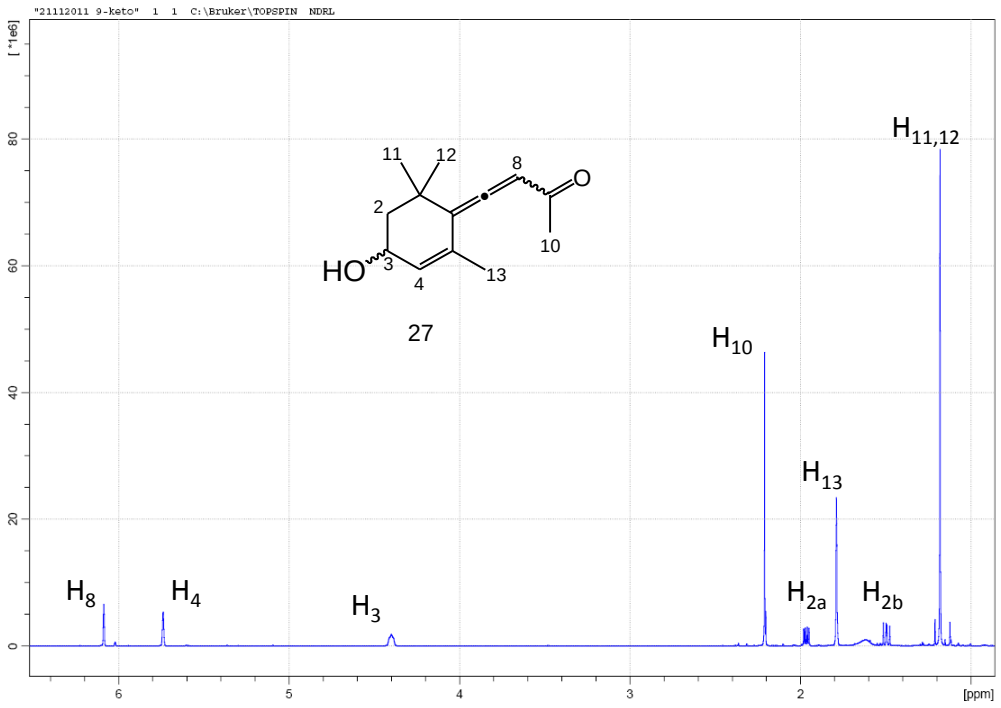


Figure 2.2 Proton NMR (600 MHz) of the first eluted diastereomer 27.

The proton NMR of the single diastereomer was directly compared to the mixture of the two diastereomers. The proton NMR of the diastereomer mixture showed two signals at 6.09 ppm and 6.02 ppm characteristic for the proton at the C_8 position, the NMR for the single diastereomer revealed one predominant signal at 6.09 ppm (*Figure 2.2*).

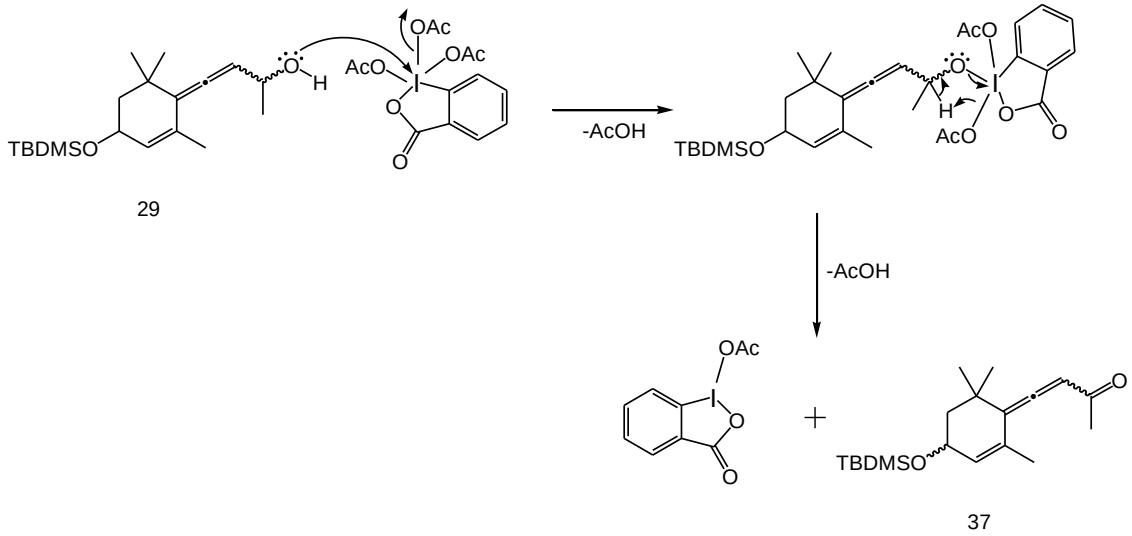
The mass spectrum of the ketone **27** had a small molecular ion peak at *m/z* 206 and showed significant ions at *m/z* 191 (100%) and *m/z* 121 for both diastereomers. The loss of a methyl group from the molecular ion followed by proton transfer produced *m/z* 191. The ion at *m/z* 149 was attributed to loss of a methyl group plus the C_9, C_{10} acetyl group analogous to the fragmentation of diketone **26**. A subsequent loss of water produced the ion at *m/z* 121.

2.3.1 Dess-Martin vs Swern oxidation

Although, the oxidation of the allene alcohols **16** and **29** were originally performed using Swern oxidation conditions, Dess-Martin oxidation of **16** was also trialled. The Dess-Martin periodinane reagent not only gave the oxidised allenes **26** and **37** in a higher yield but the reaction duration was minimised, conditions were cleaner and safer and less reagents were required.

The Swern oxidation required the reagents oxalyl chloride, DMSO, DCM and triethylamine to complete the oxidation. The reaction had to be maintained at -60 ^{o}C for long periods to enable the addition of each reagent and the workup required additional washes of the organic extract. The Dess-Martin oxidation was similar only requiring the addition of the Dess-Martin periodinane to the alcohol in DCM with stirring at room temperature.

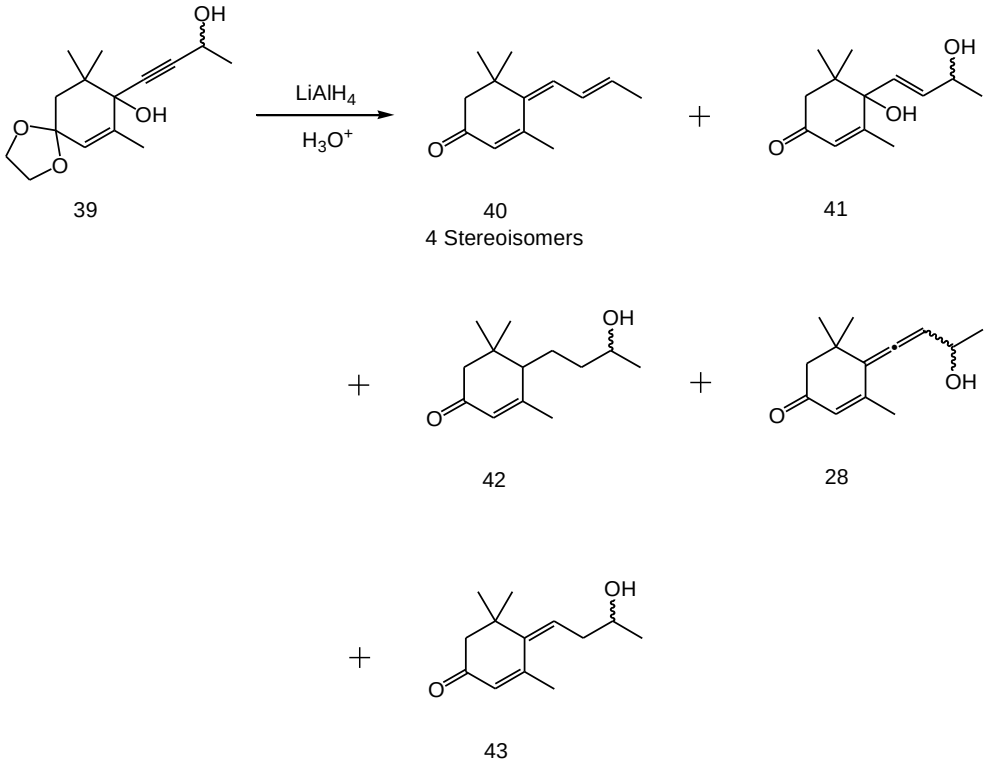
The mechanism using Dess-Martin oxidation of allenic alcohol **29** to the allene **37** can be seen below (*Scheme 2.6*). It is a selective and very mild reagent and requires a basic workup which is very suitable for these allenes which can be reactive under acidic conditions. The by-products are minimal and not toxic like the dimethyl sulfide by-product under the Swern conditions.



Scheme 2.6 Formation of the C_3 protected ketone 37 via oxidation of the alcohol 29 using the Dess-Martin periodinane reagent.

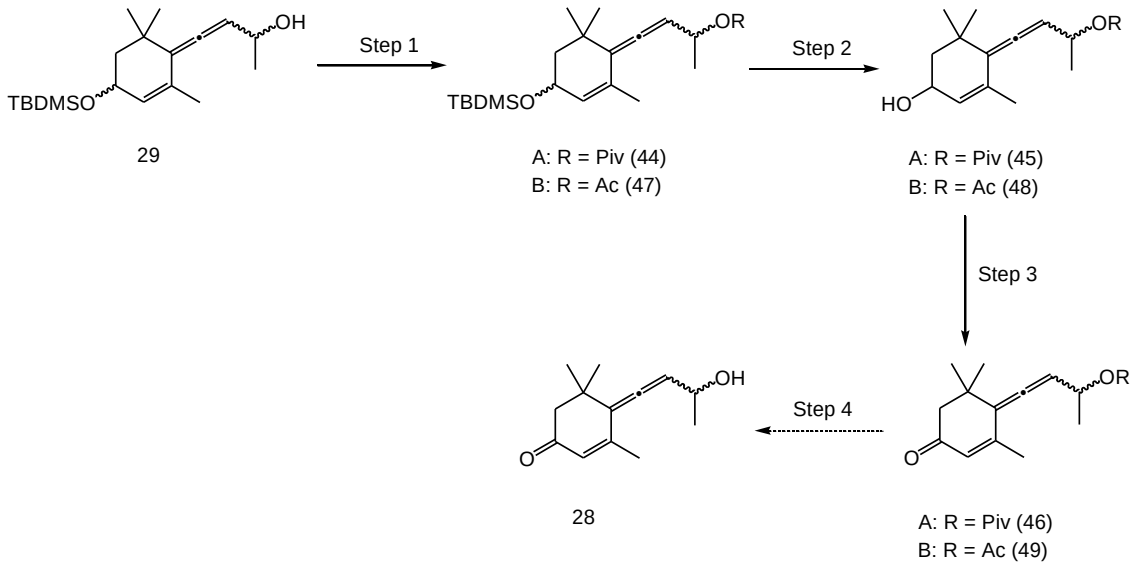
2.4 Synthesis of 9-hydroxymegastigma-4,6,7-trien-3-one (28)

The synthesis of the allenic ketone **28** had previously been achieved by Demole et al.[75] However, the reaction pathway produced a complex mixture of products and the synthesis of this ketone **28** was very low yielding (*Scheme 2.7*).



Scheme 2.7 Previous synthesis of ketone 28.

Thus, an alternative pathway was devised in the hope that the allenic ketone **28** could be produced in a higher yield. Various pathways to the ketone **28** were investigated and they are discussed in detail below (*Scheme 2.8A and Scheme 2.8B*).



Scheme 2.8 Synthetic pathway towards the synthesis of ketone 28.

Scheme 2.8A: Step 1 – Pivaloyl chloride, pyridine, DCM, reflux; Step 2 – TBAF, THF; Step 3 – Dess-Martin oxidation; Step 4 – (i)NaOH, (ii)NaOMe, (iii)Amberlite OH resin

Scheme 2.8B: Step 2 – Acetyl chloride, pyridine, DMAP, DCM; Step 2 – TBAF, THF; Step 3 – Swern oxidation, THF; Step 4 – (i)NaOH or (ii) NaOMe

Scheme 2.8A:

The first synthetic route trialled for the synthesis of ketone **28** involved 4 steps beginning with the pivaloylation of the C_3 protected allene **29**. This was followed by deprotection of the C_3 alcohol using TBAF and then oxidation of the product via Swern conditions which gave **46** in a yield of 50%.

The signal present at 4.34 ppm in the proton NMR of **45** was characteristic for the proton at the C_3 position and the proton NMR of the product **46** showed no trace of this signal confirming that the alcohol at the C_3 position had in fact been oxidised (*Figure 2.3*).

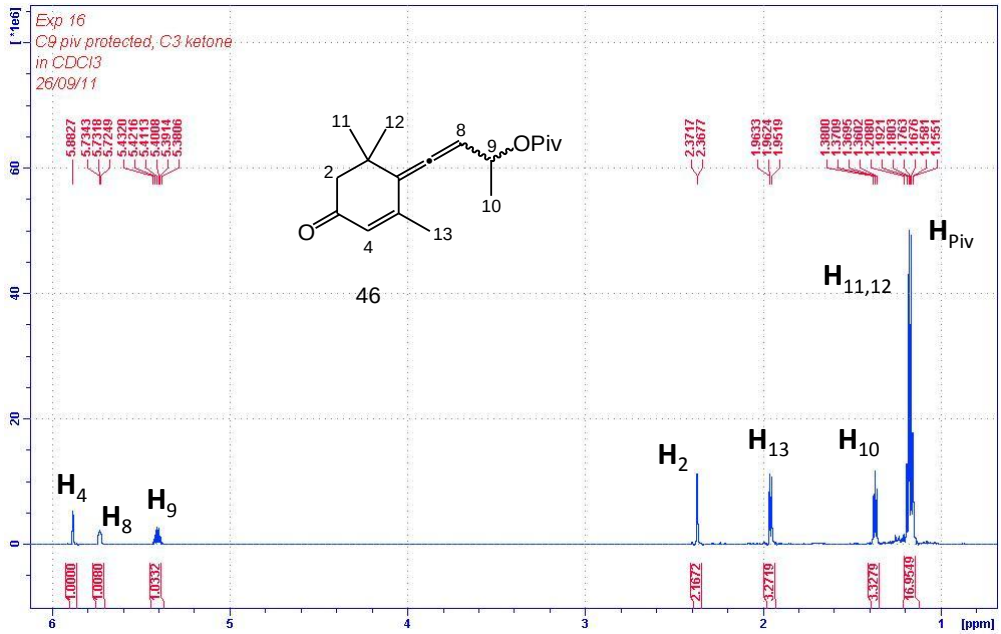


Figure 2.3 Proton NMR (600 MHz) of 46.

The final step in the reaction sequence involved removing the pivaloyl group to afford the free hydroxyl at the C_9 position. The conditions first used to remove the pivaloyl group were basic conditions with 1M sodium hydroxide. Analysis of the product by proton NMR and GCMS showed the presence of mostly the starting material and a small portion of unidentified material. It was thought a stronger base would be required for the removal of the pivaloyl group so sodium methoxide (1 equiv.) was used as according to Nicolaou et al.[76] However, NMR analysis of the product showed that the allene functional group was not remaining intact under these conditions and other reactions were occurring.

The reaction mixture was allowed to stir for 2.5 hrs and monitored by TLC. TLC indicated depivaloylation occurred efficiently over the 2 hrs but produced several products that were more polar than the starting material. Two of the fractions obtained from column chromatography of the end product appeared to be a mixture of isomers where as the last fraction appeared to be one pure isomer. Each fraction also had some other unidentified material present as indicated by GCMS which made it difficult to elucidate the structures of these unknowns.

Close examination of the NMR data did indicate that the allene functional group was no longer intact in any of the resulting products. The multiplet signal characteristic for

the allenic proton at the C_8 position is at 5.74 ppm in **46** which shifted upfield to 4.59 ppm in the product and appeared as a broad doublet. The multiplet responsible for the proton at the C_9 position also shifted upfield from 5.43 ppm in the proton NMR of **46** to 4.12 ppm in the NMR of the product. The proton NMR of the product also showed additional signals that belong to methyl groups at 3.48 ppm and 3.33 ppm.

Thus, it is likely the addition of two or more methoxy groups to the allene functional group during the reaction could have occurred. The isolated fractions from the reaction were analysed individually by NMR each producing very similar proton signals. Intensity variations of the doublet signals between 2 ppm and 3 ppm indicated the products were isomers of each other. The coupling constant for the doublets were 16 *Hz*.

The cleavage of the pivaloyl group appeared to be successful by TLC but analysis by GCMS revealed 3 major products that were not expected. Two of the products are certainly isomers of each other indicated by the mass spectrum. They both showed parent ions at *m/z* 252 and a possible loss of a methyl group to give the ion at *m/z* 237. The mass spectrum of the third product showed a parent ion at *m/z* 237. The structures of the three unknown products were not confirmed.

An amberlite-OH resin which is commonly used for the removal of isobutyryl groups was next trialled. The mixture was stirred for two days and monitored by TLC. The TLC showed the production of about 6 different products that were all more polar than the starting material **46**. Analysis by GCMS revealed that the desired ketone product **28** was not present.

The C_3 protected allene **29** and the pivaloyl protected allene **46** showed signs of degradation after about 3 weeks even storing under nitrogen, in the dark at -20 ^{o}C, indicating these types of compounds are not particularly stable. Purification was required directly before the next step in the sequence was performed.

Scheme 2.8B:

An alternative scheme was devised using an acetyl protecting group instead of the pivaloyl protecting group considering that it would be easier to remove under very mild conditions and also in an attempt to avoid rearrangement during or after the deprotection occurs.

The Swern oxidation of the hydroxyl moeity was trialled with successful production of the desired **49** with evidence by proton NMR showing the absence of the multiplet at 4.33 ppm but it was very low yielding (< 10%). The yield was increased to 23% by optimising various steps during the reaction (*Table 2.1* and *Table 2.2*). There were two steps that required the addition of the reagents DMSO and then the alcohol. The duration of reaction time after the addition of each reagent and temperature consistency was deemed important for the resulting yield. The initial parameters employed (*Table 2.1*) produced the desired product in low yield (< 10%). The optimised conditions that improved the yield involved extending the duration of the reaction time after the addition of DMSO and after the addition of the alcohol (*Table 2.2*) with the temperature sustained at -60 ^{o}C. This achieved a better yield (23%) of the desired product **49** but the total reaction time was longer.

Table 2.1 Initial conditions used that produced low yields.

Addition Reagent	**Reaction Duration (min)**
DMSO	30
Alcohol	30

Table 2.2 Conditions used that improved yields.

Addition Reagent	**Reaction Duration (min)**
DMSO	60
Alcohol	90

In contrast to the diketone **26** and the ketone **27**, Swern oxidation of the C_9 protected allene **48** was successful with adequate yields once conditions had been optimised. The Dess-Martin periodinane was not investigated as an alternative to oxidise **48** because subsequent attempts to deprotect **48** failed as described below.

The last step of *Scheme 2.8B* required the deprotection of acetate **49**. The deprotection was expected to be straightforward under mild basic conditions to afford the final product. Various attempts to deprotect **49** to cleanly isolate **28** using sodium methoxide and sodium hydroxide were trialled.

TLC analysis of the crude product from the reaction employing 1M sodium hydroxide indicated the formation of one product with some remaining starting material **49** and as expected the product was more polar. Column chromatography gave mixtures and the desired product **28** could not be identified. Analysis by GCMS also showed **28** was not present and the NMR revealed the allene functionality was no longer intact indicating addition transformations had taken place.

The basic reagent, sodium methoxide was then employed to deprotect **49**. The reaction progressed like the previous attempts with TLC revealing promising results with the formation of one product which was more polar. The R_f values matched those of the product in the previous de-acetylation reactions using sodium hydroxide. Purification by column chromatography (30% EtOAc/X4) of the crude material was performed but the fractions obtained revealed a complex mixture of products being eluted suggesting that further reaction was occurring on the silica column. The isolated fractions were analysed by GCMS and showed that the major component (90%) was not the desired product. The reactivity of the allene whilst in contact with the silica was unexpected so to avoid subjecting the allene to the mild acidic conditions in the future, buffered silica to neutral pH 7.0 was utilised for purification steps of subsequent reaction products, described below.

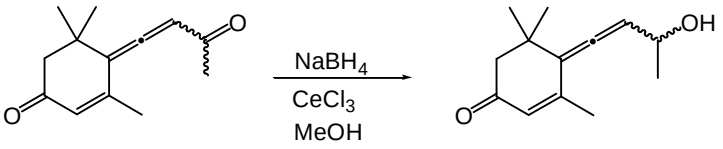
The deprotection step was attempted using an additional half equivalent of sodium methoxide. The reaction was monitored by TLC which showed the formation of one product after 2 hrs. GCMS and NMR revealed the desired product was not obtained under these conditions and other by-products had formed over the duration of the experiment.

Deprotection of the above C_9 protected allene **49** caused rearrangement of the product with the allene functionality lost in the process. An alternative pathway to synthesise the ketone **28** was required that did not allow the labile allene to react and rearrange. Selective reduction of the previously synthesised diketone **26**, was investigated. An active dry form of a baking *S. cerevisiae* strain was initially trialled as the reducing reagent[65] but failed to give any product (*Scheme 2.9*).

26 28

Scheme 2.9 Reduction of diketone 26 with S. cerevisiae.

A second reduction method was selected to reduce the diketone **26** using sodium borohydride in the presence of cerium chloride to enhance the selectivity towards the reduction of the less hindered carbonyl group (*Scheme 2.10*).



26 28

Scheme 2.10 Reduction of diketone 26 by a Luche reduction.

Reduction of the diketone **26** gave **28** in a 58% yield accompanied by an unknown product and a small amount of the diol **16** (2%). The alternative reduction product ketone **27** was not observed as a product indicating the reducing conditions using sodium borohydride with cerium chloride are very selective.

2.5 Conclusion

The synthesis of ketones **26** and **27** was relatively straight forward with optimisation required for the oxidation steps. The Dess-Martin periodinane reagent proved to be the most efficacious when oxidation of the allenic alcohols **29** and **16** was necessary. The methods utilising manganese dioxide or Swern conditions were successful in producing the desired product but the yields were relatively low.

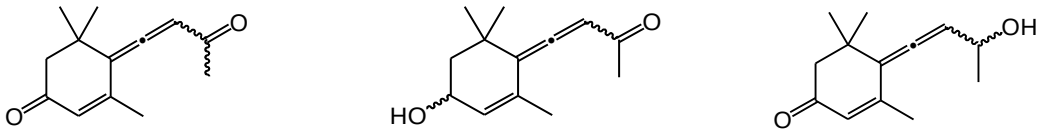
The synthesis of ketone **28** was unexpectedly challenging with the final step in the initial proposed pathway proving to be the most difficult. Deprotection did not result in the desired final product and multiple attempts gave rearranged and unwanted products. Finally, reduction of the diketone **26** was attempted and with incremental additions of the reducing agent sodium borohydride, the ketone **28** was successfully produced with high selectivity.

Chapter 3

Identification of allenic ketones as natural products

CHAPTER 3 Identification of allenic ketones as natural products

The diketone **26** (*Figure 3.1*) has been tentatively identified as a component of honey extracts based on proton NMR data.[77-80] It was not matched against an authentic standard. The diketone **26** has also been tentatively identified from mass spectral data only as a component of glycoside hydrolysates prepared from Semillon and Sauvignon Blanc juices treated with the fungal enzyme *Rohapect C*.[43,81] It was, however, suggested that **26** was an artefact of the hydrolysis and isolation procedure.[81]

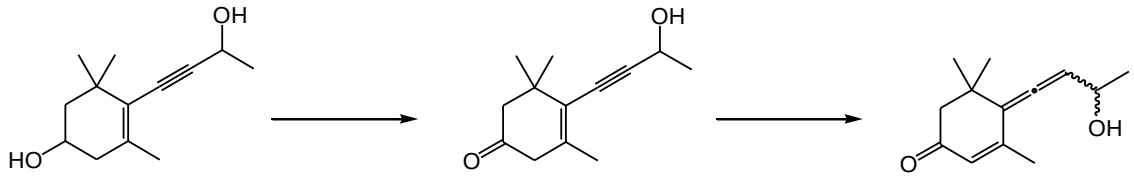


26 27 28

Figure 3.1 Potential damascenone precursors 26, 27 and 28.

The ketone **28** was identified as a trace component of American oak extract.[82] It was also identified as a grape product obtained by glycosidase enzyme hydrolysis of the grape C_{18} reverse phase isolates of the grape juice of *Vitis vinifera* cvv. Chardonnay, Semillon and Sauvignon Blanc.[47]

The formation of ketone **28** observed by Sefton et al.[83] only occurred with high concentrations of fungal glycosidase enzyme and it was believed that **28** was formed by oxidation of **15** to **38** and subsequently rearrangement of **38** to furnish the more stable end product **28** (*Scheme 3.1*).



15 38 28

Scheme 3.1 Proposed chemical conversion of 15 to 28.

The ketone **27** has not previously been identified as a natural product. It is, however, a simple dehydrated form of grasshopper ketone **12** which has previously been identified in nature including in grape extracts.[37,41]

Extracts of Riesling juice, Pinot Noir juice, glycosidase treated juice and also honey were analysed by GCMS to confirm whether compounds **26**, **27** and **28** are indeed genuine natural products.

3.1 Riesling and Pinot Noir juice (2004 vintage)

Studies conducted by Guth et al.[84] showed an increase in damascenone concentration in Gewürztraminer must after fermentation. A trace amount was detected in the must and 6.2 ppb after fermentation. Subsequently, the evolution of damascenone was monitored by others in our laboratory. We also found that various commercial fermentations of six different grape varieties (vintage 2004) also showed a significant increase in damascenone during fermentation.[85] The white grape variety that showed the largest increase in damascenone during the commercial fermentations was a Riesling must which contained up to 10 ppb of damascenone. The red variety with the largest increase in damascenone was a Pinot Noir must which contained up to 2 ppb of damascenone. Thus, the Riesling and Pinot Noir musts from this vintage were selected to search for the presence of potential oxidised damascenone precursors, ketones **26**, **27** and **28**.

The ketones **26**, **27** and **28** were also examined in extracts of Riesling and Pinot Noir juice that were treated with a glycosidase enzyme. The enzyme hydrolyses were performed using an AR 2000 enzyme which contains apiosidase, arabinosidase, rhamnosidase and β-glucosidase activities. This enzyme was used because of its ability to release the aglycones from a variety of glycosides.[86] It was important to use an enzyme that would cleave a variety of glycosides as it has been reported that such a range is present in *Vitis vinifera* varieties.[87]

Following a method used by Cox et al.[88], the must was passed down two columns packed with XAD-2 resin then eluted with DCM and MeOH. From approximately 200

mL of juice, 0.12 g of crude glycoside was obtained giving an overall yield of 0.6 mg/mL. A portion of the glycosidic fraction was treated with the AR 2000 enzyme to determine the glycosidically bound volatile constituents. The aglycone fraction, eluted with DCM prior to elution of glycosides with MeOH, was extracted to determine the free volatile constituents. The Riesling and Pinot Noir musts were also solvent extracted directly to determine if free **26**, **27** and **28** were present in the musts but not isolated by XAD-2.

The glycosidase treated extract and the aglycone extracts from must and XAD-2 isolates were all analysed by GCMS along with the authentic samples of **26**, **27** and **28** to determine the retention time of each. A full ion scan method was used initially to detect the presence of the precursors but none were observed. Characteristic ions for each compound were therefore selected to set up a more sensitive SIM method. The ions selected for monitoring the diketone **26** were the parent ion, 204, the base peak 147 and the ions, 162 and 119, for the ketone **27**, the base peak 191 and the ions, 131, 173 and 121 and for the ketone **28**, the parent ion, 206, the base peak 147 and the ion 162.

The SIM run of both the Riesling and Pinot Noir juice extracts revealed the presence of the diketone **26** but the ketones **27** and **28** could not be found. To confirm the presence of the diketone **26** in the Riesling and Pinot Noir extracts, co-injections were performed using the synthetic standard. The synthetic standard was added to the Riesling and Pinot Noir extracts and the peak assigned as the diketone showed an enhancement in the height and area by 2-fold for both the Riesling and Pinot Noir (*Figure 3.2*). The mass spectrum of the authentic standard and the corresponding peak in the extracts were shown to be identical. The extracted ion chromatogram (EIC) of the 162 ion demonstrates this enhancement (*Figure 3.2*).

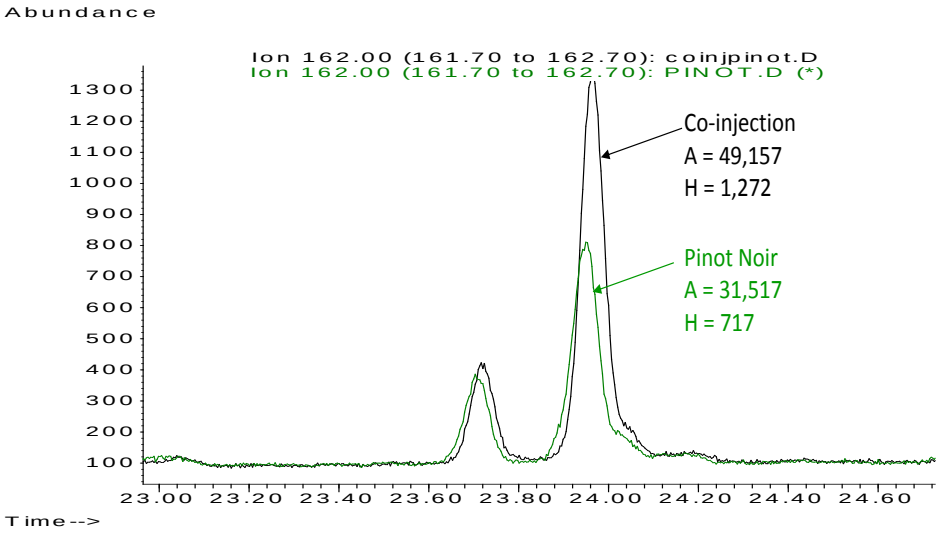


Figure 3.2 **EIC of 162 ion shows the *Pinot Noir co-injection with ketone 26.***

The diketone **26** was also identified as a trace component in the grape extracts of the glycosidic fraction of Pinot Noir that had been treated with the glycosidase enzyme. This is not surprising as the diketone **26** has no free hydroxyl to bind to glucose and the origin of **26** is uncertain.

3.2 Riesling juice (vintage 2009)

A second Riesling juice from a more recent vintage was also investigated to determine if the ketones **26**, **27** and **28** derivatives were present in higher amounts.

The glycosidase treated extracts and the solvent extracts from the Riesling must were analysed by GCMS along with the synthetic samples of **26**, **27** and **28** analogues. The compounds were again not detected in the scan mode but the diketone **26** was identified in the extracts of the solvent extracted Riesling juice using a SIM method.

3.3 Identification in honey

Many norisoprenoids have been identified in various honey extracts. Unifloral heather honey has been reported to be well-known for its high norisoprenoid content.[77,80] Tan et al.[77] identified norisoprenoids in New Zealand heather (*Calluna vulgaris*) honey and Rowland et al.[89] also identified norisoprenoids in an Australian leatherwood honey. Out of the five categories: norisoprenoids, monoterpenes, benzene derivatives, aliphatic compounds and Maillard reaction products, norisoprenoids have been

reported as the most abundant volatile secondary metabolite in yellow box (*E. Melliodora*) honey and blue gum (*E. leucoxylon*) honey. C_{13} norisoprenoids have been found up to 14.7 mg/kg in honey.[78]

In particular, Tan et al.[77] had tentatively identified the structure of the diketone **26** as a honey constituent in Heather (*Callun vulgaris*) honey samples obtained from hives kept in the central North Island of New Zealand. The diketone was also tentatively identified in other heather honeys which were from various countries in Europe.[79,80] D'Arcy et al.[78] also tentatively identified this compound in un-methylated extracts of Australian Yellow Box Honey. The identification of **26** was based on proton NMR data but was not matched against an authentic standard.

Various commercial honeys were selected and various extraction methods[78,89,90] were trialled with each honey to determine which method was likely to be the most adequate for extracting the ketones **26**, **27** and **28**. Seven commerically purchased honeys were extracted and analysed for these compounds **26**, **27** and **28**. The honey brands selected were Manuka honey, Beevital Manuka honey, Leatherwood honey, Yellow Box honey, Blue Gum honey, Honey Pure and Australian Rainforest Organic honey.

The Yellow box honey and the Blue gum honey were included because of a report by D'Arcy et al.[78] that showed the presence of monoterpenes and norisoprenoids in both of these products. The tentative identification of **26** was in a New Zealand honey[77] and so the Manuka blend honey was selected for extraction because it was from the same area. The Leatherwood honey was from Tasmania which was selected for comparative purposes.

The two commercially purchased honeys, Golden Nectar Organic Real Leatherwood Honey (Tasmania) and Manuka Blend Honey (New Zealand) were extracted according to procedures based on those previously reported by Rowland et al.[89] and Alissandrakis et al.[90], respectively.

The honey extracts were analysed by GCMS along with the authentic standards of the ketones **26**, **27** and **28**. The diketone **26** was identified in Manuka and Leatherwood honeys and the ketone **28** was identified in the Leatherwood honey. This identification was confirmed by co-injections with the synthetic standards of **26** and **28**.

The diketone standard **26** was spiked into the honey extracts at a 1:1 ratio, 1:2 ratio and 1:4 ratio of extract to standard. The peak assigned as **26** in the gas chromatograms was enhanced almost two fold on the first addition of the standard (*Figure 3.3*). The comparison between the mass spectra for the authentic standard of the diketone **26** and the corresponding peak in both of the honey extracts were identical. This confirmed the diketone **26** as a natural product in honey.

