

**APPLICATION OF FAST ATOM BOMBARDMENT
MASS SPECTROMETRY TO THE EXAMINATION
OF GLYCOCONJUGATES IN GRAPE JUICE AND
WINE**

by

Vassilios A. Marinos, BSc

**Thesis submitted for the degree of
Doctor of Philosophy
in
The University of Adelaide
(Faculty of Agricultural and Natural Resources)**

August 1992

TABLE OF CONTENTS

	page
Table of contents	i
List of figures	iii
List of tables	v
Abstract	vi
Declaration	ix
Acknowledgements	x
Publications	xii
Abbreviations	xiii
1 Introduction and general literature review	1
1.1 Wine composition and flavour	1
1.1.1 Classification of wine constituents in terms of their metabolic origin	2
1.1.2 Sensory evaluation of wine metabolites	2
1.1.3 Classification of wine flavour constituents	3
1.1.4 The accumulation of flavour components originating from grapes	4
1.1.5 The flavour precursor analysis approach	5
1.2 Volatile secondary metabolites in grape juice and wine	5
1.2.1 Terpenoids	5
1.2.2 Monoterpenes	6
1.2.3 Norisoprenoids	11
1.2.4 Shikimic acid derived volatiles	18
1.3 Glycosidically bound secondary metabolites	20
1.3.1 Biological formation of glycosides	20
1.3.2 The composition of glycoconjugated precursors in fruits	21
1.4 The analysis of flavour precursors	23
1.4.1 New dimensions in flavour precursor analysis	25
1.5 Fast atom bombardment-mass spectrometry (FAB-MS)	26
1.5.1 Instrumentation and principles	26
1.5.2 The sputtering phenomenon	28
1.5.3 Matrix support	30
1.5.4 Characteristics of FAB-MS	32
1.5.5 Mechanism of ionization	33

	page
1.5.6 Sensitivity	35
1.5.7 Application of FAB-MS to structure elucidation of glycoconjugates	36
1.6 Employment of FAB-MS in a multidimensional analysis	41
1.7 Aims of this thesis	42
2 Late-eluting, relatively non-polar wine glucosides	43
2.1 Introduction	43
2.2 Results and discussion	44
3 Early-eluting polar monoglucosides	56
3.1 Introduction	56
3.2 Results and discussion	56
4 Early-eluting disaccharide glycosides	67
4.1 Introduction	67
4.2 Results and discussion	67
5 Very early-eluting glycoconjugates	79
5.1 Introduction	79
5.2 Results and discussion	79
6 β-Carbolines: polar, non-volatile material in Riesling wine	89
6.1 Introduction	89
6.2 Results and discussion	89
7 Conclusions	96
8 Experimental	100
8.1 Chapter 1. General	100
8.2 Chapter 2. Late-eluting, relatively non-polar wine glucosides	105
8.3 Chapter 3. Early-eluting polar monoglucosides	110
8.4 Chapter 4. Early-eluting disaccharide glycosides	113
8.5 Chapter 5. Very early-eluting glycoconjugates	119
8.6 Chapter 6. β -Carbolines: polar, non-volatile material in Riesling wine	123
References	126

LIST OF FIGURES

Figure		page
1	Key volatile monoterpene compounds	6
2	Structures of some monoterpenes identified in grapes and wines	8
3	Monoterpenes induced by acid hydrolysis	10
4	<i>p</i> -Menthenediols and α -terpineol derivatives	10
5	Carotenoid degradation	11
6	Norisoprenoids identified as free volatiles in grapes and wines	12
7	TDN and vitispirane precursors in wine	14
8	β -Damascenone precursors	15
9	The actinidols and actinidol precursors	15
10	Norisoprenoids identified in the enzymic hydrolysate of glycosidic isolates of premium varieties	17
11	Shikimate-derived volatile phenols identified in juices and wines	19
12	Transglycosylation reaction for the formation of glycosidically bound secondary metabolites	20
13	Overall diagram of a FAB-MS ion source	27
14	Regimes of sputtering by elastic collisions	29
15	Positive and negative ion mode FAB-MS spectra of glycosidic mixture fl. frs. 7-12	45
16	Positive and negative ion mode FAB-MS/MS spectra of glycosides with M_r 332	47
17	<i>cis</i> -Pyran linalool oxide- β -D-glucopyranoside and its acetylated derivative	53
18	Some Riesling wine norisoprenoid constituents and their glycosidic derivatives	55
19	2-[2-Methoxy-4-(ω -hydroxypropyl)-phenoxy]-propan-1,3-diol	60
20	1,2,3,4-Tetrahydro-7-hydroxy-1-(4'-hydroxy-3'-methoxyphenyl)-6-methoxy-2,3-naphthalenedimethanol (i.e. isolariciresinol)	63
21	2,3-Bis(4'-hydroxy-3'-methoxybenzyl)butane-1,4-diol (i.e. <i>seco</i> -isolariciresinol), its glucosidic derivative and some of the positive ion mode mass spectral fragmentations of the glucosidic derivative	65
22	Benzyl alcohol- β -D-rutinoside and its per-O-acetylated derivative	70
23	(E)-2,6-Dimethylocta-2,7-diene-1,6-diol	72
24	(E,E)-2,6-Dimethylocta-2,6-diene-1,8-diol and its glycosidic derivatives	74
25	(E)-3,7-Dimethylocta-2-ene-1,7-diol and its glycosidic derivatives	75

Figure	page	
26	<i>p</i> -Menth-1-ene-7-(β -D-apiofuranosyl-(1 \rightarrow 6)- β -D-glucopyranosyl)-8-ol, vomofoliol-9-O-arabinofuranosyl-O-glucopyranoside and their per-O-acetylated derivatives	77
27	2,3-Dihydro-7-hydroxy-2-(4'-O- β -glucopyranosyl-3'-methoxyphenyl)-3-hydroxymethyl-5-benzofuranpropanol, a novel Riesling wine constituent and its per-O-acetylated derivative	84
28	2,3-Dihydro-7-hydroxy-2-(4'-hydroxyl-3'-methoxybenzyl)-3-hydroxymethyl-5-benzofuranpropanol	84
29	(E)-2,6-Dimethylocta-2,7-diene-1,6-diol-1-O- β -D-glucopyranosyl-O-arabinofuranosyl-O- β -glucopyranoside and its per-O-acetylated derivative	86
30	1-Methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid: a novel constituent of Riesling wine	90
31	β -Carboline derivatives in Riesling wine and some of their mass spectral fragmentations	94

LIST OF TABLES

Table		page
1	Glycosidically bound volatile secondary metabolites identified in grapes and wines	23
2	¹ H NMR data of compounds 85a , 85b	49
3	¹ H NMR data of compounds 86 , 30a , 30b	51
4	¹ H NMR data of compounds 90 , 90a	59
5	¹ H NMR data of compounds 91 , 91a and some of the positive ion mode mass spectral fragmentations of 91	62
6	¹ H NMR data of compounds 94 , 94a	71
7	¹ H NMR data of compounds 95a , 96a , 97a	78
8	¹ H NMR data of compounds 99a , 100a	88
9	¹ H NMR data of naturally occurring and synthetic diastereoisomers of 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid	91
10	¹³ C NMR data of naturally occurring and synthetic diastereoisomers of 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid	93

Abstract

Flavour is probably the single most important factor influencing wine quality and is related to the chemical composition of both the grape juice and the wine. Flavour-important compounds are accumulated in grapes in two different forms, i.e. as free volatile compounds and as non-volatile polyols or glycosidically bound metabolites. For the study of these compounds a technique named the flavour precursor analysis approach has been developed. The progress made in grape juice and wine compositional analysis through application of this approach has been reviewed and the limitations of the techniques involved are discussed.

The introduction of fast atom bombardment mass spectrometry (FAB-MS) together with the improved mass ranges of magnetic sector and quadrupole instruments has opened new horizons for the analysis of glycoconjugates and other polar and non-volatile compounds of biological importance.

A protocol which combines partition liquid chromatography (e.g. droplet counter current chromatography (DCCC) and high performance liquid chromatography (HPLC)) and soft ionization mass spectrometry (i.e. FAB-MS) has been developed for the direct analysis of wine and juice glycoconjugates without prior derivatization of these compounds. This protocol offers an alternative to overcome the limitations of the flavour precursor analysis approach in the investigation of polar, non-volatile and thermally labile material. Application of the protocol to pre-fractionated wine glycosidic mixtures of different degrees of polarity demonstrated that the protocol can be successfully employed as a technique to screen for flavour precursors and other glycosidically bound secondary metabolites present in wine.

In the relatively non-polar glycosidic fraction (i.e. late-eluting DCCC fractions 190-220), the β -D-glucopyranosides of ethyl indole lactate, uroterpenol and *cis*-pyran linalool oxide have been identified. The presence in the same glycosidic fraction of hexopyranosides of 3-hydroxy-5,6-epoxymegastigm-7 α -ene-9-one and of a mixture of diastereoisomeric 3,4-dihydroxy-7,8-dihydro- β -ionone, was observed.

As a consequence of these investigations, a new class of wine glucopyranosides with phenylpropanoids and lignans as aglycones has been recognized in the medium polarity

fractions (i.e. early-eluting DCCC fractions 90-100). 2-Methoxy-4-(ω -hydroxypropyl)-phenoxypropan-3-ol, isolariciresinol and *seco*-isolariciresinol β -D-glucopyranosides have been isolated from this fraction and characterized by spectroscopic and spectrometric techniques. This class of compound, although without an apparent flavour precursor role, it does contain compounds which have been suggested as precursors in the formation of biologically active compounds, e.g. *seco*-isolariciresinol- β -D-glucopyranoside.

In this same polarity group of fractions, a new class of flavour precursor material has been recognized. This class comprises a mixture of monoterpene β -D-apiosyl- β -D-glucopyranosides. These compounds include (E)-2,6-dimethylocta-2,7-diene-1,6-diol, (E,E)-2,6-dimethylocta-2,6-diene-1,8-diol and *p*-menth-1-ene-7,8-diol apiosylglucosides which have been isolated and subsequently identified by spectrometric and spectroscopic techniques. A new arabinoglucoside of (E)-3,7-dimethylocta-2-ene-1,7-diol together with two other known flavour precursors (i.e. benzyl alcohol- β -D-rutinoside and vomifoliol arabinoglucoside), were also identified in this fraction.

Investigation of the most polar glycosidic constituents of wine (i.e. very early-eluting DCCC fractions 60-75), has shown that the bulk of the material was made up of monoglucosidic derivatives of very polar aglycones such as the 4'-O- β -D-glucopyranoside of the neolignan 2,3-dihydro-7-hydroxy-2-(4'-hydroxy-3'-methoxybenzyl)-3-hydroxymethyl-5-benzofuranpropanol (i.e. cedrusin) which was isolated and structurally characterised by spectroscopic techniques. Glycosides of volatile aroma compounds were relatively minor constituents of this very polar group of fractions. The isolation and structural characterisation of a glucopyranosyl-O-arabinofuranosyl-O-glucopyranoside of (E)-2,6-dimethylocta-2,7-diene-1,6-diol, indicated that these flavour precursors can be present as polar trisaccharide derivatives.

Finally, the presence in wine of a group of polar, but not glycosidically bound, heterocyclic compounds has been established. Diastereoisomeric forms of the possible off-flavour principal 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid have been isolated from Riesling wine and structurally characterised by comparison of their spectrometric properties with those of synthetic reference samples. The presence of an incompletely characterised 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid derivative and also of a compound tentatively assigned as a 1,1-dimethyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid derivative has been observed. The presence of

these heterocyclic compounds in a grape juice (variety Semillon) was also verified by mass spectrometric means.

To summarise, this thesis has established the remarkable ability of FAB-MS and of tandem mass spectrometry (MS/MS) to provide detailed information on hitherto intransigent polar wine glycoconjugates. This study will provide a sound foundation for the future assessment of the flavour status of grape juices and wines, and for the assessment of the ageing potential, in terms of flavour release, of wines by screening for flavour precursors.

Declaration

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

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

Vassilios A. Marinos

20/08/1992

Acknowledgements

I sincerely thank my supervisors, Dr Patrick Williams, Dr Max Tate and Dr Bryan Coombe for their advice on this project and the preparation of the thesis.

Thanks are due to Dr Peter Winterhalter for performing some initial separations of glycosidic isolates from Riesling wine by droplet counter current chromatography, and Dr Greame Currie for his useful instructions for the operation of the Finnigan Mat TSQ 70 mass spectrometer, at the early stages of this study.

My colleagues of the Australian Wine Research Institute are thanked for their friendship and help, in particular, Dr Mark Sefton for his advice, especially to the interpretation of certain ^1H NMR data.

The donations of samples of (2R, 3R) *seco*-isolariciresinol from Dr Richard Cambie, Auckland, New Zealand, uroterpenol from Dr R. Carman, Queensland, Australia, 3-hydroxy-5,6-epoxymegastigm-7-ene-9-one and diastereoisomeric 3,4-dihydroxy-7,8-dihydro- β -ionones from Dr Peter Winterhalter, Würzburg, Germany, are also acknowledged.

The Director of The Australian Wine Research Institute (AWRI) Professor Terry Lee is thanked for giving me the opportunity to complete this study at the AWRI. Financial assistance from the Grape and Wine Research Council is gratefully acknowledged.

Finally, I wish to thank and also to express my deepest respect to my parents D. & A. Marinos and to my relatives, Mr S. I. Gerovasilis and his family, for their support, understanding and encouragement throughout this study.

This thesis is dedicated to my fiancée Olympia-Maria,
whose constant fortitude and loving support has
encouraged me to keep trying

V.A.M.

Publications

Part of the work described in the thesis of Vassilios A. Marinos has been published:

1. Marinos, V. A.; Tate, M. E.; Williams P. J. Glucosides of ethyl indole-3-lactate and uroterpenol in Riesling wine. *Phytochemistry* **1992**: in press.

2. Marinos, V. A.; Tate, M. E.; Williams P. J. Lignan and phenylpropanoid glycerol glucosides in wine. *Phytochemistry* **1992**: in press.

3. Williams, P. J.; Sefton, M. A.; Marinos, V. A. Hydrolytic flavour release from non-volatile precursors in fruits, wines and some other plant-derived foods. In *Recent developments in flavor and fragrance chemistry*; 3rd Haarmann and Reimer International Symposium; 12-15 April, 1992, Kyoto, Japan, 1992: in press.

ABBREVIATIONS

%	Per cent
^{13}C	Carbon isotope of atomic mass 13
α	Alpha
Ac	Acetyl-
aq.	Aqueous
Ar	Argon
β	Beta
C ₁₈	Octadecyl silyl
ca.	Approximately
CADS	Collisionally activated decomposition spectrum
CCC	Counter current chromatography
CI	Chemical ionization
cm	Centimeter
cm^{-1}	Wavenumber
C _n	n Carbon atoms
COFF	Collision offset
COSY	Correlated spectroscopy
δ	Chemical shift
DCCC	Droplet counter current chromatography
DCI	Desorption collision ionization
DI	Desorption ionization
E.C.	enzyme cataloque
e.g.	For example
EHDS	Electrohydrodynamic ionization
EI	Electron ionization
EILA	Ethyl indole lactate
<i>et al.</i>	And others
eV	Electron volts
FAB	Fast atom bombardment
FD	Field desorption
fl. frs.	Flash chromatography fractions
Frs.	Fractions
GC	Gas chromatography
HPLC	High performance liquid chromatography
hrs	Hours

Hz	Hertz
i.d.	Internal diameter
i.e.	That is
IAA	Indole acetic acid
ILA	Indole lactic acid
IR	Infra red
<i>J</i>	Coupling constant
KeV	Kilo (10^3) electron volt
Kr	Krypton
kV	Kilo (10^3) volt
λ_{\max}	Maximum absorbance
<i>m</i>	Meta
<i>m/z</i>	Mass to charge ratio
mA	Milli (10^{-3}) ampere
MBSA	Molecular beam for solid analysis
μg	Micro (10^{-6}) gram
mg	Milli (10^{-3}) gram
mg/L	Milli (10^{-3}) gram per litre
MHz	Mega (10^6) hertz
min	Minute
μL	Micro (10^{-6}) litre
mL	Milli (10^{-3}) litre
μm	Micro (10^{-6}) metre
mm	Milli (10^{-3}) metre
$\mu\text{mol/mL}$	Micro (10^{-6}) mole per milli (10^{-3}) litre
$M_{O,G}$	Relative mobility to the marker dyestuff orange G
M_r	Relative molecular weight
MS	Mass spectrometry
MS/MS	Mass spectrometry/mass spectrometry
mV	Milli (10^{-3}) volt
NDP	Nucleotide diphosphate
ng	Nano (10^{-9}) gram
nm	Nano (10^{-9}) metre
NMR	Nuclear magnetic resonance
<i>o</i>	Ortho
$^{\circ}\text{C}$	Degree Celsius
<i>p</i>	Para

ppb	Parts per billion
R _f	Retention front
SDE	Simultaneous distillation extraction
sh	Shoulder
SIMS	Secondary ion mass spectrometry
T	Temperature
TD	Thermal desorption
TDN	1,1,6-Trimethyl ^{1,2} -dihydronaphthalene
THBC	Tetrahydro-β-carboline
TLC	Thin layer chromatography
TMSi	Trimethylsilylated
UDP	Uridine diphosphate
UV	Ultra violet
UV-Vis	Ultra violet-visible
v/v	Volume by volume
w/v	Weight per volume
Xe	Xenon

Chapter 1

INTRODUCTION AND GENERAL LITERATURE REVIEW

1.1 Wine composition and flavour

Wine is a very complex liquid which is much more than just a dilute alcoholic solution. What makes wine interesting and different from other beverages is its variety: subtle nuances of colour, odour, taste, flavour and texture - which are all determined by chemical composition - create a uniqueness of character for each wine. It is the degree of excellence that each one of these sensory parameters reaches individually, as well as the proper balance between them, that defines wine quality and stimulates the one thing that provides the most enduring pleasure and appreciation - the aesthetics of wine (Amerine and Singleton 1977; Amerine and Roessler 1982).

Of all wine quality sensory parameters, flavour perception is probably the single most important factor influencing acceptability and enjoyment of wine. Flavour in wine, as with all foods and beverages, results from the interaction of the chemical constituents of the wine with the senses of taste and smell of the person consuming it (A. A. Williams 1982). It is, therefore, natural to try to relate chemical composition of grape juices and wines to flavour perception. Aspects of flavour chemistry of relevance to wines to which scientific interest has been directed include the following.

- 1) Compositional analysis of grape juice and wine from different grape varieties (Rapp *et al.* 1982, Schreier *et al.* 1982, Rapp and Ringlage 1989) and even from different clones of the same grape variety (Marais and Rapp 1991); and recognition of possible differences in composition among them.
- 2) Investigation of the contribution to varietal flavour of specific compounds or classes of chemical compounds originating from grapes, e.g. the contribution of the monoterpene alcohols linalool, geraniol, nerol and α -terpineol to the aroma of muscat grape juice or wine (Wagner *et al.* 1977) and the contribution of 3-alkyl-2-methoxypyrazines to the characteristic vegetative odours of some Cabernet Sauvignon (Bayonove *et al.* 1975) and Sauvignon Blanc wines (Allen *et al.* 1990).
- 3) The contribution to flavour and, thus, to wine quality of individual compounds which develop during wine ageing. Examples for wines from *Vitis vinifera* grapes include β -damascenone, vitispirane, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (Simpson 1979,

Simpson and Miller 1983, Winterhalter *et al.* 1990a), or methyl anthranilate, hexanol, iso-amylalcohol, benzaldehyde, 2-phenyl-ethanol in the case of wines from non-*vinifera* grapes (Stern *et al.* 1967, Amerine and Singleton 1977, Welch *et al.* 1982).

4) Recognition of factors capable of affecting chemical composition of grape juice and wine and evaluation of their affect on wine flavour and therefore quality. These factors can be classified as either pre-harvest practices, i.e. viticultural, such as grape ripening and canopy management or post-harvest practices, i.e. oenological, such as the use of glycosidase enzymes, juice extraction and ageing (Strauss *et al.* 1986a).

1.1.1 Classification of wine constituents in terms of their metabolic origin

Wine constituents can be divided into two categories: primary and secondary metabolites. Primary metabolites have been defined by plant biochemists as those compounds synthesized *in vivo* which play an essential role in the survival and well-being of the plant (Mann 1980a). Typical examples, in the case of grapes and hence of wine, are the plant derived sugars, amino-acids, common fatty acids, nucleosides and the polymers derived from them (e.g. polysaccharides and proteins).

Secondary metabolites are defined as the cellular components which frequently have no apparent utility (Mann 1980b, D. H. Williams *et al.* 1989). In the case of wine, typical examples include the plant derived non-volatile anthocyanins, flavanoids and tannins, and the volatile terpenoids (monoterpenes, sesquiterpenes, norisoprenoids) and phenolic derivatives (Mann 1980b).

1.1.2 Sensory evaluation of wine metabolites

Sensory evaluation of the components included in each category has revealed that both classes affect wine quality. Primary metabolites give sweet, acidic, and alcoholic sensations (Ribéreau-Gayon and Peynaud 1975). Secondary metabolites confer specific characteristics such as colour, aroma, flavour and texture (Ribéreau-Gayon and Peynaud 1975). More specifically, non-volatile secondary metabolites such as anthocyanins, flavanoids and tannins, are responsible for the colour, astringency and maturation potential of a wine (Somers 1975, Ribéreau-Gayon and Glories 1987). Because the major part of flavour character is perceived through the nose, volatile secondary metabolites are mainly responsible for the distinctive flavour characteristics of grape juices and wines (A. A. Williams 1982).

1.1.3 Classification of wine flavour constituents

The major volatile secondary metabolites identified in wine can be classified according to their metabolic origin into two categories.

- a) Compounds derived from mevalonic acid. This category comprises terpenoids such as monoterpenes, sesquiterpenes and norisoprenoids (Mann 1980b).
- b) Compounds derived from shikimic acid. This category embraces all the volatile phenols.

In grapes, plant derived volatile metabolites are the only flavour compounds which are apparent. However, in wine a number of other volatile constituents are also present. These compounds are formed during the alcoholic fermentation as a result of the biotransformation of primary metabolites by yeast and they also contribute to wine flavour. Thus, to obtain a more precise classification of the more than 900 volatile constituents identified in grapes and wines to date (Rapp 1988, Etiévant 1991), the volatile aroma constituents should be subdivided into groups according to whether they existed originally in grapes or were formed during the various stages of the winemaking procedure. Accordingly, wine flavour constituents can be classified into four categories.

- a) Compounds originating from the grapes. This category embraces compounds that are found in the undamaged plant cells of the grape and are, therefore, typical of the cultivar. They are transferred substantially unmodified from the grape to wine without being affected by the fermentation process, and are described as the primary or grape aroma components.
- b) Compounds formed during the processing of the grapes (destalking, crushing, pressing) by chemical and enzymatic reactions in grape must. These are described as secondary grape aroma.
- c) Compounds produced during alcoholic fermentation. They constitute the so-called fermentation bouquet of wines which imparts a vinous character to wine flavour.
- d) Compounds produced during wood maturation or bottle ageing of the wine. They result from precursors during maturation and/or ageing by chemical reactions and, equally with the primary aromatic compounds, they confer complexity to wine flavour which is described as maturation bouquet (Marais 1983).

1.1.4 The accumulation of flavour components originating from grapes

Research on flavour components derived from the grape revealed that these compounds are accumulated in the fruit in two different forms (Cordonnier and Bayonove 1974). Firstly, as volatile aglycone products having an impact effect on grape juice and wine flavour (e.g. linalool oxidation state monoterpenes). Secondly, either as hydrophilic, often polyhydroxylated, aglycones (e.g. linalool oxide oxidation state monoterpene polyols), or as glycosidically bound compounds. The latter two categories consist of compounds which have limited volatility (i.e. polyols) or are involatile (i.e. glycosides), and thus, they do not contribute directly to grape and wine flavour. However, these polar compounds have the potential to enhance the aroma content both of grape juice and wine if they are manipulated properly, i.e. they have a precursor role (Strauss *et al.* 1984). Liberation of this reserved aroma can be achieved following two separate pathways which involve chemical and/or enzymic hydrolysis (Williams 1992). Polyols are usually related metabolically to the flavour volatiles and they may be formed prior to, or after the latter compounds are biosynthesized. Importantly, the polyols can be acid labile, and thus, capable of generating volatile rearrangement products. Hence, in the case of polyols the additional aroma compounds are produced totally by acid hydrolysis (e.g. ageing can be described as a slow acid hydrolysis procedure). In the case of glycosidic precursors, the aglycone moiety may be either a volatile compound with the potential to directly contribute to grape juice and wine flavour after enzymic or acid catalysed release, or it may be a polyol which can generate additional flavour components after further acid catalyzed transformation. Thus, in the case of glycosidic precursors aroma enhancement can be achieved either directly from a glycoside to a volatile or via a polyol.

Because the concentration of the bound material in the grape is much greater than that of the material which exists as free volatiles (Wilson *et al.* 1984), and also because the nature of the aglycone moieties can dictate the appropriate manipulation technique to use in order to enhance flavour, further research on the topic of grape juice and wine glycoconjugates can be expected to provide a better understanding of the chemical behaviour of these bound secondary metabolites. Thus, it is of interest to investigate the physical and chemical properties of each metabolite, for example, its substitution pattern, its susceptibility to enzymic or acid catalysed hydrolysis and the products that are liberated under certain hydrolysis conditions.

1.1.5 The flavour precursor analysis approach

Attempts to investigate glycosidically bound flavour led to the establishment of a strategy known as flavour precursor analysis which has been developed mainly by Williams and co-workers (P. J. Williams *et al.* 1982a, Strauss *et al.* 1987a). This research strategy is a multistep methodology which involves the following procedures.

- a) Removal of the free volatile flavour material from the grape juice or dealcoholized wine by liquid-liquid extraction using dichloromethane or freon as extraction solvent.
- b) Isolation of the bound material (glycosides) using C₁₈ reversed-phase chromatography. Amberlite XAD-2 resin has also been used in place of C₁₈ reversed-phase adsorbent to isolate the glycosidic fraction from grapes and other fruits (Günata *et al.* 1985). The isolated glycosides can be, sometimes, subjected to preparative scale fractionation. This step is employed when fractions containing a limited number of glycosides are required for analytical reasons (e.g. isolation of single glycosidic component from a mixture), and for this purpose counter current chromatography (CCC) has been used with advantage (Strauss *et al.* 1987a, Winterhalter *et al.* 1990b).
- c) Elucidation of the structure of the isolated metabolites employing spectrometric techniques. Firstly, elucidation of the structures of the aglycones which result from enzymic and acid hydrolysis of glycosidic mixtures by gas chromatography-mass spectrometry (GC-MS) examination. Secondly, where possible, examination of mixtures of intact glycosides after derivatization, e.g. as acetate esters, by GC-MS and in cases of single components by ¹H NMR.

The following section describes progress in the compositional analysis of grape juice and wine from different grape varieties, in terms of volatile aroma constituents and flavour precursors. The contribution of these secondary metabolites as individual components or classes of compounds to wine flavour is also described. This review covers the literature up to early 1992.

1.2 Volatile secondary metabolites in grape juice and wine

1.2.1 Terpenoids

Terpenoids are natural products with structures made up of C₅ isoprenoid units (Mann 1980b). They occur with a variety of functional groups which include alcohols, ethers and ketones. In *Vitis vinifera* grapes the presence of monoterpene, norisoprenoid and

sesquiterpene classes have been identified. Monoterpenes may be derived from a number of sources during winemaking including grape leaves and yeast metabolism (Wildenradt *et al.* 1975, Schreier 1984a). However, these sources are minor in comparison to the grape berry itself, and only terpenoids of grape origin will be discussed. Specifically, only the classes of monoterpenes and norisoprenoids, which significantly contribute to grape juice and wine aroma, will be dealt with in this review.