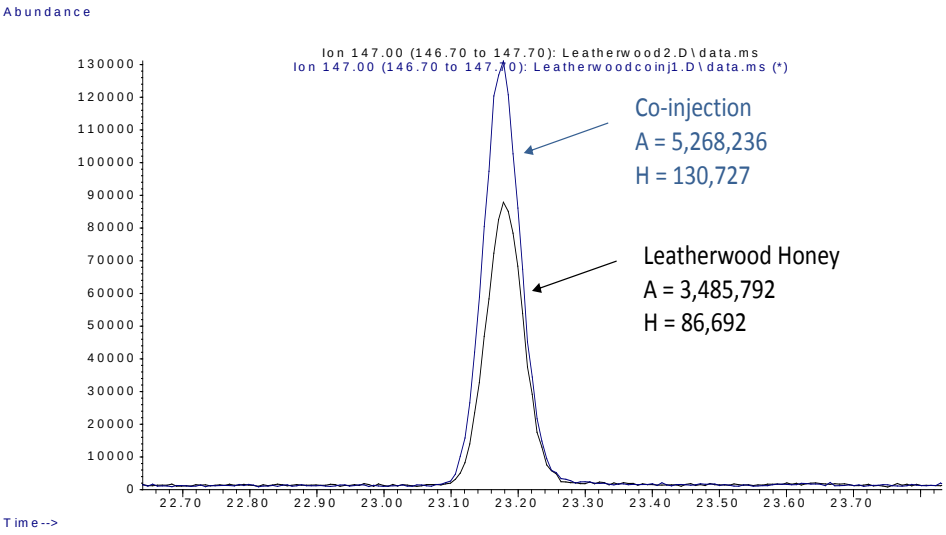


Figure 3.3 **EIC of 147 ion shows the *Leatherwood honey co-injection with ketone 26.***

The ketone standard **28** was a mixture of two diastereomers with different relative configuration at C_6 and C_9. This standard was spiked into the Leatherwood honey extract at a 1:0.5 ratio, 1:1 ratio, 1:2 ratio and 1:4 ratio of the extract to standard. The peaks assigned as **28** in the gas chromatogram of the honey extract was enhanced 2 fold on the second addition of the standard and enhanced 4 fold on the fourth addition of the standard (*Figure 3.4*). The comparison of the mass spectrum of the authentic standard **28** and the corresponding peak in the extracts were shown to be identical. This confirmed the ketone **28** as a natural product in honey and occured as two isomers.

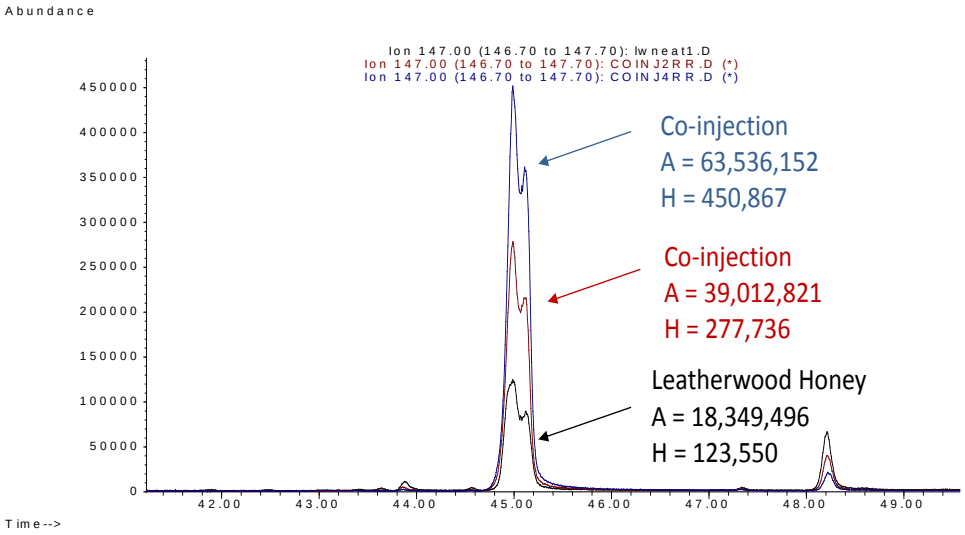


Figure 3.4 **EIC of 147 ion shows the *Leatherwood honey co-injection with ketone 28.***
NB: Adjacent peak at 48.2 min is an unknown component of honey that decreased in size during coinjections.

Analysis of the honey extracts by GCMS also showed that the ketone **27** was not present in any of the samples.

3.4 Hydrolysis studies of 3-hydroxymegastigma-4,6,7-trien-9-one (27)

As discussed in the previous section 3.3, the presence of ketone **27** in the extracts of Riesling juices, the Pinot Noir juice and in honey was evaluated. The ketone **27** could not be identified in any of the extracts, which raised the question about its stability in juice or under acidic conditions. In order to determine whether the absence of the ketone **27** from these extracts was because it was unstable in an acid environment, hydrolysis studies were conducted.

The ketone **27** hydrolysis studies were performed at a wine like pH. A pure sample of the ketone **27** consisting of 2 diastereomers in a ratio of about 2:1 (*Figure 3.5*) was subjected to acid hydrolysis conditions. Individual solutions of the ketone **27** (105 ppm, 10 mL) in 10% model wine at pH 3.0 were kept at 25 ^{o}C for 2 weeks, 45 ^{o}C for 1 week and 4 weeks and at 100 ^{o}C for 24 hrs.

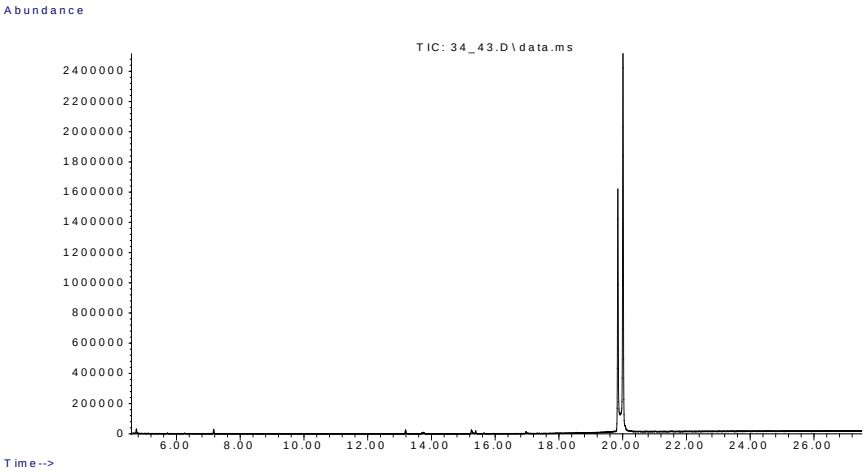


Figure 3.5 Diasteromers of 3-hydroxymegastigma-4,6,7-trien-9-one 27.
NB: This sample was used for the hydrolysis studies

The hydrolysis conducted at 25 ^{o}C for 2 weeks was selected to mimic the winemaking conditions and general duration used to produce a white wine. The hydrolysis set up at 45 ^{o}C for 1 week and 100 ^{o}C for 24 hrs were set up to determine the stability of the ketone **27** under more extreme conditions.

The extracts of the hydrolyses at 25 ^{o}C for 2 weeks revealed the formation of two main products (18% of total components), some unreacted starting material (80%) and unknown minor products (2%) (*Figure 3.6*). The products were tentatively identified as **50a** and **50b** (*Scheme 3.2*) by their mass spectra data which was compared against that for the reduced forms of the ethyl ethers **51a** and **51b** (*Figure 3.7*) that were identified by Puglisi et al.[52] during hydrolysis studies performed on the allene diol **16**.

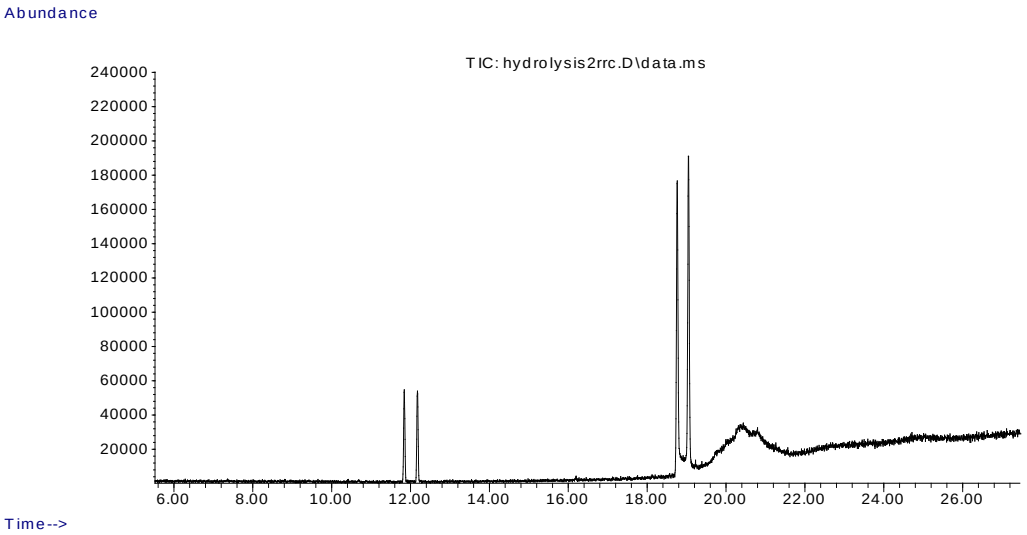
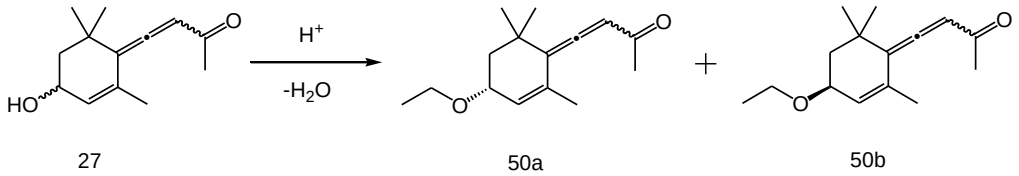


Figure 3.6 Hydrolysis performed at 25 °C for 2 weeks.

*NB: The temperature gradient was altered which caused a shift in the retention time of the starting material **27**. All samples were run at the same time as an alkane standard to confirm the starting material and products.*



Scheme 3.2 Proposed formation of the two ethyl epimers (50a and 50b) from 27.

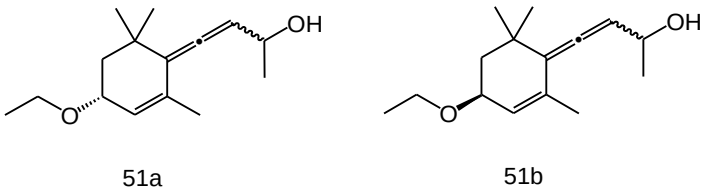


Figure 3.7 Ethyl ether epimers 51a/51b.

The products are presumed to form by a nucleophilic substitution reaction via protonation and subsequent loss of water under the acidic conditions with concomitant capture of the carbocation by ethanol to form the ethyl ethers. The mass spectra for both of the epimers showed the parent ion at *m/z* 234 with a loss of 15 resulting from the loss of a methyl moiety to give the base peak at *m/z* 219. The loss of the ethoxy group gives the ion at *m/z* 175 (*Figure 3.8*).

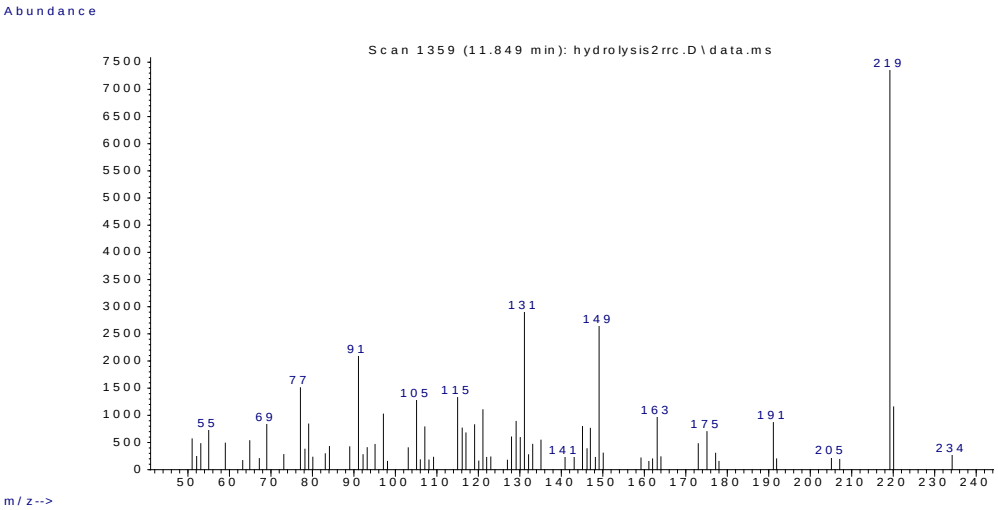


Figure 3.8 Mass spectrum of one of the ethyl ether epimers 50a/50b.

The fragment pattern tentatively identified by Puglisi and co-workers for the ethyl ether **51**[52] is almost analogous to the fragment pattern of the ketone **50**. There is a two mass unit difference for some of the ions between the spectrum for the ketone **50** and the alcohol **51**. The two mass unit difference can be identified in the parent ion, the mass from the loss of the first methyl fragment and the ethoxy fragment giving ions at, *m/z* 236, 221 (base peak) and 177 for the alcohol **51** and ions *m/z* 234, 219 (base peak) and 175 for the ketone **50**.

It was interesting to note that the diastereomeric ratio of the ketone starting material **27** was 2:1 and after hydrolysis at 25 ^{o}C, the compound epimerised to display a ratio of almost 1:1. The ethyl ether epimers produced were also in a ratio of 1:1. Given that one diastereomer was predominant in the starting material and after 1 week at 25 ^{o}C under acid hydrolysis conditions, an equal amount of the diastereomers was observed indicates that the starting material is likely to be undergoing facile nucleophilic substitution by the S_N1 mechanism at C_3 and that equilibrium is quickly reached.

The extracts from the hydrolyses at 45 ^{o}C for 1 week revealed a 28% conversion of the starting material to afford three products. These were the tentatively identified as the two ethyl ether compounds **50a** and **50b** (25%) and an additional compound at 10.7 mins (3%) (*Figure 3.9*). The diastereomeric ratio of the ketone starting material **27** also changed in the hydrolyses at 45 ^{o}C, starting with a ratio of 2:1 and during hydrolysis

epimerising to afford a mixture of 1:1. The ratio of the ethyl ether epimers formed was also 1:1.

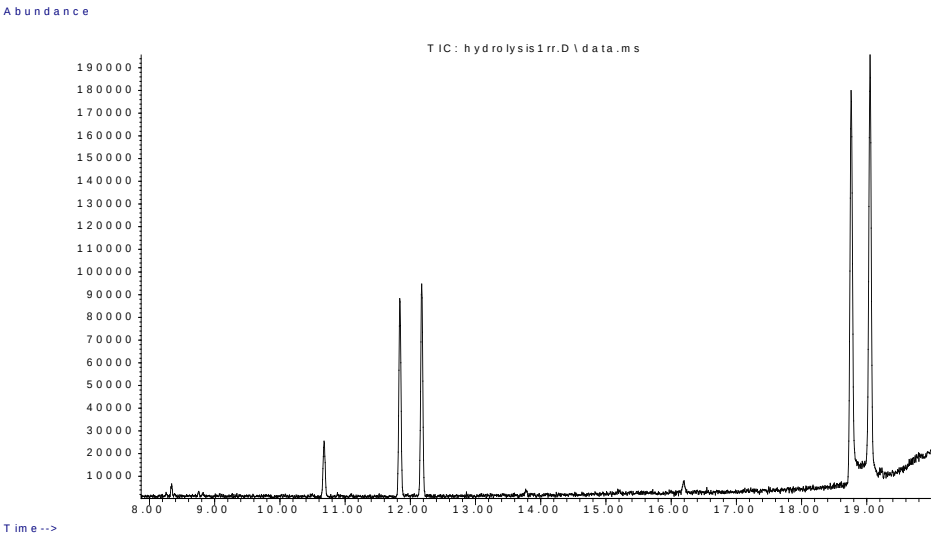
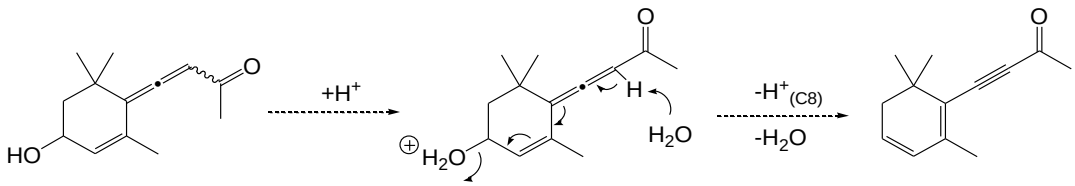


Figure 3.9 TIC of the hydrolysis performed at 45 °C for one week.

*NB: The temperature gradient was altered which caused a shift in the retention time of the starting material **27**. All samples were run at the same time as an alkane standard to confirm the starting material and products.*

It was initially hypothesised that the product at 10.7 min with *m/z* of 188 might be the acetylenic ketone **52** by analogy with similar reactions of the allene diol **16** as indicated below (*Scheme 3.3*). The formation of **52** was proposed to form by the removal of the proton at the C_8 position with concomitant loss of water.



Scheme 3.3 Hypothesised acid hydrolysis product from the ketone 27 at 45 °C for one week.

A subsequent hydrolysis of **27** was performed at 100 °C to determine if the compound at 10.7 min with *m/z* 188 would form predominantly and then could be isolated. Upon analysis of the extracts from the hydrolysis performed at 100 °C, the starting material **27** and the ethyl ether epimers **50** that were formed in the previous hydrolyses could not be detected. The chromatogram revealed the formation of one major product (*Figure 3.10*).

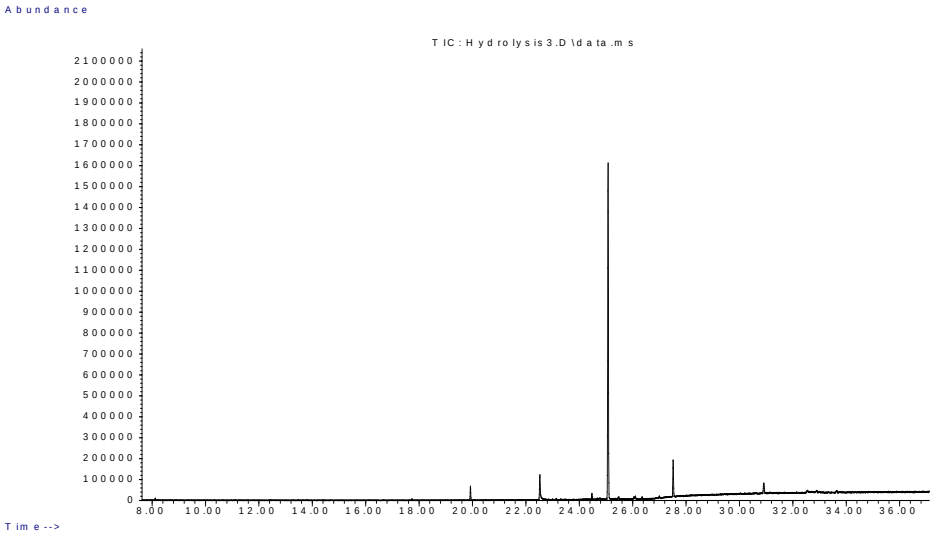
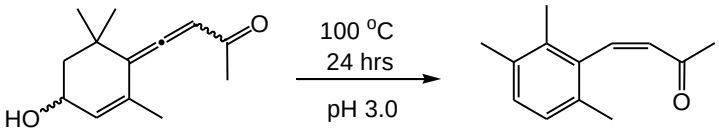


Figure 3.10 TIC of the acid hydrolysis performed at 100 °C for 24 hrs.

*NB: The temperature gradient was altered which caused a shift in the retention time of the starting material **27**. All samples were run at the same time as an alkane standard to confirm the starting material and products.*

The crude product was purified by column chromatography and analysed by ^{1}H and ^{13}C NMR to confirm the structure. The characterisation of the C_{13} norisoprenoid was made with the aid of COSY, HETCOR, HMBC and HMQC. Close examination of the NMR spectra revealed the product to be (*Z*)-4-(2,3,6-trimethylphenyl)-but-3-en-2-one **53** and not the acetylenic ketone **52** originally postulated (*Scheme 3.4*) (*Figure 3.11* and *3.12*).



27 53

Scheme 3.4 Hydrolysis of ketone 27.

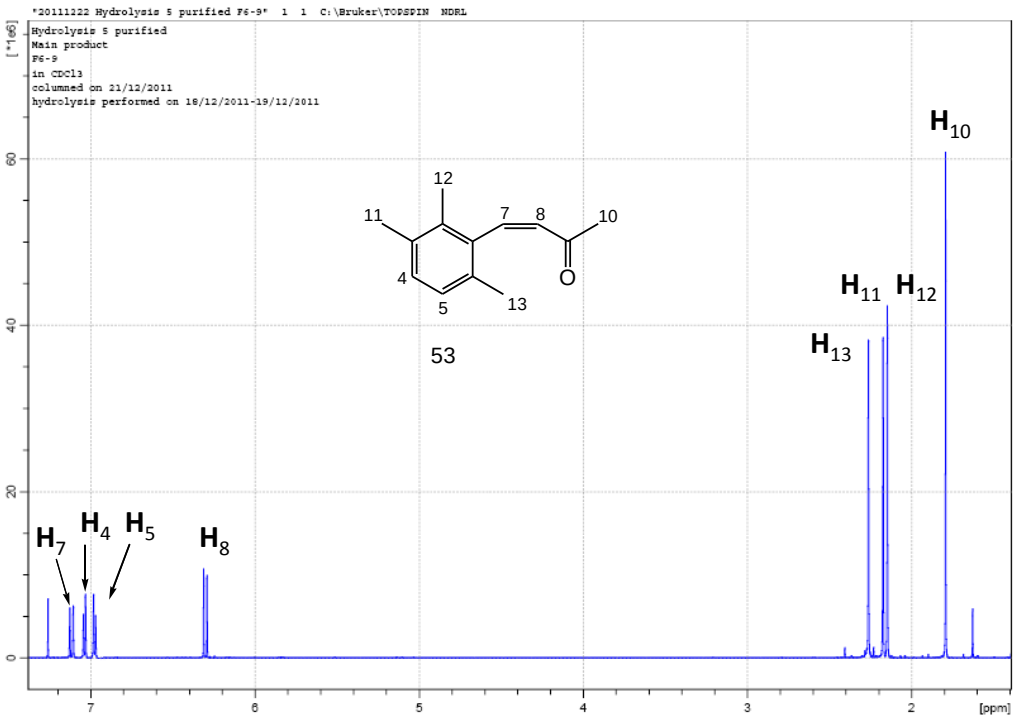


Figure 3.11 Proton NMR (600 MHz) of the aromatic ketone 53.

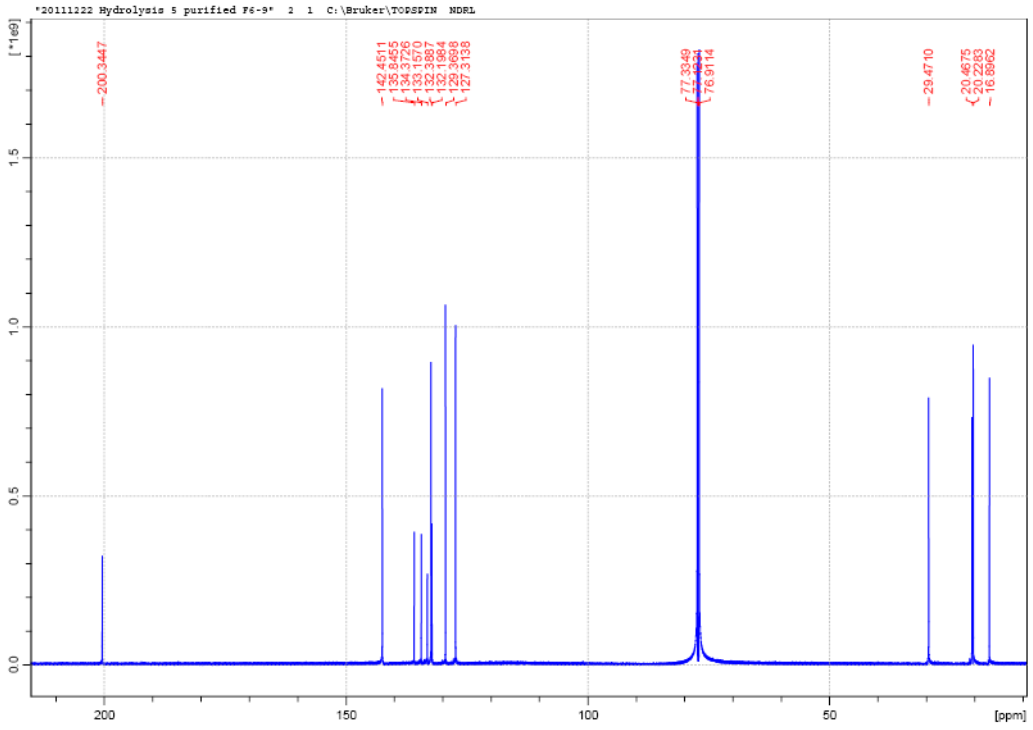


Figure 3.12 Carbon NMR of the aromatic ketone 53.

There were four individual signals for four olefinic and aromatic protons and the HMQC indicated that they were all attached to different carbons. The chemical shifts for the protons of the aromatic ring are further downfield at about 7 ppm compared to those in the starting material **27**. An AB quartet present at 7.04 ppm – 6.97 ppm, integrated for two protons and had a coupling constant of 7 *Hz*, which represented the coupling of two adjacent protons H_4 and H_5 on the aromatic ring (*Table 3.1*).

The ortho coupling between H_4 and H_5 revealed that the other substituents on the ring shared a common side. The HMBC experiment was used to confirm the position of the four substituents on the ring by assessing the long-range correlations. The coupling constant between the proton at the C_7 position and the proton at the C_8 position was identified as 12 *Hz* indicating that the $C_7 – C_8$ olefinic bond is in the *cis* configuration (*Table 3.1*).

Table 3.1 Proton NMR data for (Z)-4-(2,3,6-trimethylphenyl)but-3-ene-2-one 53.

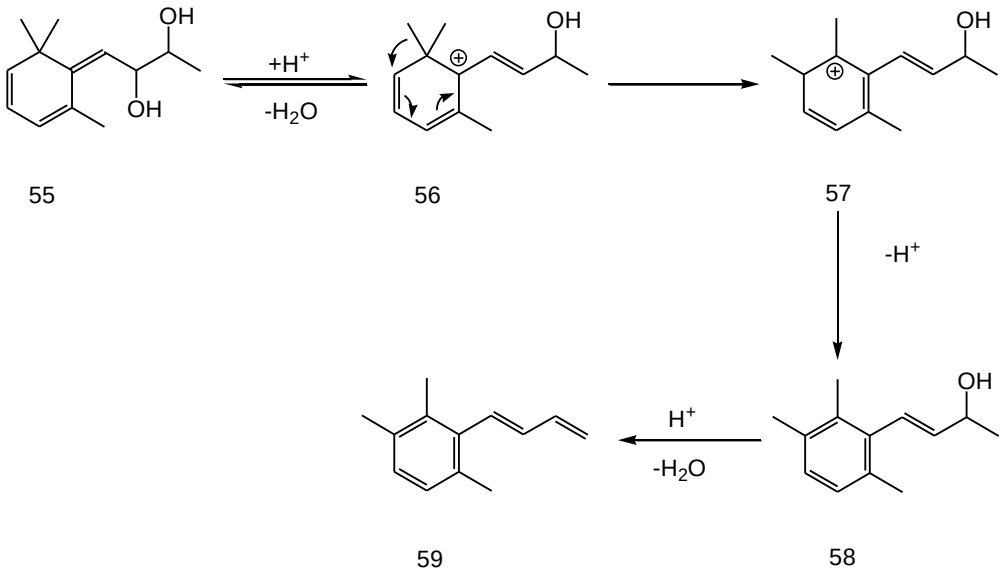
Chemical shift δ (ppm)	**Signal**	
7.13	H_7	1H, br d, *J* 12 *Hz* (*cis*)
7.04	H_4	1H, (d) A part of AB quartet, *J* 7.8 *Hz*
6.97	H_5	1H, (d) B part of AB quartet, *J* 7.8 *Hz*
6.31	H_8	1H, br d, *J* 12 *Hz* (*cis*)
2.26	H_{13}	3H, s
2.17	H_{11}	3H, s
2.15	H_{12}	3H, s
1.79	H_{10}	3H, s

The *cis* and *trans* isomers of 4-(2,3,6-trimethylphenyl)but-3-en-2-one (**53** and **54**) have both been reported previously. There are a number of studies that have tentatively reported this ketone and assumed that it is specifically the *trans* isomer **54**. Sefton and co-workers reported the *trans* isomer **54** as a grape wine product, in Chardonnay juice extracts that had been treated with acid.[45] The *trans* and *cis* isomer has also been

reported in a glycosidic fraction of Muscat juice of Alexandria grapes that had been hydrolysed at pH 1.0.[91] The stereochemistry in each of the reports was assigned based on the mass spectrum data consistent with published data.[92,93] Based on this study it is possible that this assumption was incorrect. Mikami et al.[92] isolated the *trans* isomer **54** as a product from the transformation of β-Ionone and reported the NMR and mass spectrum data.

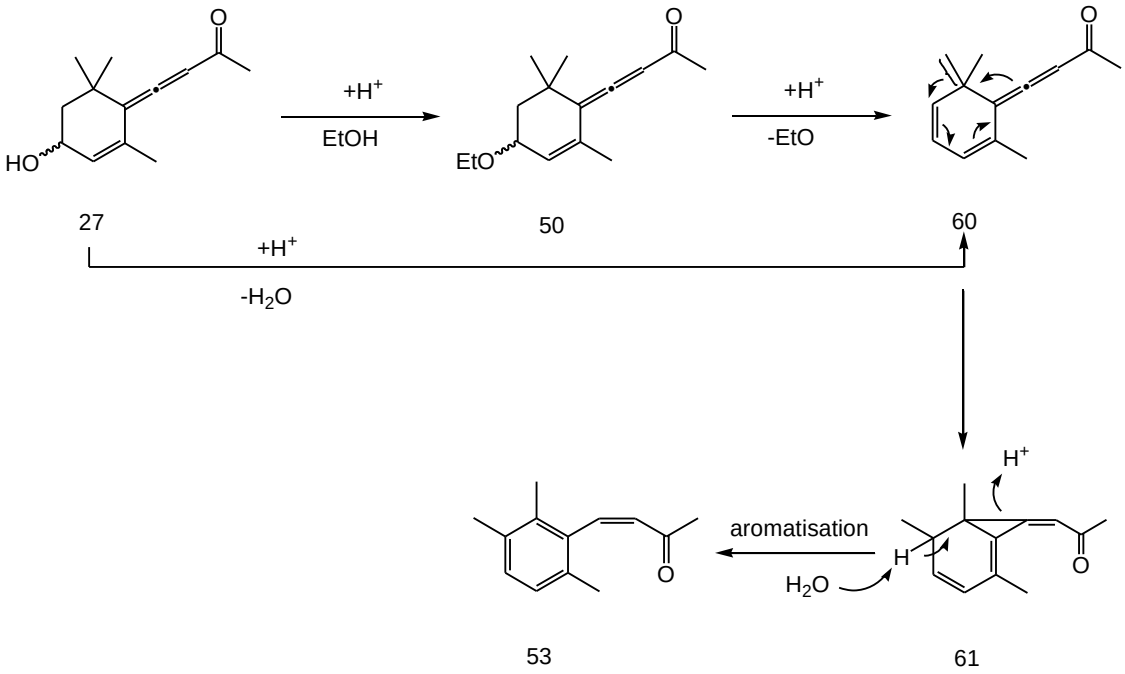
Comparison of the data for the hydrolysis product, ketone **27** with that of the reported for the *trans* isomer **54** confirmed that the hydrolyses had in fact produced the *cis* isomer **53**. Mikami eta al.[92] reports the coupling constant for the protons at C_7 and C_8 on olefinic bond was 16 *Hz* for the *trans* isomer **54** compared to 12 *Hz* for the hydrolysis product **53**.

This current hydrolysis study has revealed the formation of **53** from **27** with the migration of one of the gem dimethyl groups which was confirmed by NMR analysis. Here, **53** has been isolated and proven that the *cis* isomer is the only isomer identified. Research performed by Cox et al.[86] reported the migration of a methyl group from a 1,1-*gem*-dimethyl C_{13} norisoprenoid to form an analogous compound (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB) **59**. A mechanism involving acid catalysis was proposed which shows some similarity to the current hydrolysis study (*Scheme 3.5*).

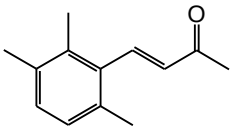


Scheme 3.5 Proposed mechanism for the formation of TPB 59.

Mechanistically, the fact that product **53** contains the less thermodynamically stable C_7-C_8 *cis* double bond is interesting and a postulated mechanism of its formation is depicted in *Scheme 3.6*. The *trans* isomer **54** (*Figure 3.13*) would be expected to be the thermodynamically preferred product.



Scheme 3.6 The proposed mechanism for the formation of the cis isomer 53 from 27.



54

Figure 3.13 Trans isomer 54 of the aromatic ketone.

In this mechanism the alcohol **27** and the ethyl ethers **50a** and **50b** under the acidic conditions will eliminate water (or ethanol) to give the cyclohexadiene **60**. Alternatively, if the ethyl ethers **50** are formed first as appeared to be the case from our hydrolytic studies at lower temperatures, then it may also undergo elimination under the acidic conditions to furnish the same cyclohexadiene **60**. At this point one of the *gem* dimethyl groups may undergo migration with concomitant disruption of the allenic moiety and formation of the bicyclic cyclopropane **61**. Simple, protolytic

induced rearrangement of **61** would be expected to form the observed aromatic ketone **53** with aromatisation being the driving force for the rearrangement. This concerted mechanism is favoured as opposed to a cationic intermediate like **56** (*Scheme 3.5*) as rapid isomerisation would be expected to form the more stable *trans* isomer of **54**.

Analysis of the hydrolysates from the hydrolysis performed at 45 ^{o}C over four weeks revealed the same three products as the hydrolyses performed at 45 ^{o}C after one week. There was now a 70% conversion of the starting material **27** into the two ethyl ether epimers (**50a** and **50b**) and the *cis* aromatic ketone **53** (*Figure 2.17*). There was also a significant change in the ratio of the products. The hydrolyses performed over four weeks now showed similar amounts of the two ethyl ether epimer products (**50a** and **50b**) and the aromatic ketone **53** (*Figure 3.14*).

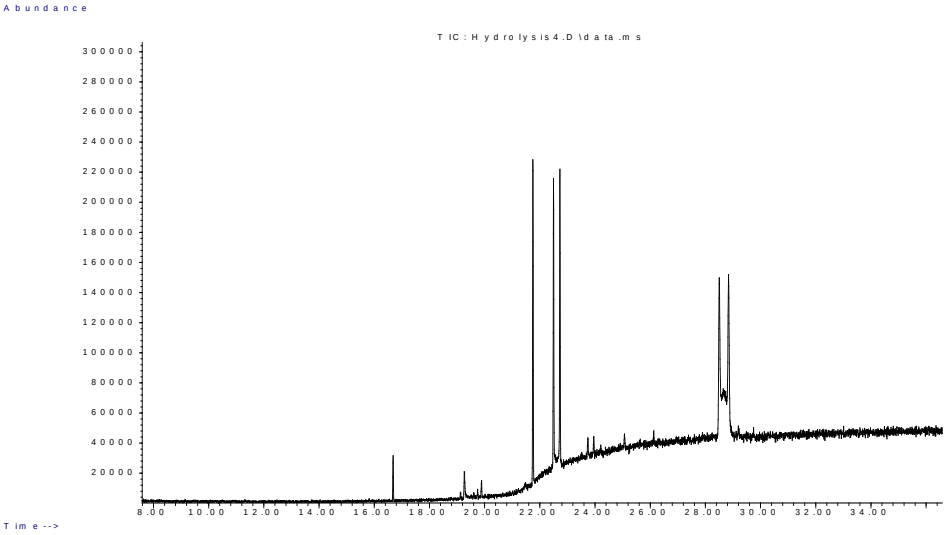
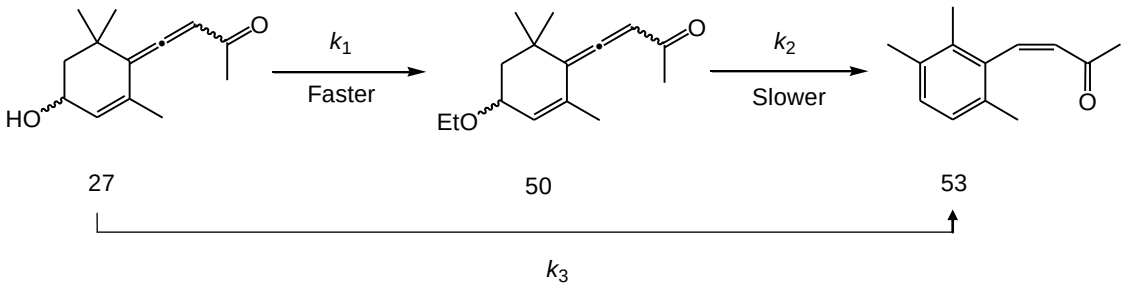


Figure 3.14 TIC of the hydrolysis performed at 45 ^{o}C for 4 weeks.

*NB: The temperature gradient was altered which caused a shift in the retention time of the starting material **27**. All samples were run at the same time as an alkane standard to confirm the starting material and products.*

Interestingly, the diminishing concentration of the ethyl ether isomers (**50a** and **50b**) and the starting material **27** and an increase in concentration of the aromatic ketone **53** was observed. The data indicates that the ethyl ethers **50** are likely to be intermediates along the pathway to the formation of the aromatic ketone **53**. The rate of formation of the ethyl ethers **50** from the starting material **27** was clearly faster than the rate of formation of the aromatic ketone **53**. The aromatic ketone might be

forming from two separate pathways. One pathway from the ketone **27**, k_3 (initial loss of H_2O) and the second pathway from the ethyl ethers **50** (loss of ethanol), however the rate of formation of the aromatic ketone **51** from the ketone **27** is also slow, which would suggest $k_1 > k_2$ and k_3 (*Scheme 3.7*).



Scheme 3.7 Acid hydrolysis of 27.

Overall, the hydrolysis studies indicated that ketone **27** reacts slowly under the conditions investigated, 25 ^{o}C for 2 weeks and 45 ^{o}C for up to 4 weeks. This indicated that the compound is reasonably stable under the acidic conditions even at slightly warmer temperatures and would survive winemaking conditions. The reactivity of the ketone **27** did not appear to be the reason for the failure in detecting this compound in grape and honey extracts.

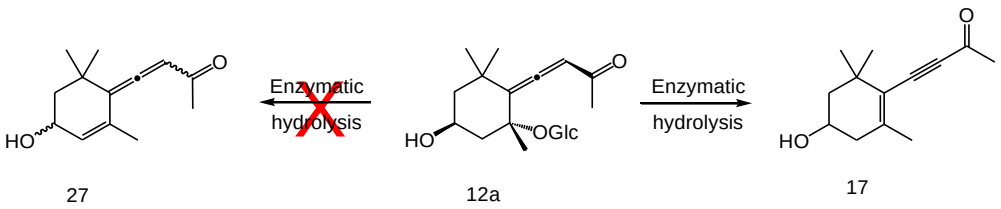
3.5 Conclusion

The ketone **28** was identified as a natural product in honey extracts for the first time. The diketone **26** was identified as a component of the extracts of Riesling juice, Pinot Noir juice and honey and thus, was also confirmed as a natural product. Unexpectedly, however, the closely related ketone **27** was not found in any of the samples. The hydrolysis studies showed that this was not because it is inherently unstable to mild acid conditions.

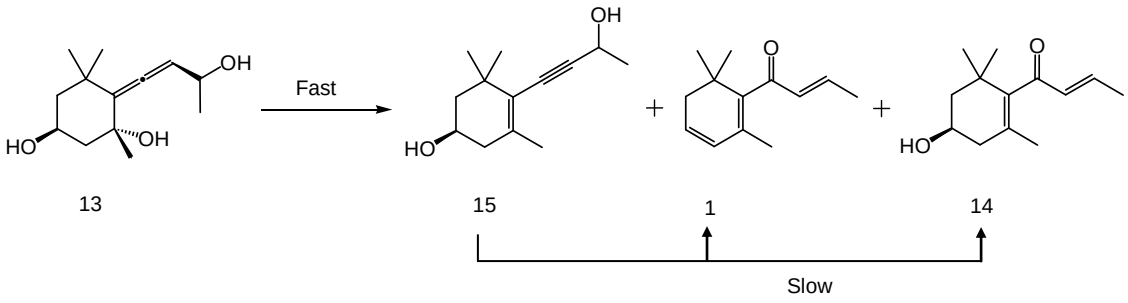
The composition of a juice post crushing is very different to that of the model wine used for the hydrolysis studies. Crushing of the grapes produces a medium with a large variety of components and/or conditions that could cause **27** to react readily. The ketone **27** may be susceptible to reaction with nucleophiles present, acid hydrolysis, reaction catalysed by trace elements, oxidation or even light induced transformations.

The naturally occurring form of norisoprenoid alcohols are predominantly in the form of the corresponding glycosides but the enzyme hydrolysis extracts of the grape juice suggested that **27** was not present in this form either.

Osorio et al.[74] showed that when the C_3 glucoside of grasshopper ketone **12a** is subjected to enzymatic hydrolysis, this C_3 glucoside **12a** rearranged to the enyne **17** (*Scheme 3.8*) and no allene such as **27** was observed. The hydroxyketone **17** is a known natural product, isolated from peelings of lulo *syn*. Naranjilla (*S. Vestissimum* D.) fruit.[48] The rearrangement (**12a** to **17**) is analogous to the formation of the enyne diol **15**, one of the two main products of hydrolysis of the allenic triol **13**, in which damascenone **1** is only a very minor product (*Scheme 3.9*). These processes are all initiated by the loss of the tertiary hydroxyl at C_5.

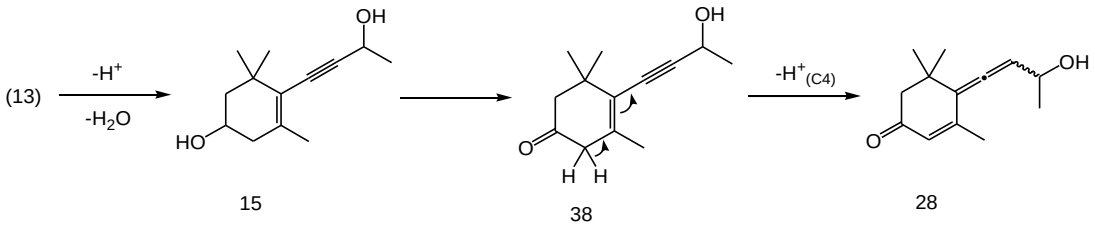


Scheme 3.8 Formation of hydroxyketone 17 from grasshopper ketone 12a via enzymatic hydrolysis.



Scheme 3.9 Formation of damascenone 1, acetylenic diol 15 and 3-hydroxy-β-damascone 14.

However, enzymatic oxidation of the C_3 hydroxyl of **15** is accompanied by rearrangement to the presumably more stable allenic ketone **28** (*Scheme 3.10*).[83] This observation might explain why the ketones **17**,[48] **26** and **28** are observed in nature but not the ketone **27**.



Scheme 3.10 Proposed pathway towards the formation of ketone 28.

Chapter 4

Fermentation studies

CHAPTER 4 Fermentation studies

The concentration of damascenone has been shown to change during various stages throughout the wine making and storage processes which has been summarised by Sefton et al.[30] In general, it has been observed that it increases in concentration post-fermentation by significant amounts, degrades over the first few months of maturation but has also been seen to increase during the ageing of wine depending on the specific conditions.

Guth[84] and Lloyd et al.[85] showed a significant quantity of damascenone forming post fermentation. The increased levels of damascenone indicated that precursors in the grapes might be converted to damascenone due to the action of yeast. This chapter focuses on this hypothesised pathway, looking at the evolution of damascenone from potential precursors during fermentation.

S. cerevisiae has been utilised as a chemical reagent for chemical syntheses. More specifically, the reduction of carbonyl compounds to their corresponding alcohols by yeast has been demonstrated.[65] Thus, the oxidised species **26**, **27** and **28** were selected for spiking into *S. cerevisiae* fermentations to determine whether they can undergo reduction by the yeast to their corresponding alcohol **16** which is known to almost exclusively give damascenone under acidic conditions (*Scheme 4.1*).

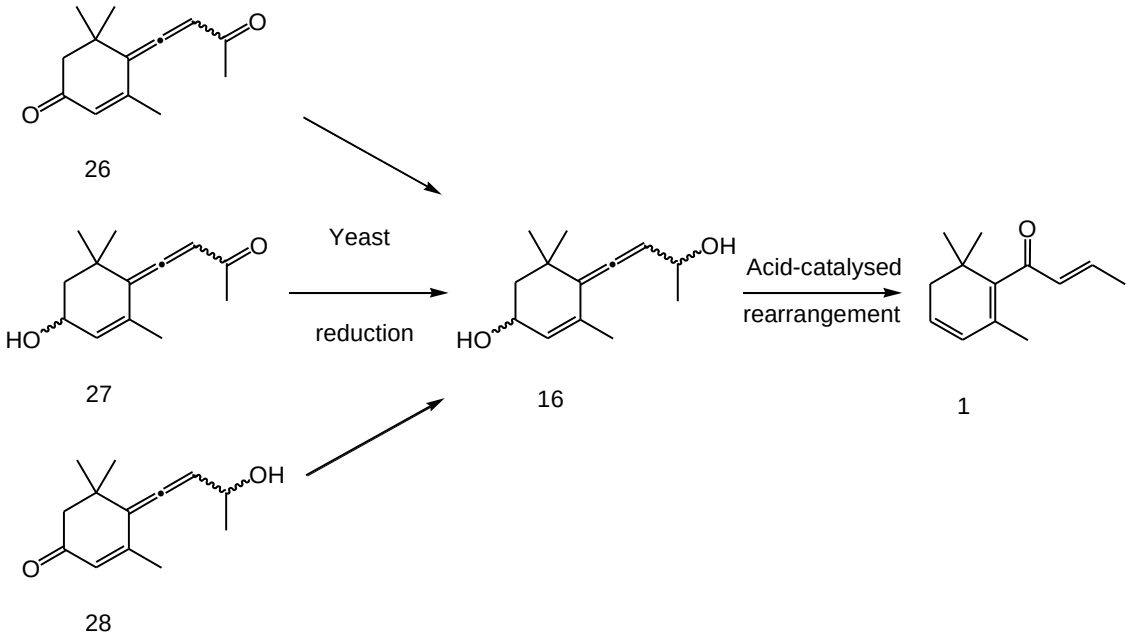
4.1 Fermentation using *S. cerevisiae*

Initial experiments were carried out using an active form of a baking *S. cerevisiae* strain following a synthetic method reported by Crump.[94] The diketone **26** and ketone **27** were spiked into CDGJ medium and inoculated with yeast to determine if **26** and **27** could form damascenone under these conditions. NMR and GCMS analysis revealed a mixture of products for both **26** and **27**. There was no starting material identified so it had apparently been completely consumed and there was no evidence by NMR and GCMS that damascenone had formed or any other norisoprenoids. Although these initial experiments were not successful in forming damascenone it was thought that the use of a winemaking strain of *S. cerevisiae*, might show more promising results.

4.2 Fermentation using yeast strain AWRI 796 and AWRI 1537

Ketones **26**, **27** and **28** were spiked into CDGJ medium and inoculated with *S. cerevisiae* yeast strains AWRI 796 and AWRI 1537 from the AWRI yeast collection. A significant property of the AWRI 796 is that low SO_2 levels are formed during fermentation.[95] Daniel et al.[96] revealed the level of SO_2 to be an important contributing factor on the concentration of damascenone and this will be further discussed in *Chapter 5*. AWRI 1537 is a yeast strain that has been shown to be involved in the hydrolysis and transformation of glycosidically bound compounds releasing volatiles during fermentation and in particular is characteristic for its low to zero production of SO_2 during fermentation.[97,98]

Model fermentations using CDGJ medium as described by Ugliano et al.[97] were performed in triplicate using the yeasts AWRI 796 and AWRI 1537. The CDGJ medium was spiked with substrates diketone **26**, ketone **27** or ketone **28** at a concentration of 1000 ppb and subsequently inoculated. Three different sets of controls were also prepared which included: ferments carried out with CDGJ medium but not spiked with the ketones **26**, **27** and **28**; CDGJ medium spiked with the ketones **26**, **27** or **28** but no yeast; and CDGJ medium without yeast and without the ketones **26**, **27** and **28**. It was hypothesised that **26**, **27** or **28** in the CDGJ medium would not form damascenone without the presence of yeast. Similarly damascenone would not form from the CDGJ medium with yeast and without the presence of the precursors. However, it was anticipated that the ferments spiked with **26**, **27** and **28** could produce damascenone **1** (*Scheme 4.1*).



Scheme 4.1 Biotransformation of ketones 26, 27 and 28.

The ferments were monitored by the consumption of sugar until they reached dryness, after which they were allowed to cold settle for 4-5 days. There was some variation in the days to reach dryness between the ferment replicates. This was recorded to determine whether this might have an effect on the final concentration of damascenone in the replicates. The ferments were racked and an aliquot was spiked with deuterium labelled damascenone and extracted to accurately quantify the final level of damascenone at the end of fermentation by GCMS.[96]

The quantification results of damascenone **1** in the samples at the end of fermentation showed that there was a significant difference in the amount of damascenone formed from each of the ketones **26**, **27** and **28**. Ferments spiked with ketone **27** produced higher levels of damascenone in all cases compared to ketones **26** and **28**. There was also a difference in the concentration of damascenone observed between the different yeast strains AWRI 796 and AWRI 1537 at the end of fermentation (*Figure 4.1*). The ferments inoculated with the yeast strain AWRI 796 resulted in higher final concentrations of damascenone compared to those ferments inoculated with the yeast strain AWRI 1537.

The final concentration of damascenone observed in the ferments using yeast strain AWRI 796 spiked with **26**, **27** and **28**, showed a 1.4%, 11.2% and 0.8% conversion to damascenone, respectively. The ferments with yeast strain AWRI 1537 showed a 0.1%, 6.0% and 0.5% conversion to damascenone for ketones **26**, **27** and **28**, respectively. The percentage conversion of the ketone **27** to damascenone was considerably more efficient when compared to the percentage conversion of the diketone **26** and the ketone **28**.

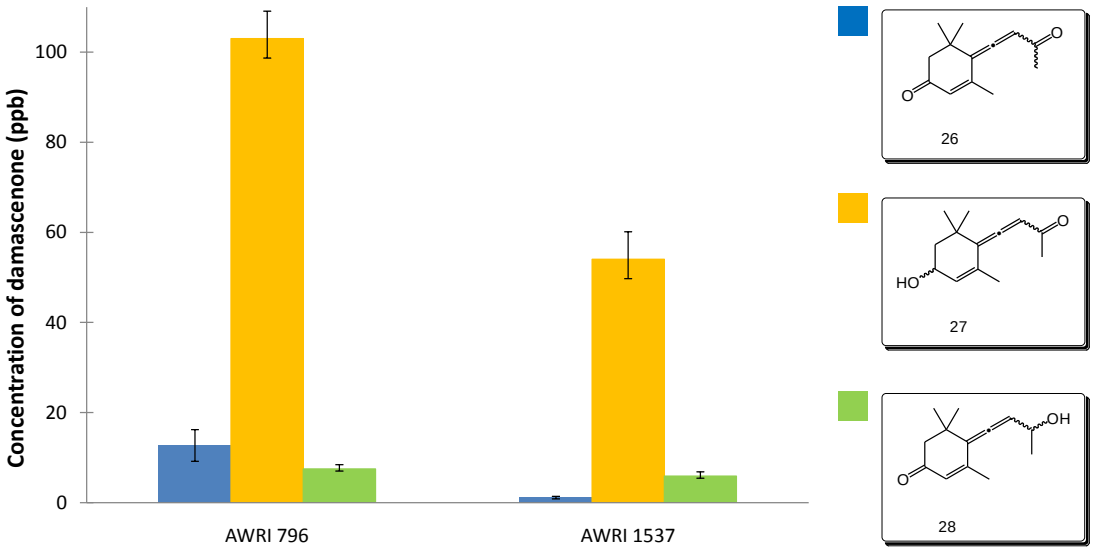


Figure 4.1 Formation of damascenone (ppb) from ferments spiked with ketones 26, 27 and 28.

Damascenone was not detected in the control containing CDGJ medium with the added ketones **26** or **27** and no yeast but a small amount of damascenone was detected in the control containing CDGJ medium with added ketone **28** and no yeast (*Table 4.1*).

Table 4.1 Controls conducted during fermentation studies.

Controls	AWRI Yeast	Damascenone (1) (ppb)
Yeast, no spike[a]	796	nd
	1537	nd
Spiked CDGJ medium (**26**)[b]	-	nd
Spiked CDGJ medium (**27**)[c]	-	nd
Spiked CDGJ medium (**28**)[d]	-	3.1
No yeast, no spike[e]	-	nd

[a]*CDGJ medium inoculated with yeast and not spiked with precursors **26, 27** and **28**.*
[b]*CDGJ medium spiked with precursor **26** at 1000 ppb but no yeast was added.*
[c]*CDGJ medium spiked with precursor **27** at 1000 ppb but no yeast was added.*
[d]*CDGJ medium spiked with precursor **28** at 1000 ppb but no yeast was added.*
[e]*CDGJ medium with no yeast and not spiked with precursors **26, 27**and **28**.*

A small amount of damascenone present in the spiked controls was expected for ketone **28** as the standard used to spike the medium contained 2% of the diol **16** which is known to give predominantly damascenone. With the addition of about 20 ppb of the diol **16** before fermentation, it was expected that approximately 18 ppb of the diol **16** would be present at the end of fermentations. Only 15% of the diol **16** could be accounted for so this might suggest that damascenone is undergoing further reactions once it is formed, as detailed further in *Chapter 5*.

The non-spiked CDGJ medium, and the CDGJ medium no spike, no yeast were also analysed but damascenone was not detected in any of these controls (*Table 4.1*). The controls showed the importance of the role of the yeast in the generation of damascenone from these ketones **26**, **27** and **28**.

In general the fermentation rate (daily sugar consumption) was consistent with 90% of the sugar consumed by day 15 for both yeast strains. The difference in time to dryness mainly occurred past day 15 when the ferments for both strains became sluggish or stuck (*Figure 4.2 – 4.4*). The effect of the yeast strain on the volatile profile of a wine and in particular the concentration of damascenone at the end of fermentation has been reported previously, but the biotransformation mechanisms responsible for this variation have not been evaluated.[24]

The types of enzymes reported to be responsible for carbonyl reductions are alcohol dehydrogenase, aldose reductase and NADPH reductase.[99] Borneman et al.[100] identified a 45kb strain-specific region in the AWRI 796 genome that encodes at least 21 Open Reading Frames (ORFs), three of which are predicted to encode aryl-alcohol dehydrogenases (AADs). AADs have previously been shown to catalyse the reversible reduction of aldehydes and ketones to aromatic alcohols.[101] Thus, one or more of these AADs present in the yeast strain AWRI 796 may well be responsible for the reduction of the ketones **26**, **27** and **28** to their corresponding alcohols during fermentation.

The differences in the final concentration of damascenone generated from the different precursors **26**, **27** and **28** could indicate the formation of damascenone is dependent on the chemical structure of the substrate. The low yield of damascenone from the ketone **26** could have been due to requiring the reduction of two carbonyl groups as opposed to one carbonyl group in precursor **27**. Thus, the biotransformation of precursor **26** might be a lot slower than that of precursor **27**. But, **28** also yielded low quantities of damascenone compared to **27**, which indicates that the specificity of the yeast may play an important role in the reduction process.

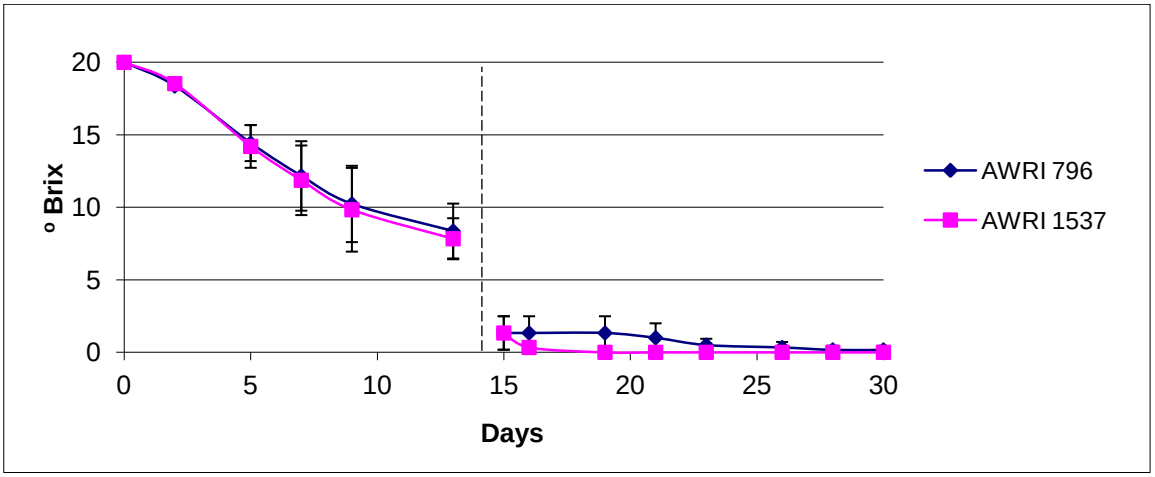


Figure 4.2 Consumption of sugar during fermentation spiked with diketone 26.
NB: The sugar levels were monitored using a refractometor from 20 Brix and below 10 ^{o} Brix Clinitest was used.

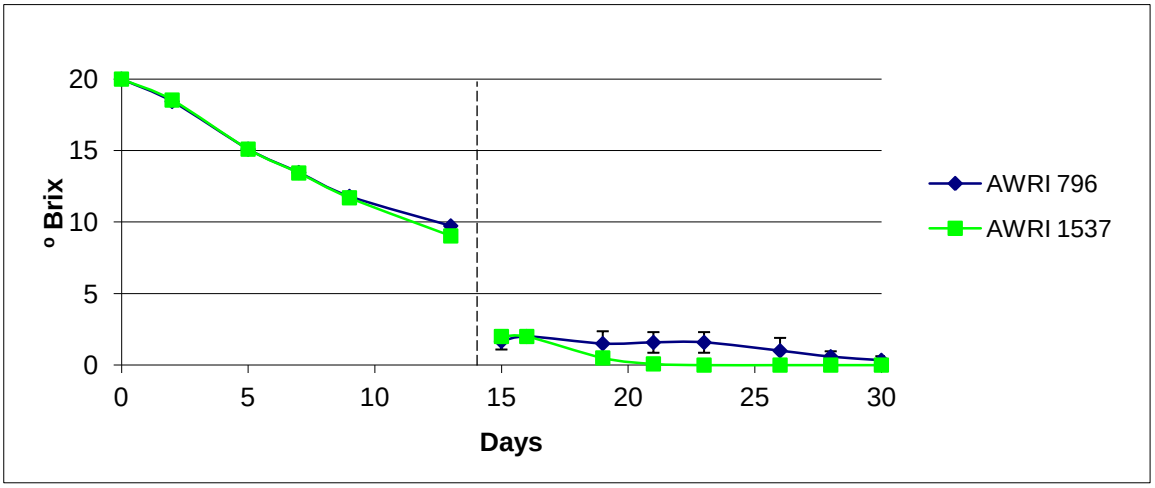


Figure 4.3 Consumption of sugar during fermentation spiked with ketone 27.
NB: The sugar levels were monitored using a refractometor from 20 Brix and below 10 ° Brix Clinitest was used.

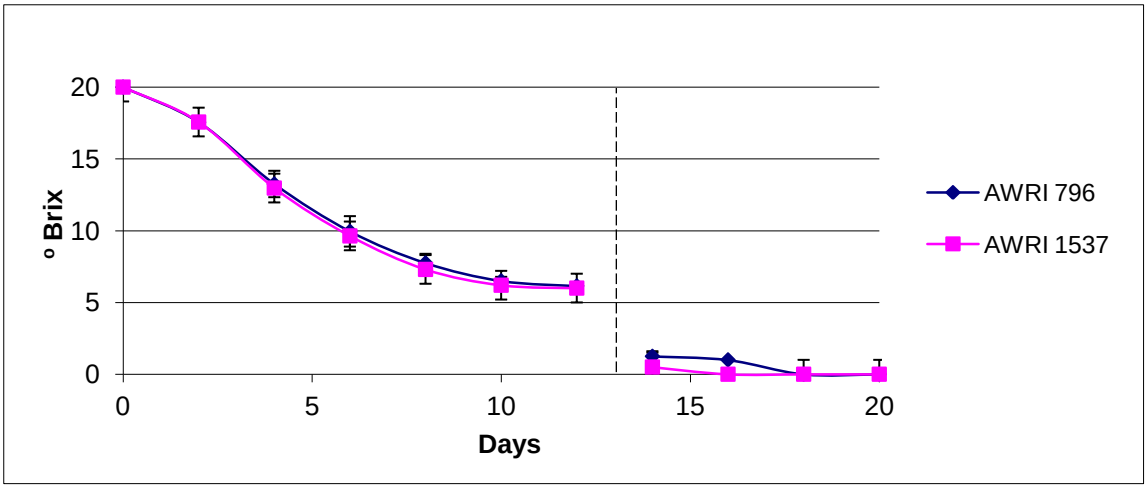
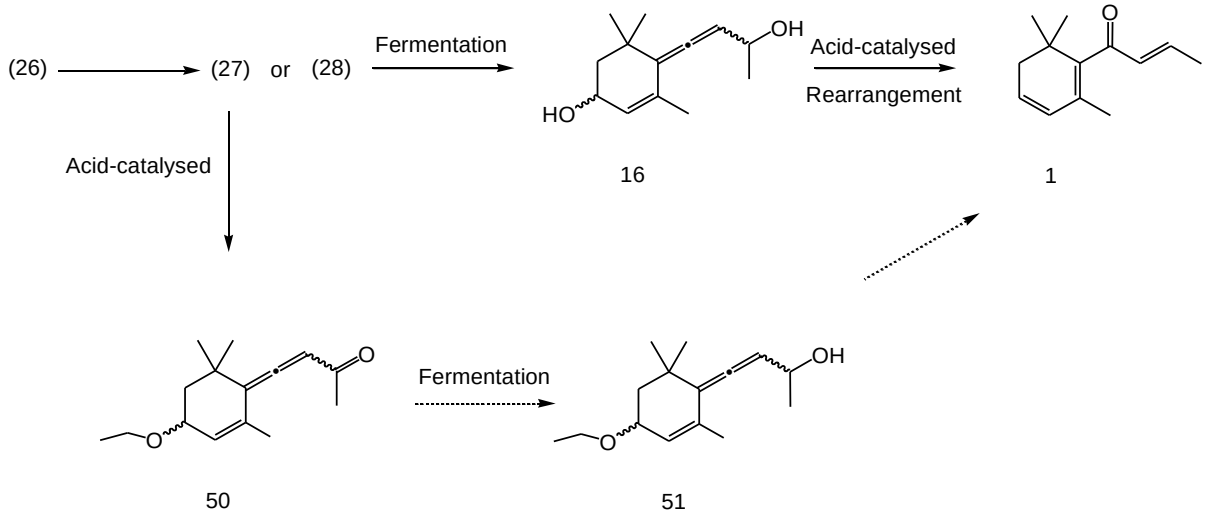


Figure 4.4 Consumption of sugar during fermentation spiked with ketone 28.
NB: The sugar levels were monitored using a refractometor from 20 Brix and below 10 ° Brix Clinitest was used.

The proposed pathway for the formation of damascenone from ketones **26**, **27** and **28** is depicted in *scheme 4.2*. The ketone **27** could be reduced readily by the yeast to form the diol **16** and then under the acidic conditions, rearrange to form damascenone. Alternatively, the ketone **27** could produce **50** under the acidic conditions and subsequently be reduced to **51**. Acid hydrolysis of **51** followed by rearrangement would afford damascenone **1** (*Scheme 4.2*).



Scheme 4.2 Proposed competing pathways during fermentation from the ketones 26, 27 and 28 to damascenone 1.

The amount of damascenone produced during fermentation in the presence of **26**, **27** and **28** might well be significantly higher than indicated by the final concentration. These fermentations show that ketones **26**, **27** and **28** were susceptible to yeast metabolism and it was thought that damascenone, itself a ketone, could also undergo biotransformation by the action of yeast. The possibility that damascenone is either reduced further by the yeast or is further metabolised to other products was investigated in *Chapter 5*.

4.3 Formation of damascenone during fermentation

The levels of damascenone observed at the end of fermentation might not reflect the total amount of damascenone that was synthesised throughout fermentation. The fermentations conducted with the additions of the naturally occurring ketones **26** and **28** which were discussed in the previous section had samples removed every two days for the first eight days of fermentation and analysed for their amount of damascenone. These days were selected in order to align with the most active stage of fermentation.

The fermentations inoculated with yeast strain AWRI 796 and with the addition of the ketone **28** revealed damascenone starts forming from the initial stages of fermentation (*Figure 4.5*). At day 2 a total of 12-13 ppb of damascenone was observed,

with an increase at day eight and then a subsequent decrease by the end of fermentation.

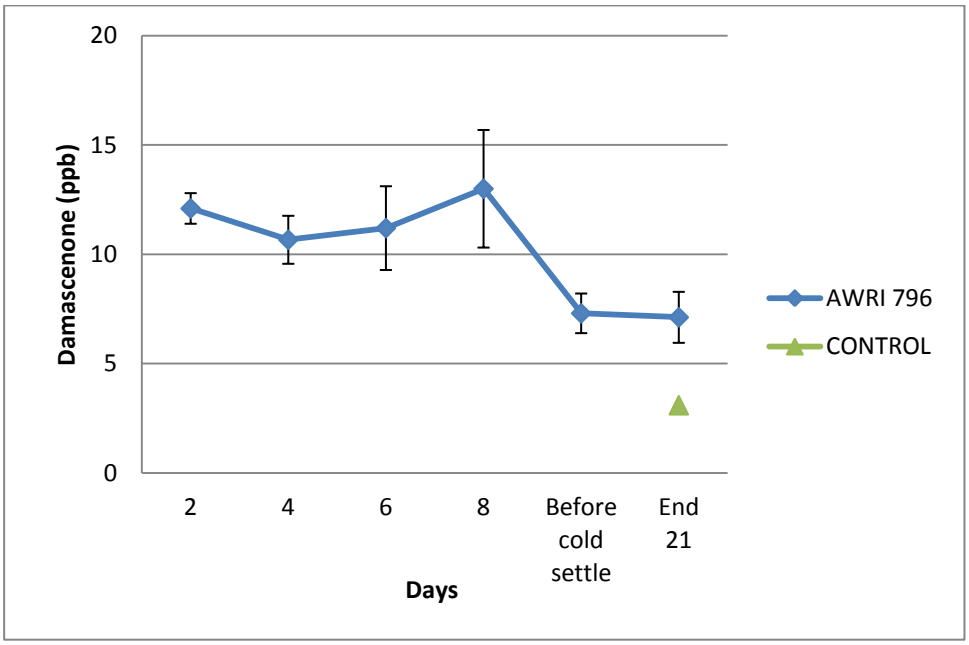


Figure 4.5 Formation of damascenone during fermentation with yeast AWRI 796.
*NB: Control – CDGJ medium spiked with ketone **28**, no yeast is present.*
The controls were not sampled to ensure they were not contaminated.

It is important to note that a small amount of damascenone was observed in the CDGJ medium control which was not inoculated with any yeast and only included the addition of **28** at 1 ppm. As discussed in the previous section the starting material used to spike the fermentation contained 2% of the diol **16**. The major product from the diol **16** under acidic conditions is predominantly damascenone **1**. The maximum concentration expected to form directly from **16** was about 18 ppb. Only 15% of this amount was recovered at the end of fermentation as indicated by the concentration of damascenone measured in the control which was 3 ppb. Given that a large portion of damascenone was lost by the end of fermentation and the concentration of **1** in the control was well below the concentration in the dry ferments, this suggests that the damascenone formed via a biochemical initiated pathways is higher than that from just chemical mediated reactions.

The fermentations inoculated with yeast strain AWRI 1537 showed a similar pattern. At day two the concentration of damascenone had already reached 12 ppb in one of

the replicates. The concentration steadily increased and then declined after day four and until the end of fermentation (*Figure 4.6*).