1.2.2 Monoterpenes

Monoterpenes comprise two C₅ isoprenoid units. Because many of them possess pleasant and often strong odours, they have long been suspected of making a significant contribution to the aroma of wine. Austerweil (1946) was the first worker to suggest that the aroma of Muscat grapes can be ascribed to terpene compounds. Cordonnier (1956) investigating the aroma of Muscat wines tentatively identified linalool, geraniol, nerol, α -terpineol and limonene by thin layer chromatography (TLC). Nevertheless, it was not until the application of sophisticated gas chromatographic techniques combined with mass spectrometry that rapid progress on the elucidation of grape and wine aroma components has been achieved.

Using these techniques Stevens *et al.* (1966) reported the presence of some 60 components in grape juice extract of Muscat varieties. The key volatile monoterpene compounds linalool (1), geraniol (2), nerol (3), α -terpineol (4), citronellol (5) and the pyran (6) and furan linalool oxide (7) isomers were amongst the compounds identified (see figure 1).

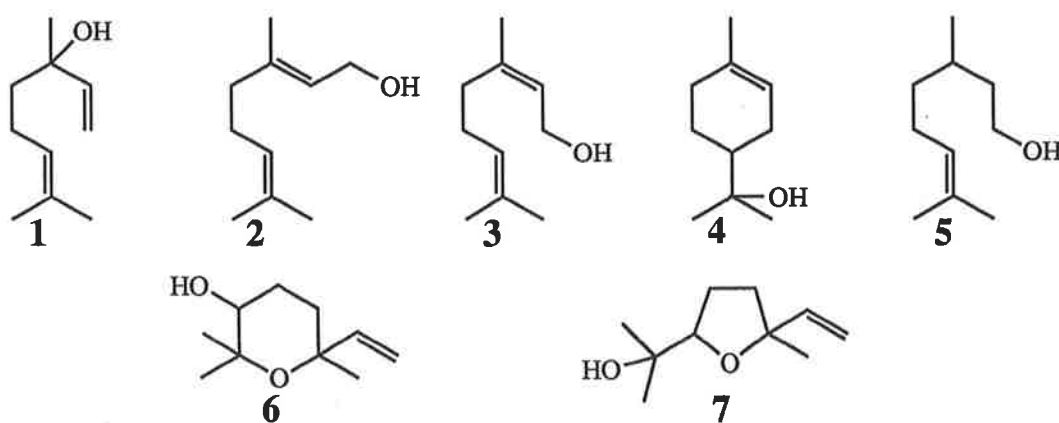


Figure 1: Key volatile monoterpene compounds.

Further work by numerous workers has led to the identification of additional alcohols, ethers, aldehydes and hydrocarbons in juice and wine. Amongst them were the compounds hotrienol (8) (Schreier *et al.* 1974, Bayonove *et al.* 1976), the rose oxide isomers (9) and nerol oxide (10) (Schreier *et al.* 1976) which have relatively low aroma threshold values (Marais 1983, J. C. Leffingwell and D. Leffingwell 1991) and, therefore, are possible contributors to the aromatic profile of grape juices and wines. The most recently identified component is iso-geraniol (11) which was detected in the *Vitis vinifera* cultivar Muscat Roy (Shosheyov *et al.* 1990). Their structures are depicted in figure 2. The free monoterpenes identified in grape juice and wine can be differentiated into two categories according to their solubility in organic solvents. The first category embraces those soluble in pentane and the second those soluble in freon (P. J. Williams *et al.* 1981). GC-MS analysis of these fractions revealed that the pentane-soluble monoterpene fraction consists of volatile compounds which have been identified several times in muscat grapes, they are made up largely of compounds at the oxidation state of linalool and constitute part of the "free monoterpenes" referred to by Cordonnier and Bayonove (1974). On the other hand, the more polar, freon-soluble fraction predominantly comprises polyols of the linalool and linalool oxide oxidation state.

Free non-volatile grape polyols were recognised by Rapp and Knipser (1979) and Rapp *et al.* (1980) who identified dienediols (12) and (13) (3,7-dimethylocta-1,5-dien-3,7-diol and 3,7-dimethylocta-1,7-dien-3,6-diol, respectively). Williams *et al.* (1980a) independently confirmed the presence of both of these compounds in addition to enediol (14) and enetriol (15). Williams *et al.* (1980a) and Rapp *et al.* (1984a) proposed the existence of additional polyol analogues which can be derived from the most abundant free monoterpene alcohols, i.e. geraniol, nerol, citronellol and linalool, by two separate pathways: a) according to the photohydroperoxide synthesis scheme (Schenck 1957) by photo-sensitized oxygen transfer onto the trisubstituted double bond (6-position) of the acyclic alcohols, followed by subsequent reduction of the corresponding hydroperoxides to diols; b) by acid catalyzed hydration of the double bond in the 6-position of the alcohols. Only a few of the proposed diol analogues have been detected up to date in grape juice and wine. These include the diol corresponding to citronellol, i.e. hydroxycitronellol (16) (Rapp *et al.* 1983), the dienediols (17)-(19) (Rapp *et al.* 1984a), the enediol (20) (Rapp *et al.* 1984a) and the diol isomers corresponding to α -terpineol, *cis*- and *trans*-1,8 terpin (21) (Williams *et al.* 1980b). The existence of the proposed diol analogues 22 and 23 could not be verified at the pH values of musts and wines, because these compounds readily cyclize to form their corresponding monoterpene ethers (Rapp *et*

al. 1984a). The monoterpenes present in *Vitis vinifera* grapes and wines have been extensively reviewed up till 1988 by a number of authors (Marais 1983, Rapp *et al.* 1984b, Strauss *et al.* 1986a, Rapp 1988). Lists of all monoterpenes identified in both grapes and wines are presented in these review papers.

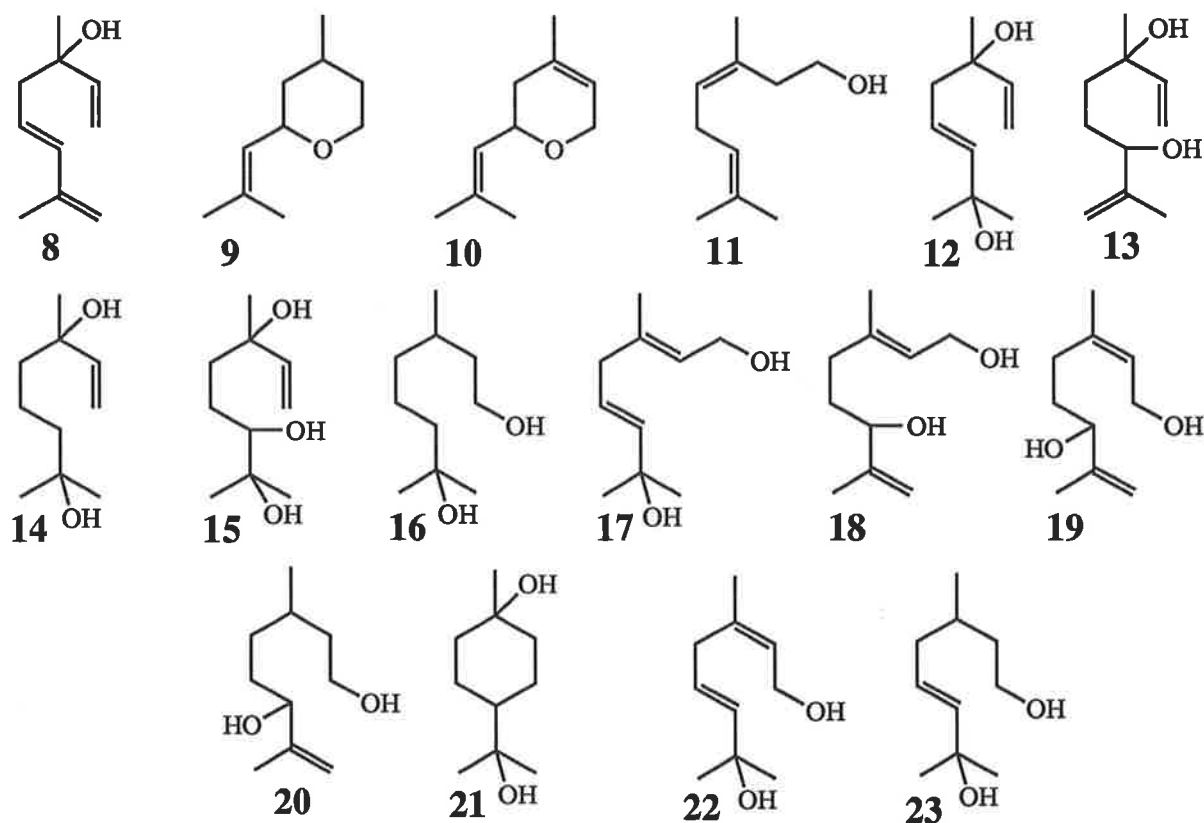


Figure 2: Structures of some monoterpenes identified in grapes and wines.

The contribution of volatile monoterpenes to the varietal flavour of a number of grape cultivars and wines has been documented by a number of authors. Bayonove and Cordonnier (1970) first observed a positive correlation between linalool concentrations in grape juices made from different varieties and their characteristic muscat odour scores. The same authors (Bayonove and Cordonnier 1971) added a mixture of the main terpene alcohols, in concentrations similar to those measured in a muscat juice, to a neutral Ugni Blanc juice. They compared the aroma of the two juices and concluded that these particular volatiles did contribute to a pleasant muscat-like odour note. Ribéreau-Gayon *et al.* (1975) studied the aroma threshold values of a number of the monoterpenoids of muscat grapes. These workers found that at the normally encountered levels the major monoterpenoids of the fruit were present in concentrations higher than their aroma thresholds. Furthermore, it was observed that whilst none of the individual compounds

studied had sensory properties identical with muscat character, a combination of these volatile monoterpenoids was essential to muscat grape aroma. The characteristic aroma of Muscat grapes and wines has been attributed to the presence of a mixture of key volatile monoterpenes 1-6 (Terrier *et al.* 1972, Ribéreau-Gayon *et al.* 1975). Because other aromatic grape varieties such as Riesling, also contain terpenoids, but in lesser amounts than muscats, it was concluded that the flavour of these varieties was also controlled by monoterpenoids.

Rapp *et al.* (1982) and Schreier (1982) used a combination of GC-MS and GC-sniff techniques along with multiple discriminant analysis applied to the concentration differences among identified compounds as a means of discriminating between varieties. The results of those analyses, together with data from work undertaken by Terrier *et al.* (1972), led to the differentiation of the grape varieties. A general classification of some *Vitis vinifera* grape varieties into categories based on their total monoterpene content has been presented by P. J. Williams *et al.* (1987). According to this classification, grape varieties can be differentiated to three broad categories.

1. Intensely flavoured muscat varieties with total monoterpenes up to 6 mg/L.
2. Non-muscat but floral varieties having a total monoterpene concentration of 1-4 mg/L.
3. More neutral varieties which are not dependent upon monoterpenes for their flavour.

The role of polyols as precursor material to important flavour compounds has been demonstrated by Williams *et al.* (1980b). Working on aqueous model solutions of polyols they observed that enediol (14), enetriol (15) and dienediol (16) were unstable under mildly acidic conditions. Each compound underwent rearrangements to form a characteristic range of products. Similar results were reported for grape juice rich in these precursor compounds such as the juices of the muscat varieties. In these cases, heating of the juice resulted in a significant enhancement of the levels of the furan linalool oxides (7), nerol oxide (10), hotrienol (8) and α -terpineol (4). In addition, levels of 2,6,6-trimethyl-2-vinyltetrahydropyran (24), the anhydrofuran linalool oxides (25), 2,2-dimethyl-5-(1-methylpropenyl)-tetrahydrofuran (26), myrcenol (27) and the ocimenol (28) isomers (figure 3) rose from trace levels to become predominant constituents. As these same transformations have been verified in aged wines (Rapp and Mandery 1986) it has been concluded that the monoterpene polyols represent a pool of precursor compounds present in the fruit which give rise to a variety of alcohols and oxides by means of facile acid hydrolysis at the juices pH.

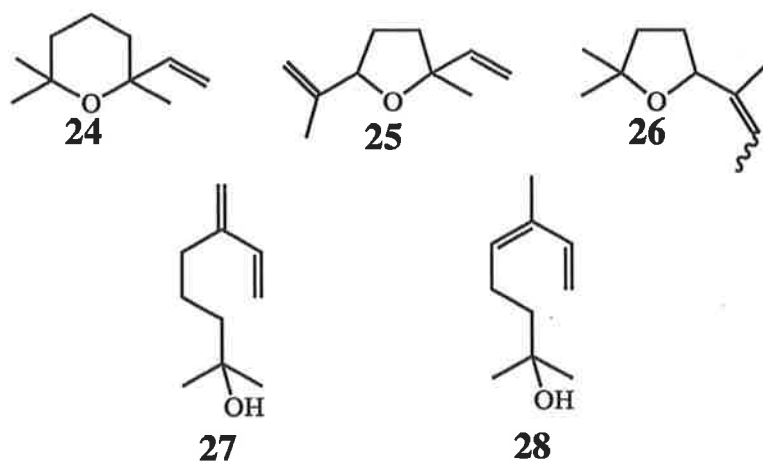


Figure 3: Monoterpenes induced by acid hydrolysis.

Recent research has established the presence of some *p*-menthenediols in must and/or wine. These compounds appear to be typical grape compounds because they were found either in free form, such as the isomers of *p*-menth-8-ene-1,2-diol (**29**) (Baumes *et al.* 1986) or, mainly, as glycosides which is the case for *p*-menth-1-ene-8,9-diol (**30**) (Winterhalter *et al.* 1990b), *p*-menth-1-ene-6,8-diol (**31**) (*cis*-isomer) and *p*-menth-1-ene-7,8-diol (**32**) (Versini *et al.* 1991). The identification of these compounds (figure 4) provides indirect evidence for the presence of allylic hydroxylase activity in grape berries. Another possible explanation for the presence of these *p*-menthenediols in grape juices and wines is the biotransformation of α -terpineol (**4**) by *Botrytis cinerea* (Rapp and Mandery 1988). When starting from α -terpineol (**4**), some *p*-menthenediols, including *p*-menth-1-ene-7,8-diol (**32**), were produced by *Botrytis cinerea* (Mandery 1986, Rapp and Mandery 1988). *p*-Menth-1-ene-7,8-diol (**32**), *p*-menth-1-ene-6,8-diols (**31**) (*cis*- and *trans*-isomers) are formed by *Nicotiana tabacum* callus suspension cells (Suga *et al.* 1980). Another possible α -terpineol derivative, 2-*exo*-hydroxy-1,8-cineole (**33**), has recently been identified (Bitteur *et al.* 1990) both free and glycosidically bound as a component of musts from different grape varieties.

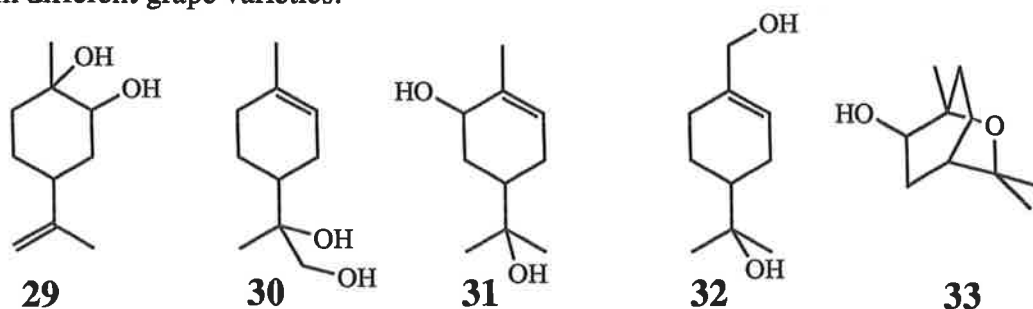


Figure 4: *p*-Menthenediols and α -terpineol derivatives.

1.2.3 Norisoprenoids

Terpenoid compounds with 9, 10, 11, and 13-carbon atoms are believed to originate from carotenoids by cleavage of 6-7, 7-8, 8-9 and 9-10 bonds (Enzell 1985; Kanasawud and Crouzet 1990) and are important to the flavour of many fruits as well as leaf products. In the intact plant, carotenoid degradation is likely to be effected by oxygenase systems (Schreier 1984b) and during post-harvest treatments by photo-oxygenations and other non-enzymatic oxidations (Enzell 1981).

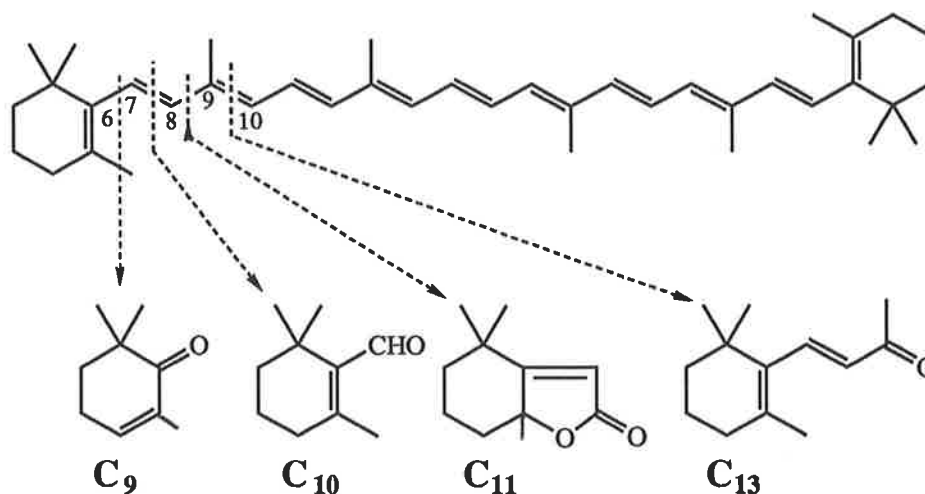


Figure 5: Carotenoid degradation.

Further enzymatic transformation of these carotenoid degradation products, or their transformation by acid hydrolysis, which may take place during the ageing of wines, can provide a diverse range of compounds which are characterized by highly attractive flavour properties often associated with low sensory threshold values (Enzell 1981). However, remarkably little is known about the immediate precursors of the norisoprenoid compounds and the reactions by which they are formed.

In contrast to the intensely flavoured muscats, the aroma of which is dependent on monoterpenes, there exist a large number of non-muscat varieties. Based upon their total content of monoterpenes, they have been differentiated into floral (e.g. Riesling), and non-floral varieties (e.g. Chardonnay). Floral varieties give juices with aromas which appear to be controlled by monoterpenes. Juices from non-floral varieties have quite subtle and often non-characteristic aromas. However, in many cases wines made from both floral and non-floral varieties develop distinctive sensory characters after

prolonged maturation. These sensory characters can be clearly distinguished from those contributed by monoterpenes, and depending on the style of wine and the maturation procedure, e.g. wood maturation or bottle ageing, are attributed to various classes of compounds (Rapp and Mandery 1986, Strauss *et al.* 1987b). Amongst the compounds regarded important to bottle bouquet are the norisoprenoids 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (34), (Bertuccioli and Viani 1976, Schreier *et al.* 1976, Simpson 1978a), vitispirane (35) (Simpson *et al.* 1977), β -damascenone (36) (Schreier and Drawert 1974), β -ionone (37) (Drawert and Rapp 1968, Schreier *et al.* 1976). These compounds as well as theaspirane (38) (Schreier *et al.* 1976), α -ionone (39) (Drawert and Rapp 1968), dihydroactinidiolide (40) (Schreier *et al.* 1976), dehydrovomifoliol (41) (Strauss *et al.* 1987c) and the recently identified, "Riesling acetal" (42) (Winterhalter *et al.* 1990c) are the norisoprenoid compounds most frequently observed as free volatiles in grape juices and wines. It should be mentioned, however, that norisoprenoids exist as free volatiles in grape juices and wines from both floral and non-floral varieties only in low absolute concentrations. The structures of the previously mentioned norisoprenoids are depicted in figure 6.

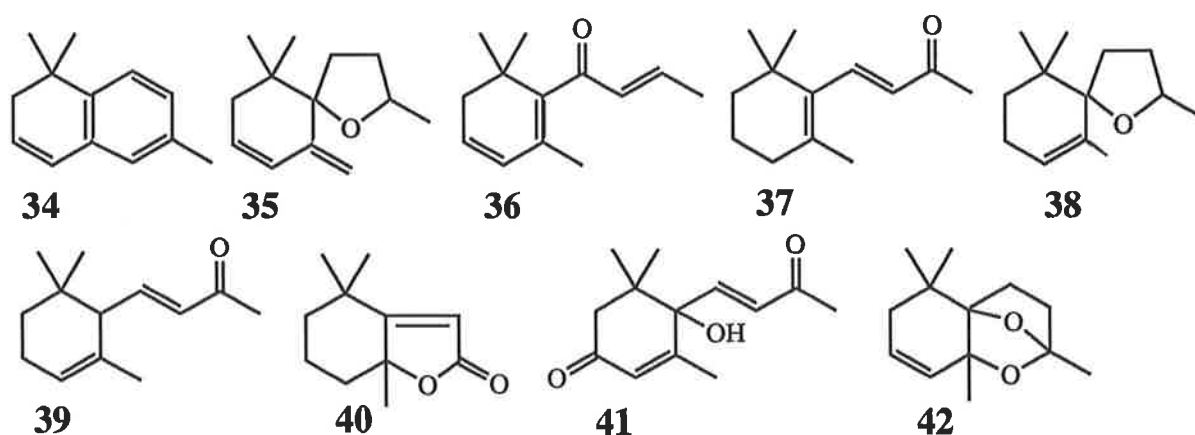


Figure 6: Norisoprenoids identified as free volatiles in grapes and wines.

The contribution of such compounds to the aromatic profile of essential oils, leaf products and fruits other than grapes has been discussed by Ohloff (1978). In the case of wines, GC-MS analysis together with GC-sniff aroma assessment experiments of the headspace aroma volatiles of Chardonnay wines from 6 consecutive vintages (Simpson and Miller 1984) showed β -damascenone (36) and β -ionone (37) to be major contributors to the aroma profile of these wines thereby imparting raisin-like and rose-like aromas, respectively. Vitispirane (35) and TDN (34) were also found present as headspace volatiles but in minor concentrations not exceeding the threshold values. TDN (34) is

considered to be a major contributor to the "bottle aged" character of aged Riesling wines as it was found to be the only norisoprenoid volatile exceeding its aroma threshold value in up to 15 year old Riesling wines (Simpson 1977, Simpson 1978b). Vitispirane (35) and β -damascenone (36) were also present as headspace volatiles of the aged Rieslings.

Unfortunately, the very low concentration in which norisoprenoids exist as free aroma compounds in juices and/or wines has only permitted the identification of a small number of them. The observation that the concentration of key-norisoprenoid volatiles (e.g. TDN, vitispirane) increases with ageing, and more dramatically in cases of wines with low pH (Simpson and Miller 1983), implied the presence of non volatile precursors. Thus, it was assumed that similar metabolic processes operate on the norisoprenoid aroma constituents of the non-floral varieties to those which transform the monoterpenes of the floral varieties, i.e. oxidative hydroxylation of some compounds, followed by conversion of these to odourless glycosides (P. J. Williams *et al.* 1989). The flavour precursor analysis approach has since been used as a strategy to investigate, indirectly, the minute quantities of free norisoprenoid flavourants which are responsible for the varietal character of these premium varieties.

The first evidence for the occurrence in wine of a pool of compounds capable of generating additional amounts of norisoprenoid volatiles came from results published by P. J. Williams *et al.* (1982a). Acid hydrolysis at pH 3.0 of non-volatile material isolated with a C₁₈ reversed phase column, revealed the existence of many unknown compounds which were assigned as norisoprenoids from mass spectral data. In addition, the results of that experiment also revealed the existence of precursors of the key norisoprenoid grape volatiles, TDN (34), vitispirane (35) and β -damascenone (36).

TDN (34) is regarded as degradation product of carotenoids and such a genesis may well be the case in wines. Nevertheless, it is possible that the formation of 34 involves extensive rearrangements under acidic conditions and its presence may not be diagnostic of specific precursors. This hypothesis has been supported by Strauss *et al.* (1987d) and Winterhalter *et al.* (1990a) who showed that the naphthalene derivative TDN (34) is formed hydrolytically from multiple glycosidic precursors. Glycosidic forms of 2,6,10,10-tetramethyl-1-oxaspiro[4.5]dec-6-ene-2,8-diol (43) (figure 7) have been demonstrated as being some of TDN precursors in Riesling wine (Winterhalter 1991).

Vitispiranes (**35**) have been identified amongst the acid hydrolysis products of a glycosidic fraction isolated by a C₁₈ reversed-phase column procedure (Strauss *et al.* 1984). These results supported the assumption that glycosidic precursors of this compound are present in grapes and wines and suggested that its formation is hydrolytic rather than oxidative. The results of hydrolytic studies on a synthetic sample of 3-hydroxytheaspirane (**44**), i.e. conversion to diastereoisomeric vitispiranes (Strauss *et al.* 1984), together with the observation of 3-hydroxytheaspirane as an aglycone after enzyme treatment of C₁₈ reversed-phase material, suggested glycosidically bound forms of 3-hydroxytheaspirane (**44**) as precursors of vitispirane. In recent studies of vitispirane precursors (Winterhalter *et al.* 1990a, Waldmann and Winterhalter 1992), the detection of unspecified bound forms of megastigma-5-ene-3,4,9-triol (**45**) and 1-(3-hydroxybutyl)-6,6-dimethyl-2-methylen-3-cyclohexen-1-ol (**46**) in C₁₈ reversed-phase isolates that gave vitispirane under simultaneous distillation extraction (SDE) conditions, indicated that both species are implicated in the formation of the latter compound in wine (figure 7).

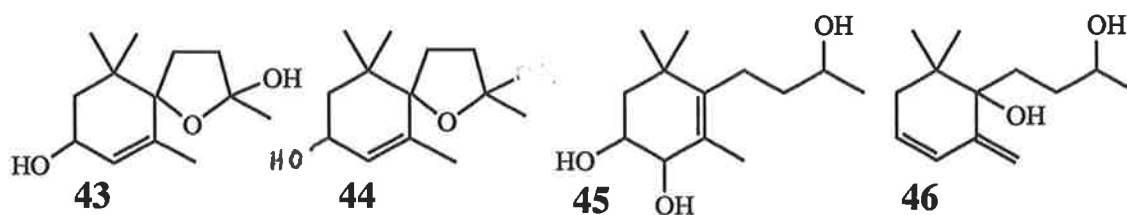


Figure 7: TDN and vitispirane precursors in wine.

Work undertaken by Masuda and Nishimura (1980) indicated the presence of a precursor of β -damascenone (**36**) in grapes. This was confirmed when acid hydrolyses of a glycosidic fraction from grape juice, isolated by C₁₈ reversed-phase column procedure, generated β -damascenone (Williams *et al.* 1982a). Ohloff *et al.* (1973) and Ohloff (1978) proposed that the chemically related compounds β -damascenone (**36**) and 3-hydroxydamascone (**47**) are presumably formed from allenic triol (**48**) via acetylene diol (**49**). Isoe and co-workers (1973) also proposed the allenic triol (**48**), which is presumably formed by reduction of the grasshopper ketone (**50**), as the precursor of β -damascenone in nature. These schemes were supported by experiments on the floral variety Riesling (Winterhalter *et al.* 1990a) and the non-floral varieties Chardonnay, Semillon and Sauvignon Blanc (Sefton *et al.* 1989) which showed the megastigmane (**49**) along with compounds (**36**), (**47**), and (**50**) as constituents of grape juices from these premium varieties (figure 8). The studies by Sefton *et al.* (1989) have also demonstrated that the acetylene diol (**49**) is a precursor to β -damascenone during wine conservation.