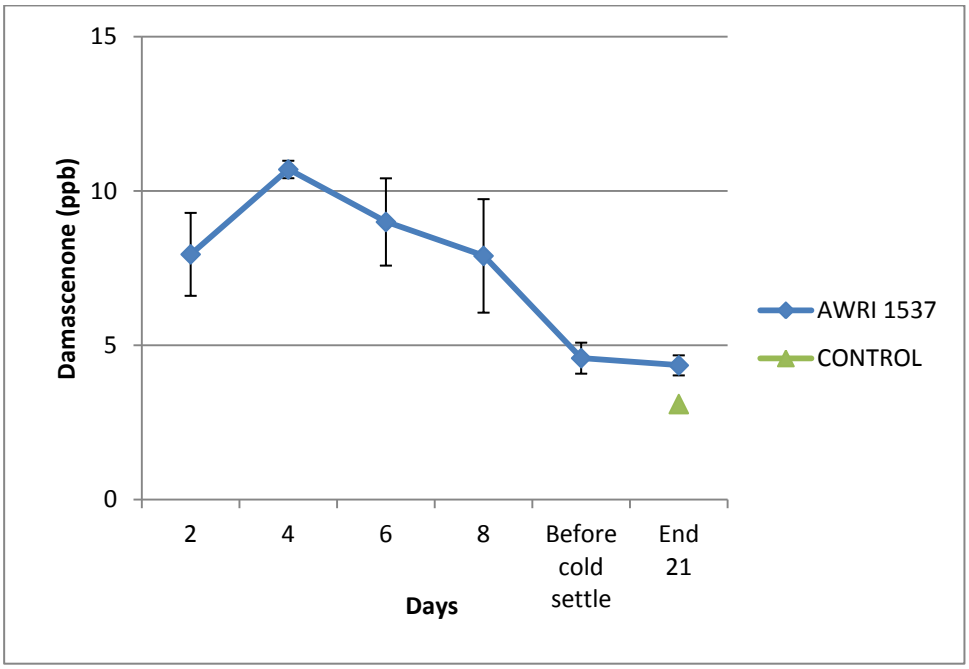
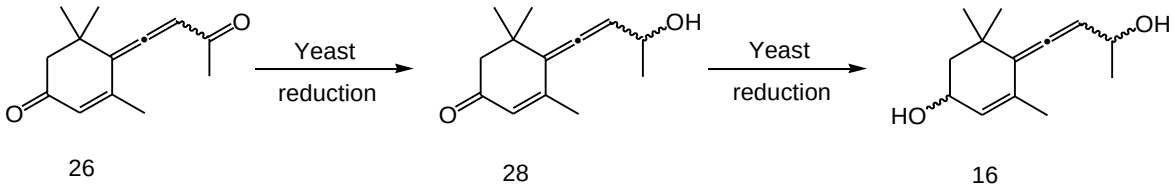


Figure 4.6 Formation of damascenone during fermentation with yeast AWRI 1537.
*NB: Controls – CDGJ medium spiked with ketone **28**, no yeast is present.*
The controls were not sampled to ensure they were not contaminated.

The difference between the concentrations of damascenone in the controls compared to the concentration of damascenone at the end of fermentation was relatively small for the ferments performed with yeast AWRI 1537. The fermentations conducted with the addition of the diketone **26** were sampled at the same time points but damascenone was not detected until the end of fermentation. This might be because a longer contact time is required in order to reduce both of the carbonyl groups.

It was deemed important to determine if the enzymes responsible for reducing the carbonyl groups in **26** have a selective preference for either the C_3 or C_9 position. The ketone **28** was identified in the samples removed from the ferments spiked with **26** with very little of the ketone **27** seen. Thus, **28** appears to be the predominant intermediate in the pathway from the diketone **26** to the diol **16** (*Scheme 4.3*).

This suggests that the enzymatic mediated reduction of a C_9 ketone is more facile than the reduction of a C_3 ketone and may explain the much higher yield of damascenone **1** from **27** compared to **28**.



26 28 16

Scheme 4.3 Reduction of diketone 28 during fermentation conditions.

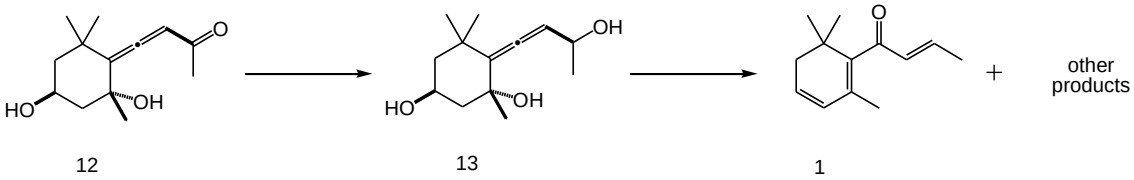
Another observation made after fermentation was that significant amounts of starting material remained in the supernatant extract for both ferments with **26** and **28**. Only a portion of the precursors were utilised by the yeast indicating that either the yeasts enzymatic activity might not be 100% efficient when reducing these types of compounds or they are not readily taken up by the yeast. The samples from the ferments spiked with **28** were analysed for other products forming during fermentation. Unexpectedly, the diketone **26** was present, even at day two so it appears that other oxidative processes are co-occurring to give rise to **26**.

4.4 Grasshopper ketone spiked fermentations

Given that grasshopper ketone **12** is the presumed precursor to the triol **13,** which, is thought to be a primary precursor to damascenone, fermentations were spiked with **12** to determine if damascenone could be produced via this pathway. The CDGJ medium was spiked with **12** at 1 ppm and inoculated with either yeast AWRI 796 or AWRI 1537.

It was hypothesised that under the reductive conditions during fermentation, grasshopper ketone **12** might be reduced to the triol **13** which would subsequently react under the acidic conditions to form damascenone **1** (*Scheme 4.6*). Previous studies reported hydrolysis of the triol resulted in about a 1% yield of damascenone. Thus, it was expected that if as little as 10% of the grasshopper ketone **12** at 1000 ppb

was transformed to the triol then about 1 ppb of damascenone would form by the end of fermentation.



12 13 1

Scheme 4.4 Proposed pathway for the reduction of grasshopper ketone 12.

Ferments spiked at 1000 ppb were carried out and the supernatant was examined for damascenone at the end of alcoholic fermentation. Damascenone could not be identified by GCMS with LOD's below 1 ppb, thus the conversion from grasshopper ketone was not confirmed. This was an unexpected result as it was presumed a small amount of damascenone would have been observed as suggested by the literature.[36] The conversion to damascenone might have been dependent on the affinity and specificity of the yeast enzymes for the substrate. This result might indicate that enzymes capable of reducing **12** to **13** were simply not present during this fermentation.

Alternatively, given the observation of damascenone loss towards the end of fermentation (*Figure 4.5* and *4.6*), it is possible that the concentration remaining at the end of this experiment was simply below the LOD.

4.5 Conclusion

This chapter shows the ketones **26**, **27** and **28** can be metabolised by yeast, transformed to the reduced species, diol **16** and then damascenone is generated under the acidic conditions. There is a clear indication that the difference in chemical structure and yeast utilised contribute to the overall concentration of damascenone after fermentation. The highest amount of damascenone produced was from ketone **27** by the action of yeast strain AWRI 796 when compared to ketones **26**, **28** and yeast strain AWRI 1537. It is apparent that these wine yeasts are reducing the carbonyl compounds to give the diol which in turn rearranges to produce damascenone **1**.

Chapter 5

Change in damascenone concentration during fermentation

CHAPTER 5 Change in damascenone concentration during fermentation

As described in the previous chapter, the generation of damascenone **1** can occur from precursors such as **26**, **27** and **28** under fermentation conditions. The aim of this work was to determine the stability of damascenone during fermentation and investigate whether the yeast or the medium influences the amount remaining in the resulting wine. It was shown that **26**, **27** and **28** are reduced by the yeast and thus it was feasible that, since damascenone **1** was also a ketone, it too could be reduced by the yeast. It was also possible that **1** might react with components of the juice and also the by-products of yeast fermentation.[96] Chevance et al.[102] reported the concentration of damascenone appeared to decrease after the fermentation of beer. Although the quantification of damascenone was performed solely by GC-FID, it is reasonable to consider that there might be multiple factors involved affecting the final concentration of damascenone after fermentation.

5.1 Damascenone spiked fermentations

To determine whether the yeast could have an impact on the concentration of damascenone, fermentations were set up with CDGJ medium and spiked at 1 ppm with damascenone. They were inoculated with AWRI 796, AWRI 1537 or AWRI QA23 and the level of damascenone was measured at the end of fermentation. This experiment revealed that a large reduction in the damascenone concentration by a total of 90%, 80% and 80% for the ferments with yeast AWRI 796, AWRI 1537 and AWRI QA23, respectively (*Figure 5.1*).

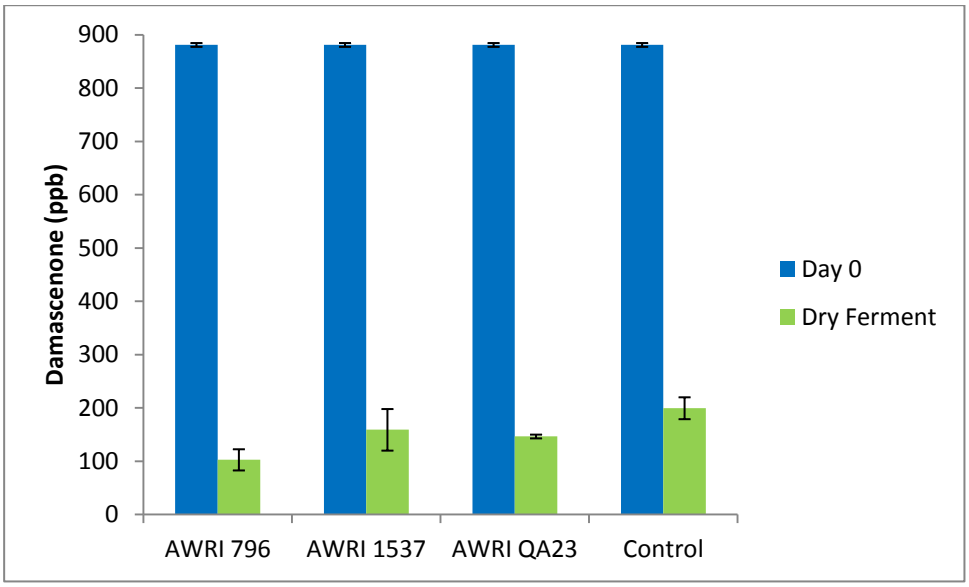


Figure 5.1 Concentration of damascenone at day 0 and at the end of fermentation.
NB: Controls removed from 17 °C at day 26 with a majority of the ferments. Two out of the three reps for ferments inoculated with AWRI 1537 reached dryness earlier at day 19.
Controls – Damascenone spiked CDGJ medium, not inoculated with yeast

In general, a higher concentration of damascenone was observed for the ferments that reached dryness sooner and those ferments that became stuck or sluggish resulted in a lower concentration. For example, two out of the three ferments inoculated with AWRI 1537 reached dryness on day 19 measuring 228 ppb of damascenone and the third replicate reached dryness on day 26 measuring 160 ppb of damascenone.

Unexpectedly, a decrease in the concentration of damascenone by 75% was also observed in the controls with no yeast present. The controls that were spiked with damascenone and not inoculated with yeast were removed from 17 °C and allowed to cold settle at 4 °C at day 26 when a majority of the ferments were dry. The concentration of damascenone remaining in the CDGJ medium inoculated with AWRI 796 was significantly less than the damascenone remaining in the control. There was no significant difference between the control and the CDGJ medium inoculated with AWRI 1537 or AWRI QA23.

Initially, it was hypothesised that not only was damascenone being metabolised by the yeast but it was also reacting with the constituents present in the CDGJ medium. The loss occurring from the CDGJ medium with no yeast and the inoculated CDGJ medium

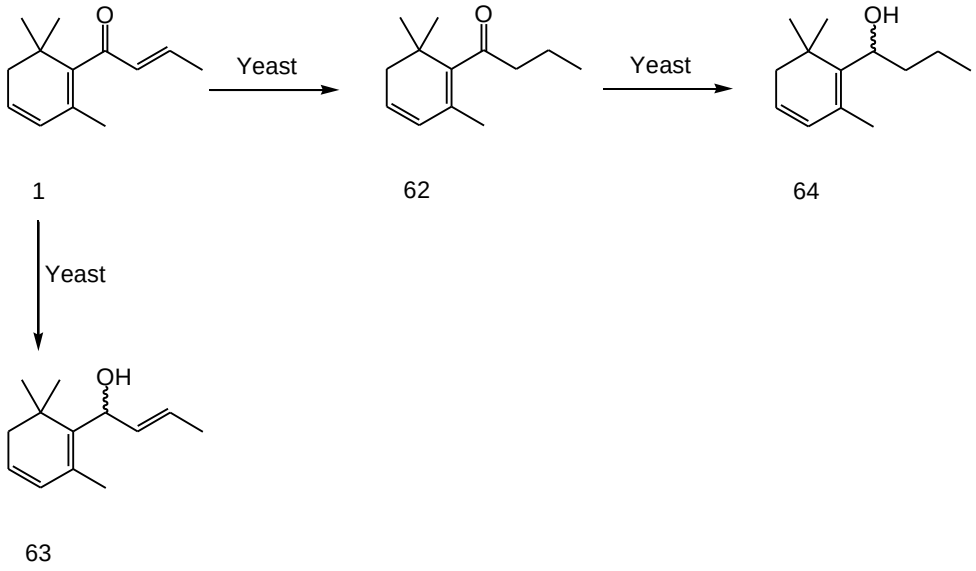
should not be directly compared with respect to the amount of damascenone remaining. As the fermentation progresses the constituents in the fermenting CDGJ medium becomes very different to the sterile CDGJ medium that was not inoculated. The control demonstrates solely the impact of possible chemical reactions that are occurring and the fermenting medium shows a combination of yeast mediated interactions as well as chemical reactions.

Overall, five different possibilities that might have caused the decrease in damascenone concentration were proposed. These include: damascenone loss through yeast metabolism, reaction with CDGJ medium constituents, volatilisation and/or by the purging effect of CO_2 released by yeast during fermentation and/or the impact of yeast lees adsorption of damascenone. All of these factors could contribute to the measured concentration of damascenone at the end of fermentation.

The unexpected loss of damascenone observed in the spiked ferments led to a review of the ferments spiked with the precursors **26**, **27** and **28**. Up to an 11% conversion from **26**, **27** and **28** to damascenone was indicated (*Chapter 4*) but in light of a possible 80% lost from a ferment, this would indicate the conversion from **26**, **27** and **28** might be significantly greater than initially thought. Further experiments were undertaken to investigate the reason behind the large reduction in damascenone concentration.

5.2 Fermentation and reduction

Fermentation provides a reducing environment and as proposed earlier in Chapter 4 pg 78, damascenone could be reduced by the action of yeast. To determine if the yeast were reducing damascenone to other reduced products, such as **62**, **63** and **64** depicted within *Scheme 5.1*, ferments were spiked with damascenone at 10,000 ppb in order to potentially isolate and characterise the reaction products. The ferments were carried out using CDGJ medium inoculated with yeast strain 796.



Scheme 5.1 Proposed reduction pathways for damascenone 1.

The ferment extracts were analysed by GCMS scans to identify possible reduced products of damascenone. Thus, ions 192 and 194 were extracted to identify a single reduced product and/or a double reduced product, respectively. Ion 121 was also extracted as it is a common base peak for many norisoprenoids. The corresponding fragments M-15 and M-18 were also extracted to identify the loss of a methyl group or water, respectively. Damascenone was identified but there was no evidence for even a trace of reduced products **62**, **63**, **64** and M-15 and M-18 were not identified.

Although reduction products of damascenone were not identified, the hydroxy adduct **65** was observed at 26.9 (*Figure 5.2*) min albeit as a small peak and the mass spectrum matched the authentic standard previously synthesised by Daniel.[28] The ethoxy adduct **66** as described by Daniel[28] was not identified in the extract (*Figure 5.3*). The hydroxy adduct had previously been identified as a minor product in the hydrolysis of the allene diol **16**.[52]

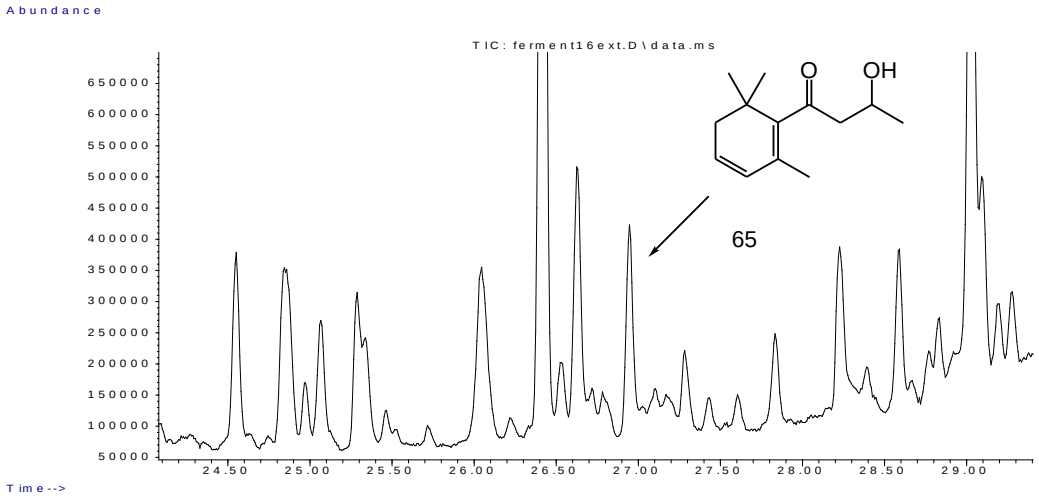
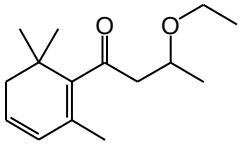


Figure 5.2 GCMS scan of the ferment extract with the hydroxy adduct 65.



66

Figure 5.3 Ethoxy adduct 66.

5.3 CDGJ medium versus Chardonnay juice

The previous experiment showed that yeast did not appear to be facilitating the formation of reduced products from damascenone **1** and this is unlikely to be a contributing factor to the loss of this compound unless such products are further transformed by the yeast. The result for the control sample indicates that the components in the matrix could be responsible for the low damascenone concentration. Constituents such as ethanol, water, amino acids and/or SO_2 could react with the conjugated alkene.

A study by Guth[84] showed that the concentration of damascenone is reduced up to 75% during the first few months of wine maturation. Furthermore, Daniel et al.[96] showed damascenone is vulnerable to react with free SO_2 in young wine which is also dependent on the concentration of SO_2 and pH of the medium. It was shown that damascenone reacts with various nucleophiles that could be present in young wine but reacts predominantly with free SO_2.[96] Thus, the amount of preservative in the

form of sulfur dioxide added to a wine or generated by yeast during the winemaking process could have a significant effect on the resulting concentration of damascenone in a young wine. Some yeast strains could have a high impact on the concentration on damascenone depending on whether they produce high or low amounts of SO_2 metabolically. It is important to understand that not only SO_2 reacts with damascenone but there are many other roaming nucleophiles that might contribute to a decrease in damascenone concentration during fermentation.

5.3.1 Stability of damascenone in CDGJ medium

This study was focussed on further investigating the changes in damascenone due to chemical interactions. The CDGJ medium was segregated into the major groups of compounds that might be responsible for reacting with and subsequently reducing the amount of damascenone. Five batches of CDGJ medium were prepared as described by Ugliano et al.[97], but with the major groups of compounds such as nitrogen based compounds, vitamins and trace elements omitted from four of the CDGJ mediums prepared. The five different stocks prepared for this study included the CDGJ medium (all components); CDGJ medium with no nitrogen sources; CDGJ medium with no trace elements; CDGJ medium with no vitamins and CDGJ medium with no ammonium chloride. At day 0 each of the batches spiked with damascenone, the concentration was measured and the solutions were maintained at 17 ^{o}C, which was the same temperature as all previous fermentations. The CDGJ medium solutions were not inoculated with yeast. At day 30 each of the batches were again analysed for their damascenone concentration.

Each of the treatments showed a decrease in damascenone concentration, but the biggest loss was observed from the CDGJ medium with all of the components. There was no observable yeast or microbial growth at the end of the experiment, indicating fermentation did not occur. The matrix composition appeared to have a direct effect on the amount of damascenone present at the end of 30 days. The reduction in damascenone might be due to a combination of multiple reactions or a ‘salting out’ effect since the matrix with all of the components showed the largest reduction in damascenone compared to the other mediums (*Figure 5.4*).

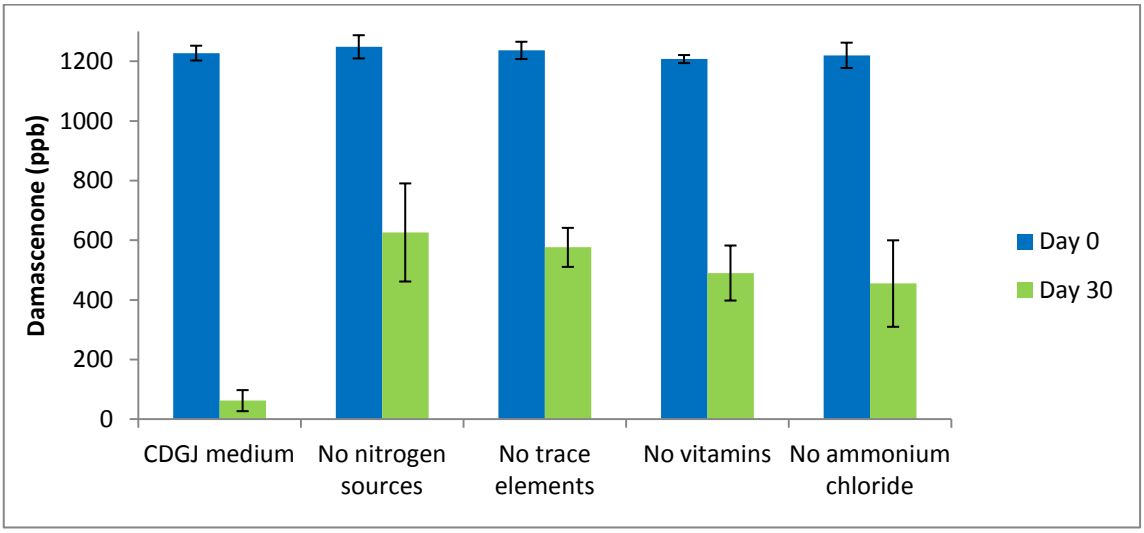


Figure 5.4 Concentration of damascenone at day 0 and day 30 in the five different mediums.
NB: The five solutions were not inoculated with yeast.

The decrease in damascenone concentration was only monitored at the beginning and end of the 30 day trial, so to determine the rate of the loss, the experiment was repeated with measurements taken more frequently. The aim was to determine if the loss was occurring rapidly at the beginning or steadily over the 30 day period. This was done by monitoring the concentration of damascenone of the spiked CDGJ medium every day for the first 10 days and then every two days till day 30. The CDGJ medium was again not inoculated with yeast.

The concentration of damascenone decreased 10% in the first 24 hrs and then decreased by 50% by day 10. From this point the concentration decreased steadily with the concentration measuring 100 ppb at day 30. This was only 5% of the spiked damascenone concentration (*Figure 5.5*).

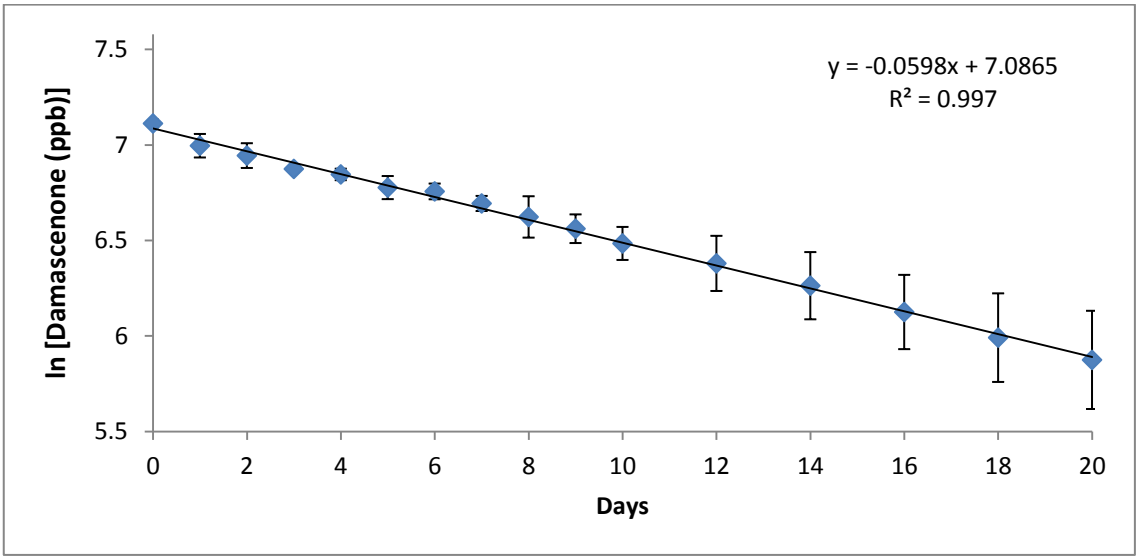


Figure 5.5 Natural log of damascenone (ppb) in CDGJ medium from day 0 – day 20
NB: The CDGJ medium was not inoculated with yeast.

The reaction rate of damascenone follows first order kinetics. It is continuously being lost from the medium until it has almost all been depleted. The concentration of damascenone remaining in the CDGJ medium at day 26 was around 200 ppb indicating a loss of 80% which corresponds closely to the initial experiment where a 75% loss of damascenone was observed in the controls (*Figure 5.1*).

This experiment does not reflect the actual amount of damascenone normally present in a juice or wine. Importantly, it does show that there are key factors that need to be considered that contribute to decreased amounts of damascenone as it is forming during fermentation. Being aware of these factors is one step towards maintaining higher levels of damascenone in wine.

5.3.2 Stability of damascenone in Chardonnay juice

After observing such a large reduction in damascenone from the sterile CDGJ medium the experiment was repeated in sterile Chardonnay juice to determine if it was only an artefact of the CDGJ medium. A large decrease in damascenone was also observed in the Chardonnay juice over the 30 day period (*Figure 5.6* and *5.7*).

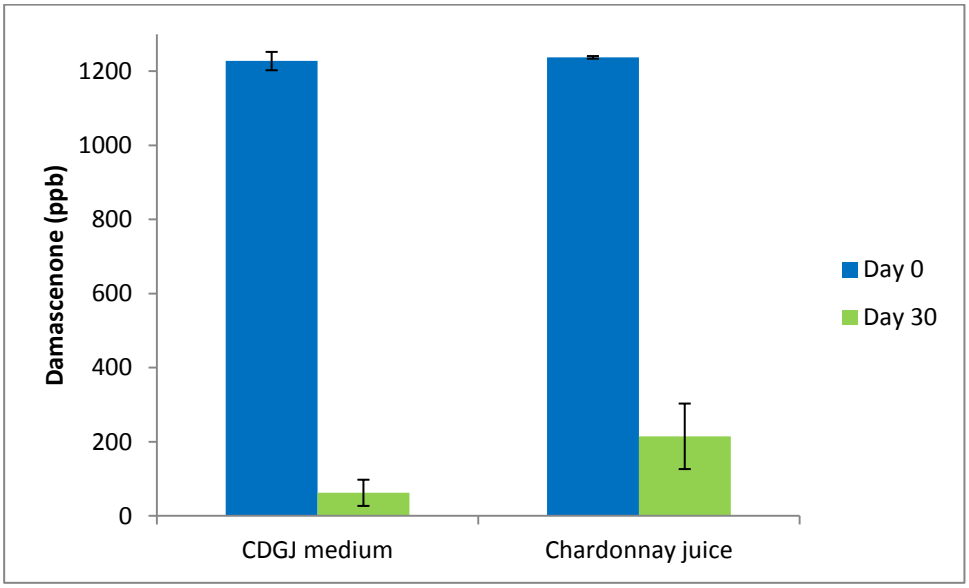


Figure 5.6 Loss of damascenone from the sterile CDGJ medium and sterile Chardonnay juice over the 30 day period.

NB: The CDGJ medium and Chardonnay juice was not inoculated with yeast.

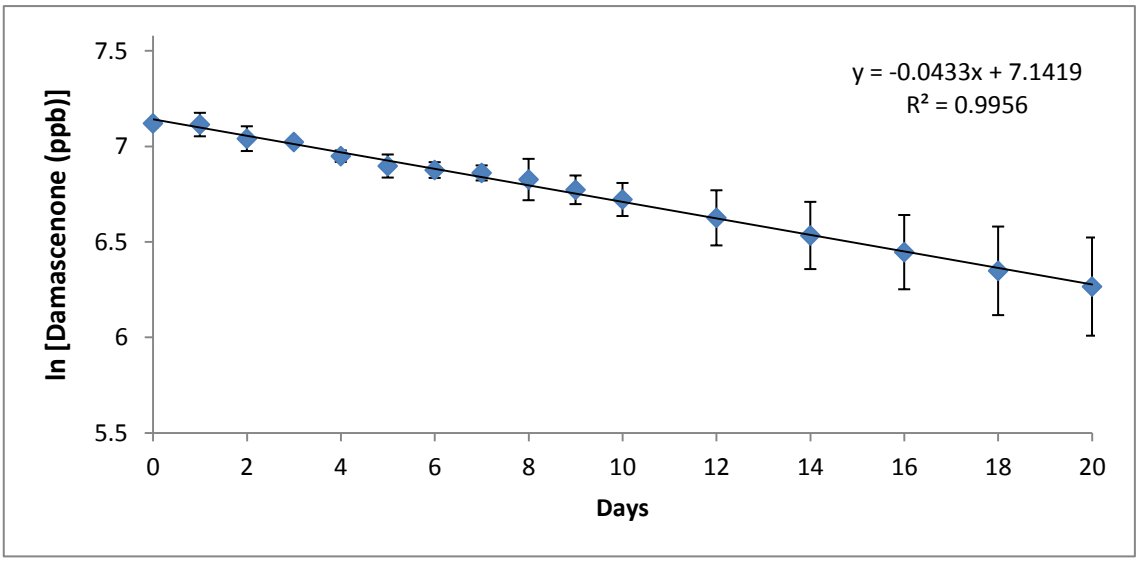


Figure 5.7 Natural log of damascenone (ppb) in CDGJ medium from day 0 – day 20.

NB: The CDGJ medium was not inoculated with yeast.

The final concentration of damascenone at day 30 in the CDGJ medium revealed a decrease by more than 95% (*Figure 5.5*) compared to the Chardonnay juice which had also reduced by a large amount and dropped by 85% (*Figure 5.7*). No observable fermentation took place during the experiment.

The rate of reaction of damascenone in the Chardonnay juice also follows a first order kinetic reaction. This indicated that the loss of damascenone wasn't just an artefact of

the CDGJ medium experiment. An unpaired t test (P value 0.1517) of the two data sets indicated that there was no significant difference in the decrease in damascenone concentration between the CDGJ medium and the Chardonnay juice.

The standard deviation for both experiments at day 30 was quite large with an error of ± 50 ppb for the CDGJ medium and ± 90 ppb for the chardonnay juice. The variation between replicates at the beginning of the experiment was very minor which indicated that as time progressed damascenone loss even between replicates can be quite different. The reason for this is unknown.

5.4 Loss of damascenone through volatilisation

Volatilisation of damascenone was also considered as a possible mechanism accounting for the observed loss in the above experiments. During these experiments, the water airlocks used to ensure the ferments remained sterile were measured for the presence of damascenone. The aroma characteristic for damascenone could be detected in the 17 ^{o}C incubator room during the experiment. The water airlocks were quantified for damascenone and about 1% of the total spiked amount was detected which revealed some damascenone was volatilising. It was thought that it might be getting partially trapped and then passing through the airlocks into the atmosphere.

It has been proposed that a range of desirable volatile compounds are lost during fermentation, which is aided by the carbon dioxide released.[103] The production of this gas can act as a purging device and that must composition and temperature can have an effect on the amount of volatiles released. Ferreira et al.[103] investigated the ability of fermentative carbon dioxide to purge important fatty acid ethyl esters and some fusel alcohol acetates, which, are synthesised during fermentation. They showed that over 80% of the synthesised esters can be lost due to the purging effect of carbon dioxide.[103]

Morakul et al.[104] looked at the modelling of the gas-liquid partitioning of the aroma compounds, isobutanol, ethyl acetate, isoamyl acetate and ethyl hexanoate to predict the aroma losses during fermentation. They found that the partitioning was not

influenced by the carbon dioxide production rate but was affected by the matrix and the temperature. They found that the amount of compound lost was dependent on its volatility and a loss of up to 70% of ethyl hexanoate at 30 ^{o}C was observed.

5.4.1 Closed experiment study

The amount of damascenone lost via volatilisation was determined by performing experiments analogous to the above CDGJ medium and Chardonnay experiments but in a closed system. The closed experiments were set up in duplicate in schott bottles with the caps lined with aluminium foil. The solutions were sterile and not inoculated with yeast.

A decrease in damascenone was observed in the closed systems and this was significantly different when compared to the open systems for all treatments. This shows the larger portion of damascenone is lost through volatilisation but there is still a portion that appears to be reacting with components in the CDGJ medium and the Chardonnay juice (*Figure 5.8*).

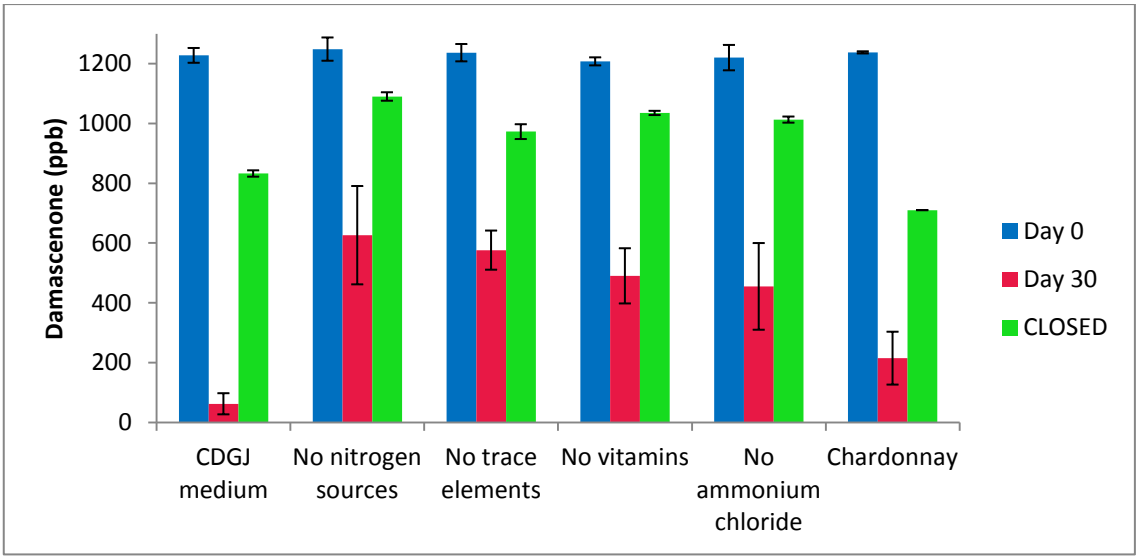


Figure 5.8 The amount of damascenone at day 0 and at day 30 in the closed sterile systems.
NB: The six solutions were not inoculated with yeast and were performed in open flasks fitted with airlocks or in a closed vessel.