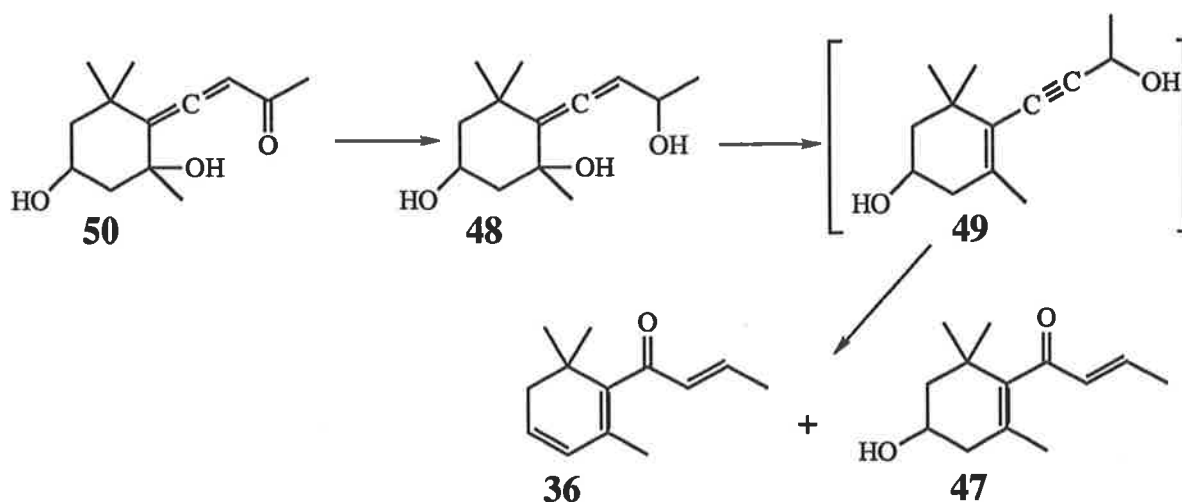


Figure 8: β-Damascenone precursors.

Another group of norisoprenoid compounds commonly observed as volatiles of bottle aged white wines, brandies and heated muscat grape juices are diastereoisomers of the actinidols (**51**) (Dimitriadis *et al.* 1985). GC-MS analyses of extracts of steam distillates of juices of *Vitis vinifera* grape varieties Muscat of Alexandria, Chardonnay and Doradillo (Dimitriadis and Williams 1984, Dimitriadis *et al.* 1985) showed that actinidols exist in four isomeric forms. Importantly, their origin in grapes can be traced to triols (**52**), via the intermediate (**53**) - figure 9 - which are present as glycosidic derivatives in the fruit (Williams *et al.* 1985, Strauss *et al.* 1986b).

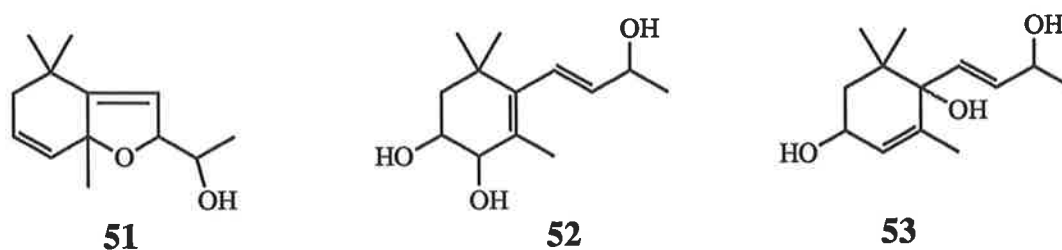


Figure 9: The actinidols and actinidol precursors.

Further applications of the flavour precursor analysis approach to the examination of C18 reversed-phase isolated glycosidic material from the grape varieties Gewürztraminer, Semillon, Shiraz, Sauvignon Blanc, Riesling, and Chardonnay (Strauss *et al.* 1987a, Strauss *et al.* 1987c, Sefton *et al.* 1989, Winterhalter *et al.* 1990b, Sefton *et al.* 1992a) led to the identification of a number of norisoprenoid compounds in both free and conjugated forms. These included compounds (**53**)-(76) the structures of which are depicted in figure

10. They were identified as grape and wine constituents for the first time during these studies. The results of these studies revealed that although Riesling is a floral variety in which monoterpenes are major volatile aroma compounds (P. J. Williams *et al.* 1987), volatile norisoprenoids were found in the conjugated fraction at a concentration 40% higher than that of the monoterpenes. Additionally, this research indicated that just as muscat grape varieties are monoterpene dependent for their flavour, non-floral premium varieties such as Chardonnay and Semillon can be categorized as norisoprenoid dependent (P. J. Williams *et al.* 1989, Sefton *et al.* 1992a). These findings, in conjunction with the well recognised aroma properties of volatile norisoprenoids (Ohloff 1978), clearly indicate that greater attention should now be directed to this group of compounds and the extent to which norisoprenoids contribute towards grape and wine aroma. Furthermore, an understanding of biogenetic origin of norisoprenoids would give a better understanding about their chemical interrelationships and also about the possibility of manipulating norisoprenoid content of grapes and consequently their contribution to wine flavour by viticultural means. A well accepted hypothesis (Razungles *et al.* 1988) for the formation of norisoprenoids suggests they arise from the degradation of carotenoids. This proposal is based on the fact that the concentration of carotenoids decreases after veraison and also during ripening. It has been postulated further that carotenoids present in the skins degrade in order to form compounds which can in turn be glycosylated to facilitate their transportation into the cell cytoplasm. A rationalization for the derivation of the major norisoprenoids observed in grape juice and wine has been given by Williams *et al.* (1992a). The proposed mechanism involves enzymatically catalyzed oxidative cleavage of the 9,10 and 9',10' double bonds of the possible parent carotenoids lutein, violaxanthin, antheraxanthin, neoxanthin (Weeks 1986, Enzell 1985, Eugster and Märki-Fischer 1991). This leads to four ketones (77), (72), (73), (50) as primary metabolites and of these only 3-hydroxy- α -ionone (77) has not been found as a grape glycoside hydrolysis product. The rest of the products that have been observed are derivable from these four ketones by a series of oxidation, dehydrogenation, reduction or elimination reactions.

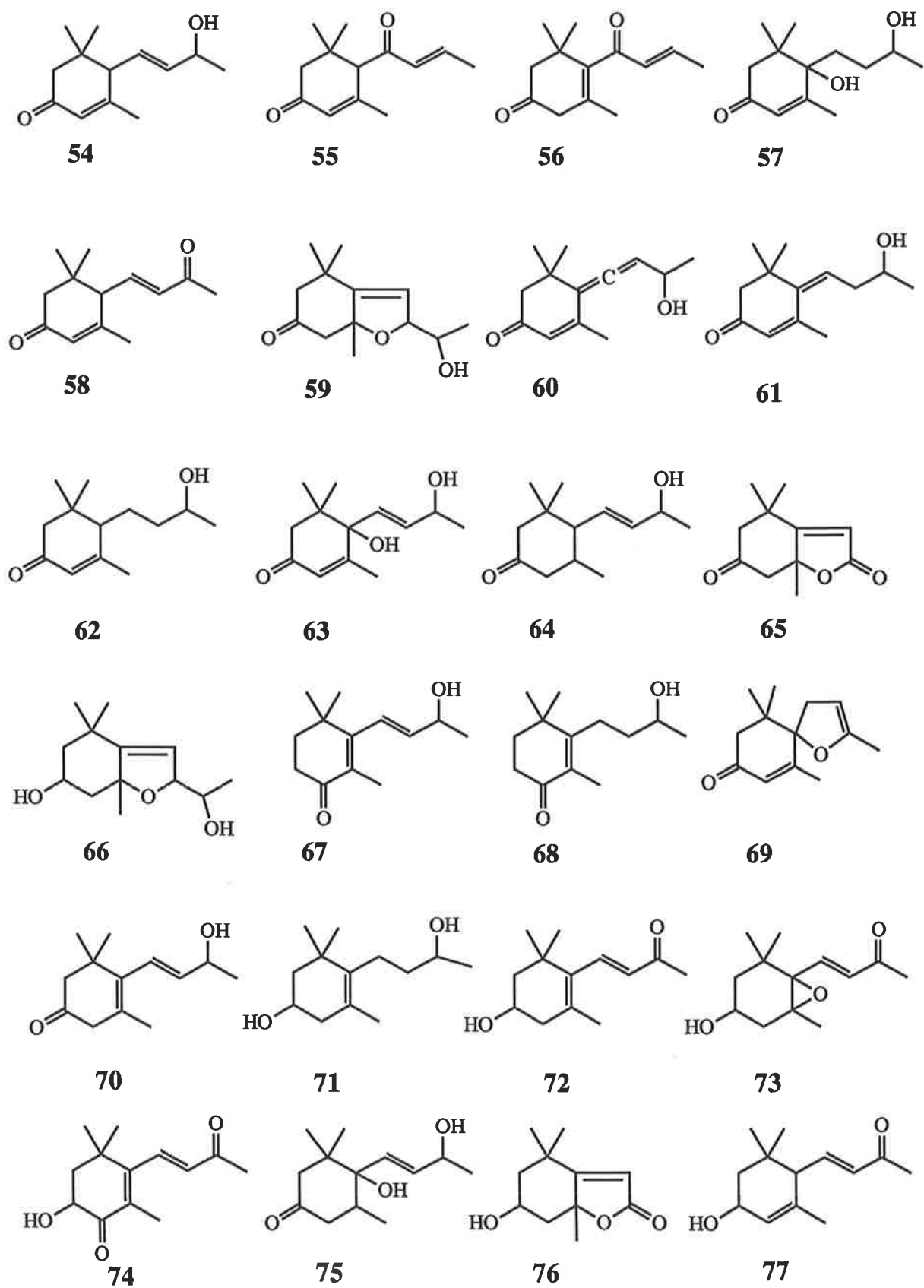


Figure 10: Norisoprenoids identified in the enzymic hydrolysate of glycosidic isolates of premium varieties.

1.2.4 Shikimic acid derived volatiles

Metabolism via the shikimate pathway gives rise to a large number of aromatic compounds. Many of these compounds are polyphenols, and usually possess a characteristic substitution pattern; *p*-hydroxy-, *o*-dihydroxy-, or 1,2,3-trihydroxy-. These products are known to be derived in plants from phenylpropanoids via side-chain degradation and elongation reactions (Mann 1980b, Gross 1981).

Volatile phenols in wines have been reviewed by numerous authors (Singleton and Noble 1976, Schreier 1979, Dubois 1983, Nykänen and Suomalainen 1983, Etiévant 1991). Since they were not, generally, detected in grape juices, the origin of shikimate-derived phenols identified in wines had been ascribed by several authors as coming from the metabolism of some precursors (Singleton & Noble 1976, Schreier 1979, Etiévant 1981, Dubois 1983). Two different pathways were described to explain the presence of volatile phenols in wines. The first involves microbiological action during fermentation to form volatile phenols from their corresponding phenolic acids (Etiévant 1991). The second pathway is chemical and explains the presence of volatile phenols as coming from the degradation of lignin in barrels. This occurs in the case of maturation of red and white wines in oak barrels (Nishimura *et al.* 1983). Experiments performed on white wines (Dzhakhua *et al.* 1978) indicated that volatile phenols such as *m*-cresol (78) could also originate from cluster stems during alcoholic fermentation. Güntert *et al.* (1986) first suggested that some volatile phenols could arise directly from the natural grape material, although their analytical data could not confirm this hypothesis. The identification of shikimate-derived volatile phenols in the hydrolysate of C₁₈ reversed-phase isolates of grape juices and wines (Strauss *et al.* 1987a, P. J. Williams *et al.* 1989, Winterhalter *et al.* 1990b) unequivocally established grapes as a source of volatile phenolic material for grape juices and wines. Figure 11 illustrates some of the volatile phenols identified in grape juices and wine.

Certain volatile phenols are considered as important contributors to wine flavour because they are known to have odorous and pungent properties (Singleton and Noble 1976, Etiévant 1981, Dubois 1983, Williams *et al.* 1988). However, the only sensory parameters reported on these compounds are odour descriptors and odour threshold values in wine or ethanolic solutions (Etiévant 1991). In the few cases where instrumental analyses were accompanied by sensory data, it was evident that the concentration of total volatile phenols in wine was sufficiently great to significantly change its aroma (Etiévant 1981)

and that the concentration of individual phenols such as 4-vinylguaiacol (**79**) or 4-ethylguaiacol (**81**) and 4-ethylphenol (**82**) could also be correlated with typical aroma properties of red wines (Versini 1985, Etiévant *et al.* 1989).

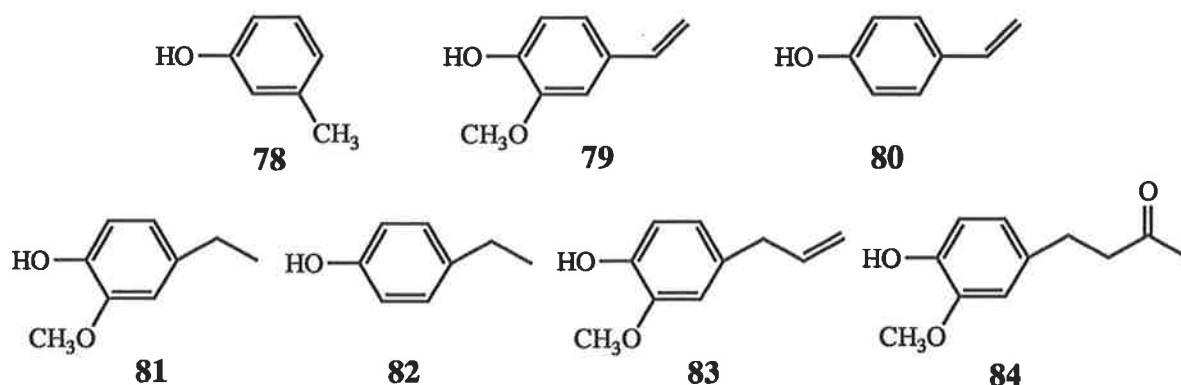


Figure 11: Shikimate-derived volatile phenols identified in juices and wines.

However, to positively establish the contribution of volatile phenols to wine flavour more sensory work needs to be performed on individual as well as mixtures of phenolic wine substances (Chatonnet *et al.* 1990). From a simple comparison of published data such as the threshold values of the volatile phenols identified in wine with their concentration levels (Etiévant 1981, Etiévant 1991), odorous phenols appear to be below their individual flavour thresholds in most wines, but because of the similarity of some of their descriptors, they are probably additive and give sensory effects as a group. According to the aroma properties of each individual volatile phenol, they can be classified into three categories (Dubois 1983). The first category includes the trimethoxyphenol and dimethoxyphenols which are present in very small amounts in addition to possessing weak odours. This category of compounds is considered as not being able to influence wine flavour. The second category embraces all phenol- derivatives, such as 4-ethylphenol (**82**), which all have strong medicinal odours. Lastly, the guaiacyl derivatives can be differentiated as a separate group. These compounds have typical clove-, smoke- or vanilla odours dependent on the substituent para to the hydroxy group. Amongst compounds of the second and third group, 4-ethylphenol (**82**), 4-vinylphenol (**80**), 4-ethylguaiacol (**81**) and 4-vinylguaiacol (**79**) have been described as the most potent contributors to wine flavour (Singleton and Noble 1976, Schreier *et al.* 1980, Etiévant 1981).

Pungency is considered a "hot", penetrating, burning sensation in the mouth which at lower levels may be "warm", spicy, sharp or harsh. Many phenols have a pungency component in their flavour and notable pungents include eugenol (83) from cloves (Singleton and Noble 1976) and zingerone (84) from ginger oleoresin (Connell 1970) both of which have also been found in wine (Williams *et al.* 1988, Winterhalter 1990b).

1.3 Glycosidically bound secondary metabolites

1.3.1 Biological formation of glycosides

Many secondary products do not occur in plants in the free form but are conjugated with a variety of monomeric or oligomeric saccharide moieties. Aliphatic and phenolic hydroxyl groups, carboxyl functions, and amino and mercapto groups of aglycones are involved in conjugation reactions. C-glycosides must also be considered in this category although they have somewhat different chemical properties (Barz and Köster 1981). The formation of glycosides in plant tissue can be described by the general transglycosylation scheme illustrated in figure 12. The conjugating agent (generally a glucopyranosyl unit) is attached through an α -glycosidic linkage to a nucleoside diphosphate (X). Transglycosylation from the glycosylation agent to the secondary metabolite aglycone A is always catalyzed by transferases utilizing nucleotide-activated sugars (NDP-sugar), usually uridine diphosphate-D-glucose (UDP-G) (Hösel 1981, Goodwin and Mercer 1983). UDP-G is an α -transfer agent which leads to the formation of a β -glucoside.

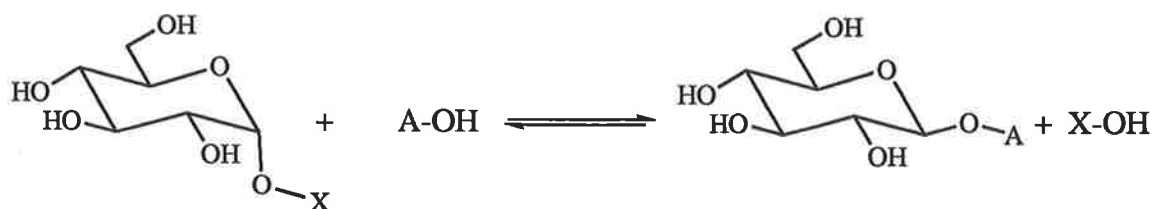


Figure 12: Transglycosylation reaction for the formation of glycosidically bound secondary metabolites.

From a physiological point of view, conjugation reactions are of importance for a number of reasons (Barz and Köster 1981): a) they greatly alter the physical (e.g. solubility and volatility) or physiological (e.g. transport through cells or membranes and biological activity) properties of secondary metabolites; b) they may result in a site of accumulation different to that occupied by the aglycone thus resulting in the formation of several

interrelated metabolic pools (Ackermann *et al.* 1989); c) the conjugated compound may enter a different metabolic pathway to the corresponding aglycone; and d) they can determine whether a compound is a metabolically active species or a metabolically inactive (end) product. Therefore, conjugation reactions are considered an important way to store materials or to detoxify unwanted material (Barz and Köster 1981, Hösel 1981, Croteau 1984).

Although the mechanism of glycosylation is understood, the trigger for this conjugation reaction in plants is not known. There is evidence that glycosylation occurs late in the biosynthetic pathway of secondary metabolites, e.g. after flowering in the leaves of peppermint plants (Croteau and Martinkus 1979, Croteau 1984) and after veraison in grape berries (Wilson *et al.* 1984). Recent evidence suggests that glycosylation takes place after extra hydroxyl groups are introduced into a monoterpene skeleton (Strauss *et al.* 1988). These observations are consistent with the hypothesis that glycosylation is the terminal step of such biosynthetic pathways (Hösel 1981). Contrary to these findings, Ackermann *et al.* (1989) have demonstrated that for two rose species, *Rosa damascena* and *Rosa gallica*, the concentration of glycosidically bound compounds was greatest at the early stage of the flowering, and decreased as flowers developed; at the same time an increase in the concentration of free volatiles was also observed. The mechanism causing these differences in the time and pattern of glycoside accumulation for these plant species is not known. Studies of the enzymes involved in the conjugation reactions would provide an understanding of how glycosides accumulate. As mentioned earlier, glycosylation alters the volatility of secondary metabolites by transforming them from volatile aroma constituents to odourless glycoconjugates. This transformation of secondary metabolites via glycosylation represents a flavour loss for fruits in general. Thus, the study of the enzymes involved in the conjugation reaction would also produce a possible means by which glycosylation and, thus, flavour loss of fruit may be controlled.

1.3.2 The composition of glycoconjugated precursors in fruits

Sensory evaluation experiments demonstrated that addition to a wine of glycosides isolated from three non-floral grape varieties did not alter the aromatic profile of the wine (P. J. Williams *et al.* 1989). Therefore, it was concluded that wine glycoconjugates are odourless. Glycosidically bound secondary metabolites may have a bitter taste, and thus, may be capable of directly affecting grape juice and wine flavour. There are examples of synthetic (Fischer and Helferich 1911), and naturally occurring monoterpene glycosides

(Hase *et al.* 1982, Sakata and Iwamura 1979) that have been described as bitter. However, sensory evaluation experiments involving the addition of the glycosidic fraction of a muscat wine which exhibited bitter characteristics to a wine medium, demonstrated that the glycosides, as a group, did not contribute to the bitter taste of that muscat wine (Noble *et al.* 1988). Similar experiments involving the glycosidic fraction of a Chardonnay wine, verified the tasteless properties of wine glycosidic fractions (Noble *et al.* 1987). Furthermore, Fong *et al.* (1991) suggested that glycosylation may well be a natural debittering process. This suggestion was based on the observation that limonoids, the cause of bitterness in a variety of citrus fruits, decreases during maturation while the concentration of limonoid glycosides, which have been quoted by Fong *et al.* (1991) as non-bitter components, increases. Although it has not been unequivocally proven whether all glycosidically bound secondary metabolites can directly influence the flavour or not, it has now been well established, at least in the case of grape and wine, that glycosidically bound secondary metabolites are capable of indirectly influencing flavour by acting as flavour precursors (P. J. Williams *et al.* 1989, Abbott *et al.* 1991). This capability of grape juice and wine glycosides justifies the increased scientific interest in their analysis because structural elucidation may lead to an understanding of their sensory aspects. Apart from this oenological perspective, structural elucidation of glycosidic secondary metabolites is dictated by another reason. The biochemical and physiological properties of many secondary products cannot be understood unless their conjugation moieties are known. Analysis of plant extracts for aglycones alone will not reveal the full complexity and possible conjugate-caused compartmentation of secondary metabolism (Barz and Köster 1981).

Studies into the bound forms of volatile secondary metabolites of fruits demonstrated that β -D-glucopyranosides predominate. Comprehensive compilations of the literature on this topic has been presented by Williams (1992) and Williams *et al.* (1992b). Glucopyranosides of grapes, apples, quince and apricot have often been shown to be further substituted at position 6 of the glucose with a second sugar unit. To date the second sugar unit of fruit disaccharides have been identified as α -L-arabinofuranose, α -L-rhamnopyranose (P. J. Williams *et al.* 1982b), β -D-apiofuranose (Voirin *et al.* 1990a) in grapes and α -L-arabinofuranose, β -D-xylopyranose (Schwab and Schreier 1990a) in apples. Recently, gentiobiosides (Güldner and Winterhalter 1991, Winterhalter *et al.* 1991) and a trisaccharide glycoside (Herderich *et al.* 1992) have been identified for the first time in quince and apple fruits, respectively. The glycosides (sugar and aglycone moieties) that have been identified in *Vitis vinifera* are listed in table 1.

Table 1: Glycosidically bound volatile secondary metabolites identified in grapes and wines.

Aglycone	β -D-glucopyranosides	α -L-arabinofuranosyl- β -D-glucopyranosides	α -L-rhamnopyranosyl- β -D-glucopyranosides	β -D-apiofuranosyl- β -D-glucopyranoside
Linalool ^a		√	√	
Geraniol ^{a, e}		√	√	√
Nerol ^a		√	√	
α -Terpineol ^a		√	√	
(E)-2,6-Dimethylocta-2,7-diene-1,6-diol ^b	√	√		
Furan linalool oxide ^{b, f}	√	√		
Benzylalcohol ^c	√	√	√	
Phenylethanol ^c	√	√	√	
Vomifoliol ^d	√			
6,9-Dihydroxymegastigm-7-en-3-one ^g	√			

Note: Data compiled from: *a.* P. J. Williams *et al.* 1982b; *b.* Strauss *et al.* 1987a; *c.* Williams *et al.* 1983; *d.* Strauss *et al.* 1988; *e.* Voirin *et al.* 1990a; *f.* Winterhalter *et al.* 1990b; *g.* Sefton *et al.* 1992b.

1.4 The analysis of flavour precursors

It is evident from the literature reviewed in the previous sections that although a great number of aroma secondary metabolites have been demonstrated to exist as glycoconjugates in grapes and wine, only a few of these glycosides have been isolated and their structure characterized unequivocally (see table 1). This is due to a number of reasons. Primarily, it is because grape juice and wine flavour precursors are present as very complex mixtures. The number of terpenoids and of shikimate derived metabolites that have already been identified as aglycone moieties of grape and wine glycosides highlights the complexity of these glycosidic mixtures. The observation in grape and wine glycosidic isolates, of aglycones being conjugated with more than one sugar moiety (Winterhalter *et al.* 1990b), adds to the complexity and the diversity of grape and wine glycosidic mixtures. The other reasons are associated with the deficiencies of the analytical techniques within the flavour precursor analysis approach. Most glycosidically

bound secondary metabolites are highly polar, in addition to being labile at the temperatures needed to volatilise them due to the presence of the sugar moiety. These properties cause the most serious problems for scientists analysing flavour precursors spectrometrically. These problems are:

- 1) difficulty in gas chromatographic analysis as the currently commercially available capillary columns are of limited polarity, making separation of very polar compounds, such as wine glycosides, impossible by GC;
- 2) difficulty in the structural analysis of glycosides using the conventional mass spectrometric techniques which require thermal volatilization of the sample prior to ionization. In the later case the problem is a combination both of the polarity and thermal lability of glycosides because they degrade at the temperatures needed for volatilization.

These problems have been partially overcome by application of derivatization techniques. Derivatization in mass spectrometry achieves mainly two objectives.

- 1) It tailors the physical properties of the analyte to the requirements of the analytical method. For direct probe or GC-MS, derivatization confers enhanced volatility and/or stability. Examples include trimethylsilylation, permethylation and peracetylation.
- 2) It introduces particular chemical features into the analyte. Examples include the introduction of a group with low ionization potential to increase molecular ion intensity, as in the case of flavazole derivatives of oligosaccharides (Johnson *et al.* 1971) or of a group of high electron affinity to promote formation of negative ions, as in the case of pentafluorobenzyl derivatives of amphetamines (Hunt and Crow 1978).

Unfortunately, derivatization is not universally applicable to all polar and thermally labile molecules because of problems associated with:

- a) the derivatization techniques, i.e. difficulties in the preparation, loss in sensitivity, increase of the molecular weight and difficulties in the interpretation of complex mass spectra;
- b) the presence of numerous secondary metabolites of such high polarity that even their derivatives are not amenable to direct probe or GC-MS examination.

Apart from the above mentioned problems, the most common MS ionization technique, electron ionization (EI), causes extensive fragmentation of molecules, and thus, rarely permits observation of the corresponding molecular ions; the latter are often critical for the structure elucidation of glycoconjugates.

1.4.1 New dimensions in flavour precursor analysis

To overcome the direct probe and GC-MS limitations described above various alternative strategies have been developed for the analysis of non-volatile or thermally labile samples. The key to these approaches is the desorption of ions directly from a condensed phase (liquid or solid). Desorption ionization (DI) is a general term which embraces the techniques of field desorption ionization (FD), electrohydrodynamic ionization (EHDS), thermal desorption ionization (TD), secondary ion mass spectrometry (SIMS) and fast atom bombardment (FAB) (Busch *et al.* 1982, Busch and Cooks 1982). Desorption ionization is markedly different from EI or CI techniques in that it dispenses with bulk sample vaporization. In essence, energy in a variety of forms is deposited in the sample causing molecules and pre-existing ions to transfer from the condensed into the gas phase. The simplicity of the underlying phenomenon and the ability of delicate biomolecules to withstand the impact of the energetic particles beam are two striking features of DI. The various DI methods differ in the rate of energy deposition, the volume of energized sample and sometimes in the use of electric fields to assist desorption. As the energy deposition is significantly less, compared to the conventional ionization techniques, the compound under examination fragments in a much "softer" way, and thus, the observation of the molecular ion is possible. It is this characteristic that led scientists to name these techniques "soft ionization techniques". To date, soft ionization techniques, other than FAB, have been applied to the elucidation of the structure of biologically important compounds such as peptides, antibiotics, and oligosaccharides (Schulten 1979, Busch and Cooks 1982, Aubagnac *et al.* 1983) which present the same features of high polarity and thermal lability as glycoconjugates in grape juice and wine. The application of these techniques has been characterized by various degrees of success in routinely achieving good mass spectra.

Further advances in the study of desorption ionization techniques and problems with their practical application led to the development of a new desorption liquid matrix assisted ionization technique which also utilizes a fast atom primary beam. It has been named by the scientists who developed it as fast atom bombardment mass spectrometry (FAB-MS) (Barber *et al.* 1982). The introduction of FAB-MS (Barber *et al.* 1981a, Barber *et al.* 1982) along with the improved mass ranges of magnetic sector and quadrupole instruments has (during the past 10 years) opened new horizons for the analysis of glycoconjugates and other polar and nonvolatile compounds of biological importance. This progress has been documented in a series of reviews (Rinehart 1982, Reinhold and

Carr 1983, Dell and Taylor 1984, Egge and Katalinic 1987). The progress has been especially remarkable in the field of glycoconjugates. With the aid of FAB-MS it has been possible to obtain data on such structural parameters as molecular mass, sequence, and the linkage type, of complex glycoconjugates. It must be stressed that these highly reproducible data were obtained on comparatively small amounts of material in a very short time. Thus, FAB-MS has set new standards for the analysis of glycoconjugates. The instrumentation, the principals and the application of this technique to the examination of glycosidic material originating from sources other than grape juice and/or wine are examined in the following section.

1.5 Fast atom bombardment-mass spectrometry (FAB-MS)

Fast atom bombardment mass spectrometry is a relatively old technique although it is often thought of as a novel mass spectral method. As early as 1966, Devienne and Grandclément presented data on a technique that they called molecular beam for solid analysis (MBSA). The idea was further developed by the same group (Devienne 1967, Devienne and Roustan 1973, Devienne 1974) but was largely ignored until the rediscovery of FAB (Surman and Vickerman 1981, Barber *et al.* 1981b).

In essence, FAB-MS is the result of the combination of two techniques whereby the sample is presented in a liquid matrix and then is bombarded by a neutral primary beam, which causes the production of a secondary ion beam. Thus, the requirement of the conventional mass spectrometric techniques for sample volatilization prior to ionization is avoided. This combination has proven successful for analytical work and, thus, has rapidly come into use.

A number of symposia and workshops devoted in whole or in part to the understanding of FAB-MS and its applications followed (see Pare *et al.* 1988 and references cited therein). Further than these efforts, only a few reports dealing with FAB-MS fundamentals have been presented up to date (Devienne and Roustan 1982, Dell 1987, Pare *et al.* 1988), although the literature describing its applications has seen an explosive growth.

1.5.1 Instrumentation and principles

In a typical FAB-MS experiment, a fast atom beam of a rare gas produced in an atom gun is fired towards the target which has been pre-loaded with the matrix containing the

sample to be analyzed (i.e. analyte). When the atom beam collides with the matrix, kinetic energy is transferred to the surface molecules, many of which are sputtered out of the liquid phase into the high vacuum of the ion source. A significant number of these molecules are ionised during the sputtering process. Thus, gas-phase ions are generated without prior volatilization of the sample. This secondary ion beam is introduced into the mass spectrometer by an extraction plate and it is focused into the molecular slit of the mass analyzer by a centering plate. The separation of ions according to their mass to charge ratio (m/z) and subsequently their detection and recording is done as in conventional mass spectrometry.

From the above discussion it is clear that the hardware for a FAB ion source must consist of the following parts.

- 1) An atom gun which could be either mounted on the source housing of the mass spectrometer or, if it is small enough, inside the housing of the source itself.
- 2) A sample inlet where a sample probe can be inserted. At the end of the probe must be attached a small metal target onto which the sample can be loaded.
- 3) Suitable source-optics for the efficient extraction of ions into the analyzer of the mass spectrometer.

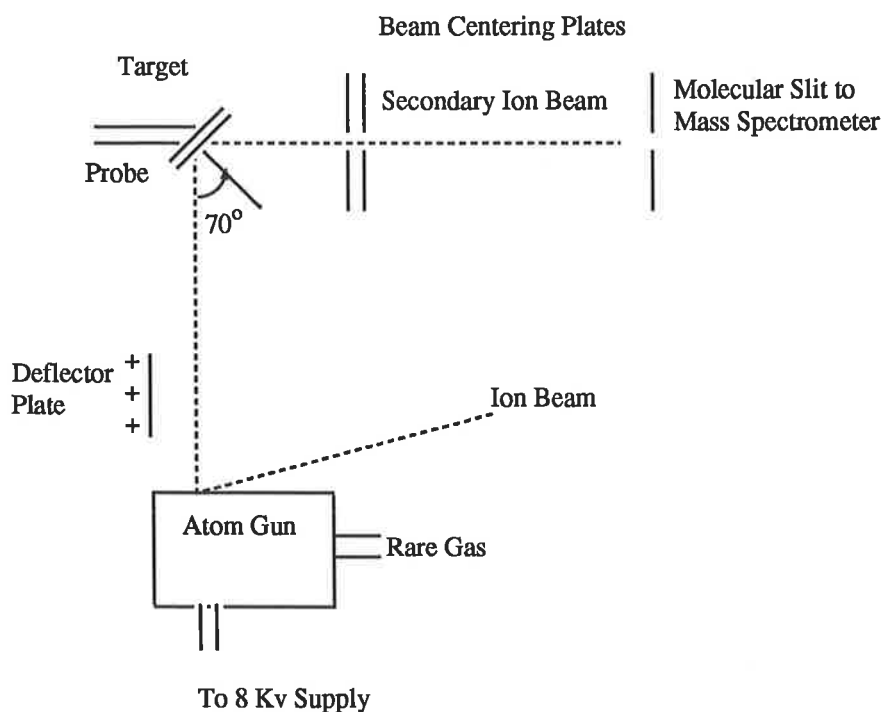


Figure 13: Overall diagram of a FAB-MS ion source (adapted from Pare *et al.* 1988).

The atom gun is made up of an evacuated chamber that encloses a plate to which a high voltage potential (nominally 8kV) is applied. The gas to be used as the bombardment gas (Xe, Ar, Kr) is allowed into the chamber where it is ionised by the high potential plate. This primary ion beam so created is focused into a high pressure (ca.10-30 torr) exchange chamber containing the same rare gas. Resonant charge exchange occurs with little loss in forward momentum of the primary ions and produces the desirable fast beam of rare gas atoms with controllable kinetic energy (Lew 1967) in the region of 3-10 keV. Electrostatic deflector plates, positioned at an angle of 90° with respect to the atom beam, cleanse the residual ions from the atom beam, i.e. the particle beam coming out of the exchange chamber contains about 30% atoms (Barber *et al.* 1983).

A typical sample probe is made of a metallic holder at the end of which is attached a copper or stainless steel tip (Martin *et al.* 1982). The sample is loaded onto the tip of the probe, along with a support matrix, which is inserted into the ion source of the mass spectrometer at a location such that it intercepts the incoming beam of fast neutrals from the atom gun (see figure 13). The angle of incidence of the beam is of importance; 70° angle (i.e. 20° with respect to the sample) is desirable (Hunt *et al.* 1981, Barber *et al.* 1981c, Martin *et al.* 1982).

Lastly, the extraction and centering plates which have been previously referred to, comprise what has been defined as suitable source-optics.

In relation to the instrumentation for the technique, it is not expected that the type of analyzer and detector used in the mass spectrometer should be critical for the successful recording of FAB spectra and, thus far, the literature supports this statement. Quadrupoles, single- and double-focusing magnetic instruments, as well as triple analyzers have been used (Busch and Cooks 1982).

1.5.2 The Sputtering phenomenon

The sputtering phenomenon always held out the promise of being used as a general solid-state ionization technique. The phenomenon was first reported by Grove in 1852 (as cited in Garler and Colligon 1968). It can be described very simply. If a solid is bombarded by high velocity particles (e.g. rare gas ions of about 8 keV energy) then material can be removed into the gas phase. This results from momentum transfer from the impinging particle to the sample atoms with the setting up of collision chains, some of which cross

the surface (Castaing and Slodzian 1962) as is illustrated in figure 14. Some of the sputtered material will be in the form of positively or negatively charged ions, and it is the mass spectrometric analysis of these species that is of interest.

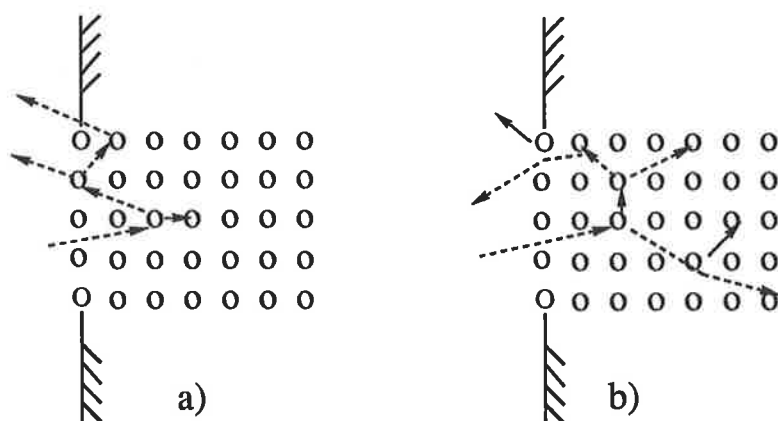


Figure 14: Two regimes of sputtering by elastic collisions: a) "direct-knock-on" regime. Recoil atoms from projectile-target collisions are ejected directly; b) "linear cascade" regime. Recoil atoms from projectile-target collisions generate higher-order cascades of recoil atoms which are then ejected (figure adopted from Magee 1983).

Although there is not a great deal of knowledge about the fundamentals of FAB-MS, the mechanism of particle emission from a surface has been studied in some detail (Heyes *et al.* 1981). The successful production of intact molecules or ionic species representative of the analyte under examination seems to involve four different processes (Cooks and Busch 1983).

- 1) Isomerization (loss of identity) of the input energy.
- 2) Desorption of preformed ions or intact molecules.
- 3) Ion/molecule reactions such as cationization occurring in the selvedge region.
- 4) Dissociation of energetic particles (metastable) ions well-removed from the surface.

However the emission of large, intact, parent molecular species is primarily dependent upon:

- 1) the nature of the momentum-transfer process (direct knock-on or linear cascade regime);
- 2) the amount of radiation damage incurred by the uppermost monolayer of the sample from which the molecules are emitted (Magee 1983).

1.5.3 Matrix support.

In early experiments charged particle sputter sources were used and the sample was deposited as a solution onto the probe and the solvent was evaporated to dryness prior to analysis. This method of preparation resulted in mass spectra of transient nature. On the other hand, the use of atom fluxes sufficient to give adequate sputter ion yields led to sample lifetimes of tenths of a second. The observation for the first time by Barber *et al.* (1982) that low vapour pressure liquids and oils give spectra that last sometimes for hours (e.g. Apiezon oils, Santovac 5 and Convalex 10) led them to study the use of solutions of analytes in low vapour pressure and viscous solvents to mimic this fluid behaviour with solids. The result of this study was the introduction of the technique of presenting solid samples dispersed or dissolved in such viscous, low vapour pressure liquids called matrices. Nowadays, it is well-known that the combination of the above technique with the sputtering phenomenon led to the introduction of FAB-MS.

The first successful matrix found was glycerol. It is a viscous, polar, low vapour pressure liquid which also has surfactant properties. Glycerol gave enhanced sensitivity compared to solid sample preparation and lifetimes of the sample which could be expanded to hours. Today, glycerol is the most commonly used matrix in FAB mass spectrometry and a number of classes of organic compounds have been successfully investigated by FAB-MS in their underivatized form using glycerol as a matrix (Barber *et al.* 1982). Compounds such as oligosaccharides (up to trisaccharides), peptide antibiotics (e.g. vancomycin), glyco-peptides (e.g. bleomycin), and glycoside antibiotics are included in those categories. Despite this expanded applicability, glycerol is not suitable for the examination of all classes of compounds, especially those that are very hydrophobic or those that are inclined to form aggregates when dissolved in a polar liquid, e.g. glycosphingolipids (Dell 1987).

Extensive research has shown that there are many other potential matrices that can be used specifically for a particular class of organic compounds, thus providing a useful back up for the relatively rare cases in which glycerol has proven ineffective. Thioglycerol, aminoglycerol, triethanolamine, diethanolamine, *p*-nitrophenylalcohols, polyethylene glycol and related compounds, have wide applicability (Gower 1985).

From the experimental data which have been collected up to date, it seems that there are three requirements that a matrix compound should fulfil (Gower 1985).

- 1) It should dissolve the compound to be analyzed with or without the aid of a co-solvent or additives such as dilute aq. HCL, sodium acetate and ammonium thiocyanate (Dell 1987). Solvation allows molecules of the analyte to diffuse to the surface layers, thus, replenishing the sample molecules that have been ionised or destroyed by interaction with the fast atom beam. An alternative explanation for the mechanism of ion formation in FAB-MS is that sputtering occurs from the bulk rather than the surface, thus, it is of vital importance for the analyte to be soluble in the matrix for homogeneity in the bulk and for continuous regeneration of the surface monolayer to occur through diffusion.
- 2) It should be of relatively low volatility under the mass spectrometric conditions. A very volatile matrix may well give spectra, but they will be of a very short lifetime.
- 3) Ideally, a matrix compound should not react chemically with the compound being analysed. If it does, it should be in a reproducible and predictable way.

The liquid matrix not only acts as a medium which expands the spectrum lifetime but also has significant spectral effects which are associated with the influence of the matrix on the secondary ion beam. The matrix appears to facilitate the formation of ions prior to desorption. The effect of pH of the matrix on the abundance of $(M+H)^+$ ions and the effect of added alkali or ammonium salts on both replacement of acidic protons and formation of cationated molecular ion species are readily rationalized in terms of known principles of solution chemistry and they are described as "reversed derivatization" (Busch *et al.* 1982, DePauw 1983). Thus acidic matrices, e.g. thioglycerol, enhance protonation of the sample and production of positive ion mode spectra. On the other hand non-acidic matrices e.g. aminoglycerol are better for negative ion mode spectra.

Another matrix effect is the possibility to isolate substrate molecules and minimise intermolecular reactions of otherwise reactive analyte molecules. A classic case is that of the quaternary ammonium compound carnitine which contains both readily donatable methyl cations and readily methylated carboxylic acid groups (Busch *et al.* 1982). The matrix also makes an important energetic contribution. When the sample is present in solution, the heat of solution contributes to lowering the energy required for desorption. This is more readily envisioned in the case of preformed ions where solvation contributes significantly to charge separation and then only desolvation is required to desorb these ions.

1.5.4 Characteristics of FAB-MS

In general, FAB spectra are characterized by abundant pseudo-molecular ions for both the sample and the matrix and a relatively high level of "chemical noise" resulting in a signal at every mass number up to the molecular ion region. Positive ion spectra may contain a variety of molecular ion species as $(M+H)^+$, $(M+Na)^+$, $(M+K)^+$ (Meili and Seibl 1984, Keough 1985) and occasionally M^+ , $(M-H)^+$ and $(M-2H)^+$ (DePauw 1983, Meili and Seibl 1984, Keough 1985, Ligon 1983, Dube 1984, Gower 1983). Negative ion spectra are simpler. Usually $(M-H)^-$ is the only molecular ion species observed. In common with other desorption techniques, species are often encountered in ^{which} acidic protons that have been replaced by alkali metal cations [e.g. $(M+Na)^+$, $(M+K)^+$, $(M+NH_4)^+$]. Alkali metal cations which have replaced protons are carried in fragment ions, as well as in molecular ion species.

Pseudo-molecular ions do not often appear as single, "clean" signals in FAB spectra. Instead, clusters of signals are always present, partly because of the presence of molecules containing the ^{13}C isotope, the natural abundance of which is 1.1%, and partly because oxidation and reduction can occur in the matrix during the FAB experiment. For example, underivatized carbohydrates frequently exhibit an intense "minus 2" signal as result of oxidation (Dell 1987). The background ions are derived from both the matrix and the sample and are probably formed from surface molecules that have disintegrated after receiving a direct hit from an accelerated atom. In addition to pseudo-molecular and background ions, two other types of signal may be present in the FAB spectrum, namely those of cluster ions and fragment ions that are important for sequencing.

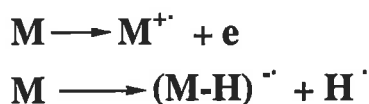
Most cluster ions are matrix derived. The appearance of a cluster matrix derived spectrum, on top of the analyte spectrum, is characteristic of FAB-MS. This may be troublesome when dealing with low molecular weight material as some analyte peaks might be masked from the matrix clusters. All matrices give their own characteristic spectrum. Glycerol for example, gives peaks at mass numbers corresponding to $(92n+1)^+$ and $(92n-1)^-$ with values of "n" up to 15 being detectable and with the relative intensities of multimers decreasing with increasing "n". Thioglycerol gives fewer cluster ions than glycerol and when it is present in a mixed matrix with glycerol, it suppresses the glycerol spectrum (Dell 1987).

Most of the fragment ions observed in a spectrum taken under FAB-MS conditions are even electron ions, anions or cations, rather than radical ions. This is true for molecular ion species and for fragment ions in both positive and negative ion mode spectra. This means that most fragmentation occurs by the loss of neutral molecules and radicals analogous to chemical ionization (Fenselau 1983).

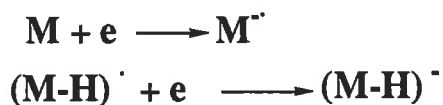
In spite of the widespread applicability of FAB-MS to the examination of large, non-volatile and thermally labile molecules, there is no systematic knowledge about fragmentation pathways of different classes of organic compounds under FAB conditions. This means that unlike electron impact spectrometry, there are not published data on fragmentation pathways by means of isotopic labelling. Whatever has been published seems to be empirically gained knowledge from observations of the spectra of compounds from the same chemical classes (Dell 1987, Domon and Costello 1988a, Pare *et al.* 1988).

1.5.5 Mechanism of ionization

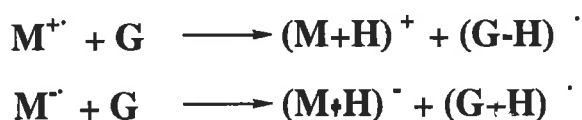
Ionization under FAB conditions appears to comprise a number of different processes so that radical cations, radical anions, anions, protonated, cationated and hydrogenated species may be observed. It has been previously stated that a FAB spectrum is characterized by abundant pseudomolecular ions of the forms of $(M+H)^+$, $(M-H)^-$, $(M+X)^+$ and $(M-X)^-$ ($X=Na, K, NH_4$) in both positive and negative mode. These ionic species are characterized as "pre-formed" ions and they are the result of solution chemistry reactions taking place in the liquid matrix phase (dissociation of intact molecules, where this is possible, is dependent upon the chemical nature of the analyte and Brønsted-Lowry reactions when the analyte is naturally present in a mixture with inorganic salts as NaCl, KCl, NH_4Cl). The degree to which they are observed is affected by the type of the matrix as an acidic one assists protonation, and thus, $(M+H)^+$ ions are predominant compared to the $(M+X)^+$ ions. Further than these usually observed ions, other ionic species as M^+ , M^- , or $(M+H)^-$ and $(M-H)^+$ are occasionally observed (Clayton and Wakefield 1984). They are desorbed directly from the bulk of the liquid phase to the high vacuum, like the "typical" ion species $(M+H)^+$, $(M-H)^-$, $(M+X)^+$ and $(M-X)^-$. These occasionally observed species seem to be the result of the interaction between the incoming particle beam and the molecules of analyte and this interaction occurs in the bulk of the matrix-analyte solution. In order to explain the presence of these occasionally observed ionic species, the incoming fast atom either ionises a molecule of sample and/or glycerol by ejecting an electron or dissociates it by the loss of a hydrogen radical:



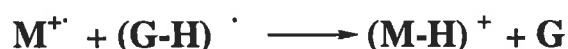
Electron capture by either a molecule or an (M-H)[•] radical gives the radical ions (M^{•-}) or the commonly observed (M-H)⁻.



Hydrogen radical abstraction by M^{•+} ions from glycerol gives the commonly observed (M+H)⁺ and by M^{•-}, ions the unusual (M+H)⁻ ions.



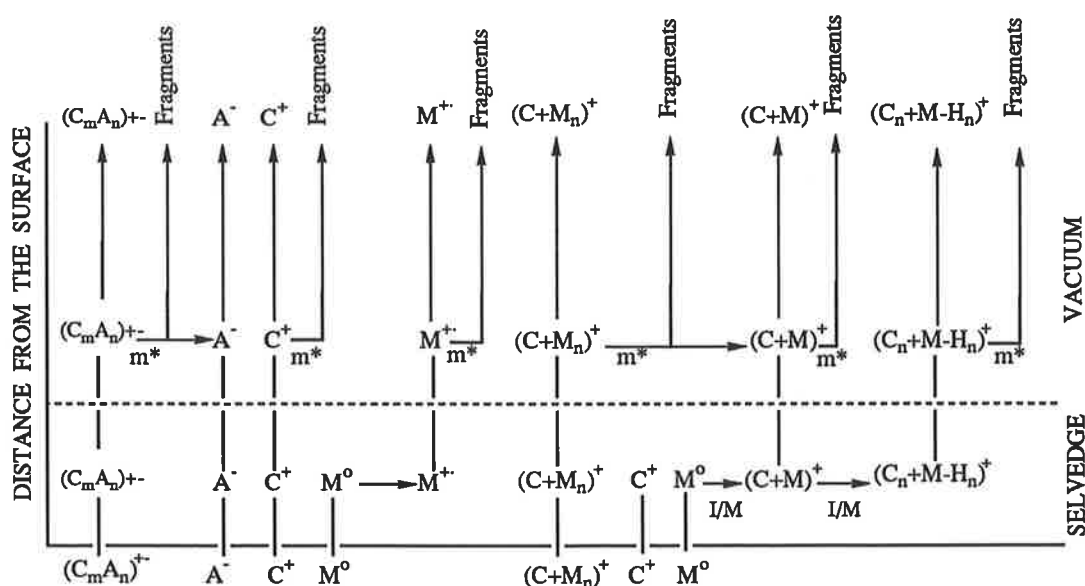
Finally abstraction of a hydrogen radical from M^{•+} gives (M-H)⁺ ions.



The presence of the above-mentioned species during a FAB experiment of different classes of organic compounds seems to be governed by factors such as: a) the nature of the analyte (i.e. volatility and existence of an active centre to accept protonation); b) the nature of the matrix from which the analyte is ionised (Clayton and Wakefield 1984); c) the operation of the experiment under positive or negative mode; and d) the presence of additives.

However, the above-mentioned details address only a part of the mechanism of ionization because, in addition the direct desorption of "pre - formed" ionic species or ionic species and radicals produced after the interaction of the incoming particle beam and the sample molecules, desorption of neutral molecules also occurs. The desorption of neutral molecules provides a secondary source for the production of ionic species detectable by the mass spectrometer as they may react with free electrons or surface electrons, generated by the impact process, to form M^{•+} or M^{•-} radical ions (Meili and Seibl 1983). Cationization of the neutral molecules may also occur as a result of bimolecular reactions (Cooks and Busch 1983). At this point it should be mentioned that these ionization and

cationization processes occur in the selvedge which is the condensed vapour liquid phase resulting from the interaction between the particle beam and atoms in the surface of the mixture of the matrix and the analyte. The radical species created and the complex $(C+M)^+$ are then extracted from the selvedge and their intact form or their unimolecular dissociation products are observed in the spectrum. The above model emphasizes that desorption of neutral species may be followed by chemical reactions of two types occurring in two distinct regions. First, in the selvedge, where fast ion/molecule reactions can occur. Second, in the free vacuum, where unimolecular dissociations occur governed by the internal energy of the parent ion. Thus, the selvedge boundary can be defined as the limit beyond which no ionization occurs. The mechanism of ionizations described above are summarized in the following scheme.



Scheme 1: Ionization reactions following desorption include electron ionization (EI) and ion/molecular reactions which occur in the selvedge. Metastable transitions (m^*) take place in free vacuum above the selvedge and are similar to those observed in other forms of mass spectrometry (scheme adopted from Cooks and Busch 1983).

1.5.6 Sensitivity

Sensitivities achieved in FAB-MS analysis are both operator and sample dependent. Correctly loading the sample into the matrix is one of the most critical steps in FAB analysis. Experiments undertaken by different groups have established different protocols for the correct loading of the sample into the matrix. The method of loading first the matrix onto the probe tip and then the sample (Barber *et al.* 1983), or that of mixing

substrate and matrix in a ratio 1:1(v/v) prior to loading (Martin *et al.* 1982), are two examples of how to load the sample onto the probe tip correctly.

In principle, however, the object is to submit the sample to the atom beam at a highly mobile surface concentration and for maximum sensitivity, the sample should form a perfect monolayer at the surface of a low volatility matrix (Barber *et al.* 1982). Monolayer formation at the surface of a dilute solution implies a constant surface excess concentration. Following Gibbs, this arises when the surface tension depends linearly on the logarithmic bulk concentration ($\log_e C$) of the solution (Kralj *et al.* 1983). The ratio of peaks attributed to the liquid matrix to those due to the sample cation falls to zero as the monolayer becomes established. Ionic groups that render compounds involatile, thus ruling out conventional methods of ionization, are also those groups that frequently lead to solubility in polar solvents and to the associated surfactant properties that facilitate good sample preparation for FAB ionization.

Studies undertaken in order to examine the relation between sample amount and absolute signal intensity, in the case of the oligopeptide angiotensin (Lehmann *et al.* 1984), revealed that there was a roughly linear relationship between sample amount and $(M+H)^+$ signal intensity for sample amounts between 1ng and 10 ng. Increase of sample amounts between 10 and 100 μg did not effect an additional increase in signal intensity of the protonated molecule. In general, however, the sensitivity of the method is such that practical structure problems can be investigated even when the amount of the material is very small ($\sim 1\mu\text{g}$) (Barber *et al.* 1982).

1.5.7 Application of FAB-MS to structure elucidation of glycoconjugates

Classes of organic compounds such as organic salts, nucleoside phosphates, antibiotics (D. H. Williams *et al.* 1981), peptides in their underivatized form (Lehmann *et al.* 1984), aminoacids (Surman and Vickerman 1981), cobalamines (Barber *et al.* 1981d), naphthalene sulphonic acids and sulphonate salts (Monaghan *et al.* 1982) and aromatic hydrocarbons (Dube 1984) constitute typical examples of large, fragile and non-volatile molecules which were examined within the first years after FAB-MS became commercially available.

Results from the examination of compounds of importance to food chemistry such as glycoalkaloids, glycosinolates, phospholipids, triglycerides, oligosaccharides (Self *et al.*

1985) and steroid conjugates (Clifford and DeLuca 1985, Rose *et al.* 1983, Rose 1983) revealed that in all cases the observation of intense pseudo-molecular ions and useful structural fragments were feasible. Those facts confirmed that FAB-MS yields information about the molecular weight, from the observation of protonated and pseudo-molecular ions $(M+H)^+$ and $(M+X)^+$ in the positive ion mode, and deprotonated molecular ions $(M-H)^-$ in the negative ion mode spectra. Fragment ions resulting mainly from the protonated (i.e. positive ion mode) and the deprotonated molecular ions (i.e. negative ion mode) are also observed. These fragments represent cleavages at each glycosidic bond, sometimes on either side of oxygen with or without transfer of two hydrogens to the neutral fragment. These latter may arise by redox processes in the glycerol matrix or by mass spectrometric rearrangements.

The application of FAB-MS in other related fields such as those of flavanoid glycosides (Crow *et al.* 1986, Domon and Hostettmann 1985, Takayama *et al.* 1987) and vegetable tannins (Self *et al.* 1986), reproduced similar data. The examination of robinin, rutin (Domon and Hostettmann 1985), quercetin, myricitrin, fraxin, arbutin, salicin (Self *et al.* 1986), quercetin-3-O- α -L-rhamnosyl-7-O- β -D-glucoside (Domon and Hostettmann 1985) and β -1,3,6-tri-O-galloyl-D-glucose (Self *et al.* 1986) revealed that upon FAB the mass spectra show the same general characteristics i.e. dominant protonated molecular ions $(M+H)^+$ and fragment ions corresponding to the sugar and aglycone parts.