The results show that the amount lost through volatilisation plus other reactions in the complete CDGJ medium can be above 95% (*Figure 5.8* or *5.5*). All components were present in this medium and the result could reflect the result from a 'salting out'

effect. The closed system with the CDGJ medium eliminates the loss through volatilisation. It shows that about 30% was lost through apparent reactions of damascenone with components in the medium, decreasing its concentration by around 40%.

The other nutrient omission experiments only show about 20% of damascenone reacting with components in the medium and only 50% lost through evaporation over the 30 days which is significantly less than the CDGJ medium and the Chardonnay juice (*Figure 5.8*). This result indicates that multiple constituents are involved in reactions with damascenone aiding in its depletion. It appears the 'salting out' effect does not have as large of an impact in the experiments with the nutrients omitted.

5.4.2 CDGJ medium/CDMW study

The previous experiments have supported the notion that the concentration of damascenone can decrease via volatilisation and by chemical interactions during and after fermentation. This experiment, however, does not take into account the change in composition of a fermenting medium as a fermentation progresses when the sugar and other nutrients decrease and alcohol content increases. Morakul et al.[105] proposed a 'salting out' effect of the medium due to the high composition of sugar at the beginning of fermentation and monitored the gas-liquid partition coefficients synthetic solutions with increasing concentrations of ethanol and decreasing concentrations of sugar. The ethanol was found to increase the solubility of the higher alcohols and esters and thus decreased their volatility.

The aim of the next experiment was to determine if the evaporation of damascenone decreased as the composition of the medium changed. Four different solutions were prepared from mixing different ratios of CDGJ medium (no alcohol) and Chemically Defined Model Wine (CDMW – 12% alcohol) giving solution A – 0% alcohol (100% CDGJ medium), solution B – 2% alcohol (1:5, CDMW:CDGJ medium), solution C – 6% alcohol (1:1, CDMW:CDGJ medium) and solution D – 12% alcohol (100% CDMW). They were spiked with damascenone and the concentration was measured every five days until day 30. The four different solutions were also set up in a closed system to

minimise the volatilisation effect. In general, approximately 90% was lost from solution A, B and C and approximately 75% lost from solution D (*Figure 5.9*). There was also an observed loss from the solutions in the closed system (*Figure 5.10*).

This open system experiment shows the amount of damascenone lost due to the combination of evaporation and/or chemical reactions (*Figure 5.9*). It was observed damascenone concentration decreases rapidly between day 0 and day 10 and then much more slowly until day 30. In solutions B and C, 10% of the starting concentration of damascenone remained at the end of the experiment. At the end of the 30 day period, approximately 20% of damascenone still remained in solution D (100% CDMW).

The solutions in the closed system eliminated damascenone loss through evaporation (*Figure 5.10*). The closed experiments showed a 30% loss in damascenone from solution A (100% CDGJ medium), a 65% loss from solution B, a 85% loss from solution C and a 55% loss from solution D (100% CDMW).

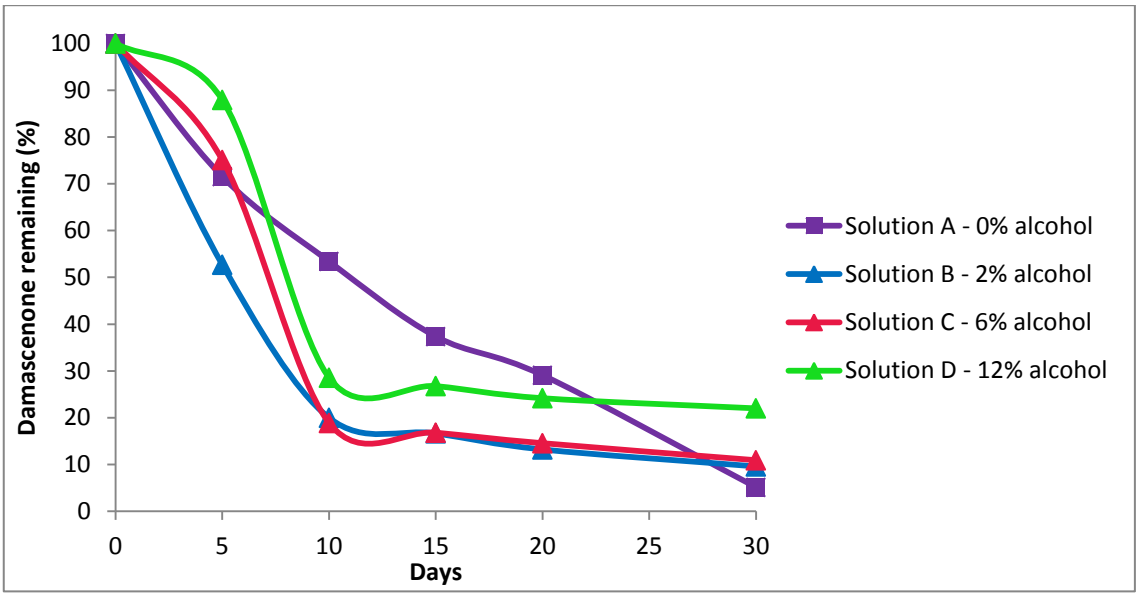


Figure 5.9 Damascenone loss from the different percentage alcohol matrices at 17 °C in open systems.

Solution A - 0% alcohol (100% CDGJ medium), Solution B - 2% alcohol (1:5, CDMW:CDGJ medium), Solution C - 6% alcohol (1:1, CDMW:CDGJ medium), Solution D - 12% alcohol (100% CDMW)

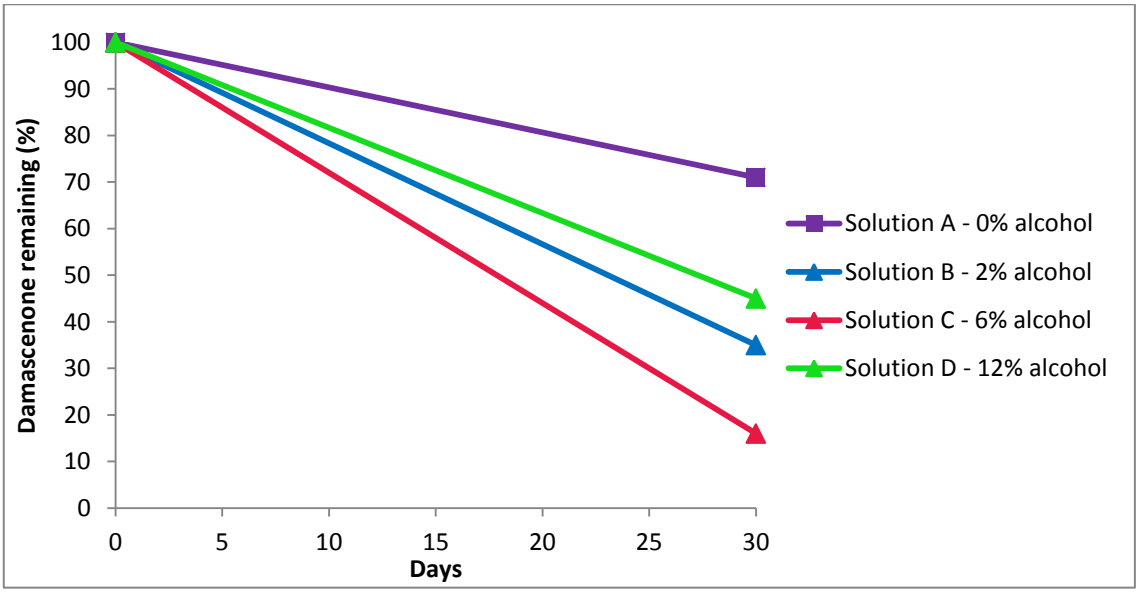


Figure 5.10 Damascenone loss from the different percentage alcohol matrices at 17 °C in closed systems.

Solution A - 0% alcohol (100% CDGJ medium), Solution B - 2% alcohol (1:5, CDMW:CDGJ medium), Solution C - 6% alcohol (1:1, CDMW:CDGJ medium), Solution D - 12% alcohol (100% CDMW)

The loss of damascenone from solution A (100% CDGJ medium) in the open system was 95% (*Figure 5.9*) and in the closed system it was 30% (*Figure 5.10*) which agrees with the results of earlier experiments where the concentration of damascenone was monitored over the 30 day period (*Figure 5.8*).

Clearly, the different proportion of the CDMW and the CDGJ medium constituents (including alcohol) affects the kinetics of damascenone loss and possibly also any equilibrium position that might be reached. As the proportion of CDGJ medium to CDMW constituents (including alcohol) change continuously during fermentation, it is not possible to extrapolate directly from these experiments to a real fermentation but, clearly, the continuous change in ferment composition will also change the rate and extent to which damascenone is lost. It is also not possible to determine from these experiments alone how much damascenone is lost due to any one process over the course of fermentation.

5.5 Adsorbent study

Various experiments were set up to investigate a suitable method to quantify the damascenone that might be lost by evaporation during fermentation. This would help to account for the actual amount of damascenone synthesised from **26**, **27** and **28** if most of this is lost through volatilisation. Adsorbing the volatiles by using a resin material and condensing the damascenone lost from a ferment using cold trapping were among the trialled methods in an effort to capture damascenone.

Resins Tenax-TA, XAD-2, XAD-7 and FX-66 were used in an attempt to try to adsorb any damascenone released from the ferment flask. Tenax-TA was thought to be the most adequate at adsorbing damascenone based on previous studies performed on similar aroma compounds.[106] Yeast strain AWRI 796 was used to inoculate the CDGJ medium (spiked with **1** at 1000 ppb) and each flask was fitted with water air locks and glass tubes filled with either, Tenax-TA, XAD-2, XAD-7 or FX-66 fitted above the airlocks. At the end of fermentation, the ferments were measured for the amount of damascenone remaining.

About 20% of the original amount of damascenone was present at the end of fermentation for each of the ferments with adsorbent resins attached. It was anticipated that at least one of the adsorbent resins would have adsorbed the damascenone being purged from the flask. The resins were solvent extracted but,

surprisingly, negligible amount of damascenone was identified in the extracts from each of the resins.

It was observed that significantly more damascenone was lost from the fermenting media compared to the amount lost from the sterile CDGJ medium at the same timepoint (*Figure 5.11*).

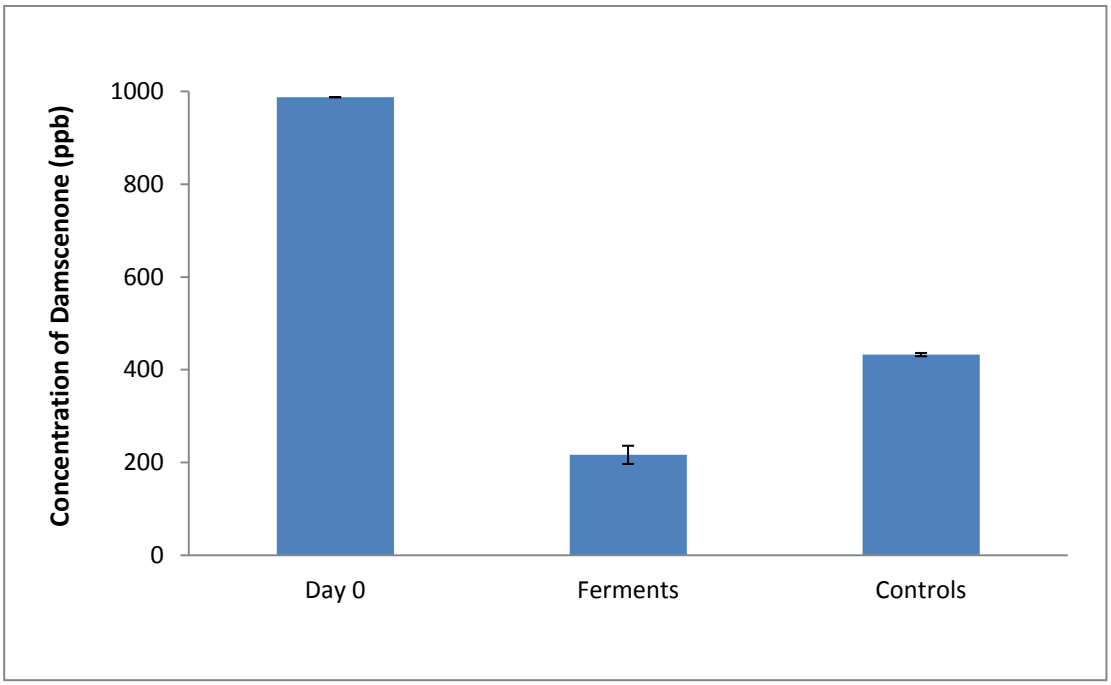


Figure 5.11 Concentration of damascenone at the day 0 and at the end of fermentation at day 21.
NB: Ferments and controls were removed from 17 °C at day 21 when the ferments reached dryness.
F1 – Ferment replicate 1 fitted with Tenax-TA; F2 – Ferment replicate 2 fitted with XAD-2;
F3 – Ferment replicate 3 fitted with XAD-6; F4 – Ferment replicate 4 fitted with FX 66
Controls – non-inoculated CDGJ medium

Thus, it appears that the CO_2 released from the yeast during fermentation could be aiding in the loss of damascenone. This supports the study by Morakul et al.[104] that suggested carbon dioxide produced during fermentation can strip the volatile compounds synthesised. However, another possibility for the greater loss in damascenone concentration in the ferment could be due to the yeast acting as a bioadsorbent of the volatile compound during or after fermentation. Once yeast cell death has occurred the intracellular constituents are released into the medium. These include hydrolytic enzymes, polysaccharides and mannoproteins.[107] It is proposed in the literature that a decrease in volatile concentration could be due to the fact that yeast cell walls can adsorb volatile compounds.[107,108] Lyophilised yeast has been shown

to act as a bioadsorbent to remove 4-ethylphenol from wine.[109] Within these studies it is a possibility that the yeast lees at the end of fermentations are also acting as a bioadsorbent, removing damascenone from the medium. Extraction of lees would be interesting to investigate in future experiments.

5.6 Cold trap fermentations

An experiment was conducted to quantify the damascenone collected in a cold trap in order to account for the amount which is lost from the ferment. Cold traps fitted to the ferment flasks were maintained at minus 15 ^{o}C for the duration of the experiment. It was considered viable that damascenone could be condensed at this temperature and lower temperatures would not be required based on its relatively high boiling point of 274 ^{o}C – 275 ^{o}C.[110]

At the end of fermentation the triplicate set of ferments all lost approximately 70% of the damascenone present. The controls revealed slightly higher levels of damascenone at the end (*Figure 5.12*), agreeing with the previous study (*Figure 5.11*).

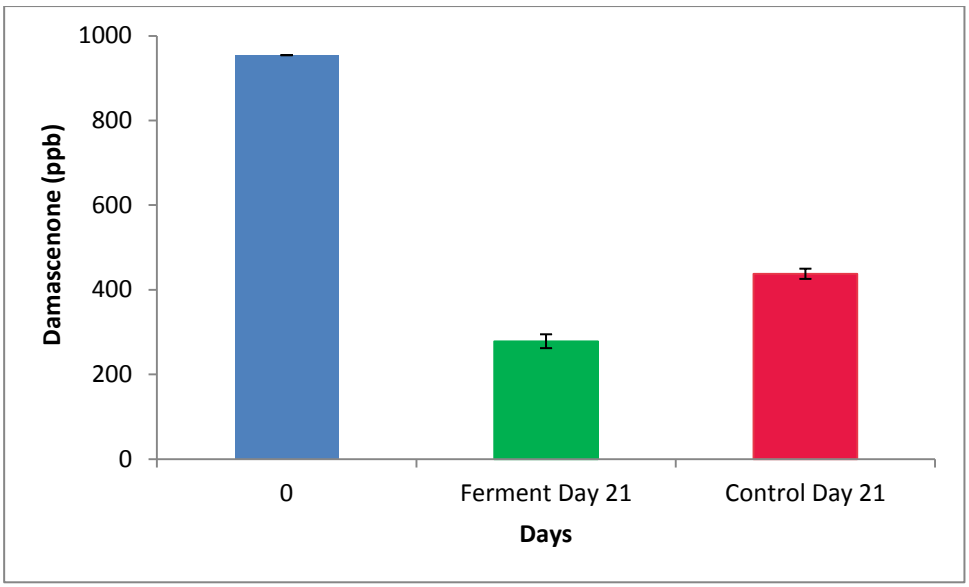


Figure 5.12 Cold trap fermentations.
Measured damascenone (ppb) at day 0 and day 21 in the ferments and the sterile controls.

Again carbon dioxide produced during fermentation appears to be acting as a purging mechanism for damascenone. The cold traps at minus 15 ^{o}C contained only traces of

damascenone indicating either that they were not adequate for trapping any sparged damascenone or that the presence of yeast, another mechanism for damascenone removal becomes more important than evaporation.

5.7 The production of damascenone - natural hydrolysis versus fermentation

Ugliano and Moio[111] observed the natural release of damascenone from Fiano grapes. They detected trace amounts of damascenone in the sterile Fiano juice at time point zero and subsequently showed that damascenone was released in the sterile juice over 18 days to produce 2 ppb. Under the same conditions they fermented the Fiano juice and showed that 3 ppm of damascenone was produced. It was concluded that the origin of damascenone formed was predominantly through acid hydrolysis.

In this study the natural evolution of damascenone in sterile Riesling juice was monitored and compared against the formation of damascenone during fermentation of the same sterile Riesling juice. It was considered important to determine the amount of damascenone released by chemical reactions versus yeast enzymatic catalysed reactions.

The Riesling juice fermentations were set up in triplicate and inoculated with the yeast strain AWRI QA23. A triplicate set of the same sterile Riesling juice utilised for the ferments were not inoculated with yeast and were used to monitor the natural evolution of damascenone under the acidic environment. The sterile juice was also allowed to stand during the length of the experiment in a closed vessel to establish if the open acid hydrolysis system versus the closed acid hydrolysis system has an effect on the final concentration of damascenone. The initial concentration of damascenone present in the Riesling juice before inoculation was less than 1 ppb for all replicates.

After 3 weeks the ferments were racked, stored at 4 ^{o}C along with the Riesling juice and replicates and all samples were analysed by GCMS. The concentration of damascenone was 5 ppb for the fermentation replicates and 5 ppb for the sterile Riesling juice replicates (*Table 5.1*). They both revealed an increase in damascenone from time zero but there was no significant difference between fermented juice and

non-fermented juice. Interestingly the sterile juice in the closed system was higher which indicates some damascenone is simply lost through volatilisation from the fermented juice and the non-fermented juice. It was confirmed at the end of the experiment that there was no visible fermentation or microbial growth occurring in experiment B or C.

Table 5.1 Damascenone formed in sterile Riesling juice (17 °C for 21 days) and Riesling juice fermentation (17 °C for 21 days).

	Damascenone (1) (ppb)	**SD**
Control t = 0	0.8	0.1
A – AWRI QA23 ferment	5	0
B – Riesling, no yeast	5	1.7
C – Riesling, no yeast, closed	9	0

Although it appears there is no difference between the amount of damascenone in the final wine and the non-inoculated juice there are a few important factors that should be considered. The closed system reveals that the maximum amount of damascenone formed is greater than or equal to 9 ppb, depending on how much is lost through reactions with the components of the medium. Therefore, more than 50% of the damascenone formed by chemical reactions in the open system was lost through volatilisation. According to the work described earlier in this chapter, yeast can contribute significantly to the loss of damascenone during fermentation (*Figure 5.11* or *5.12*), although the principal mechanism for this remains unlcear.

Damascenone loss due it its volatility is an important factor to consider when examining the effect of fermentation on the concentration measured in the resulting wine. In this case, no difference in concentration was observed between the fermented and non-fermented juice, however, considering the damascenone volatilisation losses could occur in both systems, it is likely that a higher amount would have formed during fermentation.

5.8 Conclusion

Loss in damascenone **1** concentration due to the aid of CO_2 release, reaction with medium constituents or via other mechanisms in the ferments would indicate that greater amounts of **1** could be forming during fermentation. Although these predictions were not conclusive from these experiments, there is a complex interplay of various factors that are contributing to the final concentration of damascenone **1** after fermentation and they should always be considered when measuring the amount of **1** that is present in the resulting wine.

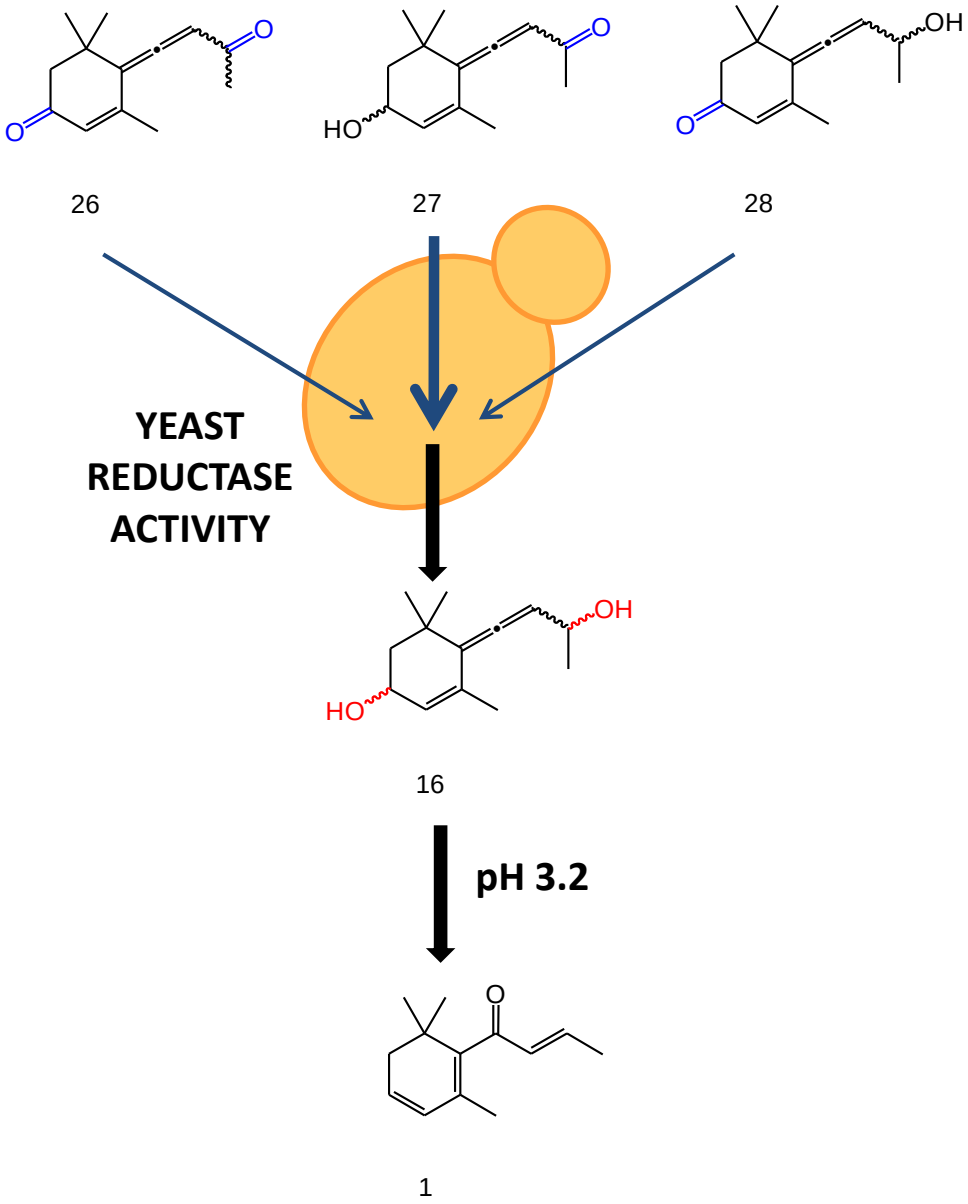
Chapter 6

Conclusion

CHAPTER 6 Conclusion

Damascenone is an important flavour and aroma compound that contributes favourably to the aroma of wine as well as many other products. Many studies have shown an increase in damascenone concentration during and at the end of fermentation but the processes responsible for this increase are not fully understood. The formation of damascenone under hydrolytic conditions has been well documented but the full extent of the role of yeast contributing to damascenone formation has not been completely elucidated. This thesis was focused on demonstrating the possibility that oxidised damascenone precursors **26**, **27** and **28** can be converted to damascenone **1** via a combination of reduction and subsequent hydrolysis.

Ferments with added ketones **26**, **27** and **28** showed that yeast was able to partially convert these to damascenone **1**. Previously, it was thought that damascenone is formed by chemical mediated reactions alone but it has now been demonstrated that oxidised damascenone precursors should also be considered in the formation of **1** (*Scheme 6.1*).



Scheme 6.1 Biotransformation of ketones 26, 27 and 28.

The ketone **27** was more efficient in the formation of damascenone compared to the other ketones **26** and **28**. This showed that yeast can be substrate specific or enzyme specificity plays a critical role towards the concentration of damascenone at the end of fermentation. A difference in the concentration of damascenone was also observed between the different yeast strains AWRI 796 and AWRI 1537. The ferments inoculated with the yeast strain 796 resulted in higher final concentrations of damascenone compared to those ferments inoculated with the yeast strain AWRI 1537 (*Figure 6.1*).

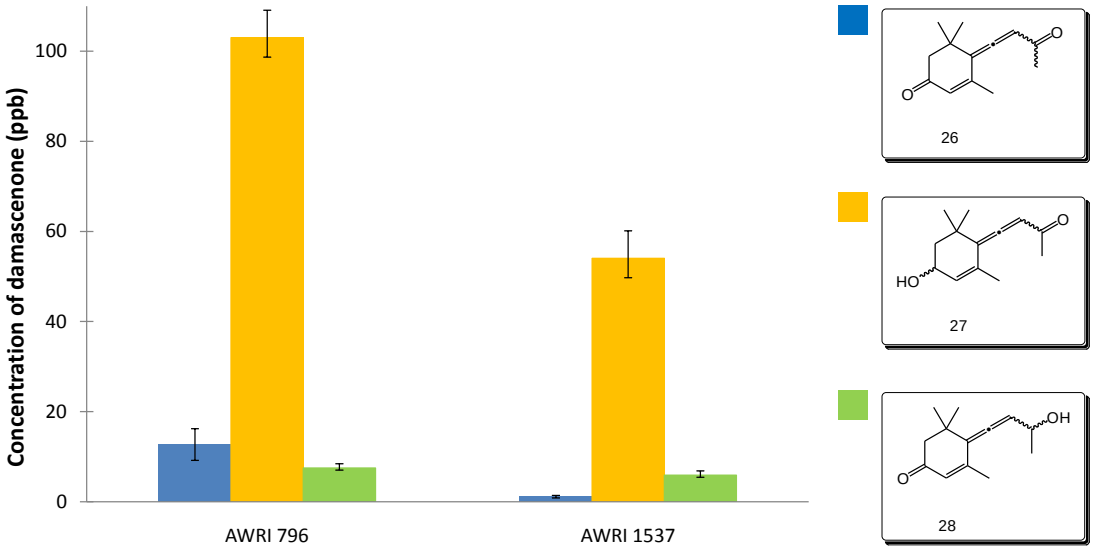
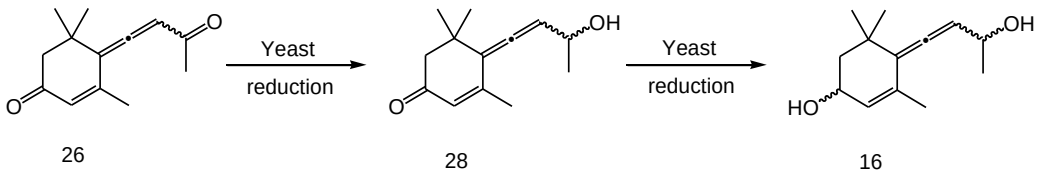


Figure 6.1 Formation of damascenone (ppb) from ferments spiked with ketones 26, 27 and 28.

The pathway by which the diketone **26** is reduced by the yeast appeared to be via the ketone **28** before undergoing a second carbonyl reduction to form the diol **16**. It was interesting that the diketone **26** and ketone **28** pathways generated similar amounts of damascenone after fermentation. In comparison, the ketone **27** generated significantly greater amounts of damascenone after fermentation. This suggested that the C_9 carbonyl was more easily reduced compared to that at C_3 and the latter process was the limiting step in forming damascenone from these oxidised precursors (*Scheme 6.2*).



***Scheme 6.2* Conversion of diketone 26 to 16 by the action of yeast.**

In ferments containing oxidised damascenone precursors **26**, **27** and **28**, it was observed that there was a decrease in damascenone concentration at the end of fermentation. A decrease in **1** was also observed when the fermentation became stuck and the number of days to reach dryness increased. A combination of various factors might be causing this decrease such as yeast cell adsorption, chemical transformations and loss through volatilisation. The experiments conducted to determine the reason

behind the large loss of damascenone from spiked ferments demonstrated that there is a complex interplay of various factors that are contributing to the final concentration of damascenone after fermentation.

During fermentation of model CDGJ medium spiked with damascenone it was observed that up to 80% is lost. This unexpected loss of damascenone observed in the spiked ferments led to a review of the ferments containing with the precursors **26**, **27** and **28**. A 1 - 11% conversion from **26**, **27** and **28** to damascenone was indicated (*Chapter 4*). In light of a possible 80% lost during fermentation, this would indicate the conversion from **26**, **27** and **28** might be significantly greater than initially thought (*Figure 6.2*).

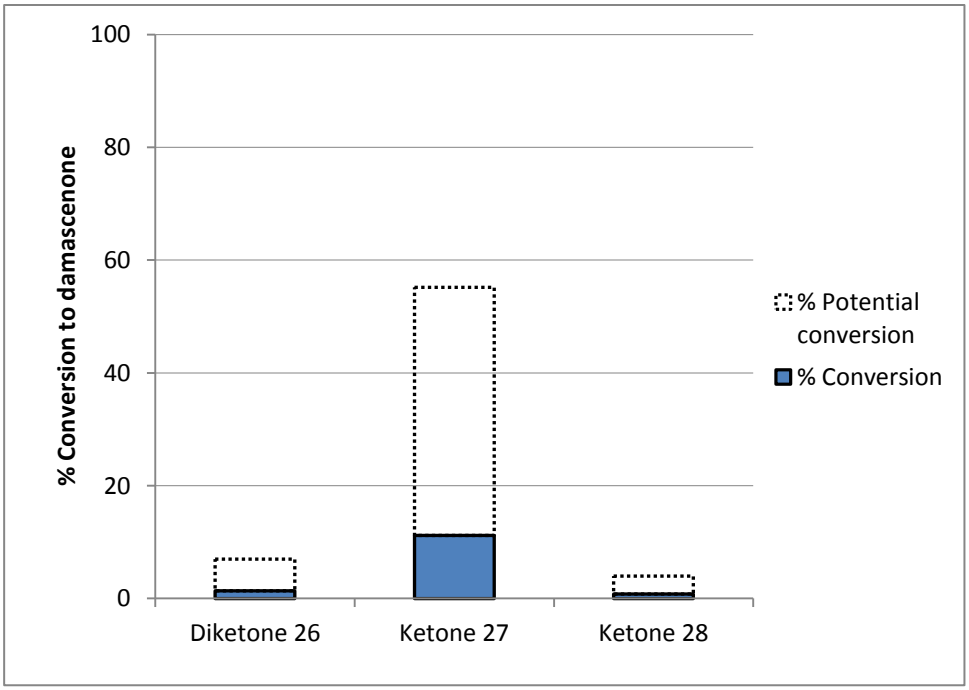


Figure 6.2 Conversion of 26, 27 and 28 to damascenone and potential percentage conversion.
NB: Potential percentage conversion is the amount remaining plus that which might be lost through volatilisation assuming that this loss is 80% of the total formed.
NB: % Conversion results from fermentations with yeast strain AWRI 796.

The concentration of **1** at the end of fermentation takes into account the amount formed and the amount lost by the purging effect of carbon dioxide, reactions with constituents in the medium and/or adsorption by yeast lees.

Previous studies were concerned solely with the chemical mechanisms involved in the formation of damascenone. The work described in this thesis has demonstrated the feasibility of a biochemical component to damascenone formation during fermentation. However, the factors affecting the loss of damascenone, once formed, might well be more important than those affecting damascenone formation in determining the final concentration in wine and other fermented products.

Chapter 7

Experimental

CHAPTER 7 Experimental

7.1 General experimental

Solvents:

The X4 that was used for column chromatography to purify synthesised compounds is a fraction obtained from distilling petroleum, containing *n*-hexane as the major component. Anhydrous diethyl ether and tetrahydrofuran (THF) used for synthesis were distilled from sodium/benzophenone directly before use. Dichloromethane (DCM) required for synthesis was distilled over CaH_2 prior to use. The anhydrous acetone used for synthesis was Suprasolv grade (Rowe Scientific). Milli-Q water – Milli-Q purification system (Millipore, North Ryde, NSW, Australia). The solvents used for juice and honey extractions were pentane (Unisolv), DCM (Suprasolv), ethyl acetate (EtOAc) (Suprasolv) and diethyl ether (Unisolv), which, were purchased from Rowe Scientific.

Reagents:

Reagents required were purchased from Sigma-Aldrich and were of high purity, ≥ 98%. The Dess-Martin periodinane reagent was purchased from Bioscientific Pty. Grasshopper ketone **12** (donated to The Australian Wine Research Institute[47]) used in the fermentations was characterised by NMR (^{1}H and ^{13}C) and GCMS. The physical and chemical properties of ketone **12** were consistent with those reported.[112]

Glassware:

The glassware used for the quantification of damascenone were volumetric glass pipettes (A grade), volumetric measuring cylinders (A grade) and glass syringes (SGE). The glassware used for the extraction of volatiles from the Riesling juice, Pinot Noir juice and honey were washed with detergent (pyroneg), rinsed with milli-Q water and rinsed with dichloromethane (DCM), pentane and ethyl acetate (EtOAc). Syringes and volumetric pipettes were cleaned with four solvents, acetone, DCM, pentane and ethanol.

Vials and ampoules:

The 15 mL glass vials with aluminium lined screw cap lids used for the extraction of damascenone were purchased from Supelco, USA. The GCMS vials with PTFE-lined crimp caps (2 mL) and glass inserts (250 μL) were purchased from Agilent Technologies. Pre-scored ampoules (1 mL, 5 mL, 10 mL and 20 mL) were purchased from Sigma-Aldrich.

Column chromatography:

Column chromatography was performed using silica gel 60 F_{254} (230-400 mesh) from Grace Davison. All solvent systems were % v/v. Analytical thin layer chromatography was performed with aluminium backed silica gel 60 F_{254} sheets from Merck.