This fragmentation probably takes place by first protonating the relevant glycosidic oxygen. As the oxygen-sugar bond breaks, hydrogen transfer takes place leaving behind a protonated alcohol or phenol (in the case of some aglycones). For most compounds with aromaticity in the aglycone portion of the molecule, the $(A-OH_2)^+$ fragment, which corresponds to the protonated aglycone part, is the most abundant. This may be due to stabilization of the positive charge by the aromatic system.

The mass spectra of negative ions contain information similar to that in the mass spectra of positive ions. The deprotonated molecular ion $(M-H)^-$ is typically the most abundant sample ion. On the other hand, ions containing sugar sequence information are due to cleavage of the glycosidic bond with charge retention on the oxygen. It is interesting to note that the examination of flavanoid glycosides under negative ion mode revealed that radical anions $(M^{\cdot-})$ were also formed. The formation of such radical anions possibly relies on the aromaticity of the aglycone part.

Another very interesting feature of FAB-MS, i.e. the potential to elucidate the configuration of the anomeric proton in glycoconjugates, became apparent when Pare *et al.* (1988) examined pairs (α - and β -) of model glycosidic monosaccharides and disaccharides. These model compounds were chosen because they do not have too high a molecular weight and the interpretation of fragment ions in their mass spectra could thus be clearly related to appropriate phenomena (i.e. protonation, cleavages of glycosidic bonds, and sugar part fragmentation).

Examination of typical representatives of both categories such as the α - and β - *p*-nitrophenylgalactose, α - and β - methylglucose, 4-O- β -D-galactopyranosyl-D-glucopyranoside (lactose) and 6-O- α -D-galactopyranosyl-D-glucopyranoside (melibiose), revealed that stereochemically dependent characteristics were exhibited in the positive ion mode spectra. A typical example is that of β -D-methylglucose which exhibits a much larger proton affinity than the α -anomer, thus, yielding a much more intense pseudo-molecular ion at m/z 195. Consequently, the peaks at m/z 163, m/z 145 and m/z 127 which result from the loss of methanol and two water molecules from the pseudomolecular ion respectively, are also prominent for the β -anomer. However, such differentiation of α - and β - anomers by FAB-MS can only be achieved when both anomers are available for analysis.

Quite often, the FAB-MS spectra contain fragment peaks which are difficult to assign as fragments originating from the parent molecule, from an impurity or from an unassigned matrix ion. The FAB-MS spectrum alone does not allow one to differentiate these possibilities. The situation becomes more unclear when one is dealing with samples that naturally occur as complicated mixtures e.g. wine glycoconjugates. The assignment of different fragments observed in the FAB spectrum becomes impossible and the necessity for a separation step before analysis is obvious. Gas-chromatography (GC) (Gallegos 1987), thin layer chromatography (TLC) (Chang *et al.* 1984), high-performance TLC (Bare and Read 1987, Tamura *et al.* 1988), high-performance liquid chromatography (HPLC) (Wang *et al.* 1984, Santikarn *et al.* 1987, Hattori *et al.* 1988, Caprioli *et al.* 1988), and counter current chromatography (CCC) (Oka *et al.* 1991) combined with FAB-MS provided the necessary pre-analysis step.

The combination of FAB and tandem mass spectrometry (Cooks and Glish 1984) can be an alternative to these separation-analysis methods. Since FAB produces primary molecular ion species, one can use the first mass analyzer of a tandem spectrometer to

select the ion or the ions of interest from a mixture from ions. Nearly all matrix ions are separated in this step. Once the ions are separated, they are subjected to collisional activation by passage through a collision cell. The activation causes transformation of some translational energy to internal energy in the ions which leads to fragmentation. Scanning the second mass analyzer of the mass spectrometry/mass spectrometry (MS/MS) instrument yields a collisionally activated decomposition spectrum (CADS). The collisionally produced fragments may not be in the normal FAB mass spectrum, because they may have been subjected to interference by other ions in the spectrum or be too low in abundance to be observed without the aid of MS/MS. Thus, the FAB-MS/MS technique provides further structural information and an additional degree of specificity.

The characteristics of mass spectra from tandem spectrometric experiments on glycosides are dominated by the $(M+H)^+$ or $(M-H)^-$ ions and by ions resulting from loss of the sugar units involved in the sugar moiety. Thus, these type of fragments, in addition to the $(A-OH_2)^+$ type fragments which are in most cases observable, clearly provide information not only about the molecular weight of each of the sugars involved but also in the case of oligosaccharides determines the sequence of sugar units in the molecule.

Extensive studies revealed that further to the above mentioned features which are observable in the direct probe FAB-MS spectrum, the combination of FAB with an MS/MS experiment is capable of generating data appropriate for:

- 1) the determination of the position of the linkage and the identification of the reducing end in linear oligosaccharides by FAB in the negative ion mode (Garozzo *et al.* 1990, Angel *et al.* 1987);
- 2) the differentiation between isomeric glycosides of the same aglycon^e and sugar parts but with a different substitution pattern;
- 3) the differentiation, between E- and Z- isomers, and diastereoisomers in some favourable cases (Unger 1985).

This success in the application of FAB and tandem mass spectrometry for the elucidation of structural problems of significance for food chemistry pointed to the possibility that these techniques could provide an alternative for the examination of the terpenoid glycosides. In early work described by Sales *et al.* (1988), geranyl- β -D-glucoside and geranyl- β -D-rutinoside were analyzed by FAB-MS in the negative ion mode. Ions at m/z 179 attributed to the glucose moiety for both compounds and at m/z 163 attributed to the rhamnose moiety of the rutinoside were found besides the molecular ions $(M-H)^-$ at m/z 315

and 461. The uncertainty concerning the order in which the sugars were linked in the geranyl disaccharide moiety was solved by desorption chemical ionization, as the ions in the positive mode spectrum at m/z 334 ($M\text{-rhamnose}+\text{NH}_4$)⁺ generated after elimination of the rhamnose segment from the pseudomolecular ion and at m/z 182 ($\text{rhamnose}+\text{NH}_4$)⁺, 164 ($\text{rhamnose}-\text{H}_2\text{O}+\text{NH}_4$)⁺ generated after subsequent eliminations of the aglycone part followed by the glucose segment, indicated that the rhamnosyl group was present in the distal position in the sequence.

More fascinating results were obtained when a crude grape extract (Muscat of Alexandria) was examined by FAB-MS in the negative ion mode (Sales *et al.* 1988). In the case of a monoglycosidic fraction, deprotonated molecular ions ($M\text{-H}$)⁻ at m/z 269, m/z 315 and m/z 331 were indicative of the presence of benzyl- β -D-glucoside, of terpenyl (α -terpenyl, geranyl, neryl and linalyl) glucosides and the linalool oxides or 3,7-dimethylocta-1,7-diene-3,6-diol and/or 3,7-dimethylocta-1,5-diene-3,7-diol glucosides. In the diglycosidic fraction, peaks at m/z 447 and 461 which were attributed to the deprotonated molecular ions ($M\text{-H}$)⁻ of the arabinoglucosides and the rutinoides of terpenols respectively, indicated the presence of these compounds among the heterosidic fraction of grapes. Further examination of that particular fraction by Desorption Collision Ionization (DCI) in positive ion mode generated further data consistent with the presence of these disaccharides. It should be emphasized, however, that such mass spectrometric assignments of glycoside structures, made in the absence of supporting data from other spectral techniques, must be considered as tentative only.

In agreement with the previously discussed examples on steroid and flavanoid glycosides, little information was available from these studies (Salles *et al.* 1988) on the nature of the aglycone part. This suggests that FAB-MS could not be employed for a full characterization of a glycoconjugate, but it could be employed as a supplementary mass spectrometric technique which can give structural information where the conventional mass spectrometric techniques fail; i.e. the determination of the molecular weight of polar molecules, the number of the sugars involved in the sugar moiety, the order in which the sugars are linked to each other, the position of the linkage and the possibility of elucidating the configuration of the anomeric proton if reference pairs of α - and β -isomers are available.

1.6 Employment of FAB-MS in a multidimensional analysis

In this research project FAB-MS has been employed as a part of a multidimensional methodology for the examination of polar, non-volatile and thermolabile material, such as glycosides, in grape juice and wine. This multidimensional methodology has been explored as a component of the flavour precursor analysis approach.

The first analytical dimension involves separation and isolation of the polar, non-volatile and, almost exclusively, glycosidic material from dealcoholized wine using C₁₈ reversed-phase chromatography. Fractionation of the glycosidic isolates according to the relative polarity of the components by DCCC was also employed within this analytical dimension.

The second dimension employs liquid-solid partition chromatography, i.e. flash chromatography and high performance liquid chromatography (HPLC), to further resolve glycosidic mixtures to fractions of a limited number of constituents. Enzymic hydrolysis and GC-MS studies of the liberated aglycones, acid hydrolysis and GC-MS studies of the resultant sugar segments as their per-trimethylsilylated (TMSi) derivatives, and GC-MS studies of the intact molecules as their per-O-acetylated derivatives, where this was possible, were also employed to assist compositional analysis of the glycosidic mixtures during the course of this analysis.

The third analytical dimension employed FAB-MS and MS/MS experiments in a multipurpose methodology. Firstly, FAB-MS is used as a detection method of individual compounds in prefractionated glycosidic mixtures by determining the molecular ions from the sequence of positive and negative ion mode experiments. Secondly, FAB-MS/MS is employed as a separation technique by performing MS/MS experiments on the molecular ions of individual compounds in prefractionated glycosidic mixtures without the need to further purify chromatographically the corresponding molecules. This was achieved by separating the individual molecular ions in the first stage of the mass spectrometer. Lastly, FAB-MS/MS is used as a structural characterization tool by giving useful structural information for the intact glycosidic molecules from the generated MS/MS data.

1.7 Aims of this thesis

This thesis had as its primary aim the examination of the applicability, as well as the limitations, of FAB-MS and tandem mass spectrometry (MS/MS) as routine analytical techniques for the detection and partial characterization of polar, non-volatile and thermally labile material from grape juice and wine. Such compounds have the potential to influence flavour. The structural information that can be obtained from the FAB-MS and MS/MS examination of wine glycoconjugates is described in section 1.5.7.

Secondly, this thesis aimed to investigate the chemical nature of a group of relatively very polar wine glycoconjugates which are early eluted off the DCCC and also act as flavour precursors. A hypothesis about their nature has been described elsewhere (Winterhalter *et al.* 1990, Williams 1992).

The last aim was to explore the presence in grapes and wine of other polar, non-volatile, and thermally labile compounds which may not have a flavour precursor role but which may have a possible biological active role.

Chapter 2

LATE-ELUTING, RELATIVELY NON-POLAR WINE GLUCOSIDES

2.1 Introduction

In almost all cases reported in the literature review, FAB-MS and FAB-MS/MS were employed as analytical tools to provide structural information on glycoconjugates which had been synthesized, isolated pure from, or were components of, simple mixtures (Takayama *et al.* 1987, Crow *et al.* 1986, Self *et al.* 1985, Domon and Hostettmann 1985). Sales *et al.* (1988, 1991) were among the first to use FAB-MS and tandem mass spectrometry as both a detection method and a separation technique to provide structural information from complex mixtures such as grape juice and an apricot glycosidic extract.

A major aim of the present work has been to explore the capabilities of FAB-MS and MS/MS to generate useful structural information for both the aglycone and the sugar moiety of glycoconjugates in pre-fractionated glycosidic mixtures isolated from an Australian Riesling wine. In suitable instances where sufficient sample was available, FAB-MS/MS data have been supplemented with data obtained after purification and examination, by other spectrometric techniques (e.g. ^1H NMR), of the compound of interest. This permitted complete or near complete structure elucidation of these compounds. A mixture of monoterpene and norisoprenoid monosaccharides isolated as a relatively non-polar mixture (i.e. late-eluting DCCC frs. 190-220), was investigated initially because it provided a relatively simple substrate to test the general applicability of the multistep research strategy outlined in page 41. This particular mixture of monosaccharides was chosen for initial analysis because low molecular weight monosaccharides give simple FAB mass spectra with fragment ions which can readily be attributed to clearly understood phenomena, i.e. protonation, cleavages of glycosidic bonds, sugar ring fragmentation (Pare *et al.* 1988). Furthermore, because of their relative low polarity and low molecular weight they could be independently studied by GC-MS as their acetate derivatives. This chapter describes the application of FAB-MS/MS technique to the analysis of these relatively non-polar glycoconjugates. Applications of the same protocol to the structural elucidation of wine glycoconjugates of increasing polarity are described in chapters 3-5. The detection and structural characterization of a group of polar but not glycosidically bound heterocyclic compounds which are present in grape juice and

wine is described in chapter 6. The aglycones described in this last chapter are less polar than the glycoconjugates described in chapters 3-5. However, because of their non-glycosidic nature, they are treated as a separate entity.

2.2 Results and discussion

a). Determination of the suitability of FAB-MS as a detection method for glycosides in a mixture

FAB-MS monitoring of monosaccharide mixtures obtained by droplet counter current chromatography (DCCC), i.e. frs. 190-220 (Winterhalter *et al.* 1990b), followed by liquid chromatography on silica gel (i.e. fl. frs. 7-12), indicated the presence of a series of glycoconjugates with apparent molecular weights (M_r s) of 332, 386, 388, 395. The M_r of these glycosides were deduced from the presence of protonated and pseudo-molecular ions in the positive ion mode at m/z 333 ($M+H$)⁺, 355 ($M+Na$)⁺, 387 ($M+H$)⁺, 389 ($M+H$)⁺, 411 ($M+Na$)⁺, and 396 ($M+H$)⁺, 418 ($M+Na$)⁺ and deprotonated molecular ions, in the negative ion mode, ($M-H$)⁻, at m/z 331, 385, 387 and 394, respectively (see figure 15). Per-O-acetylation of this glycosidic mixture and examination of the resulted derivatives by GC-EIMS, revealed that the glycosidic mixture fl. frs. 7-12 consisted almost exclusively of monoglucosides. This was deduced from the observation in the EI mass spectra of the individual chromatographic peaks in the GC trace of the derivatised fl. frs. 7-12, of fragment ions at m/z 331, 271, 211, 169 and 109 characteristic of the presence of tetra-O-acetylated hexopyranosides (Radford and Dejong 1972). Examination of the same glycosidic derivatives by GC-CIMS (NH_4) enabled the determination of the molecular weights of the derivatised constituents of the mixture from the observation in their CI mass spectra of the corresponding protonated ($M+H$)⁺ and/or pseudo-molecular ions ($M+NH_4$)⁺. From the apparent molecular weights of the derivatised glycosides, the molecular weight of the individual constituents of the mixture in their underivatised form were postulated. From each molecular weight, four acetates for the sugar moiety and one or two acetates for the aglycones according to the observed fragmentation patterns in the EI mass spectra, were subtracted. These calculations indicated that the glycosidic mixture fl. frs. 7-12 consisted of glycoconjugates with apparent molecular weights in their underivatised form of 332, 386, 388 or 395. These data were in agreement with those obtained from the FAB-MS examination of the glycosidic mixture fl. frs. 7-12 in its underivatised form. Furthermore, these data highlighted that FAB-MS is a sensitive method for the detection of glycosides even from complex mixtures. However, the fact

that in some cases the observed molecular ions corresponded to more than one compound constitutes a considerable deficiency of the employment of FAB-MS as a detection method.

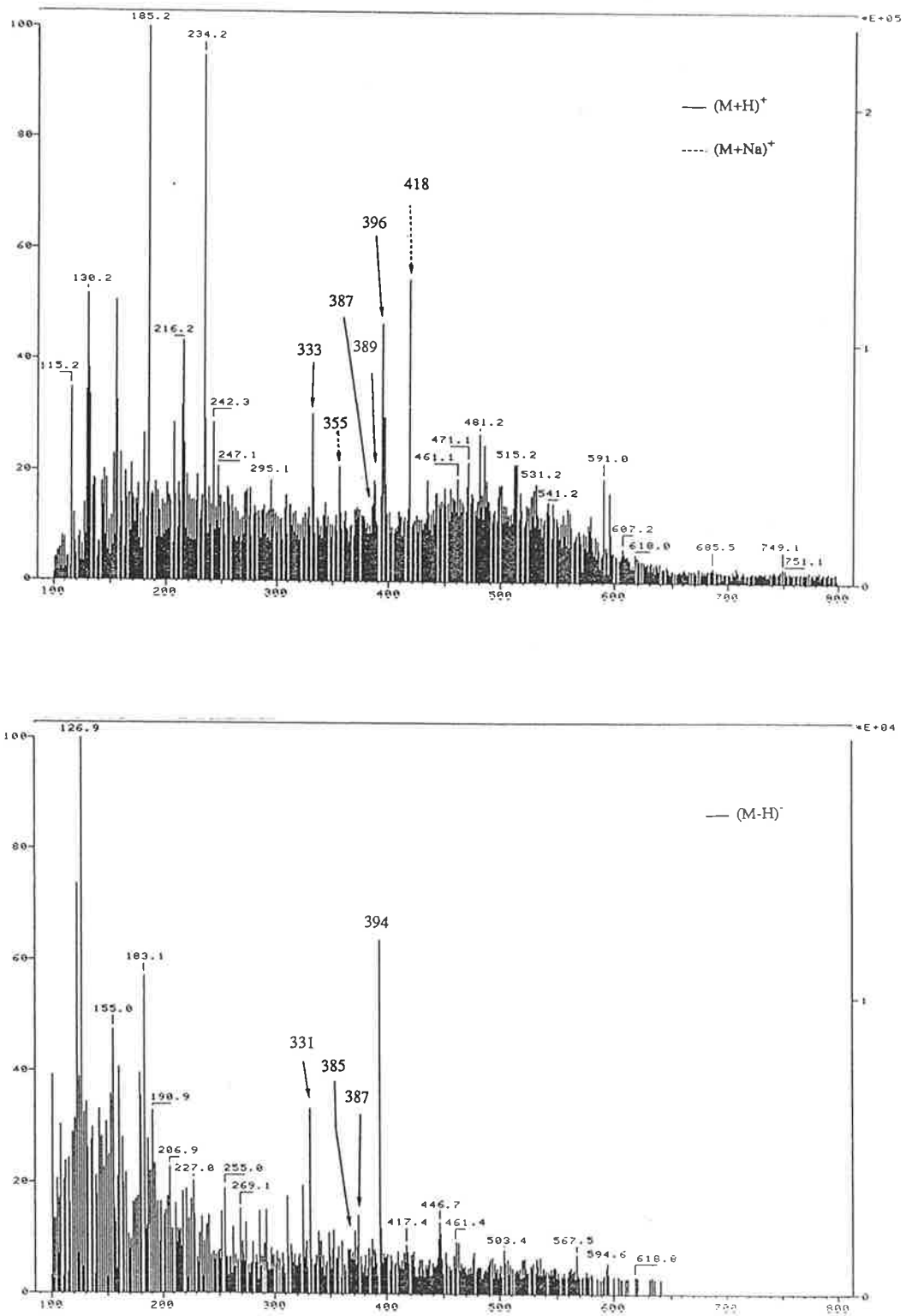


Figure 15: Positive and negative ion mode FAB-MS spectra of glycosidic mixture fl. frs. 7-12.

b). Experiments to explore the use of FAB-MS/MS as an analytical method

As might be expected from the low masses of the protonated and deprotonated molecular ions observed in the FAB spectra of fl. frs. 7-12, subsequent positive ion mode FAB-MS/MS experiments on the protonated molecular ions indicated that all glycoconjugates were mono-hexosides. These yielded $(M+H-\text{anhydrohexose})^+$ daughter ions at m/z 171, 225, 227 and 234 respectively, and/or characteristic ions at m/z 181 (protonated hexose) and hexose fragment ions at m/z 163, 145, 127 (Pare *et al.* 1988). In the negative ion mode spectrum, presence of sugar ring fragment ions at m/z 179, 161, 149, 119, 101, 89 further supported the above assignment (Domon and Costello 1988b).

Structural information for the aglycones was deduced from parameters such as their apparent M_r , calculated from the protonated and deprotonated ions after subtraction of the sugar moiety, and also the presence of certain characteristic fragment ions in the daughter ion spectra. The information gained from such parameters, when combined with information obtained after enzymic hydrolysis of the complex glycosidic mixtures and GC-MS examination of the liberated aglycones, can be indicative of the nature of the aglycone of individual glycosides and in favourable cases can even lead to tentative structural assignments. One example is provided by the glycoside (or structurally isomeric glycosides) with apparent M_r 395. The odd mass number clearly indicated that it was a nitrogenous compound with the heteroatom located on the aglycone skeleton. The existence of a strong fragment ion at m/z 130, indicated the presence of an indole nucleus bearing a methylene group (Porter 1985). These data suggested that the glycoside(s) with apparent M_r 395 was a conjugated form of ethyl indole-3-lactate which had previously been identified as a constituent of the enzymic hydrolysate of the glycosidic mixture. For glycosides with apparent M_r 332 the appearance of the protonated aglycone at m/z 171 and fragment ions at m/z 153 $[(\text{aglycone}+H)-H_2O]^+$, 135 $[(\text{aglycone}+H)-2H_2O]^+$ suggested the presence of monoterpene diols at the oxidation state of the linalool oxides.

For glycosides with apparent M_r 388 and 386 the presence of protonated ions for the aglycones $(\text{aglycone}+H)^+$ at m/z 227 and 225 suggested that they could be C_{13} norisoprenoid compounds of M_r 226 and 224, respectively. The presence of fragment ions at m/z 209 $[(\text{aglycone}+H)-H_2O]^+$, 191 $[(\text{aglycone}+H)-2H_2O]^+$ in the first case and at m/z 207 $[(\text{aglycone}+H)-H_2O]^+$, 189 $[(\text{aglycone}+H)-2H_2O]^+$ in the second case, suggested the presence of at least two oxygen atoms in the skeletons of each of these aglycones. These oxygen atoms either could be linked as hydroxyl or ether groups.

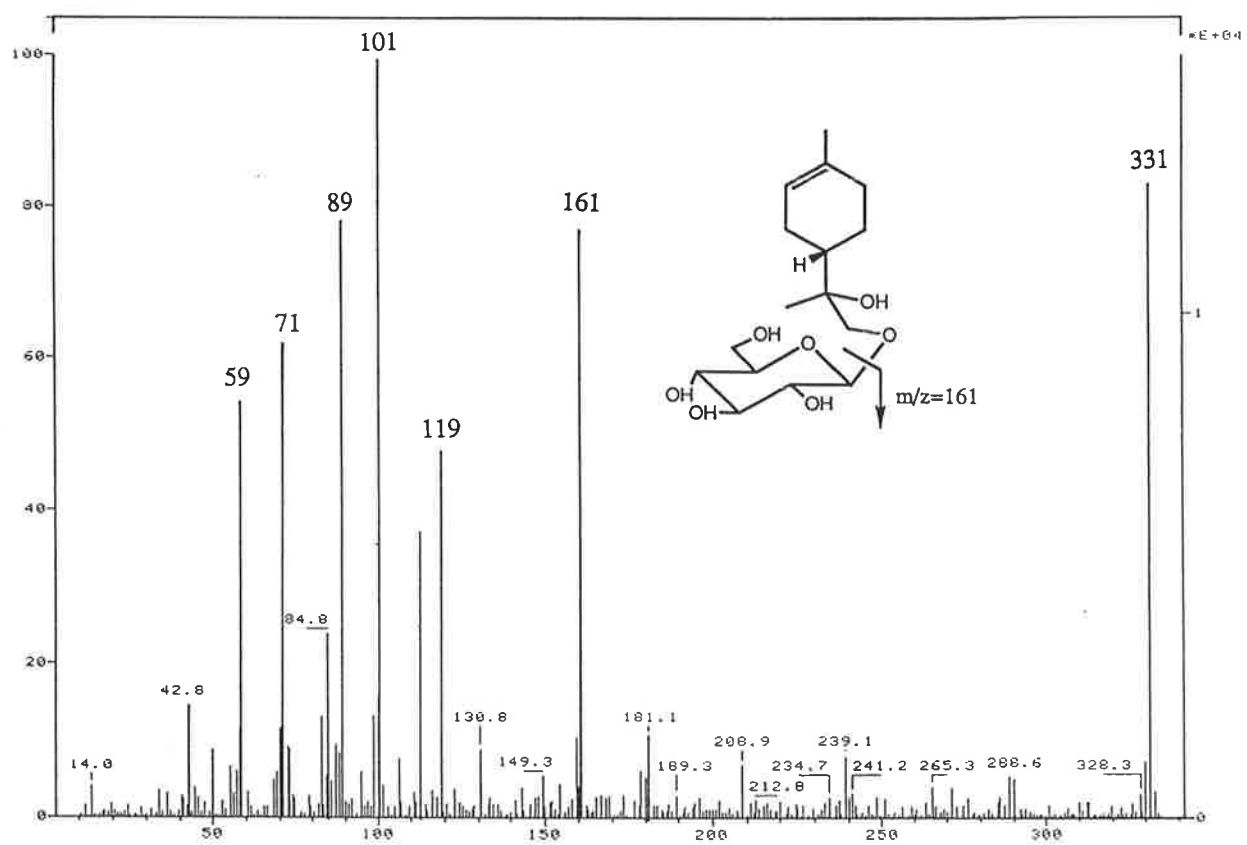
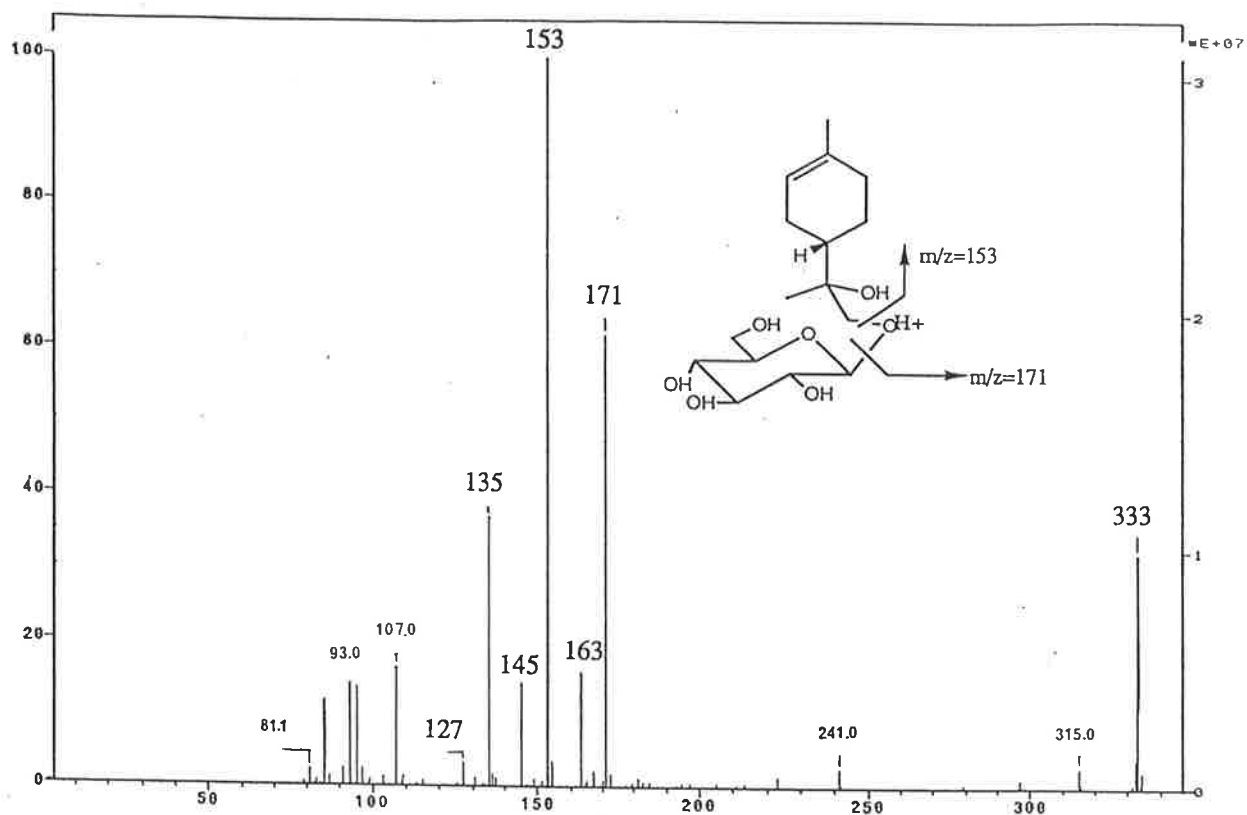


Figure 16: Positive and negative ion mode FAB-MS/MS spectra of glycosides with M_r 332

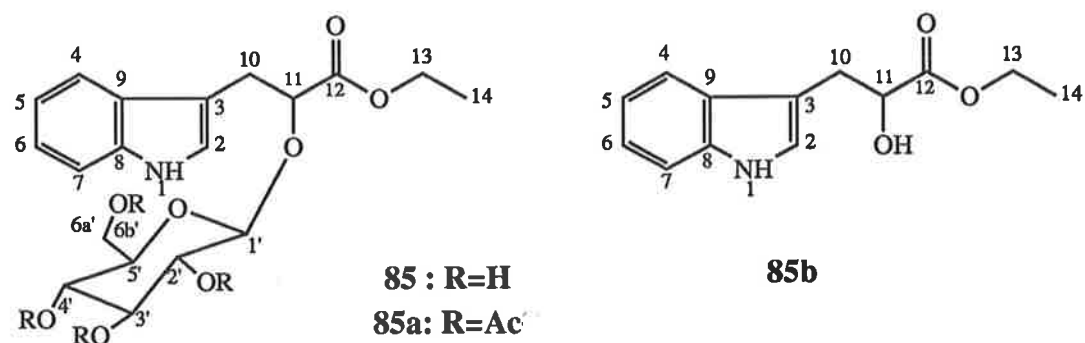
c). Isolation of glycosidic components and structural elucidation by other spectrometric techniques.

Separation of the Riesling glycosidic fraction by high performance liquid chromatography (HPLC) allowed isolation in a pure form of a glycoside with apparent M_r 395 (**85**) and two with apparent M_r 332 (i.e. glycosides **86**, **87**), showing the above FAB-MS/MS characteristics. Two other glycosides with M_r 387 and M_r 389 (i.e. glycosides **88**, **89**) were isolated as an incompletely resolved mixture with other minor components. The purity of each isolated fraction was determined by TLC and positive-negative FAB-MS examination of them.

Glycoside **85**, on TLC, gave an intense blue colour with the Van Urk-Salkowski indole reagent (Ehmann 1977) and exhibited a characteristic indole UV absorption spectrum λ_{max} 220, 280, 289 (sh) nm (D. H. Williams and Fleming 1987). Acetylation of **85** with acetic anhydride/pyridine produced a tetra-acetate **85a** [$M^+=563$ (EIMS), $(M+H)^+=564$ (FAB-MS)] the spectrum (EIMS) of which showed fragment ions at m/z 331, 271, 169, 109 characteristic of tetra-O-acetylated hexopyranosides (Radford and Dejongh 1972). The base peak in this EIMS at m/z 130 supported the presence of an indole bearing a methylene group (Porter 1985).

The 1H NMR signals of **85a** (table 2) are consistent with those for a glycosylated derivative of an ethyl indole-lactate (EILA) and the absence of a high field aromatic signal near δ 6.4 indicated that the indole nucleus was substituted at position 3 (Hiremath and Hosmane 1973). This assignment was supported by comparison of the 1H NMR data with that of a synthetic sample of racemic ethyl indole-3-lactate **85b** (see table 2). The 1H NMR spectrum of **85a** also showed signals for four acetoxy groups on the sugar moiety. A large upfield shift of one of them (δ 1.6) is consistent with that reported for the 2-OAc group of the 2,3,4,6-tetra-O-acetyl-O-(indole-3-ylacetyl)- β -D-glucopyranoside and the tetra-O-acetyl tryptophyl- β -D-glucopyranoside (Pravdic and Keglevic 1970, Magnus 1979). The splitting ($J=7.4$ Hz) of the signal for the anomeric proton suggested a β -configuration for the hexopyranoside moiety and the signals observed for the other protons of the sugar part were in good agreement with data previously published for tetra-O-acetyl-glucopyranosides (Schwab and Schreier 1988). A broadened signal at δ 8.05 in the 1H NMR of **85a**, suggesting a free imino group (Hiremath and Hosmane 1973), implied that the secondary alcohol function of the molecule was involved in the glycosidic linkage.

Table 2 : ¹H NMR data of compounds **85a**, **85b** (300 MHz, δ)



H	85a (CDCl ₃)	85b (CDCl ₃)
Aglycone moiety		
1.	8.00, br s	8.00, br s
2.	7.04, d, <i>J</i> = 2.4 Hz	7.10, s
4.	7.55, br d, <i>J</i> = 7.7 Hz	7.63, d, <i>J</i> = 7.9 Hz
5.	7.15, ddd, <i>J</i> = 7.0, 1.3, 7.8 Hz	7.16 - 7.21, ddd, <i>J</i> = 7.1, 1.1, 7.8 Hz
6.	7.09, ddd, <i>J</i> = 7.0, 1.2, 8.0 Hz	7.10, m
7.	7.32, dd, <i>J</i> = 7.8, 1.2 Hz	7.36, d, <i>J</i> = 7.9 Hz
10.	3.16, d, <i>J</i> = 6.6 Hz	3.10 - 3.30, AB part of an ABX system <i>J</i> _{AB} = 14.8, <i>J</i> _{AX} = 6.1, <i>J</i> _{BX} = 4.4 Hz
11.	4.26, t, <i>J</i> = 6.6 Hz	4.47, X part of an ABMX system <i>J</i> _{AX} = 6.1, <i>J</i> _{BX} = 4.5, <i>J</i> _{MX} = 6.2 Hz
13.	4.12, q, <i>J</i> = 7.2 Hz	4.14, AB part of an ABX ₃ system <i>J</i> _{AB} = 10.2, <i>J</i> _{AX} = 7.1, <i>J</i> _{BX} = 7.1 Hz
14.	1.20, t, <i>J</i> = 7.2 Hz	1.23, t, <i>J</i> = 6.4 Hz 2.79, 1H, d, <i>J</i> = 6.4 Hz -OH.
Sugar moiety		
1'.	4.50, d, <i>J</i> = 7.4 Hz	
2'.	5.00 - 5.10, m	
3'.	5.00 - 5.10, m	
4'.	5.00 - 5.10, m.	
5'.	3.60 - 3.70, ddd, <i>J</i> = 2.5, 5.0, 9.8 Hz	
6a'.	4.10 - 4.20, dd, <i>J</i> = 5.1, 12.2 Hz	
6b'.	4.01 - 4.05, dd, <i>J</i> = 2.5, 12.2 Hz	
Acetates: 1.60 (1s), 1.94 (1s), 1.97 (1s), 2.05 (1s)		

Prolonged (5 days) enzymic hydrolysis of **85** with β-D-glucosidase (Serva, E.C. 3.2.1.21) afforded an aglycone **85b** which, on gas chromatography-mass spectrometry (GC-EIMS), co-chromatographed with and gave an identical mass spectrum to, a synthetic sample of racemic ethyl-indole lactate (EILA). The sugar moiety of **85** was demonstrated to be β-D-glucose by an enzymatic assay which coupled the specificity of β-D-glucosidase activity of emulsin with a glucose oxidase and peroxidase system (Mizukami *et al.* 1982). Acid

hydrolysis of **85** and GC examination of the liberated carbohydrates as their TMSi derivatives provided independent evidence that the sugar involved in the glycoconjugate was glucose.

The glucoside **85** proved to be highly resistant to β -D-glucosidase hydrolysis under the conditions employed (enzyme/substrate ratio: 1/10; pH=5.0; T=37°C) and the half time for the hydrolysis was 100 hrs. Hydrolysis of **85** was readily effected with a non-specific glycosidase enzyme preparation (*Rohapect C*), however this reaction yielded indole-3-lactic acid (ILA) indicating the additional presence of esterase activity in the enzyme preparation as well as the glucosidase activity.

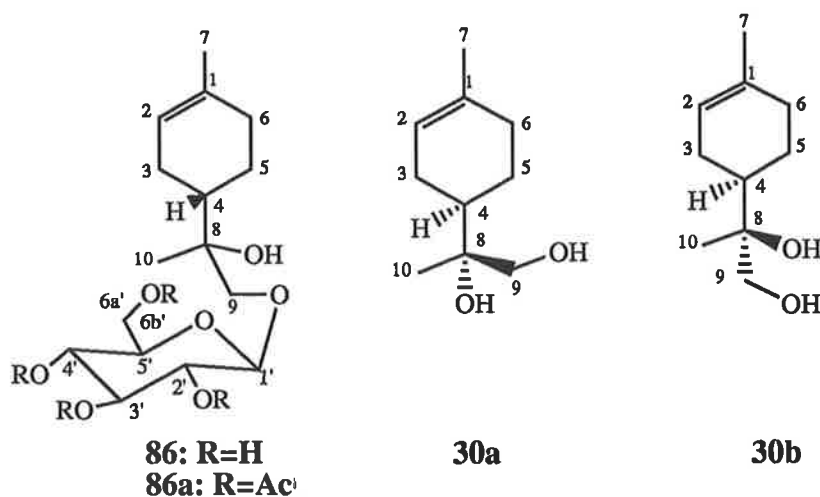
During GC-MS analysis of the acetylated DCCC group of fractions 190-220 an earlier eluting and minor isomeric EILA hexoside was observed accompanying glycoside **85a**. This suggests that the aglycone of **85** is derived from one of the two possible enantiomers of ILA or that an isomeric hexoside of EILA was present. Because the isomeric EILA hexoside was only a minor constituent, the data did not permit these alternatives to be distinguished.

It is possible that EILA- β -D-glucoside is formed by acid-catalyzed esterification of ILA- β -D-glucoside with the ethanol produced during wine fermentation. The presence of indoles in wines was first reported by Ehmann (1976) who identified indole-3-ethanol, indole-3-lactic acid (ILA), ethyl indole-3-lactate (EILA), and indole-3-acetic acid (IAA). The presence of glycosidic derivatives of indoles in grape juice and/or wine was first reported by Strauss *et al.* (1987a) who observed a glucosidic derivative of indole-3-lactic acid as a constituent of the glycosidic mixture of a grape juice (cultivar Riesling). The role of IAA as a plant growth regulator is well documented (Takahashi 1986) and ILA has long been implicated as an intermediate in the biosynthesis of IAA from tryptophan (Cohen and Bialek 1984).

Glucoside **86** (apparent M_r 332) was obtained as a hygroscopic solid which showed only end absorption in its UV spectrum, consistent with the presence of a non-conjugated olefinic system (D. H. Williams and Fleming 1987). The 1H NMR spectrum (see table 3) showed signals for both the carbohydrate and the aglycone parts of the molecule. For the aglycone there were signals at δ 1.10 and δ 1.13 assigned to methyl groups adjacent to a carbon bearing oxygen; these two signals, with relative intensities of ca 5:1 in favour of

the first signal, integrated for three protons suggesting that the sample was a mixture of diastereoisomers.

Table 3 : ^1H NMR data of compounds **86**, **30a**, **30b***



H	86 (300 MHz, D ₂ O)	30a (4R, 8R)	30b (4R, 8S)
Aglycone moiety			
2.	5.45, m, $W_{1/2} = 10.0$ Hz	5.41, m, $W_{1/2} = 9.0$ Hz	5.36, m, $W_{1/2} = 9.0$ Hz
3.	2.01, m		
4.	1.76, m		
5.	1.32, m		
6.	2.01, m		
7.	1.65, s	1.64, br s	1.64, br s
9.	3.63 - 3.53, ABq, $J=11.7$ Hz		3.39-3.54, ABq, $J=11.0$ Hz
9.	3.65 - 3.55, ABq, $J=11.4$ Hz	3.45-3.60, ABq, $J=11.0$ Hz	
10.	1.10, s		1.07, s
10.	1.13, s	1.12, s	
Sugar moiety			
1'.	4.44, d, $J = 7.8$ Hz ⁺		
2'.	3.30 - 3.45, m		
3'.	3.40, t, $J=8.8$ Hz		
4'.	3.30 - 3.40, m		
5'.	3.30 - 3.40, m		
6a'.	3.70, dd, $J = 12.1, 5.6$ Hz		
6b'.	3.80, m		

*Data for isomers **30a** and **30b** are from Carman *et al.* (1986).

+Accompanying this signal was a minor doublet at δ 4.43, $J=7.8$ Hz. cf text.

Additionally the spectrum showed two unequal AB quartets (δ 3.53-3.65) for methylene protons geminally coupled on a carbon bearing oxygen, a singlet at δ 1.65 assigned to a methyl group on a double bond, and an unresolved signal for an olefinic proton of a tri-

substituted double bond (δ 5.45, $W_{1/2}$ =10.0 Hz). The observation in the ^1H NMR spectrum of **86** of only one olefinic proton suggested the presence in the aglycone of only one double bond. This implied that the aglycone, presumably a monoterpene diol at the linalool oxide oxidation state from the FAB-MS/MS data, was monocyclic. These data support a *p*-menthenediol structure for the aglycone which is also consistent with the mass fragmentation pattern and in good agreement with data published for the isomeric *p*-menth-1-ene-8,9-diols (Carman *et al.* 1986).

For the sugar moiety, the splitting (J =7.8 Hz) of the signal for the anomeric proton (δ 4.44) indicated a β -glucosidic linkage. The presence of an accompanying minor signal with similar characteristics (δ 4.43, d, J =7.8 Hz) suggested the presence of more than one diastereoisomer. Signals for the other sugar protons were similar to those previously published for non-derivatized glucopyranosides (Gao *et al.* 1991, Shiraga *et al.* 1988).

Uncertainty about which -OH of the monoterpene diol was glycosylated was resolved by per-O-acetylation of the isolate and examination of the acetates by FAB-MS, FAB-MS/MS and GC-MS. This experiment established that the derivatized product (**86a**) was a tetraacetate (FAB-MS: $(\text{M}+\text{H})^+=501$, $(\text{M}+\text{Na})^+=523$), and that the acetates were attached to the sugar moiety, i.e. FAB-MS and EIMS: fragment ions at m/z 331, 271, 169, 109 characteristic of a tetra-O-acetylated hexopyranoside (Radford and Dejong 1972). These data implied the presence of a tertiary -OH on the aglycone which was resistant to acetylation and allowed a tentative assignment of the compound as **86**.

Enzymic hydrolysis of the isolated glycosidic fraction with the β -D-glucosidase readily afforded an aglycone (**30**) which co-chromatographed with and gave a spectrum identical to, a mixture of reference diastereoisomeric *p*-menth-1-ene-8,9-diols (**30a**, **30b**). The latter reference diastereoisomers were themselves inseparable under the GC conditions. As the concentration of the substrate in the hydrolysis mixture (0.35 $\mu\text{mol/mL}$) was below the detection limit for the enzymic glucose assay, the liberated sugar was examined by GC as its TMSi derivative and found to be identical to an authentic sample of TMSi glucose. Thus, the isolated compound has been characterized as a diastereoisomeric mixture of *p*-menth-1-ene-9-(β -D-glucopyranosyl)-8-ol.

Two alternative routes to the formation of uroterpenol diastereoisomers have been proposed. Carman *et al.* (1986) have suggested a pathway from limonene, via an epoxide

intermediate, giving two optically pure diastereoisomers of uroterpenol i.e. the (4R, 8R) **30a** and the (4R, 8S) **30b** diastereoisomers. Winterhalter *et al.* (1990b) have proposed that, in wine, an acid catalyzed cyclization of 2,6-dimethylocta-2,7-diene-1,6-diol would yield the (4S,8R) and (4S,8S) enantiomers in addition to **30a** and **30b**. The present data are consistent with both proposals. The apparent cyclisation of 2,6-dimethylocta-2,7-diene-1,6-diol to yield uroterpenol has been observed in independent experiments that are reported in chapter 4.

Glycoside **87** also showed end absorption only in its UV spectrum. Per-O-acetylation of **87** with pyridine/acetic anhydride yielded a tetra-O-acetate derivative **87a** (FAB-MS: $(M+H)^+=501$, $(M+Na)^+=523$) the spectrum of which (FAB-MS, EIMS) contained the sequence of fragment ions at m/z 331, 271, 211, 169 characteristic of a monosubstituted tetra-O-acetylated hexopyranoside (Radford and Dejongh 1972). The adoption by the glycoside of only four acetates suggested the presence of either a tertiary -OH on the aglycone which was resistant to acetylation, or of an ether group in the aglycone moiety. Glycoside **87a** when examined under GC-MS conditions in parallel with a reference sample of isomeric *cis*- and *trans*- tetra-O-acetylated- β -D-glucopyranosides of pyran linalool oxide (Strauss 1983) was characterized as the *cis*- isomer. On enzymic hydrolysis with β -D-glucosidase **87** yielded an aglycone **6** which was identified by its chromatographic and mass spectrometric characteristics as the *cis*- pyran linalool oxide.

On acid hydrolysis, **87** liberated a sugar which after trimethylsilylation was examined by GC and found indistinguishable from glucose. Thus, **87** has been characterized as the *cis*-2,2,6-trimethyl-2-vinyl-4-(β -D-glucopyranosyl)-tetrahydropyran.

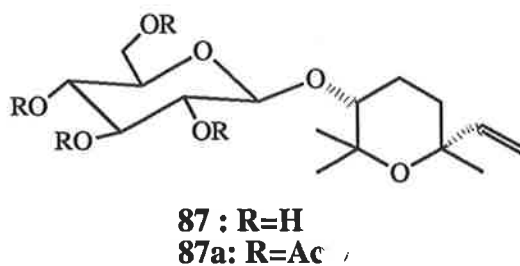


Figure 17: *cis*-Pyran linalool oxide- β -D-glucopyranoside and its acetylated derivative.

Glucoside **87** was previously identified in a glycosidic mixture from Muscat juice by Strauss (1983) and this is the first time that it has been isolated from grape juice and/or wine. This molecule, together with glucoside **86**, provide a substrate to test the ability of

FAB-MS/MS to differentiate between isomeric compounds. A comparison of these two glucosides under the same mass spectrometric conditions (i.e. FAB-MS/MS) gave similar spectral data which did not allowed the two molecules to be differentiated. This observation suggests that FAB-MS/MS spectrometry has a distinct limitation and, presumably, can only differentiate between structural isomers in some favourable cases.

The glycosidic mixture containing compounds with apparent M_r 387 (i.e. glycoside **88**) and 389 (i.e. glycoside **89**) after per-O-acetylation and FAB-MS, CI-MS examination, showed the presence of protonated molecular ions and pseudo-molecular ions at m/z $555=(M+H)^+$, $572=(M+NH_4)^+$ and $599=(M+H)^+$, $616=(M+NH_4)^+$ and $621=(M+Na)^+$ corresponding to a tetra-O-acetylated glycoside of an aglycone with molecular weight 224 (**88a**) and a penta-O-acetylated glycoside of an aglycone with molecular weight 226 (**89a**), respectively. The GC-EIMS spectra of both glycosides contained the sequence of fragment ions at m/z 331, 271, 211, 169 consistent with the presence of monosubstituted tetra-O-acetylated hexopyranosides (Radford and Dejongh 1972). In the case of **88**, this implied the aglycone had two resistant to acetylation oxygen atoms. For glycoside **89**, the presence of a fifth acetate implied the presence of an additional primary or a secondary hydroxyl group in the aglycone of **89**, further than the one involved in glycosylation.

Enzymic hydrolysis of the glycosidic mixture with β -D-glucosidase liberated three aglycones. Under GC-EIMS conditions, one of them co-chromatographed with and gave a spectrum identical to, a reference sample of 3-hydroxy-5,6-epoxymegastigm-7-ene-9-one (**73**) (Winterhalter and Schreier 1988). The other two aglycones were assigned from their mass spectra as diastereoisomers of 3,4-dihydroxy-7,8-dihydro- β -ionone (**89b**) (Winterhalter 1991) and under co-chromatography conditions they found indistinguishable from a reference diastereoisomeric mixture of **89b**. Thus, compound **88** was characterized as the D-hexopyranoside of 3-hydroxy-5,6-epoxymegastigm-7-ene-9-one and **89** as the D-hexopyranosides of a mixture of diastereoisomeric 3,4-dihydroxy-7,8-dihydro- β -ionone. This was the first observation of these glycosidic derivatives in wine.

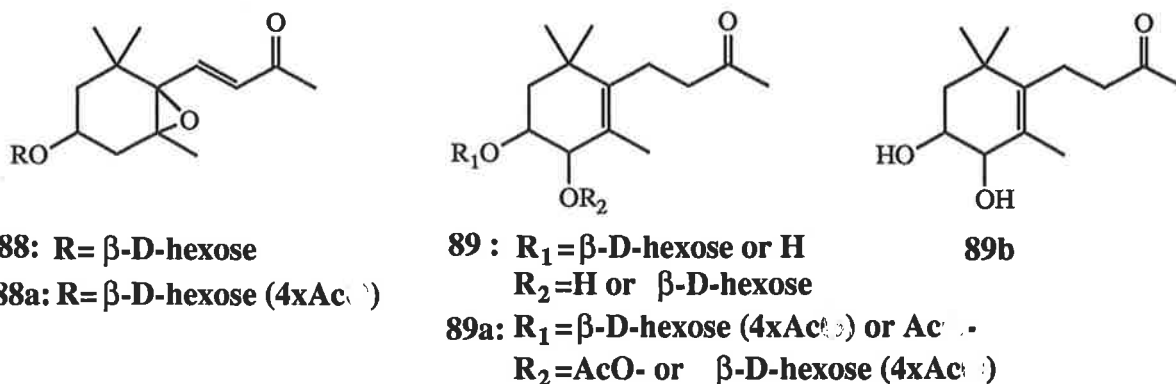


Figure 18: Some Riesling wine norisoprenoid constituents and their glycosidic derivatives.

Because **88** and **89** were present in the Riesling wine in only trace amounts, it was not possible to isolate sufficient material for a ^1H NMR studies and, thus to unequivocally establish the structure of the sugar moieties, the position of glycosylation in the case of **89**, or the anomeric configuration. However, because both glycosides were hydrolysed after incubation with β -D-glucosidase (almond emulsin, E.C. 3.2.1.21), it would be reasonable to suggest that both **88** and **89** are β -D-hexopyranosides.

These experiments demonstrate both the power and the limitations of FAB-MS and MS/MS experiments for the detection and structural characterization of glycosidic components in complex mixtures. Although structural assignments based solely on mass spectral data are only tentative, FAB-MS/MS experiments provide an extremely time-efficient protocol for the study of glycosidic mixtures by avoiding the necessity of preliminary, laborious and often only partially effective, chromatographic separations. The examination of the spectra of individual constituents in a mixture can be achieved on-line by making use of the separation power of the tandem mass analysers. However, this focus on individual constituents of mixtures is not possible in cases where diastereoisomeric compounds, or more than one structural isomer, are present as a mixture. In these cases, the presence of only a common pseudo-molecular ion for all isomers will be observed in the FAB mass spectra.

Chapter 3

EARLY-ELUTING POLAR MONOGLUCOSIDES

3.1 Introduction

Work presented in the previous chapter demonstrated that FAB-MS and tandem mass spectrometry (MS/MS) experiments can be used with advantage for the study of wine glycosides. This part of the work was undertaken to determine if the protocol could be applied to more polar DCCC fractions which may contain glycoconjugates of polar aglycones, not amenable to GC-MS analysis following enzyme hydrolysis, and therefore not previously detected in earlier studies (Strauss *et al.* 1987a, P. J. Williams *et al.* 1989, Winterhalter *et al.* 1990b). Furthermore, these more polar glycoconjugates may include higher molecular weight constituents which would test the general applicability of the protocol. Application of these techniques to the analysis of a polar, early-eluting DCCC glycosidic fraction, has permitted the detection and structural characterization of two new classes of phenolic derivatives in wine.

3.2 Results and discussion

A series of polar glycosidic fractions was obtained from a Riesling wine by droplet counter current chromatography (DCCC, fr. 90-100), followed by flash chromatography on silica gel (Winterhalter *et al.* 1990b). Monitoring these flash fractions by positive and negative FAB-MS indicated the presence of three glycoconjugates with apparent M_r s of 418, 522 and 524. The M_r of each of these glycoconjugates was deduced from the presence of protonated and pseudo-molecular ions in the positive ion mode at m/z 419 (M+H)⁺, 441 (M+Na)⁺ for the first compound, 523 (M+H)⁺, 545 (M+Na)⁺ for the second, and 525 (M+H)⁺ for the third compound as well as deprotonated molecular ions in the negative ion mode at m/z 417 (M-H)⁻, 521 (M-H)⁻ and 523 (M-H)⁻ for the first, second and third compound, respectively. FAB-MS/MS experiments on these protonated and deprotonated molecular ions provided the following structural information.

For the glycoside with apparent M_r 418, (glycoside 90) MS/MS data suggested a monohexoside, which showed positive and negative mode fragment ions for the hexose ring at m/z 163 [(hexose+H)-H₂O]⁺, 145 [(hexose+H)-2H₂O]⁺, 127 [(hexose+H)-

$3\text{H}_2\text{O}]^+$, and $161 [(\text{hexose-H})-\text{H}_2\text{O}]^-$, $119 [(\text{hexose-H-H}_2\text{O})-\text{CH}_2=\text{C}=\text{O}]^-$, 101 , 89 , respectively (Pare *et al.* 1988, Domon and Costello 1988b). The fragmentation pattern of the glycoside in the positive ion mode showed subsequent losses from the protonated molecular ion of 162 (loss of anhydrohexose) and of 74 leading to a protonated ion at m/z 183 . In the negative mode losses were observed from the deprotonated molecular ion of 182 and 74 leading to an ion of a deprotonated anhydrohexose at m/z 161 . These patterns are consistent with a molecule comprising three individual parts, i.e. the hexose moiety, a segment of 182 mass units, and a third segment of 92 mass units; the latter losing water to yield the observed fragment of 74 . Furthermore, either the hexose moiety or the segment of 182 mass units was lost from the protonated and the deprotonated molecular ions, respectively, leaving alternatively the segment of 182 mass units or the hexose moiety as the ultimate daughter ion. In either case the penultimate daughter ion showed a loss of 74 mass units. These data suggested a linear pattern of substitution with the segment of 92 mass units being a disubstituted species. A candidate for this central disubstituted species of 92 mass units was glycerol and a possible structure for **90** was, therefore, a glycosylated glycerol with a further substituent of apparent M_r 182 . The fact that the protonated ion at m/z 183 in the positive mode daughter ion spectrum further generated a strong fragment ion at m/z 137 , which is characteristic of a hydroxy-methoxy-substituted benzylium ion, suggested that the substituent could be dihydroconiferyl alcohol.

In the case of compounds **91** and **92** with apparent M_r 522 and 524 , respectively, the positive and negative mode daughter ion mass experiments indicated that they were also monohexosides. Thus, glycoside **91** showed daughter ions in the positive ion mode at m/z $361 [(\text{M+H})-\text{anhydrohexose, i.e. aglycone+H}]^+$, $163 [(\text{hexose+H})-\text{H}_2\text{O}]^+$, $145 [(\text{hexose+H})-2\text{H}_2\text{O}]^+$ and in the negative ion mode daughter ions at m/z $359 [(\text{M-H})-\text{anhydrohexose}]^-$ and $161 [(\text{hexose-H})-\text{H}_2\text{O}]^-$. For glycoside **92** a loss of an anhydrohexose unit from the protonated molecular ion in the positive mode, and a loss of a hexose unit from the deprotonated molecular ion in the negative mode, were also observed.