NMR analysis:

^{1}H and ^{13}C NMR spectra were recorded with a Bruker spectrometer operating at frequencies of 400 MHz or 600 MHz and 100 MHz, respectively. All spectra were run in deuterated chloroform ($CDCl_3$) or deuterated acetone ($(CD_3)_2CO$). Chemical shifts are quoted in ppm and coupling constants in *Hz*.

The numbering system for the included NMR data is based on the numbering scheme used for the megastigmane skeleton (*Figure 7.1*).

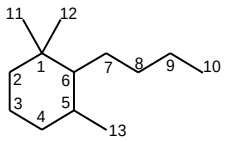


Figure 7.1 Basic C_{13} megastigmane skeleton.

Mass spectra:

Mass spectra were recorded using Hewlett-Packard (HP) 6890 gas chromatograph fitted with Gerstel autosampler and coupled to a HP 5973N mass spectrometer.

Accurate mass analysis:

Accurate mass was performed by the University of Tasmania, Hobart, Tasmania, Australia.

Model wine:

The 10% model wine was freshly prepared before each use by preparing a solution of 10% redistilled ethanol and milli-Q water (v/v), saturating the mixture with potassium hydrogen tartrate and adding a saturated solution of tartaric acid dropwise until the required pH was reached.

Phosphate citrate buffer:

The phosphate citrate buffer solution was prepared by dissolving a phosphate citrate buffer tablet (pH 5.0) (Sigma Aldrich) in 100 mL milli-Q water.

pH measurements:

pH measurements were made with an Ecoscan pH 5/6 meter (Eutech Instruments, Singapore) which was calibrated before each use.

Alkane standards:

C_8-C_{24} standard alkanes were purchased from Altech, Brookhollow Ave, Baulkham Hills, NSW and were run for the determinations of Kovats Indices.

Statistical analysis:

Statistics data were analysed using GraphPad Prism 6.01 (GraphPad Software Inc. U.S.A.). For analyses involving two and three or more groups paired t-test and ANOVA were used, respectively with $\alpha = 0.05$ for significance.

Chemically defined grape juice medium (CDGJ medium):

The CDGJ medium was prepared from stock solutions of nitrogen sources, trace elements, vitamins, salts with the addition of sugars, glucose and fructose, as described by Ugliano et al.[97]

Chemically defined model wine (CDMW):

The CDMW was obtained from a fermentation carried out using CDGJ medium and the resulting wine was sterile filtered through 0.8 μm/0.2 μm filter.

Adsorbent resins:

Tenax-TA was purchased from Phenomenex. The XAD-2, XAD-7 and FX66 resins were purchased from Sigma-Aldrich.

7.1.1 Quantification of damascenone

Preparation of samples:

A 10 mL aliquot of the sample in a 15 mL glass screw cap vial with an aluminium-lined cap (Supelco) was spiked d_4-damascenone (100 μL, 10 μg/mL) using a glass syringe (100 μL, SGE). Pentane:EtOAc (2:1, 3 mL) was added and the mixture was shaken vigorously. The samples were allowed to stand for 1 hr and the organic layer was then transferred to a 2 mL vial for GCMS analysis. For calculating the concentration of the analytes in the samples, replicate standards were prepared at the same time as the samples to be analysed, by adding the internal standard solution (100 μL, 5 μg/mL) and a solution of damascenone in ethanol (100 μL, 5 μg/mL) to DCM (1.8 mL), and analysing the mixture by GCMS to calculate the relative response factors. The method used to prepare and analyse the samples and standards was that described by Daniel et al.[96]

GCMS conditions:

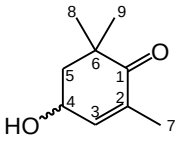
The liquid injector was operated in fast liquid injection mode with a 10 μL syringe (SGE) fitted. The gas chromatograph was fitted with an approximately 30 m x 0.25 mm i.d. J&W fused silica capillary column DB-WAX, 0.25 μm film thickness. The carrier gas was helium (BOC Gases, ultrahigh purity), and the flow rate was 1.6 mL/min. The oven temperature, started at 50 °C, was held at this temperature for 1 min, then increased to 240 °C at 10 °C/min and held at this temperature for 10 min. The injector was held at 200 °C and the transfer line at 240 °C. The sample volume injected was 2 μL, and was introduced in pulsed splitless mode with an inlet pressure of 25.0 psi maintained until splitting. The glass liner (Agilent Technologies) was borosilicate glass with a plug

of resilanized glass wool (2-4 mm) at the tapered end to the column. The ions monitored in SIM runs were: *m/z* 73, 193 and 194 for d_4-damascenone and 69, 175 and 190 for unlabelled damascenone. Selected fragment ions were monitored for 30 ms each. The underlined ion is the ion that was used for quantification. The other ions were used as qualifiers.

7.2 Synthesis of the allene precursors (Chapter 2)

7.2.1 Synthesis of the allenic diol (16)

4-Hydroxy-2,6,6-trimethylcyclohex-2-en-1-one (31)

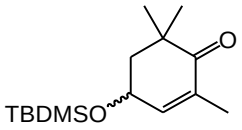


31

Sodium borohydride (0.88 g, 23.2 mmol) was added over 10 mins to a mixture of 4-oxoisophorone (2,6,6-trimethylcyclohex-2-ene-1,4-dione) (10.37 g, 68.14 mmol) and $CeCl_3$ (5.604 g, 22.74 mmol) in MeOH (100 mL) at 0 ^oC under nitrogen. The mixture was stirred for 1 hr at 0 ^oC and a cold aqueous solution of NH_4Cl (50 mL, 20% aq.) was then added to the mixture. The solution was extracted with ether (3 x 100 mL), washed with $NaHCO_3$ (75 mL, sat. aq.), brine (75 mL), dried ($MgSO_4$) and the solvent evaporated. The product was purified by column chromatography (20% EtOAc/DCM) yielding 5.48 g (61%) of the ketone **31** as a pale yellow oil, R_f (**31**) = 0.53 (20% EtOAc/DCM).

^1H NMR δ: 6.61 (1H, m, H_3), 4.61 (1H, m, H_4), 2.17 (1H, ddd, *J* 12.8, 5.4 and 2, H_{5eq}), 1.85* (1H, br s, OH), 1.85* (1H, dd, *J* 12.8 and 10.2, H_{5ax}), 1.79 (3H, dd, *J* 2.0 and 1.5, H_7), 1.15, 1.11 (6H, 2s, $H_{8,9}$). **Hydroxyl and H_{5ax} overlapping signals. Similar to data reported.*[28]

4-{[*tert*-Butyl(dimethyl)silyl]oxy}-2,6,6-trimethylcyclohex-2-en-1-one; (4-*tert*-Butyldimethylsilyloxy-2,6,6-trimethyl-4-hydroxycyclohex-2-en-1-one) (32)

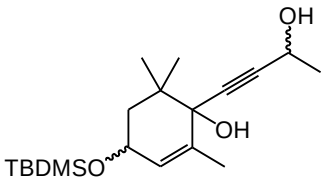


32

To a solution of the alcohol **31** (5.17 g, 33.50 mmol) in pyridine (40 mL) was added TBDMSCl (7.60 g, 50.41 mmol) and the resulting mixture stirred at ambient temperature for 3 days. Water (10 mL) was added and the mixture stirred for a further 30 mins. The solution was diluted with EtOAc (20 mL) and washed with $CuSO_4$ (3 x 50 mL, sat. aq.). The organic layer was extracted with EtOAc (4 x 100 mL) the organic extracts washed with $NaHCO_3$ (2 x 100 mL), brine (2 x 100 mL) and the solvent evaporated. The product was purified by column chromatography (30% X4/DCM → 5% EtOAc/X4 → 20% EtOAc/X4) yielding 8.79 g (98%) of **32** as a clear oil, R_f = 0.73 (20% EtOAc/X4).

^{1}H NMR δ: 6.50 (1H, m, H_3), 4.53-4.57 (1H, m, H_4), 2.01 (1H, ddd, *J* 13.0, 5.4 and 1.8, H_{5eq}), 1.90 (1H, dd, *J* 12.9 and 9.9, H_{5ax}), 1.76-1.77 (3H, m, H_7), 1.13, 1.11 (6H, 2s, $H_{8,9}$), 0.92 (9H, s, *t*-Bu), 0.12, 0.11 (6H, 2s, SiMe). *Similar to data reported.*[28]

4-{[*tert*-Butyl(dimethyl)silyl]oxy}-1-(3-hydroxybut-1-yn-1-yl)-2,6,6-trimethylcyclohex-2-en-1-ol; (3-*tert*-Butyldimethylsilyloxy-6,9-dihydroxymegastigma-4-en-7-yne) (33)

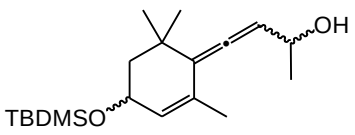


33

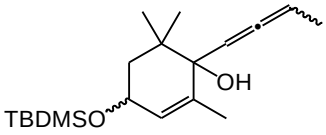
(±)-But-3-yn-2-ol (3.35 mL, 40.36 mmol) in anhydrous THF (150 mL) at -78 ^{o}C was treated with *n*-BuLi (2.5 M in hexanes, 30.67 mL, 76.68 mmol) under nitrogen and the mixture stirred at ambient temperature for 1 hr. To this solution was added the protected ketone **32** (5.42 g, 20.18 mmol) in THF (40 mL) and the reaction mixture was stirred for 2 days. The reaction was quenched with NH_4Cl (100 mL), diluted with ether (100 mL), washed with brine (4 x 100 mL) and dried ($MgSO_4$) and the volatiles were evaporated. The product was purified by column chromatography (10% EtOAc/X4 → 20% EtOAc/X4 → 30% EtOAc/X4) yielding 6.49 g (97%) of the protected dienyne alcohol **33** as a yellow/orange oil, R_f = 0.62 (30% EtOAc/X4) and was a mixture of stereoisomers in a ratio of approx. 2:1.

^{1}H NMR δ(mixture of stereoisomers): 5.46-5.47†, 5.40-5.41* (1H, m, H_4), 4.53-4.61 (1H, m, H_3), 4.18-4.28*† (1H, m, H_9), 1.86 (3H, m, H_{13}), 1.71-1.74*† (2H, m, H_2), 1.46*,1.47† (3H, d, *J* 6.6, H_{10}), 1.15, 0.99*† (6H, 2s, $H_{11,12}$), 0.89*† (9H, s, *t*-Bu), 0.08, 0.07*† (6H, 2s, SiMe). **Major isomer,* †*minor isomer,* *†*mixture of both isomers. Similar to data reported.*[28]

4-(4-{[*tert*-Butyl(dimethyl)silyl]oxy}-2,6,6-trimethylcyclohex-2-en-1-ylidene)but-3-en-2-ol; (3-*tert*-Butyldimethylsilyloxy-9-hydroxymegastigma-4,6,7-triene) (29) and 1-(Buta-1,2-dien-1-yl)-4-{[*tert*-butyl(dimethyl)silyl]oxy}-2,6,6-trimethylcyclohex-2-en-1-ol; (3-*tert*-Butyldimethylsilyloxy-6-hydroxymegastigma-4,7,8-triene) (34)



29



34

To a solution of the dienyne alcohol **33** (4.90 g, 14.48 mmol) in anhydrous ether (200 mL) at 0 ^{o}C was added $LiAlH_4$ (3.85 g, 96.27 mmol) in ether (100 mL). The mixture was heated under reflux for 5 hrs, quenched with acetone (100 mL), diluted with ether (150 mL), washed (10 % NaOH solution (2 x 100 mL), brine (2 x 100 mL)), dried ($MgSO_4$) and the solvent evaporated. The product was purified by column chromatography (X4 → 5% EtOAc/X4 → 10% EtOAc/X4) to yield two major products **29** (2.69 g, 58%) as a viscous yellow oil along with the regioisomer **34** (0.40 g, 9%) as a viscous yellow oil, each a mixture of 2 steroisomers only in a ratio of approx. 2:1 for **29** and approx. 1:1 for **34**. R_f (**29**) = 0.47 (30% EtOAc/X4), R_f (**34**) = 0.43 (10% EtOAc/X4).

^{1}H NMR δ (mixture of steroisomers **29**): 5.64-5.65*, 5.55-5.56† (1H, br d, *J* 5.4, H_8), 5.51*† (1H, m, H_4), 4.33-4.36*† (2H, m, $H_{3,9}$), 1.73*† (3H, br s, H_{13}), 1.47-1.57*† (1H, m, $H_{2a,b}$), 1.31*, 1.30† (3H, d, *J* 6.2, H_{10}), 1.11, 1.07*† (6H, 2s, $H_{11,12}$), 0.91*† (9H, s, *t*-Bu), 0.10, 0.09*† (6H, 2s, $SiMe_2$). **Major isomer, †minor isomer, *†mixture of both isomers. Similar to data reported.*[28]

^{1}H NMR δ (mixture of steroisomers **34**): 5.40-5.44*† (1H, m, H_9), 5.36*† (1H, br s, H_4), 5.28-5.32*† (1H, m, H_7), 4.25-4.29*† (1H, m, H_3), 1.90*, 1.86† (1H, br s, OH), 1.70-1.72*† (3H, m, H_{10}), 1.66*, 1.64† (3H, s, H_{13}), 1.57-1.61*† (1H, m, $H_{2a,b}$), 1.01, 1.00, 0.95, 0.92*† (6H, 2s, $H_{11,12}$), 0.89*† (9H, s, *t*-Bu), 0.07, 0.06, 0.06, 0.05*† (6H, 2s, $SiMe_2$). **Major isomer, †minor isomer, *†mixture of both isomers.*

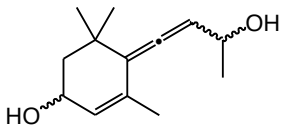
^{13}C NMR δ (**34**): 201.2, 201.0 (C_8), 138.9, 138.4 (C_5), 127.5, 127.3 (C_7), 97.6, 97.2 (C_4), 92.2, 91.8 (C_9), 76.3, 76.0 (C_6), 66.5, 66.4 (C_3), 44.8, 44.7 (C_2), 39.6, 39.3 (C_1), 26.1, 26.1, 25.6, 25.4 (*t*-Bu), 22.8, 22.7, 18.5, 18.4 ($C_{11,12}$), 17.7, 17.6 (C_{13}), 14.8 (C_{10}), -4.3, -4.4, -4.4, -4.4 (SiMe).

MS (**34**)*m/z* (%): (Isomer 1 – KI 2250, 60 m VF-WAX column) 322 (M^+, 0.1), 307 (3), 269 (100), 265 (34), 251 (8), 209 (18), 191 (9), 175 (22), 149 (19), 137 (23), 121 (16), 109 (25), 95 (13), 75 (67), 73 (65), 57 (7), (Isomer 2 – KI 2268, 60 m VF-WAX column) 322 (M^+, 0.1), 307 (2), 269 (100), 265 (34), 251 (8), 209 (18), 191 (9), 175 (22), 149 (19), 137 (23), 121 (16), 109 (25), 95 (13), 75 (67), 73 (65), 57 (8).

HRMS (ESI) (**34**): found 322.2323, $C_{19}H_{34}O_2Si$ requires 322.2328.

7.2.2 Synthesis of megastigma-4,6,7-triene-3,9-dione (26)

4-(3-Hydroxybut-1-en-1-ylidene)-3,5,5-trimethylcyclohex-2-en-1-ol; (3,9-Dihydroxymegastigma-4,6,7-triene) (16)



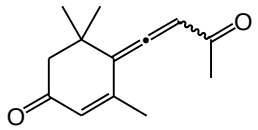
16

To a solution of the protected allene diol **29** (2.15 g, 6.67 mmol) in anhydrous THF (100 mL) was added TBAF (1.0 M in THF, 1M) under nitrogen and the mixture stirred for 3 days at ambient temperature. The reaction was quenched with $NaHCO_3$, diluted with EtOAc (20 mL), washed with brine, dried (Na_2SO_4) and the solvent evaporated. The product was purified by column chromatography (40% EtOAc/X4), yielding 0.69 g (50%) of a yellow/orange oil, as a mixture of stereoisomers in a ratio of approx. 2:1, R_f = 0.21 (30% EtOAc/X4).

^{1}H NMR δ(mixture of steroisomers): 5.67*† (1H, br d, *J* 5.2, H_8), 5.57-5.60*† (1H, m, H_4), 4.32-4.39*† (2H, m, $H_{3,9}$), 2.03*† (2H, br s, OH), 1.93*† (1H, dd, *J* 12.4 and 5.7, H_{2a}), 1.75*† (3H, m, H_{13}), 1.45*† (1H, dd, *J* 12.0 and 9.0, H_{2b}), 1.31*, 1.31† (3H, d, *J* 6.3, H_{10}), 1.13, 1.09*† (6H, 2s, $H_{11,12}$). **Major isomer,* †*minor isomer,* *†*mixture of both isomers. Similar to data reported.*[28]

3,5,5-Trimethyl-4-(3-oxobut-1-en-1-ylidene)cyclohex-2-en-1-one;

(3,9-Diketomegastigma-4,6,7-triene) (26)



26

Method 1:

Oxalyl chloride (276.6 μL, 3.17 mmol) was dissolved in anhydrous DCM (20 mL) and chilled to -60 ^{o}C under nitrogen. DMSO (460 μL, 6.49 mmol) was dissolved in anhydrous DCM (10 mL) and added to the above solution over a period of 15 mins with further stirring for 30 mins while maintaining the temperature at -60 ^{o}C. The diol **16** (300 mg, 1.44 mmol) was dissolved in anhydrous DCM (10 mL) and added to the mixture over a period of 15 mins, with further stirring for another 90 mins while maintaining the mixture at -60 ^{o}C. Triethylamine (2.01 mL, 14.41 mmol) was added to the reaction mixture over a period of 15 mins. The mixture was then allowed to reach ambient temperature before the addition of water (15 mL) and the mixture was subsequently stirred for a further 30 mins. The organic layer was extracted with DCM (3 x 25 mL) and washed with 10% aq. HCl, water, saturated $NaHCO_3$, brine, dried ($MgSO_4$) and the solvent evaporated *in vacuo*. The product was purified by column chromatography (30% EtOAc/X4) to yield 127 mg (43%) of a very viscous yellow oil, R_f = 0.23 (20% EtOAc/X4).

Method 2:

To a solution of the allene diol **16** (108 mg, 0.518 mmol) in DCM (7 mL) was added the Dess-Martin periodinane (329 mg, 0.776 mmol). The reaction mixture was stirred for 2 hrs at RT and monitored by TLC until completion. The reaction was quenched with sodium thiosulphate (15 mL), extracted with EtOAc (3 x 10 mL), the extract was washed with brine (2 x 15 mL), dried ($MgSO_4$) and the solvent evaporated. The product was purified by column chromatography (10% EtOAc/X4 → 20% EtOAc/X4) to yield a clear oil (86 mg, 81%).

^{1}H NMR δ: 6.17 (1H, br s, H_8), 5.96 (1H, br s, H_4), 2.42 (2H, s, H_2), 2.24 (3H, s, H_{10}), 2.00 (3H, br s, H_{13}), 1.25, 1.22 (6H, 2s, $H_{11,12}$).

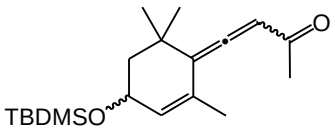
^{13}C NMR δ: 214.3 (C_7), 197.5, 197.1 ($C_{3,9}$), 148.5 (C_5), 127.3 (C_4), 117.0 (C_6), 102.5 (C_8), 50.8 (C_2), 37.0 (C_1), 28.7, 28.6 ($C_{10,12}$), 27.2 (C_{11}), 21.5 (C_{13}).

MS *m/z* (%): (KI 1826, 30 m DB-5 column) 204 (M^+, 9), 162 (66), 147 (100), 133 (5), 119 (11), 91 (9), 77 (10), 43 (43).

HRMS (ESI): found 205.1225 (M + H^+), $C_{13}H_{17}O_2$ requires 205.1223, found 227.1044 (M + Na^+) (major peak), $C_{13}H_{16}O_2Na$ requires 227.1043 and found 243.0991 (M + K^+), $C_{13}H_{16}O_2$ requires 243.0782.

7.2.3 Synthesis of 3-hydroxymegastigma-4,6,7-trien-9-one (27)

4-(4-{[*tert*-Butyl(dimethyl)silyl]oxy}-2,6,6-trimethylcyclohex-2-en-1-ylidene)but-3-en-2-one; (3-*tert*-Butyldimethylsilyloxymegastigma-4,6,7-trien-9-one) (37)



37

Method 1:

Oxalyl chloride (1.02 g, 8.04 mmol) in anhydrous DCM (40 mL) was chilled to -60 ^{o}C under nitrogen. DMSO (1.29 g, 16.6 mmol) in anhydrous DCM (20 mL) was added to the above solution over a period of 15 mins with further stirring of the mixture for 30 mins at -60 ^{o}C. The allene alcohol **29** (1.19 g, 3.69 mmol) in DCM (20 mL) was added to the mixture over a period of 15 mins with further stirring of the mixture for 45 mins at -60 ^{o}C. Triethylamine (3.71 g, 36.7 mmol) was added and the mixture was then allowed to reach ambient temperature. Water (90 mL) was added and the mixture was stirred for a further 30 mins. The organic layer was extracted DCM (3 x 50 mL) and the combined organic extracts were washed with 10% aq. HCl, water, sat. aq. $NaHCO_3$, brine, dried ($MgSO_4$) and the solvent evaporated. The product was purified by column chromatography (5% EtOAc/X4 → 10% EtOAc/X4) to yield 986 mg (84%) of a yellow oil, R_f = 0.67 (30% EtOAc/X4).

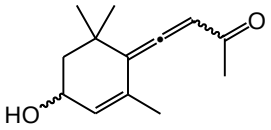
Method 2:

To a solution of the C_3 protected allene alcohol **29** (209.4 mg, 0.65 mmol) in anhydrous DCM (12 mL) under nitrogen was added Dess-Martin periodinane (400 mg, 0.942 mmol). The reaction mixture was stirred for 2 hrs at ambient temperature and monitored by TLC until completion. The reaction was quenched with sodium thiosulphate (15 mL), extracted with EtOAc (3 x 10 mL), the extract was washed with brine (2 x 15 mL), dried ($MgSO_4$) and the solvent evaporated. The product was purified

by column chromatography (10% EtOAc /X4 → 20% EtOAc /X4) to yield a clear oil (195 mg, 94%).

^{1}H NMR δ (mixture of stereoisomers): 6.07*, 6.00† (1H, br s, H_8), 5.64*† (1H, m, H_4), 4.37-4.43*, 4.24-4.27† (1H, m, H_3), 2.20*, 2.19† (3H, s, H_{10}), 1.77*† (3H, br s, H_{13}), 1.57-1.64*† (1H, m, $H_{2a,b}$), 1.20, 1.17*† (6H, 2s, $H_{11,12}$), 0.92*† (9H, s, *t*-Bu), 0.12, 0.11*† (6H, 2s, $SiMe_2$). **Major isomer, †minor isomer, *†mixture of both isomers. Similar to data reported.*[28]

4-(4-Hydroxy-2,6,6-trimethylcyclohex-2-en-1-ylidene)but-3-en-2-one; (3-Hydroxymegastigma-4,6,7-trien-9-one) (27)



27

To a solution of the allene **37** (460 mg, 1.44 mmol) in anhydrous THF (40 mL) was added TBAF (2.87 mL of 1M solution in THF, 2.87 mmol) under nitrogen and the mixture was stirred for 16 hrs at ambient temperature. The reaction was quenched with $NaHCO_3$ (50 mL) sat. aq., diluted with EtOAc (25 mL), washed with brine (3 x 25 mL), dried ($MgSO_4$) and the solvent evaporated. The residue was purified by column chromatography (10% EtOAc/X4 → 20% EtOAc/X4) to afford **27** (280 mg, 94%) as a pale yellow oil and as a mixture of stereoisomers in a ratio of approx. 1:1, R_f = 0.20 (30% EtOAc/X4).

^{1}H NMR δ (mixture of stereoisomers): 6.09*, 6.02† (1H, br s, H_8), 5.74*† (1H, m, H_4), 4.38-4.42*† (1H, m, H_3), 2.21, 2.20*† (3H, s, H_{10}), 1.94-2.00*† (1H, m, H_{2a}), 1.79*† (3H, br s, H_{13}), 1.46-1.56*† (1H, m, H_{2b}), 1.21, 1.18, 1.18, 1.13*† (6H, 4s, $H_{11,12}$). **Major isomer, †minor isomer, *†mixture of both isomers.*

^{13}C NMR δ: 213.5* (C_7), 199.0, 198.7 (C_9), 130.3, 130.2, 116.99, 116.96 ($C_{5,6}$), 128.15, 128.10, 102.6, 102.4 ($C_{4,8}$), 65.8, 65.7 (C_3), 45.9, 45.8 (C_2), 35.2, 35.1 (C_1), 29.8, 29.6, 27.9, 27.8, 26.8, 26.7 ($C_{10,11,12}$), 20.9, 20.8 (C_{13}). **signals for the two stereoisomers coincident.*

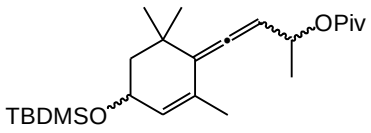
MS *m/z* (%): (Isomer 1 – KI 1845, 30 m DB-5 column) 206 (M^+, 2), 192 (15), 191 (100), 173 (18), 163 (10), 149 (23), 131 (71), 121 (42), 105 (21), 91 (26), 77 (20), 69 (11), 43 (93), (Isomer 2 – KI 1858, 30 m DB-5 column) 206 (M^+, 2), 192 (14), 191 (100), 173 (15), 163 (9), 149 (23), 131 (45), 121 (35), 105 (17), 91 (22), 77 (16), 69 (11), 43 (71).

MS Isomer 1 – KI 2769, 60 m VF-WAX, isomer 2 – KI 2787, 60 m VF-WAX.

HRMS (ESI): found 229.1198 (M +Na^+), $C_{13}H_{18}O_2Na$ requires 229.1199.

7.2.4 Synthesis towards 9-hydroxymegastigma-4,6,7-trien-3-one (28)

4-(4-{[*tert*-Butyl(dimethyl)silyl]oxy}-2,6,6-trimethylcyclohex-2-en-1-ylidene)but-3-en-2-yl 2,2-dimethylpropanoate;
(3-*tert*-Butyldimethylsilyloxy-9-pivaloyloxymegastigma-4,6,7-triene) (44)

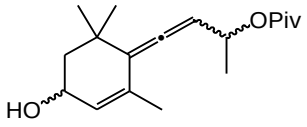


44

To a stirred solution of **29** (51.4 mg, 0.159 mmol) in pyridine (3 mL) and DCM (3 mL) at 0 ^{o}C under nitrogen was added pivaloyl chloride (115 μL, 0.956 mmol). The reaction was heated under reflux for 1 hr, cooled and quenched with water (10 mL), diluted with ether (20 mL), washed with water (20 mL), $CuSO_4$ (2 x 20 mL) and brine (2 x 20 mL), dried ($MgSO_4$) and the solvent evaporated *in vacuo*. The product was purified by column chromatography (X4 → 1.5% EtOAc/X4) yielding a yellow oil, 51 mg (79%), as a mixture of stereoisomers in a ratio of approx 2:1, R_f = 0.78 (30% EtOAc/X4).

^{1}H NMR δ (mixture of stereoisomers): 5.59*, 5.58† (1H, d, *J* 5.7, H_8), 5.49*† (1H, br s, H_4), 5.37*† (1H, quintet, *J* 6.3, H_9), 4.33-4.36*† (1H, m, H_3), 1.72-1.75*† (1H, m, H_{2a}), 1.71*† (3H, br s, H_{13}), 1.46-1.53*† (1H, m, H_{2b}), 1.28-1.31*† (3H, m, H_{10}), 1.18, 1.17*† (9H, s, PivH), 1.11, 1.08*† (6H, 2s, $H_{11,12}$), 0.90*† (9H, s, t-Bu), 0.10, 0.08*† (6H, 2s, SiMe). **Major isomer, †minor isomer, *†mixture of both isomers. Similar to data reported.*[28]

4-(4-Hydroxy-2,6,6-trimethylcyclohex-2-en-1-ylidene)but-3-en-2-yl 2,2-dimethylpropanoate; (3-Hydroxy-9-pivaolyloxymegastigma-4,6,7-triene) (45)



45

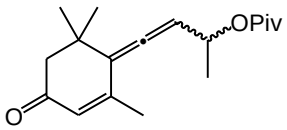
Method A: To a solution of the allene **44** (91.2 mg, 0.22 mmol) in THF (10 mL) was added TBAF (0.67 mL, 0.67 mmol, 1.0 M in THF) and the mixture was stirred at RT for 24 hrs. The reaction was quenched with $NaHCO_3$ (10 mL), diluted with EtOAc (20 mL), washed with brine (2 x 20 mL), dried ($MgSO_4$) and the solvent evaporated *in vacuo*. The product was purified by column chromatography (20% EtOAc/X4) to yield a colourless oil, 16 mg (24%), R_f = 0.31 (30% EtOAc/X4).

Method B: To a solution of **44** (518.1 mg, 1.27 mmol) in THF (10 mL) was added TBAF (2.55 mL, 2.55 mmol, 1.0M in THF) and the mixture was stirred at RT for 24 hrs. A second portion of TBAF (2.55 mL, 2.55 mmol, 1.0 M in THF) was added to the reaction mixture and the mixture stirred for a further 24 hrs. The reaction was quenched with $NaHCO_3$ (15 mL), diluted with EtOAc (30 mL), washed with brine (2 x 30 mL), dried ($MgSO_4$) and the solvent evaporated *in vacuo*. The product was purified by column chromatography (20% EtOAc/X4 → 30% EtOAc/X4) to yield a colourless oil, 220 mg (59%) as a mixture of stereoisomers in a ratio of approx. 1:1.

^{1}H NMR δ (mixture of stereoisomers): 5.63*, 5.61† (1H, d, *J* 5.6, H_8), 5.58*† (1H, br s, H_4), 5.32-5.38*† (1H, m, H_9), 4.34*† (1H, m, H_3), 1.89-1.92*† (1H, m, H_{2a}), 1.72-1.74*†

(3H, m, H_{13}), 1.60*† (1H, br s, OH), 1.40-1.43*† (1H, m, H_{2b}), 1.30-1.32*† (3H, m, H_{10}), 1.18-1.17*† (9H, br s, Piv), 1.11, 1.08*† (6H, 2s, $H_{11,12}$). **Major isomer,* †*minor isomer,* *†*mixture of both isomers. Similar to data reported.*[28]

4-(2,6,6-Trimethyl-4-oxocyclohex-2-en-1-ylidene)but-3-en-2-yl 2,2-dimethylpropanoate;
(3-Keto-9-pivaloyloxymegastigma-4,6,7-triene) (46)



46

To a solution of the C_9 protected allene alcohol **45** (49.4 mg, 0.17 mmol) in DCM (3 mL) was added Dess-Martin periodinane (109.3 mg, 0.26 mmol). The mixture was stirred for 2 hrs at ambient temperature and monitored by TLC until completion. The reaction was quenched with aqueous sodium thiosulphate (2 mL), extracted with EtOAc (4 x 2 mL), washed with brine (2 x 10 mL), dried ($MgSO_4$) and the solvent evaporated *in vacuo*. The product was purified by column chromatography (10% EtOAc/X4 → 20% EtOAc/X4) to yield a clear oil (24.0 mg, 50%), R_f = 0.46 (30% EtOAc/X4).

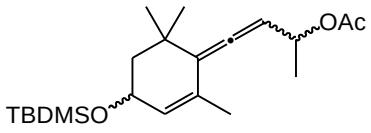
^{1}H NMR δ: 5.88 (1H, s, H_4), 5.72-5.73 (1H, m, H_8), 5.43-5.38 (1H, quintet, *J* 6.3, H_9), 2.37 (2H, m, H_2), 1.97,1.96 (3H, m, H_{13}), 1.36-1.38 (3H, m, H_{10}), 1.18, 1.17* (9H, s, Piv), 1.18, 1.16* (6H, 2s, $H_{11,12}$). **Piv and $H_{11,12}$ overlapping signals.*

^{13}C NMR δ: 204.37, 203.89 (C_7), 198.53, 198.51 (C_3), 177.84, 177.74 (C=O Piv), 151.68, 151.58 (C_6), 125.95, 125.92 (C_4), 116.08, 115.81 (C_5), 98.72, 98.44 (C_8), 67.63, 67.40 (C_9), 51.11 (C2), 38.85 (C Piv), 36.07, 36.02 (C_1), 28.96, 28.95, 28.71, 28.70 ($C_{11,12}$), 27.23, 27.22 (Piv), 21.49 (C_{13}), 20.33, 20.20 (C_{10}).

MS *m/z* (%): 290 (M^+,5), 206 (25), 191 (15), 173 (30), 145 (11), 133 (36), 85 (10), 77 (11) 69 (23), 57 (100).

HRMS (ESI): found 290.1882, $C_{18}H_{26}O_3$ requires 290.1882.

4-(4-{[*tert*-Butyl(dimethyl)silyl]oxy}-2,6,6-trimethylcyclohex-2-en-1-ylidene)but-3-en-2-yl acetate; (3-*tert*-Butyldimethylsilyloxy-9-acetoxymegastigma-4,6,7-triene) (47)

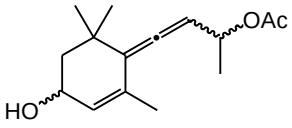


47

To a solution of the alcohol **29** (2.21 g, 6.85 mmol) in anhydrous pyridine (665 μL, 8.22 mmol), anhydrous DCM (40 mL) and DMAP (100 mg, 0.82 mmol) under nitrogen at 0 ^{o}C was added acetyl chloride (535 μL, 7.54 mmol). The reaction mixture was stirred for 24 hrs at ambient temperature. The reaction was quenched with water (100 mL), diluted with ether (150 mL), washed (water (2 x 100 mL), $CuSO_4$ (2 x 100 mL), brine (3 x 100 mL)), dried ($MgSO_4$) and the solvent evaporated. The reaction yielded **47**, 2.29 g (92%) as a yellow oil and as a mixture of stereoisomers in a ratio of approx. 1:1, R_f = 0.94 (20% EtOAc/X4).

^{1}H NMR δ (mixture of stereoisomers): 5.61*, 5.58† (1H, d, *J* 5.2, H_8), 5.50*† (1H, br s, H_4), 5.40*† (1H, quintet, *J* 6.2, H_9), 4.35*† (1H, m, H_3), 2.03*† (3H, s, Ac), 1.76*† (1H, m, H_{2a}), 1.72*† (3H, br s, H_{13}), 1.52*† (1H, m, H_{2b}), 1.33*† (3H, m, H_{10}), 1.09, 1.08*† (6H, 2s, $H_{11,12}$), 0.91*† (9H, s, *t*-Bu), 0.10, 0.08*† (6H, 2s, $SiMe_2$). **Major isomer,* †*minor isomer,* *†*mixture of both isomers. Similar to data reported.*[28]

4-(4-Hydroxy-2,6,6-trimethylcyclohex-2-en-1-ylidene)but-3-en-2-yl acetate; (3-Hydroxy-9-acetoxymegastigma-4,6,7-triene) (48)

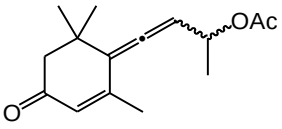


48

To a solution of the allene **47** (0.50 g, 1.37 mmol) in anhydrous THF (40 mL) was added TBAF (1.0 M in THF, 2.74 mL, 2.74 mmol) and the mixture was stirred for 3 days. The reaction mixture was quenched with $NaHCO_3$ solution, diluted with EtOAc (100 mL), washed with brine (2 x 50 mL), dried ($MgSO_4$) and the solvent evaporated. The product was purified by column chromatography (10% EtOAc/X4 → 20% EtOAc/X4) yielding 244 mg (72%) of a light yellow oil, R_f = 0.19 (20% EtOAc/X4).

^{1}H NMR δ: 5.54-5.59 (1H, m, H_8), 5.59 (1H, br s, H_4), 5.40 (1H, m, H_9), 4.33 (1H, m, H_3), 2.03 (3H, s, Ac), 1.92 (1H, m, H_{2a}), 1.74 (3H, br s, H_{13}), 1.42 (1H, m, H_{2b}), 1.33 (3H, d, *J* 6.4, H_{10}), 1.11, 1.08 (6H, 2s, $H_{11,12}$). *Similar to data reported.*[28]

4-(2,6,6-Trimethyl-4-oxocyclohex-2-en-1-ylidene)but-3-en-2-yl acetate; (9-Acetoxymegastigma-4,6,7-trien-3-one) (49)



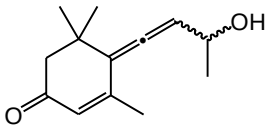
49

Oxalyl chloride (259 μL, 2.97 mmol) in anhydrous DCM (20 mL) was chilled to -60 ^{o}C under nitrogen. DMSO (458 μL, 6.45 mmol) in DCM (20 mL) was then added to this solution over a period of 15 mins with further stirring of the mixture for 60 mins at -60 ^{o}C. The alcohol **48** (0.68 g, 2.70 mmol) in DCM (20 mL) was next added to the mixture over a period of 15 mins with further stirring for 90 mins at -60 ^{o}C. Triethylamine (1.88

mL, 13.49 mmol) was then added neat to the reaction and the mixture and was allowed to reach ambient temperature. Water (10 mL) was added and the mixture was stirred for a further 30 mins. The organic layer was extracted with DCM (3 x 50 mL) and the combined organic extracts were washed with 10% aqueous HCl, water, saturated $NaHCO_3$, brine, dried ($MgSO_4$) and the solvent evaporated *in vacuo*. The product was purified by column chromatography (1% EtOAc/X4 → 2% EtOAc/X4 → 5% EtOAc/X4) to yield 153 mg (23 %) of a yellow oil, R_f = 0.74 (30% EtOAc/X4).

^{1}H NMR δ: 5.91-5.96 (1H, m, H_8), 5.93 (1H, br s, H_4), 5.34-5.38 (1H, m, H_9), 2.04 (3H, s, Ac), 1.90 (1H, m, H_{2a}), 1.76 (3H, br s, H_{13}), 1.61 (1H, m, H_{2b}), 1.28 (3H, m, H_{10}), 1.17, 1.16 (6H, 2s, $H_{11,12}$).

7.2.5 Synthesis of 9-hydroxymegastigma-4,6,7-trien-3-one (28); (4-(3-hydroxybut-1-en-1-ylidene)-3,5,5-trimethylcyclohex-2-en-1-one)



28

*Method 1: Removal of the pivaloyl group from **46** using NaOH*

To a solution of **46** (275.2 mg, 0.95 mmol) in aqueous MeOH (1 mL) was added a solution of NaOH (950 μL, 1 M, 0.95 mmol) in MeOH (10 mL) with stirring at RT overnight. The product was extracted with DCM (3 x 50 mL) and the organics were dried ($MgSO_4$) and concentrated. TLC analysis showed the presence of starting material and some minor unidentified products. Analysis by GCMS and proton NMR further showed that the starting material **46** was still present as the major component.

*Method 2: Removal of the Pivaloyl group from **46** using NaOMe*

To a solution of the allene **46** (16.5 mg, 0.0568 mmol) in MeOH (1 mL) was added NaOMe in MeOH (1.0 M, 40 μL). The reaction mixture was allowed to stir for 2.5 hrs and then concentrated *in vacuo*. The product was purified by column chromatography

(10% EtOAc/X4 → 20% EtOAc/X4) to furnish an oil. NMR and GCMS analysis of the fractions obtained from column chromatography revealed the desired product was not obtained and multiple unidentified compounds were present.

Unknown 1

MS *m/z* (%): 252(M^+,1), 237 (7), 220 (93), 205 (23), 179 (17), 165 (16), 153 (20), 137 (100) 123 (50), 115 (26), 91 (23), 83 (29), 73 (60), 59 (57).

Unknown 2

MS *m/z* (%): 252(M^+,1), 237 (6), 220 (97), 205 (24), 179 (21), 165 (17), 153 (21), 137 (100) 123 (47), 115 (23), 91 (23), 83 (27), 73 (66), 59 (61).

Unknown 3

MS *m/z* (%): 237(M^+,1), 220 (29), 205 (1), 163 (11), 149 (46), 136 (94) 121 (100), 105 (16), 91 (40), 77 (37).

*Method 3: Removal of the pivaloyl group from **46** using Amberlite (OH) resin*

To a solution of the C_3 ketone C_9 piv protected allene **46** (19.4 mg, 0.067 mmol) in 3 mL of MeOH was added the dry amberlite (OH) resin (187 mg). The reaction mixture was stirred gently for 2 days after which the mixture was filtered, washed with MeOH (5 x 1 mL) and the filtrate evaporated to dryness. Analysis by GCMS revealed multiple products formed with the desired product not detected.

*Method 4: Removal of the acetate group from **49** using NaOH*

To an aqueous solution of ethanol (200 μL, 25% ethanol/water, v/v) was added NaOH in ethanol (164 μL, 1 M, 0.16 mmol). The allene **49** (40.8 mg, 0.16 mmol) was dissolved in aqueous ethanol (200 μL) and added to the above solution. The reaction mixture was stirred for 5 hrs and the organic portion was then extracted with EtOAc (3 x 10 mL), the extract was washed with brine, dried ($MgSO_4$) and solvent was evaporated *in vacuo*. TLC analysis revealed the formation of one product that was more polar than the starting material **49**. R_f (**49**) (30%EtOAc/X4) = 0.78, R_f (Product) (30%EtOAc/X4) =

0.59. Further analysis by GCMS indicated that the desired product was not present in the mixture.

*Method 5: Removal of the acetate group from **49** using NaOMe*

To a solution of the allene **49** (19.7 mg, 0.079 mmol) in MeOH (1 mL) was added 1M NaOMe in methanol (79.4 μL, 0.079 mmol) and the mixture was stirred for 2 hrs. The reaction mixture was then diluted with EtOAc (15 mL), washed with brine (2 x 5 mL) and the solvent evaporated. The crude product revealed a mixture of the starting material and the formation of a product that was more polar than the starting material. Column chromatography (30% EtOAc/X4) of the crude mixture revealed a complex mixture of products being eluted. Analysis by GCMS indicated that desired product was not present.

*Method 6: Reduction of the diketone **26** using S. cerevisiae*

An active dry form of a baking *S. cerevisiae strain*, Type II (2.5 g) and sucrose (5 g) was stirred vigorously in water (50 mL) at 30 ^{o}C until fermentation commenced (30 mins). The diketone **26** (30 mg, 0.15 mmol) was dissolved in ethanol (1 mL) and added to the mixture and the mixture was stirred for 2.5 hrs. An additional amount of sucrose (5 g) was added and the ferment was stirred for a further 24 hrs. The fermented mixture was filtered through Celite and washed with water. The filtrate was acidified with 10% HCl to pH 3.0. The filtrate was allowed to stir at ambient temperature overnight. The filtrate was saturated with NaCl, the product was extracted with ether (3 x 50 mL) and the solvent was evaporated. The crude product was analysed by NMR and GCMS but the ketone **28** was not identified.

*Method 7: Reduction of the diketone **26** using $NaBH_4$*

Sodium borohydride in MeOH (100 μL, 550 mmol) (Suprasolv) was added to a mixture of the allene diketone **26** (20 mg, 98.4 μmol) and $CeCl_3$ (14.5 mg, 39 mmol) in MeOH (1.5 mL) at 0 ^{o}C. The mixture was stirred for 1 hr and monitored by TLC. A cold aqueous solution of NH_4Cl (2 mL, 20% aq.) was added to the mixture, which was then extracted with ether (5 x 1 mL), the ether solution was washed with $NaHCO_3$ (1 mL, sat. aq.), brine and dried ($MgSO_4$). The product was purified by column

chromatography (20% EtOAc/X4) to yield a colourless oil, 12 mg (58%), R_f (**28**) (30%EtOAc/X4) = 0.19.

^{1}H NMR δ (mixture of stereoisomers): 5.90, 5.89*† (1H, s, H_4), 5.79*† (1H,d, *J* 5.4, H_8), 4.45-4.49*† (1H, m, H_9), 2.39*† (2H, s, H_2), 1.99, 1.98*† (3H, s, H_{13}), 1.37-1.36*† (3H, d, *J* 6.6, H_{10}), 1.19, 1.19, 1.18, 1.17*† (6H, 2s, $H_{11,12}$). **Major isomer,* †*minor isomer,* *†*mixture of both isomers.*

^{13}C NMR δ: 202.9, 202.8 (C_7), 198.6* (C_9), 151.8, 151.7 (C_5), 126.0, 125.9 (C_4), 116.1, 116.0 (C_6), 102.4, 102.4 (C_8), 66.2, 66.2 (C_9), 51.2, 51.1 (C_2), 36.1, 36.1 (C_1), 29.2, 29.2, 28.8 ($C_{11,12}$), 23.8, 23.7 (C_{10}), 21.7, 21.7 (C_{13}). **signals for the two stereoisomers coincident.*

MS *m/z* (%): (KI 1917, 60 m DB-FFAP) 206 (M^+,9), 191 (4), 173 (2), 162 (65), 147 (100), 132 (8), 133 (8) 119 (27), 106 (61), 91 (24), 77 (31), 65 (9), 51 (13), 45 (56), 43 (19), 39 (14).

HRMS (ESI): found 207.1383 (M +H^+), $C_{13}H_{19}O_2$ requires 207.1380.

7.3 Identification of precursors as natural products (Chapter 3)

7.3.1 General method for the extraction of glycosides using amberlite XAD-2

The Riesling and Pinot Noir musts obtained from 2004 vintage were from a local winery, Adelaide Hills, South Australia.

XAD-2 conditioning and column preparation:

Amberlite XAD-2 (50-80 mesh) dry resin was covered with MeOH, stirred for one min and allowed to stand for 15 mins. The MeOH was decanted and the resin covered with milli-Q water, stirred, allowed to stand for 10 mins and then the water was decanted. The amberlite XAD-2 was again covered with milli-Q water and poured into a glass column (30 x 3 cm I.D.) with a PTFE tap and a glass wool stopper. The packed column contained about 15 cm of resin. A second smaller column (20 x 1 cm I.D.) was packed with the amberlite XAD-2 in milli-Q water. The packed column contained about 8 cm of resin. Several portions of milli-Q water were passed through the columns to pack the resin.[113-115]

A sample of must (approximately 200 mL) at room temperature was passed through the XAD-2 packed column 1 and then passed through packed column 2. Several portions of milli-Q water were used to wash the columns to remove the sugars, acids and water soluble material from the columns.[113-115]

Free aglycone fraction:

The aglycone fraction was eluted using DCM (400 mL) at a flow rate of 2 mL/min. The aglycone extract was dried with anhydrous sodium sulfate and concentrated *in vacuo* to about 200 μL. The concentrate was analysed by GCMS.[113-115]

Bound fraction (Glycosides):

After the free fraction, the bound fraction was eluted with 800 mL MeOH. The extract was dried with anhydrous sodium sulfate and concentrated *in vacuo* yielding 120 mg of dark red viscous oil.[113-115]

7.3.2 Riesling and Pinot Noir juice extractions – vintage 2004

Enzyme hydrolysis of bound fraction:

A portion of the crude bound fraction (approximately 25 mg) from XAD-2 was dissolved in citrate-phosphate buffer, pH 5.0 (2-3 mL) and Rapidase (AR 2000) enzyme added (15 mg). The mixture was placed on a water bath incubator at 38 ^{o}C for 18 hrs.

The enzyme hydrolysates were extracted with pentane:DCM (2:1, 5 x 1 mL) and then diethyl ether:DCM (3:1, 2 x 1 mL). Emulsions formed were broken with 3-4 drops of redistilled ethanol. The extracts were dried with anhydrous sodium sulfate and concentrated using a stream of nitrogen to about 200 μL for analysis by GCMS.

Solvent extraction of Riesling and Pinot Noir juice 2004:

The Riesling must and the Pinot Noir must (approximately 200 mL of each) were solvent extracted in a 200 mL volumetric flask using pentane:DCM (2:1, 3 x 5 mL). The extracts were dried (Na_2SO_4) and concentrated using a stream of nitrogen to about 200 μL for each juice extract.

Co-injections:

The authentic standard of diketone **26** was diluted from a 1 mg/mL solution to a 1 μg/mL solution in ethanol. The Riesling and Pinot Noir extracts were concentrated further to about 100 μL for the co-injections using a SIM run analysis by GCMS. The ion that was selected for analysis for the co-injections was the ion at *m/z* 162. A (1 in 50) dilution was made of the 1 μg/mL diketone solution and used for the Riesling co-injection and a (1 in 20) dilution was made of the 1 μg/mL diketone solution and used for the Pinot Noir co-injection. The authentic mixture of the diketone was added to the Riesling and Pinot Noir extracts to enhance the corresponding diketone peak and confirm its presence as a natural product.

GCMS conditions:

The liquid injector was operated in fast liquid injection mode with a 10 μL syringe (SGE) fitted. The gas chromatograph was fitted with an approximately 51.7 m x 0.25 mm i.d. J&W fused silica capillary column DB-WAX, 0.25 μm film thickness. The carrier

gas was helium (BOC Gases, ultrahigh purity), and the flow rate was 1.6 mL/min. The oven temperature, started at 50 °C, was held at this temperature for 1 min, then increased to 170 °C at 20 °C/min and further increased to 240 °C at 3 °C/min, and held at this temperature for 10 min. The injector was held at 200 °C and the transfer line at 240°C. The sample volume injected was 2 μL, and was introduced in pulsed splitless mode with an inlet pressure of 45.0 psi maintained until splitting. The glass liner (Agilent Technologies) was borosilicate glass with a plug of resilanized glass wool (2-4 mm) at the tapered end to the column. The ions monitored in SIM runs were: *m/z* 119, 147, <u>162</u> and 204 for diketone the **26** and 121, 131, <u>191</u> and 206 for ketone the **27**. Selected fragment ions were monitored for 30 ms each. The underlined ion is the ion that was used during the co-injections. The other ions were used as qualifiers. Additional ions 206, 162 and 147 were included in the SIM run to detect the ketone **28**.

7.3.3 Riesling juice extractions – vintage 2009

The Riesling grapes from April 2009 harvest were handpicked from the Henshcke Wines, Lenswood vineyard, corner of Coldstore Rd and Crofts Rd, 198 Riesling clone, Adelaide Hills, South Australia. They were picked in April 2009, frozen at -20 ^{o}C and they were processed in September 2009.

The Riesling must (vintage 2009) was processed in a similar fashion as the Riesling and Pinot Noir musts from vintage 2004. From about 200 mL of juice, 0.0853 g of crude glycoside was obtained giving an overall yield of 0.43 mg/mL. A portion of the glycosidic fraction was treated with the AR 2000 enzyme and the aglycones were extracted using pentane:EtOAc (2:1), concentrated to about 200 μL and analysed by GCMS.

Processing of grapes:

The Riesling grapes were processed in batches in a 4 ^{o}C cool room. The grapes (500 g) were separated from the stems and homogenised. PMS (30.6 mg) was added to the must homogenate. The must was centrifuged for 30 mins at 4 ^{o}C at 8000 rpm (8.09984 x 10^5 g (force)). Solids were washed with milli-Q water (300 mL) and centrifuged a

second time with the same parameters. The Riesling juice and the aqueous wash of the Riesling juice were stored under nitrogen in a 4 ^{o}C cool room.

Enzyme hydrolysis of crude glycosidic extract:

The crude glycosidic extract from XAD-2 was concentrated and 10.4 mg was dissolved in pH 5 phosphate-citrate buffer (1 mL) and AR 2000 glycosidase enzyme added (10.3 mg). The glycosides were hydrolysed in the incubator at 38 ^{o}C overnight, extracted with pentane:EtOAc and analysed by GCMS.

Solvent extraction:

The Riesling juice (350 mL) and aqueous wash of the Riesling juice (300 mL) were both solvent extracted. The Riesling juice (250 mL) was extracted using a 250 mL volumetric flask shaken with pentane:DCM (2:1) (3 x 5 mL) and pentane:EtOAc (2:1) (3 x 5 mL). A second portion of the Riesling juice (100 mL) was also extracted using a 100 mL measuring cylinder fitted with a glass stopper, shaken with pentane:DCM (2:1) (3 x 5 mL) and pentane:EtOAc (2:1) (3 x 5 mL).

The aqueous wash (about 250 mL) of the marc was also solvent extracted using a 250 mL volumetric flask shaken with pentane:DCM (2:1) (3 x 5 mL) and pentane:EtOAc (2:1) (3 x 5 mL). The remaining aqueous wash (about 50 mL) was solvent extracted using a 100 mL measuring cylinder with pentane:DCM (2:1) (3 x 5 mL) and pentane:EtOAc (2:1) (3 x 5 mL).

The Riesling juice extracts were combined, concentrated under a stream of nitrogen to about 300 μL and stored in a -20 ^{o}C freezer until analysis by GCMS.

GCMS conditions:

The GCMS conditions are the same as per section 7.3.2.

7.3.4 Honey extractions

Commercially purchased honeys:

Manuka blend honey, New Zealand, Bee Products Active (500 g), Golden nectar organic real leatherwood honey, Mole Creek Tasmania, (500 g), Beevital Manuka honey (500 g), Yellow Box honey (500 g), Blue Gum honey (500 g), Honey Pure (500 g), and Australian Rainforest organic honey (500 g).

Extraction of Manuka honey (New Zealand):

The Manuka honey extracts were prepared based on a procedure reported by Alissandrakis et al.[90] To a sample of the honey (40 g) was added 22 mL of distilled water and 1.5 g of $MgSO_4$. The solution was extracted by sonication at 25 ^{o}C for 15 mins with pentane:DCM, 2:1 (15 mL). This procedure was repeated in a second flask. The samples were transferred to a separation funnel, saturated NaCl (20 mL) was added and the mixture was well shaken and then left to rest at room temperature. When the two layers separated, the overlying emulsion was collected in a beaker, the separating funnel was rinsed with pentane:DCM, 2:1 (15 mL) and the emulsion was sonicated until the layers had separated. The organic extract was concentrated by a vigoroux column to approximately 1-2 mL and dried ($MgSO_4$). The extract was transferred to a 2 mL vial (using DCM) and concentrated with a gentle stream of nitrogen to approximately 1.5 mL. The sample was sealed and kept in the fridge until GCMS analysis.

Extraction of Leatherwood honey (Tasmania):

The Leatherwood honey extracts were prepared based on a procedure reported by Rowland et al.[89], with some modifications. The honey sample (30 g) was placed in a beaker and extracted by sonication for 20 mins at 25 ^{o}C (5 x 20 mL, pentane:DCM (2:1)) and (2 x 30 mL, EtOAc). The combined decanted extract was dried over $MgSO_4$ and concentrated by a vigoroux column. When the volume was reduced to approximately 1-2 mL, the extract was transferred (using DCM) to a 2 mL vial and concentrated with a gentle stream of nitrogen to approximately 1.5 mL. The sample was sealed and kept in the fridge until the GCMS analysis.

Co-injections:

The authentic standard of the diketone **26** or **28** was diluted from a 1 mg/mL solution to a 0.5 μg/mL solution. The honey extracts were concentrated further to about 100 μL for the co-injections using a scan mode analysis by GCMS. The ion that was selected for analysis of the co-injections for **26** and **28** was the ion at *m/z* 147. The authentic mixture of the diketone **26** was added to Leatherwood honey extract and the Manuka honey extract to enhance the corresponding diketone peak in the extract to enhance 2-fold. For co-injection 1, 20 μL of the authentic diketone solution was added to 20 μL of the honey extracts. For co-injection 2, 30 μL of the authentic diketone **26** solution was added to 15 μL of the honey extracts. And finally for co-injection 3, 40 μL of the authentic diketone solution was added to 10 μL of the honey extracts. The ketone standard **28** was spiked into the Leatherwood honey extract at a 1:0.5 ratio, 1:1 ratio, 1:2 ratio and 1:4 ratio of the extract to standard.

GCMS conditions:

The GCMS conditions used for the coinjections of **26** were the same as per section 7.3.2 with the following exceptions. The oven temperature, started at 50 ^{o}C, was held at this temperature for 1 min, then increased to 200 ^{o}C at 15 ^{o}C/min and further increased to 245 ^{o}C at 5 ^{o}C/min, and held at this temperature for 20 min.

The coinjections for the ketone **28** were perfomed using a 59 m x 0.25 mm i.d. J&W fused silica capillary column FFAP, 0.25 μm film thickness. The oven temperature, started at 45 ^{o}C, was held at this temperature for 2 min, then increased to 170 ^{o}C at 10 ^{o}C/min and further increased to 240 ^{o}C at 2 ^{o}C/min, and held at this temperature for 8 min. The injector was held at 200 ^{o}C and the transfer line at 240 ^{o}C.

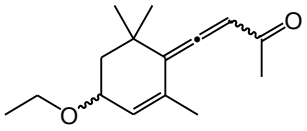
7.3.5 Hydrolysis of 3-hydroxymegastigma-4,6,7-trien-9-one (27)

Four separate samples of the ketone **27** (105 ppm, 10 mL) in 10% model wine at pH 3.0 were heated at 45 ^{o}C for 1 week and 4 weeks, at 25 ^{o}C for 2 weeks and at 100 ^{o}C for 24 hrs. All solutions were stored under nitrogen in 10 mL ampoules in the dark. Samples were extracted with pentane: EtOAc (2:1) (3 mL) and allowed to stand for 1 hr. The organic extract was placed into a 2 mL GCMS vial and analysed by GCMS.

GCMS conditions:

The GCMS conditions used were the same as per section 7.1.1 with the following exceptions. The gas chromatograph was fitted with an approximately 30 m x 0.25 mm i.d. J&W fused silica capillary column VF-WAX, 0.25 μm film thickness. The oven temperature, started at 50 °C, was held at this temperature for 1 min, then increased to 240°C at 10 °C/min and held at this temperature for 8 min.

4-(4-Ethoxy-2,6,6-trimethylcyclohex-2-en-1-ylidene)but-3-en-2-one;
3-Ethoxy-9-ketomegastigma-4,6,7-triene (50)

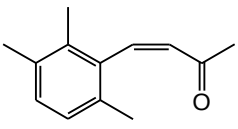


50

The extracts of the hydrolysis of ketone **27** (105 ppm, 10 mL) in 10% model wine at pH 3.0 heated at 45 ^{o}C for 1 week contained 2 products which were tentatively assigned as isomers of the ethyl ethers **52**.[52]

MS *m/z* (%) (Isomer 1 – KI 2317, 60 m VF-WAX) 234 (1), 219 (100), 205 (1), 191 (10), 175 (10), 163 (10), 149 (32), 131 (33), 115 (17), 105 (17), 91 (23), 77 (17), 69.1 (15), 55 (7), (Isomer 2 – KI 2339) 234 (1), 219 (100), 205 (3), 191 (10), 175 (13), 163 (12), 149 (35), 131 (26), 115 (22), 105 (20), 91 (23), 77 (19), 69 (15), 55 (7).

(*Z*)-4-(2,3,6-Trimethylphenyl)-but-1-en-3-one (53)



53

A solution of the ketone **27** (100 mg, 0.48 mmol) in 10% model wine (10 mL) at pH 3.0 was heated at 100 ^{o}C for 24 hrs under nitrogen in the dark. The organics were extracted with pentane: EtOAc (2:1) (10 x 1 mL). The extracts were washed with brine, dried ($MgSO_4$) and solvent evaporated *in vacuo* to yield **53**, 20 mg (20%) as a yellow oil, R_f = 0.64 (30% EtOAc/X4).

^{1}H NMR δ: 7.13 (1H, br d, *J* 12, H_7), 7.04 (1H, d (A part of AB quartet), *J* 7.8, H_4), 6.97 (1H, d (B part of AB quartet), *J* 7.8, H_5), 6.31 (1H, br d, *J* 12, H_8), 2.26 (3H, s, H_{13}), 2.17 (3H, s, H_{11}), 2.15 (3H, s, H_{12}), 1.79 (3H, s, H_{10}).

^{13}C NMR δ: 200.3 (C_9), 142.5 (C_7), 132.4 (C_4), 129.4 (C_5), 127.3 (C_8), 135.8 (C_6), 134.4 (C_2), 133.2 (C_1), 132.2 (C_5), 29.5 (C_{10}), 20.5 (C_{11}), 20.2 (C_{13}), 16.9 (C_{12}).

MS *m/z* (%): (2248, 60 m VF-WAX) 188 (3), 173 (100), 158 (7), 145 (8), 129 (20), 115 (13), 105 (4), 91 (3), 77 (3), 65 (3), 51 (3).

The mass spectral data was consistent with the reported mass spectral data.[93] *The NMR data for the cis isomer obtained also matched closely against the trans isomer* ***54*** *reported by Valla et al.*[116]

7.4 Fermentation studies (Chapter 4 and 5)

7.4.1 General fermentations

The three yeasts used in the laboratory fermentations, *S. cerevisiae* strains AWRI 796, AWRI 1537 and AWRI QA23, were maintained on yeast-malt extract (YM) medium (Amyl Media, Dandenong, Australia) supplemented with 1.5% agar and stored at 4 ^{o}C. All strains were obtained from The Australian Wine Research Institute Culture Collection (Adelaide, Australia).

Fermentations were carried out in a chemically defined grape juice (CDGJ medium) as described by Ugliano et al.[97] The medium was sterilized by filtration through a 0.2 μm sterile membrane and, after spiking with either megastigma-4,6,7-trien-3,9-dione (**26**), 3-hydroxymegastigma-4,6,7-trien-9-one (**27**), 9-hydroxymegastigma-4,6,7-trien-3-one (**28**), grasshopper ketone **12** or damascenone **1** at concentrations of 1000 ppb, was divided into 200 mL aliquots.

The inoculation procedure was as follows. A loopful of yeast cells was incubated in 10 mL of YM medium with shaking for 24-48 hrs at 28 ^{o}C. Pre-adaptation of the cells to the fermentation medium was carried out by inoculating 100 μL of the cultures into 20 mL of 50% (v/v) CDGJ medium. These subcultures were grown at 28 ^{o}C until a biomass of (1-2) x 10^{8} cells/mL, determined microscopically using a hemocytometer, was reached, after which they were inoculated in the CDGJ medium at a final concentration of 1 x 10^{6} cells/mL. Fermentations were carried out at 17 ^{o}C in 250 mL Erlenmeyer flasks sealed with fermentation water locks and shaken at 180 oscillations per min. Flasks were kept in a thermostatically controlled water bath with recirculating water acting as coolant, to ensure optimal dispersion of the heat generated during fermentation.

Samples (1 mL) for monitoring the progress of alcoholic fermentation were removed daily or every second day under sterile conditions with a needle and syringe via a sample port closed with a rubber suba seal. The ferments were analysed using a refractometer to monitor the brix and once they were below about 10 ^{o}brix, the

ferments were then monitored using Clinitest tablets to measure residual glucose/fructose. The samples were analysed enzymatically for total reducing sugar concentration (Roche Molecular Biochemicals) using an automated Cobas FARA centrifugal analyzer. For each yeast strain, triplicate fermentations of CDGJ medium with and without spiked compounds were performed. Non-inoculated samples of CDGJ medium containing the precursor compounds was used to evaluate acid-catalyzed hydrolysis of ketones **26**, **27** and **28**. Upon completion of fermentation (residual sugars < 1 g/L), samples were cold-settled for 5 days at 5 ^{o}C, with sterile nitrogen supplied at low pressure to prevent the ingress of air into the flasks. The wines were then racked off gross yeast lees and submitted to extraction and analysis of damascenone.

Day 0 quantification of damascenone ferments:

After spiking the CDGJ medium with damascenone **1** at a concentration of 1000 ppm, 10 mL aliquots (in triplicate) were removed and spiked with 100 μL of internal standard, d_4-damascenone (10 μg/mL). The samples were stored in the freezer at 20 ^{o}C until ready for analysis by GCMS.

Clinitest:

To 200 μL of sample combined with 400 μL of milli-Q water was added half a Clinitest tablet and the mixture shaken. The colour change was compared to the Clinitest colour chart to determine the sugar level in g/L.

Measuring dry cell weight:

A homogeneous 10 mL aliquot was removed from the ferment once it had reached dryness. The aliquot was shaken, filtered through a 0.45 μm pre-weighed filter (Phenomenex) and rinsed with a 10 mL aliquot of milli-Q water. Using a mini oven, the filter paper was dried and the weight of the yeast cells.

7.4.2 *S. cerevisiae* fermentations

An active dry form of a baking *S. cerevisiae* strain, Type II (Sigma Aldrich) (2.5 g) and sucrose (5 g) was stirred vigorously in water (50 mL) at 30 ^{o}C until fermentation

commenced (30 mins). The diketone **26** (30.67 mg, 0.15 mmol) or ketone **27** (5.0 mg, 0.024 mmol) was dissolved in ethanol (1 mL) and was added to the mixture with stirring for 2.5 hrs. An additional amount of sucrose (5 g) was added and the ferment was stirred for a further 24 hrs. The fermented mixture was filtered through Celite and washed with water. The filtrate was acidified with 10% HCl to pH 3.00. The filtrate was allowed to stir at ambient temperature overnight. The filtrate was then saturated with NaCl and the product extracted with ether (3 x 50 mL) and the solvent evaporated. The crude product was analysed by NMR and GCMS.

7.4.3 Riesling fermentations

Grape processing:

The Riesling grapes were processed in batches in a 4 ^{o}C cool room. The grapes (2.5 kg) were separated from the stems and crushed by hand. The must was sonicated for 2.5 hrs in an ice bath. The must was centrifuged for 30 mins at 4 ^{o}C at 8000 rpm (8.09984 x 10^5 g (force)). Potassium metabisulfite (170 mg) was added to the must. The juice was filtered under vacuum and filter sterilised through 0.8 μm/0.2 μm filters. The sugar content of the Riesling juice was measured at 22.6 ^{o}Brix using a refractometer.

Fermentations:

The yeast used in the Riesling fermentations was a *S. cerevisiae* strain, AWRI QA23. A loopful of yeast cells was incubated in 10 mL of YM medium with shaking for 24-48 hrs at 28 ^{o}C. Pre-adaptation of the cells to the fermentation medium was carried out by inoculating 100 μL of the cultures into 20 mL of 50% (v/v) Riesling juice. These subcultures were grown at 28 ^{o}C until a biomass of (1-2) x 10^8 cells/mL. Triplicate fermentations were carried out using the sterile filtered (0.02 μm) Riesling juice in 250 mL Erlenmeyer flasks and inoculated with yeast QA23 at a final concentration of 1 x 10^6 cells/mL. Flasks were left shaking at 180 oscillations per minute at 17 ^{o}C in the dark.

Non-inoculated Riesling juice controls were also set up in a closed schott bottle. Upon completion of fermentation (residual sugars < 1 g/L), samples were cold-settled for 5 days at 5 ^{o}C, with sterile nitrogen supplied at low pressure to prevent the ingress of air

into the flasks. The ferments and controls were then racked off gross yeast lees and submitted to extraction and analysis of damascenone.

Day 0 quantification of Riesling juice:

A 10 mL aliquot of the juice (in triplicate) was spiked with 100 μL of the internal standard, d_4-damascenone (10 μg/mL). The samples were stored in the freezer at -20 ^{o}C until ready for analysis by GCMS.

7.4.4 Fermentations to identify reduction products of damascenone

The yeast used in the fermentations was a *S. cerevisiae* strain, AWRI 796. The fermentations were carried out in sterile CDGJ medium (200 mL) in triplicate and spiked at 10,000 ppb with unlabelled damascenone. Once they reached dryness the ferments were allowed to cold settle for 4-5 days at 4 ^{o}C and racked off the lees. A 10 mL aliquot of the supernatant from one of the ferments was extracted with pentane:EtOAc (2:1) (5 x 1 mL) and concentrated to 500 μL. The sample was analysed by GCMS. A 1 mL aliquot of the supernatant from each of the triplicate ferments was taken and diluted up to 10 mL with milli-Q water for quantitation.

GCMS conditions:

The GCMS conditions are the same as per section 7.1.1 with the following exceptions. The flow rate was 1.1 mL/min. The oven temperature, started at 50 ^{o}C, was held at this temperature for 1 min, then increased to 240 ^{o}C at 5 ^{o}C/min and held at this temperature for 10 min.

7.5 Damascenone stability studies (Chapter 5)

7.5.1 Stability of damascenone in CDGJ medium

Five separate stock solutions of the CDGJ medium were made up, with four out of the five solutions prepared with at least one group of compounds omitted. The five stock solutions included: 500 mL CDGJ medium (all compounds included), 500 mL CDGJ mediium – with no nitrogen sources; 500 mL CDGJ medium – with no trace elements;

500 mL CDGJ medium – with no vitamins and 500 mL CDGJ medium – with no ammonium chloride. All stock solutions were adjusted to pH 3.20. Each sterile stock solution (200 mL) (in duplicate) was placed into a sterile ferment flask, fitted with an airlock and allowed to shake in the dark over 30 days at 17 ^{o}C. The remaining portion of each of the stock solution was stored in a closed glass bottle with an aluminium lined lid for the 30 days. All solutions were monitored over the 30 days to ensure microbial growth was not occurring.