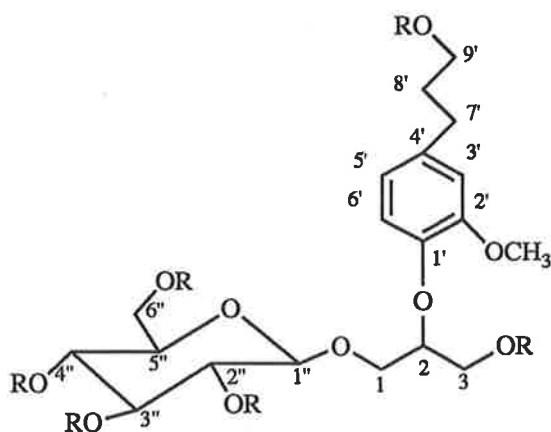
Preparative separation by HPLC of the Riesling fraction which contained glycosides **90** and **91** (i.e. fl. frs. 10-12), allowed isolation of these two glycosides in pure form. This was determined by TLC and positive-negative FAB-MS examination of the isolates. The UV absorbance of glycoside **90** showed λ_{max} at 278 nm, consistent with the presence of a substituted benzene ring (D. H. Williams and Fleming 1987). The ^1H NMR spectrum (table 4) showed the presence of three aromatic protons (δ $6.86-7.07$), six protons characteristic of a phenylpropanol side chain (two broad triplets at δ 2.64 , and 3.60 and a

multiplet at δ 1.84), signals for a glycerol moiety (δ 3.27-4.55), a methoxyl singlet at δ 3.86, and a signal for an anomeric proton of β -configuration (δ 4.47, $J=6.6$ Hz). The above spectral data are essentially identical with those reported for 1-*O*-(β -D-glucopyranosyl)-2-[2-methoxy-4-(ω -hydroxypropyl)-phenoxy]-propan-3-ol (Miki and Sasaya 1979a).

Acetylation of **90** with acetic anhydride/pyridine produced the hexaacetate **90a** [$M^+=670$ (EIMS), $(M+H)^+=671$ (FAB-MS), $(M-H)^-=669$ (negative ion NH_4 CIMS)], the positive ion spectra (EIMS, FAB-MS) of which contained a sequence of fragment ions at m/z 331, 271, 169, 109 characteristic of a monosubstituted tetra-*O*-acetylated hexopyranoside (Radford and Dejongh 1972). The observation in the EIMS and FAB-MS/MS spectra of **90a** of ions at m/z 447 and 448 was consistent with fragmentation resulting from loss of the dihydroconiferyl alcohol monoacetate moiety from the molecular ion and from the protonated molecular ion, respectively, as was suggested by the MS/MS study of **90**.

The 1H NMR signals of the hexaacetate **90a** are given in table 4; absence of a low field signal near δ 2.3 for a phenolic acetate indicated that the dihydroconiferyl alcohol moiety was linked to the glycerol via the phenolic hydroxyl. The absence of a significant downfield shift, after acetylation, for the C-2 proton of the glycerol skeleton, indicated that the hydroxyl group on this centre was substituted. Furthermore, its chemical shift (δ 4.45) suggested a CH-O-Ar system (D. H. Williams and Fleming 1987, Miki and Sasaya 1979a), and therefore it was concluded that the dihydroconiferyl alcohol was attached to glycerol at position 2 as an aromatic ether. The signals observed for the sugar part of the molecule were in agreement with those previously published for tetra-*O*-acetylated glucopyranoses (Schwab and Schreier 1988) and clearly established the sugar part of **90a** as tetra-*O*-acetylated glucopyranose with the anomeric proton having a β -configuration. The above spectral data for **90a** are in good agreement with those published previously by Miki and Sasaya (1979a) for the per-*O*-acetylated 1-*O*-(β -D-glucopyranosyl)-2-[2-methoxy-4-(ω -hydroxypropyl)-phenoxy]-propan-3-ol. From 1H - 1H -COSY of **90a** the coupling relationships of the individual protons were observed and these, together with data from decoupling experiments of the signals at δ 4.08 (see table 4), were found to accommodate the proposed structure.

Table 4: ^1H NMR data of compounds **90**, **90a** (300 MHz, δ)



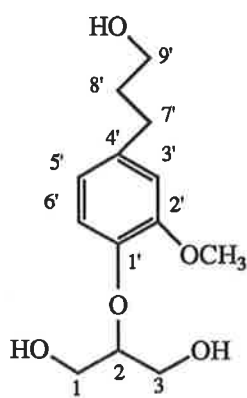
90 : R=H

90a: R=Ac

H	90 (D ₂ O)	90a (CDCl ₃)	90a ^1H - ^1H COSY
Aglycone moiety			
1a.	3.27, brt, $J=8.9\text{Hz}$	4.08, brt, $J=6.5\text{Hz}^*$	H-1b, H-2
1b.	3.27, brt, $J=8.9\text{Hz}$	3.80, dd, $\Sigma J=10.6\text{Hz}$	H-1a, H-2
2.	4.55, m	4.45, m	H-1a/H-1b/H-3a/H-3b
3a.	3.39, m	4.08, brt, $J=6.5\text{Hz}^*$	H-3b/H-2
3b.	3.39, m	4.31, brt, $J=5.7\text{Hz}$	H-3a/H-2
3'.	6.98, brs	6.71, brs	
5'.	7.07, d, $J=6.8\text{Hz}$	6.67, dd, $J=7.6, 1.5\text{Hz}$	H-6'/H-3'
6'.	6.86, d, $J=7.0\text{Hz}$	6.90, d, $J=7.6\text{Hz}$	H-5'
7'.	2.64, brt, $J=8.0\text{Hz}$	2.63, brt, $J=7.6\text{Hz}$	H-8'
8'.	1.84, m	1.93, m	H-7'/H-9'a/H-9'b
9'a.	3.60, brt, $J=6.2\text{Hz}$	4.08, brt, $J=6.5\text{Hz}^*$	H-9'a/H-8'
9'b.	3.60, brt, $J=6.2\text{Hz}$	4.31, brt, $J=5.7\text{Hz}$	H-9'a/H-8'
Sugar moiety			
1''.	4.47, d, $J=6.6\text{Hz}$	4.65, d, $J=7.9\text{Hz}$	H-2''
2''.	3.40-4.10, m	5.00, dd, $J=8.1, 9.5\text{Hz}$	H-1''/H-3''
3''.	3.40-4.10, m	5.22, brt, $J=9.5\text{Hz}$	H-2''/H-4''
4''.	3.40-4.10, m	5.07, brt, $J=9.6\text{Hz}$	H-3''/H-5''
5''.	3.40-4.10, m	3.65, m	H-4''/H-6''a/H-6''b
6''a.	3.40-4.10, m	4.26, obsc.	H-5''/H-6''b
6''b.	3.40-4.10, m	4.07, obsc.	H-5''/H-6''a
CH ₃ O-	3.86, s	3.83, s	
Acetates:		2.01 (1s), 2.02 (1s), 2.06 (2s), 2.17 (2s)	

Footnote : * Irradiation of the signals at δ 4.08 showed them to be coupled with signals at δ 1.93 (8'), δ 3.65 (5''), δ 3.80 (1b), and δ 4.31 (3b, 9'b).

Enzymic hydrolysis of **90** with β -D-glucosidase liberated an aglycone (**90b**) with an apparent molecular weight at m/z 256. This was deduced from the observation of the molecular ion in the probe EI spectrum and from the observation of the protonated $(M+H)^+$, pseudomolecular $(M+Na)^+$, and the deprotonated $(M-H)^-$ molecular ions in the positive and negative FAB spectra, respectively. The MS/MS examination of the molecular ion (probe EI mode) produced a spectrum in agreement with that published for 2-[2-methoxy-4-(ω -hydroxypropyl)-phenoxy]-propan-1,3-diol (Miki and Sasaya 1979a). Furthermore, the FAB-MS/MS spectrum of the protonated molecular ion generated fragment ions similar to those observed in the MS/MS spectrum of **90** for the aglycone portion.



90b

Figure 19: 2-[2-Methoxy-4-(ω -hydroxypropyl)-phenoxy]-propan-1,3-diol.

Finally, acid hydrolysis of **90**, followed by GC and GC-MS examination of the acid hydrolysis products as their TMSi derivatives, independently confirmed the sugar part as glucose. Thus, it was concluded that **90** is the 1-*O*-(β -D-glucopyranosyl)-2-[2-methoxy-4-(ω -hydroxypropyl)-phenoxy]-propan-3-ol.

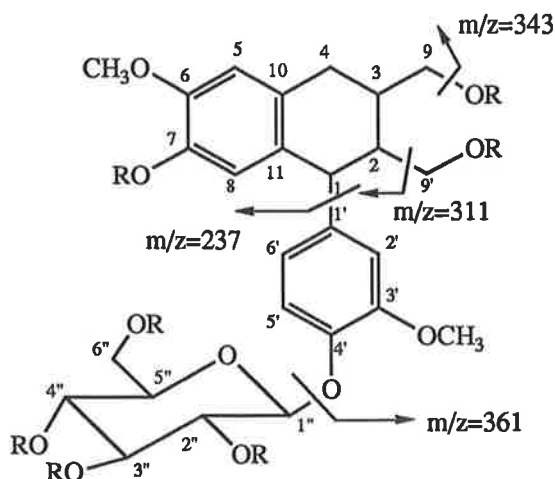
Phenylpropanoid-glycerol glycosides have been reported to be components of lily bulbs (Shimomura *et al.* 1988), and 1-*O*-(β -D-glucopyranosyl)-2-[2-methoxy-4-(ω -hydroxypropyl)-phenoxy]-propan-3-ol has been isolated from the inner bark of *Larix leptolepis* Gord (Miki and Sasaya 1979a).

Glycoside **91** showed absorption in its UV spectrum at λ_{max} =280 nm, consistent with the presence of a substituted benzene ring (D. H. Williams and Fleming 1987). The 1H NMR

spectrum (table 5), showed signals for five aromatic protons (δ 6.43-6.92) with only two of them showing a coupling relationship ($J=8.0$ Hz, ortho-coupling), indicating the probable presence of two aromatic systems with one aromatic ring being tri- and the other one tetrasubstituted. Additionally, signals for two benzylic protons (δ 2.83), one homobenzylic (δ 2.05), one bis-homobenzylic (δ 1.81), one anomeric proton with β -configuration (δ 4.52, $J=7.5$ Hz), and two methoxyl groups were observed. The data support the assignment of **91** as a glycoside of the isomeric lignans (+) and/or (-) 1,2,3,4-tetrahydro-7-hydroxy-1-(4'-hydroxy-3'-methoxyphenyl)-6-methoxy-2,3-naphthalenedimethanol (i.e. isolariciresinol), and were in good agreement with data published previously for glycosylated derivatives of both these compounds (Lundgren *et al.* 1981, Popoff and Theander 1977). At this stage the data did not permit an assignment of the position of glycosylation, because the four hydroxy methylene protons of the aliphatic side chains of isolariciresinol could not readily be distinguished from the hydroxy methylene group of the carbohydrate portion of the molecule (δ 3.32-3.75, 9H).

Acetylation of **91** with acetic anhydride/pyridine produced a heptaacetate **91a** [(M+NH₄)⁺=834, (M-H)⁻=815 positive and negative ion mode NH₄ CIMS]. The ¹H NMR data are given in table 5. The observation of only one phenolic acetate (δ 2.29) suggested that **91a** was glycosylated via one of its phenolic hydroxyls. The upfield shift for one of the methoxyls (δ 3.79 from 3.83), and one of the aromatic protons (δ 6.61 from 6.92) as a consequence of acetylation, implied that they both were situated next to the acetylated phenolic hydroxyl. The absence of any detectable coupling between that aromatic proton which had shifted and any other proton in the ¹H-¹H NMR COSY experiment indicated that the aromatic proton was proximal to the fused ring. This suggested that the phenolic acetate was located at position C-7 of the tetrasubstituted ring of **91a**, and that the isolariciresinol was therefore glycosylated at position C-4' of the other aromatic ring. Other signals observed for the aglycone part of **91a** were similar to those previously published for isolariciresinol acetate (Popoff and Theander 1977). Furthermore, the exact chemical shifts and coupling relationships as deduced from the COSY ¹H-¹H NMR experiment verified **91a** as a glycosidic derivative of isolariciresinol. The signals observed for the saccharide portion of **91a** were second order and the coupling constants for the protons of the sugar portion could not be determined.

Table 5: ^1H NMR data of compounds **91**, **91a** (300 MHz δ) and some of the positive ion mode mass spectral fragmentations of **91**



91 : R=H

91a: R=Ac

H	91 (D ₂ O)	91a (CDCl ₃)	91a ^1H - ^1H COSY
Aglycone moiety			
1.	3.32-3.75, m, obsc.	3.91, d $J=10.7\text{Hz}$	H-2
2.	1.81, brt, $J=9.1, 11.0\text{Hz}$	2.05, m (overlap with acetates)	H-1/H-3/H-9'a/H-9'b
3.	2.05, m	2.20, m (overlap with acetates)	H-4/H-2/H-9a/H-9b
4.	2.83, m	2.85, m	H-3
5.	6.43, brs	6.47, brs	
8.	6.92, brs	6.61, s	
9a.	3.32-3.75, m	4.25, dd, $J=4.1, 11.1\text{Hz}$	H-9b/H-3
9b.	3.32-3.75, m	4.10, m	H-9a/H-3
9'a.	3.32-3.75, m	4.25, dd, $J=4.1, 11.0\text{Hz}$	H-9'b/H-2
9'b.	3.32-3.75, m	4.10, m	H-9'a/H-2
2'.	6.79, brs	6.70, d, $J=1.7\text{Hz}$	H-6'
5'.	6.90, d, $J=8.1\text{Hz}$	6.96, d, $J=8.1\text{Hz}$	H-6'
6'.	6.74, d, $J=8.0\text{Hz}$	6.65, dd, $J=8.1, 1.7\text{Hz}$	H-5'/H-2'
Sugar moiety			
1''.	4.52, d, $J=7.5\text{Hz}$	4.53, m	H-2''
2''.	3.32-3.75, m	5.11-5.26, m	H-1''/H-3''
3''.	3.32-3.75, m	5.11-5.26, m	H-2''/H-4''
4''.	3.32-3.75, m	5.11-5.26, m	H-3''/H-5''
5''.	3.02, m	3.45, m	H-4''/H-6''a/H-6''b
6''a	3.32-3.75, m	3.82, dd, $J=10.0, 1.9\text{Hz}$	H-6''b/H-5''
	3.32-3.75, m	4.10, m	H-6''a/H-5''
CH ₃ O-	3.83, s	3.79, s	
CH ₃ O-	3.78, s	3.78, s	
Acetates		2.00 (1s), 2.02 (1s), 2.05 (1s), 2.08 (1s), 2.17 (2s), 2.29 (1s)	

Glycoconjugate **91** was resistant to enzymic treatment with β -D-glucosidase, but on enzymic hydrolysis with a fungal enzyme preparation (*Novoferm* 12) liberated an aglycone (**91b**) with an apparent molecular ion at $m/z=360$ (probe EIMS). Trimethylsilylation of the liberated aglycone and GC-MS examination of the derivative showed it as a single peak with a mass spectrum in close agreement with that previously published for the per-trimethylsilylated derivative of isolariciresinol (Kraus and Spitteller 1990).

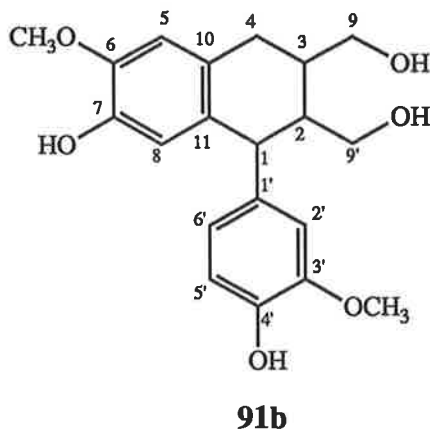


Figure 20: 1,2,3,4-Tetrahydro-7-hydroxy-1-(4'-hydroxy-3'-methoxyphenyl)-6-methoxy-2,3-naphthalenedimethanol (i.e. isolariciresinol).

On acid hydrolysis, **91** liberated a sugar which after trimethylsilylation co-chromatographed with and gave an identical ^{EIMS} spectrum to, an authentic sample of TMSi glucose. Thus, glycoside **91** was characterized as the 1,2,3,4-tetrahydro-7-hydroxy-1-(4'- β -glucopyranosyl-3'-methoxyphenyl)-6-methoxy-2,3-naphthalenedimethanol (i.e. isolariciresinol- β -4'-*O*-glucopyranoside).

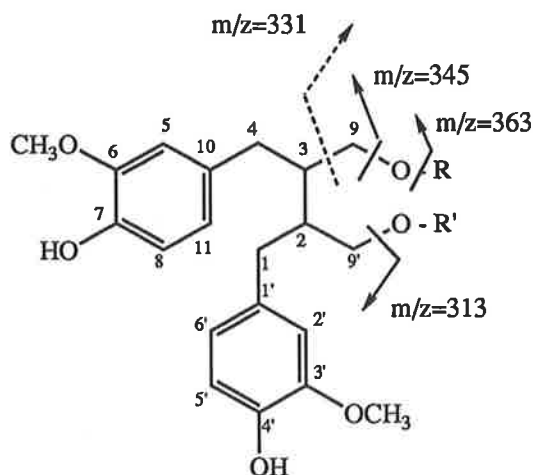
From a study of the fragmentation pathways of **91** under FAB-MS/MS conditions it was possible to assign the position of glycosylation in the molecule from that data also. Thus, parent ion experiments on the fragments observed in the positive mode daughter ion spectrum of **91** showed that only ions at m/z 361 and m/z 237 were directly associated with the protonated molecular ion, suggesting formation of these ions after elimination of anhydroglucose or methoxyphenylglucose, respectively, from the protonated molecular ion. This was consistent with the isolariciresinol being glycosylated on the C-4' phenolic hydroxyl. Further support for the assignment was provided by the negative mode daughter ion spectrum in which elimination of anhydroglucose but not of glucose from the

deprotonated molecular ion of **91** was observed. This latter fragmentation was attributed to the fact that the negative charge was preferably stabilized on the phenolic hydroxyl rather than the aromatic ring, leading to cleavage of the glycoside between glucose and the phenolic oxygen only and to elimination of anhydroglucose.

Isolariciresinol glycosides have previously been isolated from the needles of *Pinus sylvestris* (Popoff and Theander 1977), *Pinus massoniana* Lamb. (Lundgren *et al.* 1985), *Picea abies* (Lundgren *et al.* 1981) and *Populus nigra* L. (Benecke *et al.* 1989). In all cases reported, the glycosylation has involved the hydroxyls of the aliphatic side chains rather than the phenolic hydroxyls. This appears to be the first time that isolariciresinol has been identified as a phenolic glycoside.

The crude glycosidic flash chromatography fractions (i.e. fl. frs. 6-7) containing the glycoside **92** were enzymically hydrolyzed with β -D-glucosidase. The liberated aglycones when examined by probe EIMS indicated the presence of a constituent with apparent molecular weight 362 (**92a**). Probe EI-MS/MS examination of that ion produced a daughter ion spectrum with the same fragments as those seen in the spectrum of a reference sample of (2R, 3R) 2,3-bis(4'-hydroxy-3'-methoxybenzyl)butane-1,4-diol, i.e. *seco*-isolariciresinol (Cambie *et al.* 1985), when analysed under the same conditions. Trimethylsilylation of the aglycones and GC-EIMS examination revealed the existence of a peak which gave an identical EI spectrum to, and co-chromatographed with, the TMSi derivative of the reference *seco*-isolariciresinol. Thus, it was concluded that the glycoconjugate **92** was a β -hexoside of *seco*-isolariciresinol. HPLC separation of fl. frs. 6-7 yielded pure glycoside **92**. The positive ion MS/MS experiment on the protonated molecular ion of **92** under FAB conditions showed, in addition to ions corresponding to fragmentation of the sugar ring, other ions at m/z 363, 345, 331 and 313, which appeared to be associated with the aglycone part of the molecule. It was unclear, however, whether these ions were derived from the protonated aglycone or the protonated molecular ion. Parent ion experiments on these four ions showed that only those at m/z 363 and m/z 331 were directly associated with the protonated molecular ion, arising after elimination of 162 (anhydrohexose) and 194 (methylhexoside), respectively (see figure 21). This implied glycosylation of the aglycone via one of the aliphatic side chain hydroxyls rather than via one of the phenolic hydroxyls. This deduction was supported by the negative mode daughter ion spectrum where elimination of both anhydrohexose and hexose were observed suggesting that fragmentation occurred on either side of the glycosidic oxygen. Such a fragmentation pathway was not observed in the negative ion spectrum of phenolic

glycoside **91**. For the ions at m/z 345, 313 in the positive mode, the parent ion experiments suggested that they were formed after elimination of H_2O from ions at m/z 363 and 331, respectively.



92: R=H or β -D-glucose, R'= β -D-glucose or H
92a: R=R'=H

Figure 21: 2,3-Bis(4'-hydroxy-3'-methoxybenzyl)butane-1,4-diol (i.e. *seco*-isolariciresinol), its glucosidic derivative and some of the positive ion mode mass spectral fragmentations of the glucosidic derivative.

On acid hydrolysis, **92** liberated a sugar which after trimethylsilylation co-chromatographed with a reference sample of TMSi glucose.

Because **92** was present in the Riesling wine in only trace amounts (ca. 2 ppb based on the amount isolated), it was not possible to isolate sufficient material for 1H NMR study, and hence to confirm the position of glycosylation. Thus, compound **92** was tentatively assigned as the β -D-glucoside of *seco*-isolariciresinol with the sugar moiety being conjugated via one of the aliphatic rather than the phenolic hydroxyls.

Seco-isolariciresinol glycosides have previously been isolated from the needles of *Pinus sylvestris* (Popoff and Theander 1977) and from *Berchemia racemosa* (Inoshiri *et al.* 1987). In both cases, the glycosylation has involved the hydroxyls of the aliphatic chains as appears to be the case here. Although *seco*-isolariciresinol has never been detected in mammals, its glycoside has been implicated as a dietary precursor of *trans*-2,3-bis(3-hydroxybenzyl)- γ -butyrolactone (enterolactone) and 2,3-bis(3-hydroxybenzyl)butane-1,4-diol (enterodiol) (Axelson *et al.* 1982) in urine. The latter are formed in the intestinal tract

by bacteria (Axelson *et al.* 1982) and their possible biological activities have been discussed (Axelson *et al.* 1982, Adlercreutz 1984).

The spectrometric (¹H NMR, UV) verification of the initial assignments, which were based on an interpretation of FAB-MS/MS data for the molecules with M_r 418 and 522, demonstrates the utility of FAB-MS/MS for the detection and partial characterization of polar glycosidic compounds.

This is the first time that the phenylpropanoid-glycerol glycoside (**90**) and the lignan glycosides (**91**) and (**92**) have been reported as wine constituents. It was observed that the enzymic hydrolysate of **90** had a strong caramel, buttery-like aroma raising the possibility that phenylpropanoid glycerols may have an impact on wine flavour.

Chapter 4

EARLY-ELUTING DISACCHARIDE GLYCOSIDES

4.1 Introduction

The occurrence of disaccharide glycosides of secondary metabolites in grapes was first reported by Williams *et al.* (1982b, 1983) who identified β -rutinosides and 6-O- α -L-arabinofuranosyl- β -D-glucopyranosides of terpenoid and shikimate-derived secondary metabolites. An indication that another sugar was also involved in glycosylation of grape juice or wine aroma volatile constituents came from the identification of apiose as a sugar constituent of the acid hydrolysates of glycosidic fractions from varietal grape juices (Brillouet *et al.* 1989). Identification of the geranyl-6-O- β -D-apiofuranosyl- β -D-glucopyranoside "accuminoside" as a constituent of the unhydrolysed glycosidic fractions isolated from these varietal grape juices, verified that suggestion (Voirin *et al.* 1990a). This was the first report of an apiosylglucoside in grape juice, although these compounds are commonly found in nature (Beck and Hopf 1990).

This chapter describes the isolation and characterization of three new monoterpene apiosylglucosides in Riesling wine. These apiosylglucosides were found together with a new arabinoglucoside and known rutinoside and arabinoglucoside derivatives.

4.2 Results and discussion

Fractions of early-eluting DCCC disaccharide glycosides were obtained as described in chapter 3. Monitoring these flash fractions by positive-negative FAB-MS indicated the presence of a series of glycoconjugates with apparent M_r s of 416, 464, 466, and 518. These M_r were deduced from the presence of protonated $(M+H)^+$ and pseudo-molecular ions $(M+Na)^+$ in the positive ion mode and corresponding deprotonated molecular ions $(M-H)^-$ in the negative ion mode spectra for each case. FAB-MS/MS experiments on these protonated and deprotonated molecular ions provided substantial structural information.

For glycosides with apparent M_r 416, MS/MS data suggested they were deoxyhexosyl-hexosides (e.g. rutinosides). The assignment was based on the observation in the positive mode daughter ion spectrum of a fragment ion at m/z 309 corresponding to a protonated

anhydrodeoxyhexosyl-hexose (Domon and Hostettmann 1985). The spectrum also showed fragment ions for the individual sugar rings at m/z 181 [hexose+H]⁺, 163 [(hexose+H)-H₂O]⁺, 147 [(deoxyhexose+H)-H₂O]⁺, and 129 [(deoxyhexose+H)-2H₂O]⁺ (Pare *et al.* 1988, Domon and Hostettmann 1985). The observation in the negative mode daughter ion spectrum of fragment ions at m/z 269 [(M-H)-anhydrodeoxyhexose]⁻, 163 [deoxyhexose-H]⁻, 145 [(deoxyhexose-H)-H₂O]⁻ and 161, 119, 101, 89 (hexose ring fragment ions) further supported the above assignment (Domon and Costello 1988a, Domon and Costello 1988b). For glycosides with apparent M_r s of 464, 466 and 518, positive ion mode MS/MS experiments on the protonated molecular ions indicated that all glycoconjugates were pentosyl-hexosides. These yielded daughter ions at m/z 295 corresponding to a protonated anhydropentosyl-hexose and fragment ions for the individual sugar rings at m/z 163, 145 (hexose), 133, 115 (pentose) (Pare *et al.* 1988). These positive mode daughter ion assignments were supported by the observation in the negative mode daughter ion spectra of fragments characteristic for both sugar rings at m/z 161, 119, 113, 101 (hexose) and 149, 131 (pentose) (Domon and Costello 1988b, Domon and Costello 1988a).

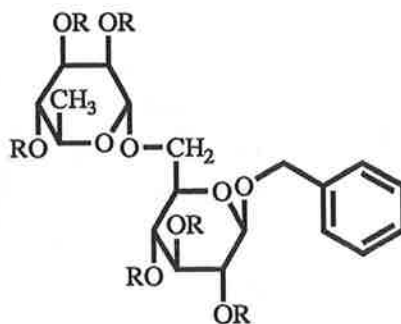
For the sequence of the sugar moieties the fragmentation pathways of the glycosides in the positive and negative mode daughter ion spectra suggested that both deoxyhexose and pentose were terminal units. This was deduced from the observation of anhydrodeoxyhexose and anhydropentose being either readily lost as fragment ions from the protonated or the deprotonated molecular ions or being the ultimate daughter ions after subsequent elimination of the aglycone and the remaining sugar segment. The penultimate daughter ion in all cases in the positive ion mode experiments showed losses of hexose or anhydrohexose. These data suggested a linear pattern of substitution with the hexose sugar part being disubstituted.