Samples were taken at day 0 (2 x 1 ml and 2 x 5 mL) and at day 30 (2 x 1 ml and 2 x 5 mL) from each of the stock solutions and closed systems. Samples were taken daily from the CDGJ medium and then every two days from day 10 to day 30 (2 x 5 ml and 2 x 10 mL). The pH of all stock solutions at day 30 was measured. The CDGJ medium samples were extracted and damascenone was quantified as per section 7.1.1.

7.5.2 Stability of damascenone in Chardonnay juice

Sterile ferment flasks were filled with sterilised Chardonnay juice (200 mL) that had been spiked with damascenone at 1 ppm, fitted with water airlocks and allowed to shake in the dark over 30 days at 17 ^{o}C. A 50 mL sterile Schott bottles with the caps lined with aluminium foil was filled with approximately 20 mL of the spiked Chardonnay juice and allowed to stand in the dark at 17 ^{o}C for the 30 day period. Samples of the juice were removed as described for the stability study of damascenone in CDGJ medium (Section 7.5.1). The Chardonnay juice samples were extracted and damascenone was quantified as per section 7.1.1.

7.5.3 CDGJ medium/CDMW study

Four separate solutions containing 0% alcohol (CDGJ medium, solution A), 2% alcohol (solution B), 6% alcohol (solution C) and 12% alcohol (CDMW, solution D) were utilised in this study. Sterile CDGJ medium and sterile CDMW were used to produce solutions B and C. Solution B (2% alcohol) was prepared using CDGJ medium : CDMW (5:1) and solution C (6% alcohol) was prepared using CDGJ medium : CDMW (1:1).

Sterile ferment flasks were filled with the solutions 1 – 3 (in duplicate) that had been spiked with damascenone at 1 ppm, fitted with water airlocks and allowed to shake in the dark over 30 days at 17 ^{o}C. Three Schott bottles were filled with each of the stock solutions 1 – 3 and allowed to stand in the dark at 17 ^{o}C over the 30 day period. Samples were taken from each of the ferment flasks at day 0 (2 x 1 mL), every 5 days till day 20 (2 x 1 mL) and at day 30 (2 x 1 mL). The CDGJ medium/CDMW samples were extracted and damascenone was quantified as per section 7.1.1.

7.5.4 Adsorbent study ferments

Glass tubes (6 mm I.D., 9 mm O.D., 100 mm in length) (Emerald Scientific) were packed with resin and plugged at each end with glass wool. All glass tubes and glass wool were solvent cleaned with DCM and EtOAc and MeOH and oven dried before use. The resins utilised in this experiment were Tenax-TA, XAD-2, XAD-7 and FX66. Two glass tubes were packed with Tenax-TA (*circa* 300 mg), two glass tubes were packed with XAD-2 (*circa* 500 mg), one glass tube was packed with XAD-7 (*circa* 500 mg) and one glass tube was packed with FX66 (*circa* 500 mg).

Four 200 mL fermentations were set up using CDGJ medium that had been spiked with unlabelled damascenone at 1 ppm and inoculated with yeast 796 at 1 x 10^{6} cells/mL. A duplicate sample (1 mL) of the spiked juice was taken at day 0 for the quantification of damascenone. Ferment 1 was set up and attached to the water airlock was a glass tube packed with XAD-2 followed by an attached glass tube packed with Tenax-TA. Ferment 2 was set up and attached to the water airlock was a glass tube packed with Tenax-TA followed by an attached glass tube packed with XAD-2. Ferment 3 was set up and a glass tube packed with XAD-7 was attached to the water airlock and ferment 4 was set up and a glass tube packed with FX66 was attached to the water airlock.

Once the ferments reached dryness they were allowed to cold settle in a 4 ^{o}C room, with sterile nitrogen supplied at low pressure to prevent the ingress of air into the flasks. The wines were then racked off gross yeast lees and submitted to extraction and analysis of damascenone.

The resins were solvent extracted with a 10 mL aliquot of each solvent and dried with $MgSO_4$. The XAD-2, XAD-7 and FX66 resins were extracted with three solvent systems, pentane:EtOAc (2:1) (10 mL), DCM (10 mL) and MeOH (10 mL). The Tenax-TA resin was extracted with pentane:EtOAc (2:1) (10 mL) and MeOH (10 mL).

A 5 mL aliquot of each extract was spiked with labelled standard, d_4-damascenone (50 μL). A portion of this extract was put into a 250 μL glass insert in a 2 mL GCMS vial and stored at -20 ^{o}C until GCMS analysis.

7.5.5 Cold trap ferments

Ferments were set up in triplicate using sterile CDGJ medium and inoculated with yeast strain 796. Three controls were also set up using the sterile CDGJ medium. The sterilised CDGJ medium was spiked at 1 ppm with unlabelled damascenone **1**.

The ferments and controls were fitted with airlocks which were connected to a glass 100 mL round bottom containing 50 mL of ethanol and fitted with a condenser and a second airlock. The coolant running through the condenser was maintained at -15 ^{o}C. A portion of the CDGJ medium that was spiked with **1** was placed in a closed system, in a 50 mL Schott bottle and placed with the ferments at 21 ^{o}C.

An aliquot of 1 mL was taken using a glass bulb pipette of spiked CDGJ medium and made up to 10 mL with milli-Q water in a 15 mL glass aluminium screw cap vial. Labelled d_4-damascenone (100 μL, 10 μg/mL) was added and the sample frozen at -20 ^{o}C until ready for extraction and analysis by GCMS.

The water airlock attached to the condenser attached to ferment 1 was transferred into a 15 mL glass aluminium screw cap vial. Ferment 1 was then transferred to a sterile 200 mL Schott bottle. The water airlock attached to the ferment was transferred to a second 15 mL glass aluminium screw cap vial. The contents of the round bottom flask were then transferred to a measuring cylinder and made up to 200 mL in a 200 mL Schott bottle. All parts of the apparatus were rinsed with redistilled

ethanol (6 x 1 mL) and then milli-Q water (10 x 1 mL).This process was repeated for each of the other two ferments and the three controls.

The ferments were allowed to cold settle in the fridge at 4 ^{o}C along with the other aqueous solutions collected. The CDGJ medium spiked with damascenone at day 0 in a closed Schott bottle was also removed from the ferment room and placed in the fridge at 4^{o}C. The ferment samples were extracted and damascenone was quantified as per section 6.1.1.

References

1. Rankine, B., *Making good wine - A manual of winemaking practice for Australia and New Zealand*. Second ed. Sydney **2004**; pp. 1, 113-114.
2. Jackson, R. S., *Wine science: Principles practice perception*. Third ed. Academic Press: San Diego, California, **2000**; pp. 15.
3. Iland, P.; Gago, P., *Australian wine from the vine to the glass*. Patrick Iland Wine Promotions: Adelaide, South Australia, **1997**; pp. 15.
4. Iland, P.; Gago, P., *Australian wine: Styles and tastes*. Patrick Iland Wine Promotions: Adelaide, **2002**; pp. 11-13, 16.
5. Australian Government, Australian Wine and Brandy Coorporation, *Wine export approval report*, Wine Australia, September **2011**; www.wineaustralia.com, (Accessed 27/12/2011).
6. Wine Australia; Australian Wine and Brandy Coorporation, *Wine export approval report*, December **2011**; www.wineaustralia.com, (Accessed 21/01/2012).
7. Anderson, K.; Nelgen, S., *Global wine markets, 1961 to 2009: a statistical compendium*. University of Adelaide Press: Adelaide, **2011**.
8. 2012 Australia Top, *Top 10 Australian wines*. **2011**; www.australiatop.com, (Accessed 16/01/2012).
9. Winetitles Pty Ltd, Winebiz: Wine industry statistics - Wine producers - *Largest wine companies by total wine production*, **2012**; http://www.winebiz.com.au/statistics/wineriestable29.asp, (Accessed 05/03/2012).
10. Australian Bureau of Statistics, *Australian Wine and Grape Industry.* **2010**, (Report 1329.0).
11. Rapp, A.; Mandery, H., Wine aroma. *Experientia* **1986**, 42, 873-884.
12. Ribereau-Gayon, P.; Glories, Y.; Maujean, A.; Dubourdieu, D., *Handbook of Enology Volume 2: The chemistry of wine stabilization and treatments*. Second ed. John Wiley & Sons, Ltd: West Sussex, England, **2000**; Vol. 2, pp. 205-227.
13. Francis, I. L.; Newton, J. L., Determining wine aroma from compositional data. *Aust. J. Grape Wine Res.* **2005**, 11, 114-126.
14. Regodón Mateos, J. A.; Pérez-Nevado, F.; Ramírez Fernández, M., Influence of *Saccharomyces cerevisiae* yeast strain on the major volatile compounds of wine. *Enzyme Microb. Technol.* **2006**, 40, (1), 151-157.

15. Hornsey, I., *The chemistry and biology of winemaking*. The Royal Society of Chemistry: Cambridge, UK, **2007**; pp. 1-10, 114-117.
16. Baumes, R., *Wine aroma precursors: Wine chemistry and biochemistry*. Springer, New York, **2009**; pp. 251-274.
17. Fleet, G. H., Yeast interactions and wine flavour. *Int. J. Food Microbiol.* **2003**, 86, (1-2), 11-22.
18. Baumes, R.; Wirth, J.; Bureau, S.; Gunata, Y.; Razungles, A., Biogeneration of C_{13}-norisoprenoid compounds: experiments supportive for an apo-carotenoid pathway in grapevines. *Analytica Chimica Acta* **2002**, 458, (1), 3-14.
19. Howell, K. S.; Klein, M.; Swiegers, J. H.; Hayasaka, Y.; Elsey, G.; Fleet, G. H.; Hoj, P. B.; Pretorius, I. S.; de Barros Lopes, M. A., Genetic determinants of volatile-thiol release by *Saccharomyces cerevisiae* during wine fermentation. *Appl. Environ. Microbiol.* **2005**, 71, (9), 5420-5426.
20. Howell, K. S.; Swiegers, J. H.; Elsey, G. M.; Siebert, T. E.; Bartowsky, E. J.; Fleet, G. H.; Pretorius, I. S.; Barros Lopes, M. A., Variation in 4-mercapto-4-methyl-pentan-2-one release by *Saccharomyces cerevisiae* commercial wine strains. *FEMS Microbiol. Lett.* **2004**, 240, (2), 125-129.
21. Iland, P.; Dry, P.; Proffitt, T.; Tyerman, S., *The grapevine from the science to the practice of growing vines for wine*. Patrick Iland Wine Promotions Pty Ltd: Campbelltown, Adelaide, South Australia, **2011**; pp. 148-149.
22. Tominaga, T.; Peyrot des Gachons, C.; Dubourdieu, D., A new type of flavor precursors in *Vitis vinifera* L. cv. Sauvignon Blanc: *S*-Cysteine conjugates. *J. Agric. Food Chem.* **1998**, 46, (12), 5215-5219.
23. Grant-Preece, P. A.; Pardon, K. H.; Capone, D. L.; Cordente, A. G.; Sefton, M. A.; Jeffery, D. W.; Elsey, G. M., Synthesis of wine thiol conjugates and labeled analogues: Fermentation of the glutathione conjugate of 3-mercaptohexan-1-ol yields the corresponding cysteine conjugate and free thiol. *J. Agric. Food Chem.* **2010**, 58, 1383-1389.
24. Loscos, N.; Hernandez-Orte, P.; Cacho, J.; Ferreira, V., Release and formation of varietal aroma compounds during alcoholic fermentation from nonfloral grape odorless flavor precursors fractions. *J. Agric. Food Chem.* **2007**, 55, (16), 6674-6684.

25. Rodriguez-Bustamante, E.; Sanchez, S., Microbial production of C_{13}-norisoprenoids and other aroma compounds via carotenoid cleavage. *Crit. Rev. Microbiol.* **2007**, 33, 211-230.
26. Lund, S. T.; Bohlmann, J., The molecular basis for wine grape quality - A volatile subject. *Science* **2006**, 311, (5762), 804-805.
27. Mendes-Pinto, M. M., Carotenoid breakdown products – the norisoprenoids – in wine aroma *Arch. Biochem. Biophys.* **2009**, 483, (2), 236-245.
28. Daniel, M. A. Formation and fate of damascenone in wine. Flinders University, Adelaide, **2005**; pp. 146-150.
29. Williams, A., Rose ketones: Celebrating 30 years of success. *Perfum. Flavor.* **2002**, 27, 18-31.
30. Sefton, M. A.; Skouroumounis, G. K.; Elsey, G. M.; Taylor, D. K., Occurrence, sensory impact, formation, and fate of damascenone in grapes, wines, and other foods and beverages. *J. Agric. Food Chem.* **2011**, 59, (18), 9717-9746.
31. Demole, E.; Enggist, P.; Säuberli, U.; Stoll, M.; Kováts, E., Structure et synthèse de la damascénone (trimethyl-2,6,6-*trans*-crotonoyl-1-cyclohexadiene-1,3), constituant odorant de l'essence de rose bulgare (rosa damascena Mill. *Helv. Chim. Acta* **1970**, 53, (3), 541-551.
32. Schreier, P.; Drawert, F.; Junker, A., Identification of volatile constituents from grapes. *J. Agric. Food Chem.* **1976**, 24, (2), 331-336.
33. Buttery, R. G.; Teranishi, R.; Ling, L. C.; Turnbaugh, J. G., Quantitative and sensory studies on tomato paste volatiles. *J. Agric. Food Chem.* **1990**, 38, (1), 336-340.
34. Guth, H., Quantitation and sensory studies of character impact odorants of different white wine varieties. *J. Agric. Food Chem.* **1997,** 45, (8), 3027-3032.
35. Ferreira, V.; Ortin, N.; Escudero, A.; Lopez, R.; Cacho, J., Chemical characterization of the aroma of Grenache rose wines: Aroma extract dilution analysis, quantitative determination, and sensory reconstitution studies. *J. Agric. Food Chem.* **2002**, 50, (14), 4058-4054.
36. Isoe, S.; Katsumura, S.; Takeo, S., The synthesis of damascenone and β-damascone and the possible mechanism of their formation from carotenoids. *Helv. Chim. Acta* **1973**, 56, 1514-1526.

37. Meinwald, J.; Erickson, K.; Hartshorn, M.; Meinwald, Y. C.; Eisner, T., Defensive mechanisms of arthropods. XXIII. An allenic sesquiterpenoid from the grasshopper *Romalea microptera*. *Tetrahedron Lett.* **1968**, 25, 2959-2962.
38. Dembitsky, V. M.; Maoka, T., Allenic and cumulenic lipids. *Prog. Lipid Res.* **2007**, 46, 328-375.
39. Burns, J.; Fraser, P. D.; Bramley, P. M., Identification and quantification of carotenoids, tocopherols and chlorophylls in commonly consumed fruits and vegetables. *Phytochemistry* **2003**, 62, 939-947.
40. Ohloff, G.; Rautenstrauch, V.; Schulte-Elte, K. H., Model reactions in the biosynthesis of compounds in the damascone series and their preparative application. *Helv. Chim. Acta* **1973**, 56, 1503-1513.
41. Russell, S. W.; Weedon, B. C. L., The allenic ketone from grasshoppers. *Chem. Commun.* **1969**, (3), 85-86.
42. Boido, E.; Lloret, A.; Medina, K.; Farina, L.; Carrau, F.; Versini, G.; Dellacassa, E., Aroma composition of *Vitis vinifera* Cv. Tannat: the typical red wine from uruguay. *J. Agric. Food Chem.* **2003**, 51, (18), 5408-5413.
43. Sefton, M. A., The free and bound volatile secondary metabolites of *Vitis vinifera* grape cv. Semillon. *Aust. J. Grape Wine Res.* **1996**, 2, (3), 179-183.
44. Sefton, M. A., Hydrolytically-released volatile secondary metabolites from a juice sample of *Vitis vinifera* grape cvs Merlot and Cabernet Sauvignon. *Aust. J. Grape Wine Res.* **1998**, 4, (1), 30-38.
45. Sefton, M. A.; Francis, O. L.; Williams, P. J., The volatile composition of Chardonay juices: A study by flavour precursor analysis. *Am. J. Enol. Vitic* **1993**, 44, (4), 359-370.
46. Ohloff, G., Importance of minor components in flavors and fragrances. *Perfum. Flavor.* **1978**, 3, 11-22.
47. Sefton, M. A.; Skouroumounis, G. K.; Massywestropp, R. A.; Williams, P. J., Norisoprenoids in *Vitis vinifera* white wine grapes and the identification of a precursor of damascenone in these fruits. *Aust. J. Chem* **1989**, 42, (12), 2071-2084.

48. Winterhalter, P.; Full, G.; Herderich, M.; Schreier, P., Application of on-line HRGC-FTIR spectroscopy to the analysis of acetylenic flavour precursors. *Phytochem. Anal.* **1991**, 2, (2), 93-96.

49. Winterhalter, P.; Sefton, M. A.; Williams, P. J., Volatile C_{13}-Norisoprenoid Compounds in Riesling Wine Are Generated From Multiple Precursors. *Am. J. Enol. Vitic.* **1990**, 41, (4), 277-283.

50. Skouroumounis, G. K.; Massy-Westropp, R. A.; Sefton, M. A.; Williams, P. J., Precursors of damascenone in fruit juices. *Tetrahedron Lett.* **1992**, 33, (24), 3533-3536.

51. Puglisi, C. J.; E. G. M.; Prager R.H.; Skouroumounis G.K.; Sefton, M.A., Identification of a precursor to naturally occurring β-damascenone. *Tetrahedron Lett.* **2001**, 42, 6937-6939.

52. Puglisi, C. J.; Daniel, M. A.; Capone, D. L.; Elsey, G. M.; Prager, R. H.; Sefton, M. A., Precursors to damascenone: Synthesis and hydrolysis of isomeric 3,9-dihydroxymegastigma-4,6,7-trienes. *J. Agric. Food Chem.* **2005**, 53, (12), 4895-4900.

53. Skouroumounis, G. K.; Sefton, M. A., Acid-catalyzed hydrolysis of alcohols and their β-D-glucopyranosides. *J. Agric. Food Chem.* **2000**, 48, (6), 2033-2039.

54. Daniel, M. A.; C.J., P.; Capone, D. L.; Elsey, G. M.; Sefton, M. A., Rationalizing the formation of damascenone: Synthesis and hydrolysis of damascenone precursors and their analogues, in both aglycone and glycoconjugate forms. *J. Agric. Food Chem.* **2008**, 56, (19), 9183-9189.

55. Cordente, A. G.; Curtin, C. D.; Varela, C.; Pretorius, I. S., Flavour-active wine yeasts. *Appl. Microbiol. Biotechnol.* **2012**, 96, 601-618.

56. Romano, P.; Fiore, C.; Paraggio, M.; Caruso, M.; Capece, A., Function of yeast species and strains in wine flavour. *Int. J. Food Microbiol.* **2003**, 86, (1-2), 169-180.

57. Lilly, M.; Bauer, F. F.; Lambrechts, M. G.; Swiegers, J. H.; Cozzolino, D.; Pretorius, I. S., The effect of increased yeast alcohol acetyltransferase and esterase activity on the flavour profiles of wine and distillates. *Yeast* **2006**, 23, (9), 641-659.

58. Ribereau-Gayon, P.; Dubourdieu, D.; Doneche, B.; Lonvaud, A., *Handbook of Enology Volume 1: The microbiology of wine and vinifications*. Second ed. John Wiley & Sons, Ltd: West Sussex, England, **2000**; Vol. 1, pp. 429.

59. Chatonnet, P.; Dubourdieu, D.; Boidron, J.-N.; Pons, M., The origin of ethylphenols in wines. *J. Sci. Food Agric.* **1992,** 60, 165-178.

60. Edlin, D. A. N.; Narbad, A.; Dickinson, J. R.; Lloyd, D., The biotransformation of simple phenolic compounds by *Brettanomyces anomalus*. *FEMS Microbiol. Lett.* **1995**, 125, 311-316.

61. Dubourdieu, D.; Tominaga, T.; Masneuf, I.; Peyrot des Gachons, C.; Murat, M. L., The role of yeasts in grape flavor development during fermentation: The example of Sauvignon blanc. *Am. J. Enol. Vitic.* **2006**, 57, (1), 81-88.

62. Swiegers, J. H.; Capone, D. L.; Pardon, K. H.; Elsey, G. M.; Sefton, M. A.; Francis, I. L.; Pretorius, I. S., Engineering volatile thiol release in *Saccharomyces cerevisiae* for improved wine aroma. *Yeast* **2007**, 24, (7), 561-574.

63. Swiegers, J. H.; Pretorius, I. S., Modulation of volatile sulfur compounds by wine yeast. *Appl. Microbiol. Biotechnol.* **2007**, 74, 954-960.

64. Hernández-Orte, P.; Cersosimo, M.; Loscos, N.; Cacho, J.; Garcia-Moruno, E.; Ferreira, V., The development of varietal aroma from non-floral grapes by yeasts of different genera. *Food Chem.* **2008**, 107, (3), 1064-1077.

65. Csuk, R.; Glanzer, B. I., Baker's yeast mediated transformations in organic chemistry. *Chem. Rev.* **1991**, 91, (1), 49-97.

66. Stewart, J. D., Organic transformations catalyzed by engineered yeast cells and related systems. *Curr. Opin. Biotechnol.* **2000**, 11, (4), 363-368.

67. Crocq, V.; Masson, C.; Winter, J.; Richard, C.; Lemaitre, G.; Lenay, J.; Vivat, M.; Buendia, J.; Prat, D., Synthesis of trimegestone: The first industrial application of Bakers' yeast mediated reduction of a ketone. *Org. Process Res. Dev.* **1997**, 1, (1), 2-13.

68. Koslitz, S.; Renaud, L.; Kohler, M.; Wüst, M., Stereoselective formation of the varietal aroma compound rose oxide during alcoholic fermentation. *J. Agric. Food Chem.* **2008**, 56, (4), 1371-1375.

69. Carballeira, J. D.; Valmaseda, M.; Alvarez, E.; Gago, J. V. S., *Gongronella butleri*, *Schizosaccharomyces octosporus* and *Diplogelasinospora grovesii*: novel

microorganisms useful for the stereoselective reduction of ketones. *Enzyme Microb. Technol.* **2004**, 34, (6), 611-623.

70. van Dyk, M. S.; van Rensburg, E.; Rensburg, I. P. B.; Moleleki, N., Biotransformation of monoterpenoid ketones by yeasts and yeast-like fungi. *J. Mol. Catal. B: Enzym.* **1998**, 5, (1-4), 149-154.
71. Marcantoni, E.; Alessandrini, S.; Malavolta, M.; Bartoli, G.; Bellucci, M. C.; Sambri, L.; Dalpozzo, R., Reversed stereochemical control in the presence of $CeCl_3$ and $TiCl_4$ in the lewis acid mediated reduction of α-alkyl-β-keto esters by metal hydrides. A general methodology for the diastereoselective synthesis of syn- and anti-α-alkyl-β-hydroxy esters. *J. Org. Chem.* **1999**, 64, (6), 1986-1992.
72. Skouroumounis, G. K. β-Damascenone precursors in grapes and wines. *PhD thesis*. University of Adelaide, Adelaide, **1991**, pp. 66.
73. Caddick, S.; Cheung, S.; Doyle, V. E.; Frost, L. M.; Soscia, M. G.; Delisser, V. M.; Williams, M. R. V.; Etheridge, Z. C.; Khan, S.; Hitchcock, P. B.; Pairaudeau, G.; Vile, S., Stereoselective synthesis of polyfunctionalised hydroxylated cyclopentanes from dihydroxylated 2-cyclopentenone derivatives. *Tetrahedron* **2001**, 57, (29), 6295-6303.
74. Osorio, C.; Duque, C.; Fujimoto, Y., C_{13}-norisoprenoid glucoconjugates from Lulo (*Solanum quitoense* L.) leaves. *J. Agric. Food Chem.* **1999,** 47, (4), 1641-1645.
75. Demole, E.; Enggist, P., Novel synthesis of 3,5,5-trimethyl-4-(2-butenylidene)-cyclohex-2-en-1-one, a major constituent of *Burley* tobacco flavour. *Helv. Chim. Acta* **1974**, 57, (7), 2087-2091.
76. Nicolaou, K. C.; Caulfield, T. J.; Kataoka, H.; Stylianides, N. A., Total synthesis of the tumor-associated Lex family of glycosphingolipids. *J. Am. Chem. Soc.* **1990**, 112, (9), 3693-3695.
77. Tan, S. T.; Wilkins, A. L.; Holland, P. T.; McGhie, T. K., Extractives from New Zealand unifloral honeys. 2. Degraded carotenoids and other substances from heather honey. *J. Agric. Food Chem.* **1989**, 37, (5), 1217-1221.
78. D'Arcy, B. R.; Rintoul, G. B.; Rowland, C. Y.; Blackman, A. J., Composition of Australian honey extractives. 1. Norisoprenoids, monoterpenes, and other natural volatiles from blue gum (*Eucalyptus leucoxylon*) and yellow box (*Eucalyptus melliodora*) honeys. *J. Agric. Food Chem.* **1997**, 45, (5), 1834-1843.

79. Guyot-Declerck, C.; Chevance, F.; Lermusieau, G.; Collin, S., Optimized extraction procedure for quantifying norisoprenoids in honey and honey food products. *J. Agric. Food Chem.* **2000**, 48, (12), 5850-5855.

80. Guyot, C.; Scheirman, V.; Collin, S., Floral origin markers of heather honeys: *Calluna vulgaris* and *Erica arborea*. *Food Chem.* **1999**, 64, (1), 3-11.

81. Sefton, M. A.; Francis, I. L.; Williams, P. J., Free and bound volatile secondary metabolites of *Vitis vinifera* grape cv. Sauvignon Blanc. *J. Food Sci.* **1994**, 59, (1), 142-147.

82. Sefton, M. A.; Francis, I. L.; Williams, P. J., Volatile norisoprenoid compounds as constituents of oak woods used in wine and spirit matruation. *J. Agric. Food Chem.* **1990**, 38, 2045-2049.

83. Sefton, M. A.; Williams, P. J., Generation of oxidation artifacts during the hydrolysis of norisoprenoid glycosides by fungal enzyme preparations. *J. Agric. Food Chem.* **1991**, 39, (11), 1994-1997.

84. Guth, H., Comparison of different white wine varieties in odor profiles by instrumental analysis and sensory studies. In *The Chemistry of Wine Flavor*, Waterhouse, A. L., Ebeler, S. E. Eds; ACS Symposium Series 714, American Chemical Society: Washington D.C., **1999**; pp 39-54.

85. Lloyd, N. D. R.; Capone, D. L.; Ugliano, M.; Taylor, D. K.; Skouroumounis, G. K.; Sefton, M. A.; Elsey, G. M., Formation of damascenone under both commercial and model fermentation conditions. *J. Agric. Food Chem.* **2011**, 59, (4), 1338-1343.

86. Cox, A.; Skouroumounis, G. K.; Elsey, G. M.; Perkins, M. V.; Sefton, M. A., Generation of (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene from C_{13}-norisoprenoid precursors. *J. Agric. Food Chem.* **2005**, 53, (14), 6777-6783.

87. Voirin, S. G.; Baumes, R. L.; Bitteur, S. M.; Gunata, Z. Y.; Bayonove, C. L., Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes. *J. Agric. Food Chem.* **1990**, 38, (6), 1373-1378.

88. Cox, A. (*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene: A new, potent, grape derived aroma compound in wine. *PhD thesis*. Flinders University, Adelaide, **2004**; pp.124-125.

89. Rowland, C. Y.; Blackman, A. J.; D'Arcy, B. R.; Rintoul, G. B., Comparison of organic extractives found in leatherwood (*Eucryphia lucida*) honey and leatherwood flowers and leaves. *J. Agric. Food Chem.* **1995**, 43, 753-763.

90. Alissandrakis, E.; Daferera, D.; Tarantilis, P. A.; Polissiou, M.; Harizanis, P. C., Ultrasound-assisted extraction of volatile compounds from citrus flowers and citrus honey. *Food Chem.* **2003**, 82, 575-582.

91. Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A., Use of C_{18} reversed-phase liquid chromatography for the isolation of monoterpene glycosides and norisoprenoid precursors from grape juice and wines. *J. Chromatogr., A* **1982**, 235, (2), 471-480.

92. Mikami, Y.; Fukunaga, Y.; Arita, M.; Kisaki, T., Microbial transformation of β-ionone and β-methylionone. *Appl. Environ. Microbiol.* **1981**, 41, (3), 610-617.

93. Thomas, A. F.; Willhalm, B.; Müller, R., Mass spectrometry and organic analysis—XIII. The mass spectra of doubly unsaturated carbonyl compounds. *Org. Mass Spectrom.* **1969**, 2, (2), 223-239.

94. Crump, D. R., Synthesis of (2*S*)-2-propylthietan. *Aust. J. Chem* **1982**, 35, 1945-1948.

95. The Australian Wine Research Institute In *Technical Review No. 159,* Technical note, Daniel, C., Ed. December **2005**; p 5.

96. Daniel, M. A.; Elsey, G. M.; Capone, D. L.; Perkins, M. V.; Sefton, M. A., Fate of damascenone in wine: The role of SO_2. *J. Agric. Food Chem.* **2004**, 52, (26), 8127-8131.

97. Ugliano, M.; Bartowsky, E. J.; McCarthy, J.; Moio, L.; Henschke, P. A., Hydrolysis and transformation of grape glycosidically bound volatile compounds during fermentation with three *Saccharomyces* yeast strains. *J. Agric. Food Chem.* **2006**, 54, (17), 6322-6331.

98. Anchor Wine Yeast VIN 13 http://www.anchorwineyeast.com/envin13.html (accessed 21/02/2012).

99. Vesely, P.; Duncombe, D.; Lusk, L. B., G.; Seabrooks, J.; Ryder, D., The impact of fermentation temperature on yeast reductase activity. *Tech. Q. Master Brew. Assoc. Am.* **2004**, 41, 282-292.

100. Borneman, A. R.; Desany, B. A.; Riches, D.; Affourtit, J. P.; Forgan, A. H.; Pretorius, I. S.; Egholm, M.; Chambers, P. J., Whole-genome comparison reveals novel genetic elements that characterize the genome of industrial strains of *Saccharomyces cerevisiae*. *PLos Genet.* **2011**, 7, (2), 1-9.
101. Reiser, J.; Muheim, A.; Hardegger, M.; Frank, G.; Fiechter, A., Aryl-alcohol dehydrogenase from the white-rot fungus *Phanerochaete chrysosporium*. Gene cloning, sequence analysis, expression, and purification of the recombinant enzyme. *J. Biol. Chem.* **1994**, 269, (28152-28159).
102. Chevance, F.; Guyot-Declerck, C.; Dupont, J.; Collin, S., Investigation of the β-Damascenone Level in Fresh and Aged Commercial Beers. *J. Agric. Food Chem.* **2002**, 50, (13), 3818-3821.
103. Ferreira, V.; Peña, C.; Escudero, A.; Cacho, J., Losses of volatile compounds during fermentation. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A* **1996**, 202, (4), 318-323.
104. Morakul, S.; Mouret, J.-R.; Nicolle, P.; Trelea, I. C.; Sablayrolles, J.-M.; Athes, V., Modelling of the gas-liquid partitioning of aroma compounds during wine alcoholic fermentation and prediction of aroma losses. *Process Biochemistry* **2011**, 46, (5), 1125-1131.
105. Morakul, S.; Athes, V.; Mouret, J.; Sablayrolles, J., Comprehensive study of the evolution of gas-liquid partitioning of aroma compounds during wine alcoholic fermentation. *J. Agric. Food Chem.* **2010**, 58, 10219-10225.
106. Krings, U.; Kelch, M.; Berger, R. G., Adsorbents for the recovery of aroma compounds in fermentation processes. *Journal of Chemical Technology & Biotechnology* **1993**, 58, (3), 293-299.
107. Loscos, N.; Hernández-Orte, P.; Cacho, J.; Ferreira, V., Fate of grape flavor precursors during storage on yeast lees. *J. Agric. Food Chem.* **2009**, 57, 5468-5479.
108. Voilley, A.; Lamer, C.; Dubois, P.; Feuillat, M., Influence of macromolecules and treatments on the behaviour of aroma compounds in a model wine. *J. Agric. Food Chem.* **1990**, 38, 248-251.
109. Palomero, F.; Ntanos, K.; Morata, A.; Benito, S.; Suárez-Lepe, J. A., Reduction of wine 4-ethylphenol concentration using lyophilised yeast as a bioadsorbent:

influence on anthocyanin content and chromatic variables. *Eur. Food Res. Technol.* **2011**, 232, 971-977.

110. Rodríguez-Bencomo, J. J.; Muñoz-González, C.; Andújar-Ortiz, I.; Martín-Álvarez, P. J.; Moreno-Arribas, M. V.; Pozo-Bayón, M. Á., Assessment of the effect of the non-volatile wine matrix on the volatility of typical wine aroma compounds by headspace solid phase microextraction/gas chromatography analysis. *J. Sci. Food Agric.* **2011**, 91, (13), 2484-2494.
111. Ugliano, M.; Moio, L., Free and hydolytically released volatile compounds of *Vitis vinifera* L. cv Fiano grapes as odour-active constituents of Fiano wine. *Anal. Chim. Acta* **2008**, 621, 79-85.
112. Shiraga, Y.; Kanemichi, O.; Toshiaki, A.; Chikara, F.; Kazujasa, Y.; Shigeo, T.; Hiroshi, F.; Mamoru, T., Structures of potent antiulcerogenic compounds from *Cinnamomum cassia*. *Tetrahedron* **1988**, 44, (15), 4703-4711.
113. Gunata, Y. Z.; Bayonove, C. L.; Baumes, R. L.; Cordonnier, R. E., The aroma of grapes I. Extraction and determination of free and glycosidically bound fractions of some grape aroma components. *J. Chromatogr., A* **1985**, 331, 83-90.
114. Groyne, J.; Lognay, G.; Marlier, M., Accumulation of glycosidically bound compounds in *Fragaria x ananassa* cv. elsanta fruits at various developmental stages. *Biotechnol. Agron. Soc. Environ.* **1999**, 3, (1), 5-9.
115. Baderschneider, B.; Winterhalter, P., Isolation and characterization of novel stilbene derivatives from Riesling wine. *J. Agric. Food Chem.* **2000**, 48, 2681-2686.
116. Valla, A.; Andriamialisoa, Z.; Valla, B.; Labia, R.; Le Guillou, R.; Dufossé, L.; Cartier, D., A new biomimetic-like aromatization of the cyclic end groups of terpenoids with stereospecific migration of one of the methyl groups: A convenient route to isorenieratene (ϕ,ϕ-carotene). *Eur. J. Org. Chem.* **2007**, (4), 711-715.