Structural information for the aglycones was, again, deduced from parameters such as their apparent M_r , calculated from the protonated and deprotonated ions after subtraction of the sugar moieties, and the presence in the daughter ion spectra of certain diagnostic fragment ions. In the case of the glycoside(s) with apparent M_r 416 the calculated molecular weight for the aglycone moiety (416-308=108) and the existence of a strong fragment ion at m/z 91 (tropylium ion) (Williams *et al.* 1983) suggested that glycoside(s) with apparent M_r 416 is a conjugated form of benzyl alcohol. The latter alcohol had previously been identified as a constituent of the enzymic hydrolysate of this glycosidic mixture after GC-EIMS examination. In the cases of glycoconjugates with apparent M_r s of 464 and 466 the appearance of ions for protonated aglycones at m/z 171 and 173 and

fragment ions at m/z 153 [(aglycone+H)-H₂O]⁺, 135 [(aglycone+H)-2H₂O]⁺, 155 [(aglycone+H)-H₂O]⁺, 137 [(aglycone+H)-2H₂O]⁺, suggested the presence of monoterpene diols of molecular weights 170 and 172, respectively. For the glycoside(s) with apparent M_r 518, the presence of a protonated ion for the aglycones at m/z 225 [aglycone+H]⁺ suggested one or more C₁₃ norisoprenoids of molecular weight 224. The observation of fragment ions at m/z 207 [(aglycone+H)-H₂O]⁺, 189 [(aglycone+H)-2H₂O]⁺, and 161 [(aglycone+H)-2H₂O-CO]⁺, suggested the presence of two hydroxyl and a carbonyl group in at least one aglycone skeleton. This was consistent with the assignment of glycoside(s) with apparent M_r 518 as including a conjugated form of vomifoliol (**63**) which was the only norisoprenoid of that molecular mass identified in the hydrolysate of the glycosidic mixture after GC-EIMS examination.

After the preliminary FAB-MS/MS examination, the flash chromatography fractions under investigation were further separated by chromatography on HPLC. The sub-fractions so obtained were monitored by positive-negative FAB-MS and those which contained glycoconjugates with the previously mentioned mass spectrometric characteristics were derivatized (i.e. per-O-acetylated by pyridine/acetic anhydride) and further purified by flash chromatography on silica gel. These chromatographic steps enabled isolation of disaccharide glycosides **93a-96a** in a pure form, while glycosides **97a**, **98a** were isolated as a mixture. This was determined by TLC examination of the isolates and by the ¹H NMR data.

FAB-MS examination of **93a** suggested that it was a hexaacetate [(M+H)⁺=669]. The observation in the EI and FAB mass spectra of fragment ions at m/z 273, 561 indicated the presence of a monosubstituted hexa-O-acetylated deoxyhexosyl-hexoside (P. J. Williams *et al.* 1982a). This glycoside was tentatively identified as the rutinoside of benzyl alcohol by means of its gas chromatographic and mass spectrometric characteristics which were in agreement with those reported for **93a** elsewhere (Williams *et al.* 1983). Glycoside **93a** was not further characterized here as it had already been previously identified as a grape juice constituent (P. J. Williams *et al.* 1982a). Our interest about this molecule is based on the fact that **93** provides a case to study the behaviour of rutinosides under FAB-MS/MS conditions.



93 : R= H

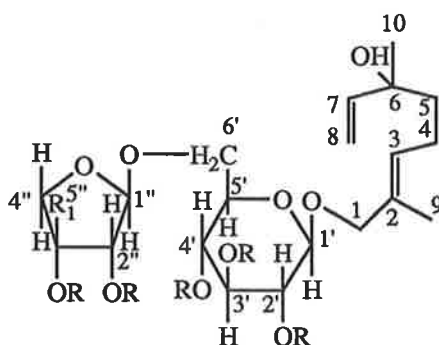
93a: R=Ac

Figure 22: Benzyl alcohol- β -D-rutinoside and its per-O-acetylated derivative.

Acetylation of **94** produced a hexaacetate (**94a**) [(M+H)⁺=717, (M+Na)⁺=739, (M+K)⁺=755] the spectrum of which contained the sequence of fragment ions at *m/z* 139, 259, 331, 547 characteristic of a monosubstituted hexa-O-acetylated pentosyl-hexoside (P. J. Williams *et al.* 1982a). The adoption of all six acetates by the sugar moiety, indicated the presence in the aglycone part, presumably a monoterpene diol from the FAB-MS/MS data, of a free tertiary hydroxyl. The ¹H NMR spectrum of **94a** (see table 6) showed signals at δ 1.27 and δ 1.57 assigned to a methyl group on a carbon bearing an oxygen atom and an olefinic methyl group, respectively. Additionally the spectrum showed an ABX system for a mono-substituted double bond (δ 5.89, 5.21, and 5.06), an unresolved signal for an olefinic proton of a tri-substituted double bond (δ 5.32), and an AB quartet system, centered at δ 4.16, for methylene protons geminally coupled on a carbon bearing oxygen. These data are consistent with the identification of the aglycone as 2,6-dimethylocta-2,7-diene-1,6-diol. The presence of four doublets characteristic of the protons on positions four and five of a tri-O-acetylated apiofuranoside (δ 3.93, 4.12, 4.55, 4.74) and also signals observed for a bis substituted at positions one and six tri-O-acetylated glucose, suggested that the sugar moiety was a per-O-acetylated apiofuranosyl-(1 \rightarrow 6)-glucopyranoside. The spectrometric data observed for the sugar moiety were in agreement with those previously published for per-O-acetylated apiofuranosyl-(1 \rightarrow 6)-glucopyranosides (Nishimura *et al.* 1990). The coupling constants observed for the anomeric protons of apiose (δ 5.02, *J*=0-1.0 Hz) and glucose (δ 4.45, *J*=8.0 Hz) indicated that both have the β -configuration. The high field position of the AB quartet system centered at δ 4.16, and the presence of a free tertiary hydroxyl on the aglycone part of the molecule deduced from the FAB-MS data, suggested glycosylation of the aglycone via the C-1 primary hydroxyl. From a ¹H-¹H-COSY spectrum of **94a** the coupling relationships of the individual protons were observed and they were found to accommodate the proposed structure (see table 6).

The stereochemical purity of C-3 of the apiose skeleton was checked by performing an acetolysis experiment on **94a**. Acetolysis of a stereochemical pure apiofuranosylglucopyranoside would liberate only the two anomers (α and β) of D- or L- apiose.

Table 6 : ^1H NMR data of compounds **94**, **94a** (300MHz, δ ppm).



94: R=H, $\text{R}_1=\text{CH}_2\text{OH}$

94a: R=Ac $\text{R}_1=\text{CH}_2\text{OAc}$

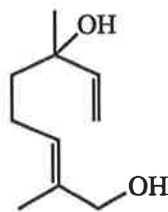
H	94 (D_2O)	94a (CDCl_3)	^1H - ^1H COSY correlated with
Aglycone moiety			
1.	3.98, 4.06, ABq, $J=9.3\text{Hz}$	4.12-4.20, ABq, $J=10.6\text{Hz}$	H-1b/H-1a
3.	obsc.*	5.40, brt, $J=7.0\text{Hz}$	
4.	2.15-2.05, m	obsc.	
5.	1.63-1.58, m	obsc.	
7.	5.96, dd, $J=17.4, 11.1\text{Hz}$	5.90, dd, $J=10.7, 17.6\text{Hz}$	H-8a/H-8b
8a.	obsc.*	5.05, dd, $J=1.3, 10.7\text{Hz}$	H-7/H-8b
8b.	obsc.*	5.21, dd, $J=1.3, 17.6\text{Hz}$	H-7/H-8a
9.	1.66, brs	1.57, brs	
10.	1.29, brs	1.27, brs	
Sugar moiety			
1'.	4.39, d, $J=8.5\text{Hz}$	4.45, dd, $J=8.0\text{Hz}$	H-2'
2'.	3.30-3.23, m	4.95, dd, $J=8.0, 9.4\text{Hz}$	H-1'/H-3'
3'.	3.50-3.39, m	5.16, t, $J=9.4\text{Hz}$	H-2'/H-4'
4'.	3.50-3.39, m	4.90, t, $J=9.4\text{Hz}$	H-3'/H-5'
5'.	3.50-3.39, m	3.65-3.54, m	H-4'/H-6a'/H-6b'
6a'.	3.30-3.23, m	3.65-3.54, m	H-5'/H-6b'
6b'.	3.71-3.73, m	3.65-3.54, m	H-5'/H-6a'
1''.	obsc.*	5.02, brs, $J<1.0\text{Hz}$	
2''.	3.99, brs	5.32, brs, $J<1.0\text{Hz}$	
4a''.	3.89, d, $J=10.3\text{Hz}$	3.93, d, $J=11.9\text{Hz}$	H-4b''
4b''.	4.07, d, $J=10.5\text{Hz}$	4.12, d, $J=11.9\text{Hz}$	H-4a''
5a''.	4.12, d, $J=12.3\text{Hz}$	4.55, d, $J=12.5\text{Hz}$	H-5b''
5b''.	4.21, d, $J=12.2\text{Hz}$	4.74, d, $J=12.5\text{Hz}$	H-5a''
Acetates:		1.99 (1s), 2.00 (1s), 2.01 (1s), 2.01 (1s), 2.06 (1s), 2.08 (1s)	

* Underneath H_2O peak

The observations of Bishop and Cooper (Can. J. Chem., **41**, 2743, [1963]) on the rates of anomerisation of pentofuranosides suggested a method for a determination of the stereochemistry of C-3 of the apiose moiety of disaccharide **94a**. On the basis of their data it was expected that a mild, kinetically-controlled, acetolysis of a stereochemically-pure apioside, e.g. apiin (the D-erythrofuranoside configuration was established by Halyalkar *at al.*, Can. J. Chem. **43**, 2085, [1965]) would liberate the peracetate esters of only two isomers, i.e. the α - and β -anomers of 3-C-(hydroxymethyl)-D-erythrofuranose. In contrast, the acetylation of the thermodynamically-equilibrated isomers from an acid hydrolysis of 1,2:3,5-di-O- isopropylidene- α -D-apiose would give the two anomers of 3-C-(hydroxymethyl)-D-erythrofuranose plus the the two anomers of 3-C-(hydroxymethyl)-L-threofuranose.

A reference sample of 1,2:3,5-di-O- isopropylidene- α -D-apiose, after acid hydrolysis, equilibration and acetylation, showed on GC analysis that all four of the isomers could be separated. Thus, by using GC-MS to monitor the acetolysis products of **94a** in parallel with those from the acetolysis of a reference sample of apiin and of the tetraacetates of the four isomers from 1,2:3,5-di-O- isopropylidene- α -D-apiose above, it was found that the two isomeric forms of the pentose liberated from **94a** were identical to the two from apiin.

Enzymic hydrolysis of **94** with a fungal enzyme preparation (*Novoferm* 12) liberated an aglycone which under GC-EIMS conditions co-chromatographed with and gave an identical spectrum to, a reference sample of (E)-2,6-dimethylocta-2,7-diene-1,6-diol (**94b**). These data established the structure of **94** as (E)-2,6-dimethylocta-2,7-diene-1-(β -D-apiofuranosyl-(1 \rightarrow 6) - β -D-glucopyranosyl)-6-ol.



94b

Figure 23: (E)-2,6-Dimethylocta-2,7-diene-1,6-diol.

Acid hydrolysis of **94** and GC and GC-MS examination of the hydrolysates as their TMSi derivatives independently proved that the glycon consisted of an apiose and a glucose moiety. This was confirmed by co-injection with TMSi derivatives of authentic apiose and glucose. Examination by GC-EIMS of the CH_2Cl_2 -soluble acid hydrolysates revealed the presence of uroterpenol (**30**) and diastereoisomeric *p*-menth-1-en-9-als together with some (E)-2,6-dimethylocta-2,7-diene-1,6-diol (**94b**). This finding supports the hypothesis that,

in wine, uroterpenol (all four possible diastereoisomers), can be formed via acid catalyzed cyclization of 2,6-dimethylocta-2,7-diene-1,6-diol (Winterhalter *et al.* 1990b). Uroterpenol was previously seen as an unknown compound in the reaction mixture when the diol **94b** was hydrolysed under mild acidic conditions (pH 3.0) (Strauss *et al.* unpublished data). Uroterpenol was not reported as a product when diene-diol (**94b**) was hydrolysed under strong acidic conditions (SDE, pH 1.0) by Schwab and Schreier (1990b). A possible explanation for the discrepancy between the published data (Schwab and Schreier 1990b) and those obtained during the course of this study, is that the experimental conditions under which **94b** and **94** were hydrolysed, were different. Under the vigorous acid hydrolysis conditions employed by Schwab and Schreier (1990b), it is likely that uroterpenol (**30**) was further transformed to give diastereoisomeric *p*-menth-1-en-9-als. Another, more specific pathway for the formation of certain uroterpenol diastereoisomers (Carman *et al.* 1986) has been discussed previously in chapter 2.

The positive ion mode FAB-MS examination of **95a** suggested a heptaacetate [(M+H)⁺=759], the spectrum of which contained the sequence of fragment ions at *m/z* 259, 331, 547 which are ions given by a monosubstituted hexa-O-acetate of a pentosyl-hexoside (P. J. Williams *et al.* 1982a). Hence, the seventh acetate had to be accommodated on the aglycone.

The ¹H NMR signals of **95a** are given in table 7. The presence of signals for two olefinic methyl groups (δ 1.40, 1.70), an AB quartet system for two protons geminally coupled on a carbon bearing oxygen (centered at δ 4.25), an C=CH-CH₂-O system (δ 4.6, 2H, brd; δ 5.7, 1H, dd) and an olefinic proton on a tri-substituted double bond (δ 5.4), suggested 2,6-dimethylocta-2,6-diene-1,8-diol as a likely candidate for the aglycone. The signals observed for the sugar moiety were again in agreement with data previously published for per-O-acetylated apiofuranosyl-(1→6)-glucopyranosides as well as those observed for glycoside **94a**. The coupling constants observed for the anomeric protons of apiose and glucose suggested that both have the β -configuration (δ 4.4, d, *J*=8.0 Hz, glucose-H1; δ 5.01, brs, *J*<1.0 Hz, apiose-H1). The upfield chemical shift of the AB quartet system centered at δ 4.25 in relation to the chemical shift of the two protons of the C=CH-CH₂-O system at δ 4.60, suggested that the aglycone was glycosylated via the C-1 hydroxyl rather than that on C-8. From the above data **95a** was assigned as the hepta-O-acetylated 2,6-dimethylocta-2,6-diene-1,8-diol-1-O- β -apiofuranosyl-(1→6)- β -glucopyranoside.

Acetolysis of **95a** liberated only two anomers of per-O-acetylated apiose which were identified by GC and GC-MS comparison with a reference sample as those of D-apiose.

De-acetylation of **95a** produced a disaccharide glycoside with M_r 464 and MS/MS characteristics similar to those described for glycosides with apparent M_r 464 in the original flash chromatography fractions.

Enzymic hydrolysis of **95** with a fungal enzyme preparation (*Novoferm* 12) liberated an aglycone which under GC-EIMS conditions gave a spectrum identical to, and co-chromatographed with a reference sample of (E,E)-2,6-dimethylocta-2,6-diene-1,8-diol (**95b**). The data allowed **95** to be assigned as the (E,E)-2,6-dimethylocta-2,6-diene-1-(β -D-apiofuranosyl-(1 \rightarrow 6)- β -D-glucopyranosyl)-8-ol.

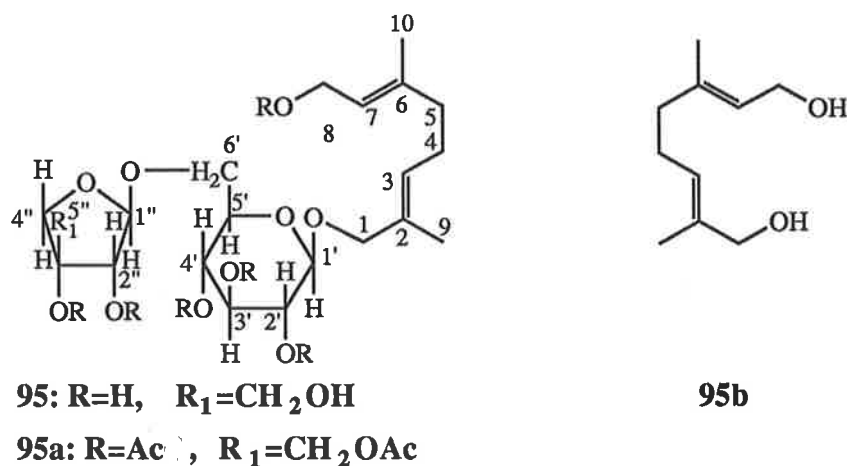


Figure 24: (E,E)-2,6-Dimethylocta-2,6-diene-1,8-diol and its glycosidic derivatives.

For glycoside **96a** the FAB mass spectrum suggested a hexaacetate [(M+H)⁺=719]. The presence of the sequence of fragment ions at m/z 259, 331, 547 similar to these seen in the cases of glycosides **94a** and **95a** again implied that this molecule was a monosubstituted hexa-O-acetate pentosyl-hexoside (P. J. Williams *et al.* 1982a). The adoption of all six acetates by the sugar moiety, indicated the presence of a tertiary hydroxyl in the aglycone, which was presumed to be a dihydro-monoterpene diol from the FAB-MS/MS data.

The ¹H NMR signals of **96a** are given in table 7; the presence of an ABX system for a C=CH-CH₂-O group (δ 4.30, dd; 4.15, dd; 5.6, m), of a six proton singlet for a CH₃-C(OH)-CH₃ system (δ 1.23, 6H), and of a methyl on a double bond (δ 1.63) suggested that the aglycone was 3,7-dimethylocta-2-ene-1,7-diol. For the sugar moiety, signals for a tri-

O-acetylated glucopyranoside unit bis-substituted at positions one and six were observed. Absence of the four doublets characteristic of protons on position four and five of apiose (δ 3.93, 4.12, 4.55, 4.74) which were observed in the previous cases (see tables 6,7) excluded the possibility of apiose being the reducing end unit. Instead, signals indicative for the presence of arabinofuranose (δ 5.01, arabinose H1; 5.13, arabinose H2; 4.15-4.30, arabinose H5a, H5b), were observed (Voirin *et al.* 1990b). The coupling constants of the anomeric protons of glucose and of the second sugar unit which appeared to be arabinose implied β - and α -configuration, respectively (δ 4.5, d, $J=8.0$ Hz, glucose-H1; δ 5.01, brs, $J<1.0$ Hz, arabinose-H1). The upfield chemical shift of the two protons on position one of the aglycone, as well as the observation of only six acetates, implied that the monoterpene was glycosylated via the primary hydroxyl.

De-acetylation of **96a** produced a disaccharide glycoside of M_r 466 [(M+H) $^+=$ 467, (M+Na) $^+=$ 489] with the same MS/MS characteristics as those observed in the original glycosidic mixture for glycosides with apparent M_r 466.

Enzymic hydrolysis of **96** with a fungal enzyme preparation (*Novoferm* 12) liberated an aglycone which under GC-EIMS conditions co-chromatographed with and gave a spectrum identical to, a reference sample of (E)-3,7-dimethylocta-2-ene-1,7-diol (**96b**). Finally, acid hydrolysis of **96** liberated only two sugars which were identified by GC and GC-MS (TMSi derivatives) comparison of reference sugars as arabinose and glucose. Thus glycoside **96** was assigned as (E)-3,7-dimethylocta-2-ene-1-(α -arabinofuranosyl-(1 \rightarrow 6)- β -glucopyranosyl)-7-ol.

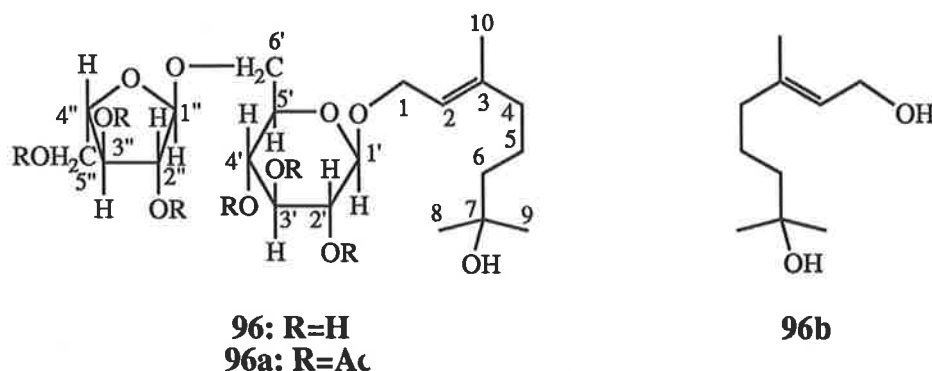


Figure 25: (E)-3,7-Dimethylocta-2-ene-1,7-diol and its glycosidic derivatives.

Compounds **97a** and **98a** were isolated as a mixture. This was also deduced from the observation in the FAB-MS spectrum of the isolate of two protonated molecular ions

(M+H)⁺ at *m/z* 717 (M+H)⁺ and 771 (M+H)⁺. These protonated ions corresponded to the hexa-O-acetylated derivatives of the glycosides with apparent M_r in their underivatized form of 464 (**97**) and 518 (**98**), respectively. The observation of fragment ions (i.e. FAB-MS/MS) at *m/z* 259, 331, 547 characteristic of the presence of hexa-O-acetylated pentosyl-hexosides, indicated that in both cases (i.e. glycosidic derivatives with apparent M_r 716 and 770) all six acetates were adopted by the sugar moiety. These data implied that in case of **97a** the aglycone moiety, presumably a monoterpene diol from the FAB-MS/MS data, had a tertiary hydroxyl. The data for **98a** could similarly be interpreted as being that for a glycoside of a C₁₃ norisoprenoid of molecular weight 224 with two resistant to acetylation oxygens in addition to that involved in the glycosylation.

The ¹H NMR spectrum of the mixture of **97a** and **98a** (see table 7) showed signals for two tertiary methyls on a carbon bearing oxygen (δ 1.23), an AB quartet system for geminally coupled protons on a carbon bearing oxygen centered at δ 4.16 and an unresolved signal for an olefinic proton of a tri-substituted double bond (δ 5.67, W_{1/2}=9.5 Hz). The observation in the ¹H NMR spectrum of only one olefinic proton suggested the presence in the aglycone of **97a** of only one double bond. This implied that the aglycone, presumably a monoterpene diol at the linalool oxide oxidation state from the FAB-MS/MS data, was monocyclic. These data suggested a *p*-menthenediol structure for the aglycone of the glucoside with M_r 716. The presence of minor signals assigned to another methyl on a carbon bearing oxygen (δ 1.40) and an olefinic methyl (δ 1.60) were attributed to glycoside **98a**. The ratio of the major to minor signals of approximately 4:1 indicated that the sample comprised mainly **97a**. The signals observed for the sugar moiety were in agreement with those previously observed for the per-O-acetylated apiofuranosyl-glucopyranosides **94a** and **95a**. The observation of only two signals for two anomeric protons, one for glucose (δ 4.47) and another for apiose (δ 5.01), in a ratio of 1:1, as well as the 1 to 1 ratio of the signals for both the sugar moiety and the aglycone with the *p*-menthenediol structure, suggested that only signals for the sugar moiety of **97a** were observable in the spectrum. Thus, **97a** was tentatively assigned as a hexa-O-acetylated-β-apiofuranosyl-β-glucopyranoside of a *p*-menthenediol. The chemical shift of the AB quartet system (centered at δ 4.16) and the presence of only six acetates (i.e. the aglycone has a free tertiary hydroxyl) suggested that the *p*-menthenediol was glycosylated via the C-7 hydroxyl.

De-acetylation of the mixture produced two disaccharide glucosides with apparent M_r's 464 and 518 [(M+H)⁺=465, (M+H)⁺=519]. Their MS/MS characteristics were similar to

those described above for glycosides with apparent M_r 's of 464 and 518 in the original flash chromatography fractions.

Enzymic hydrolysis of the mixture with a fungal enzyme preparation (*Novoferm* 12) liberated two aglycones which were assigned by their mass spectra as *p*-menth-1-ene-7,8-diol (**32**) and vomifoliol (**63**) by comparison with published data (Versini *et al.* 1991, Strauss *et al.* 1987c). Acid hydrolysis of the mixture and examination of the liberated sugars as the TMSi derivatives by GC-MS, showed anomers of two major sugars, apiofuranose (presumably the D-form) and glucopyranose, and a minor one, arabinofuranose. Thus, glycoside **97a** was assigned as the *p*-menth-1-ene-7-(β -apiofuranosyl-(1 \rightarrow 6)- β -glucopyranosyl)-8-ol. The minor constituent of the sample (i.e. glycoside **98a**) because of the incomplete ^1H NMR data, could only be tentatively assigned as a vomifoliol-9-O-arabinofuranosyl-O-glucopyranoside. The position of glycosylation was deduced by the observation in the FAB spectrum of **98a** of only six acetates (i.e. $(M+H)^+=771$) suggestive of the presence of a free tertiary -OH in the aglycone skeleton. Because of the common occurrence of 6-O- α -L-arabinofuranosyl-(1 \rightarrow 6)- β -D-glucopyranosides in grapes, it seems possible that **98a** is also a (1 \rightarrow 6) linked disaccharide glycoside.

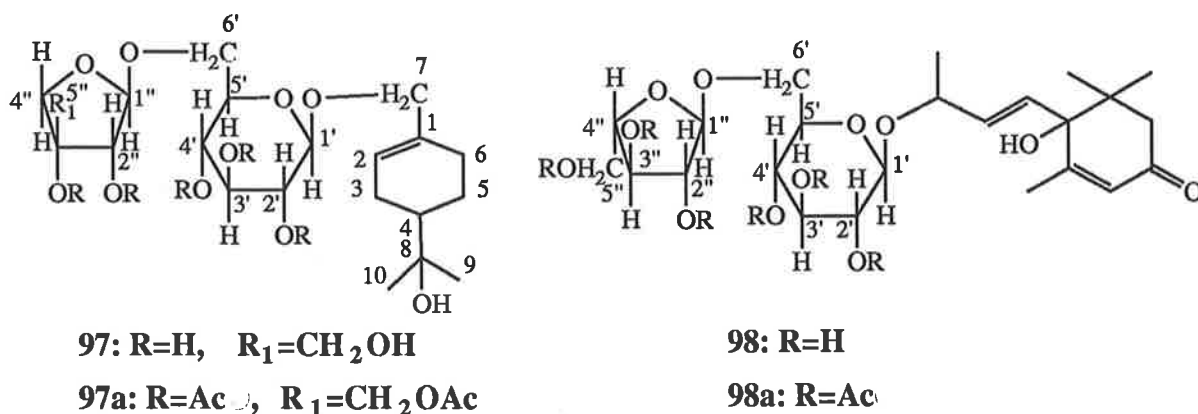


Figure 26: *p*-Menth-1-ene-7-(β -apiofuranosyl-(1 \rightarrow 6)- β -glucopyranosyl)-8-ol, vomifoliol-9-O-arabinofuranosyl-O-glucopyranoside and their per-O-acetylated derivatives. (Note: In case of glycosides **98**, **98a** the intersugar linkage has not been determined. Arabinose might also be linked to glucose via hydroxyls on glucose positions 2, 3, 4 and not only via position 6, as it is illustrated in figure 26 for reasons of simplicity).

The observation of apiosyl-glycosides in wine, a fermented product, indicates that, like the other glycosides found in wine, apiosylglycosides survive the wine making process.

Table 7 ; ¹H NMR data of compounds **95a**, **96a**, **97a** (300MHz, δ ppm).

H	95a (CDCl ₃)	96a (CDCl ₃)	97a (CDCl ₃)
Aglycone moiety			
1a.	4.20-4.30, ABq, <i>J</i> =10.5Hz	4.15, dd, <i>J</i> =10.8, 2.1Hz	
1b.	4.20-4.30, ABq, <i>J</i> =10.5Hz	4.30, dd, <i>J</i> =10.8, 13.2Hz	
2.		5.6, m	5.67, m, <i>W</i> _{1/2} =9.5Hz
3.	5.40, m		
4.	obsc.	obsc.	
5.	obsc.	obsc.	1.43, m
6.		obsc.	1.43, m
7.	5.68, m		4.12-4.20, d, <i>J</i> =10.5Hz
8a.	4.60, brd, <i>J</i> =8.0Hz	1.23, s	
8b.	4.60, brd, <i>J</i> =8.0Hz		
9.	1.70, brs	1.23, s	1.23, s
10.	1.40, brs	1.63, s	1.23, s
Sugar moiety			
1'.	4.45, d, <i>J</i> =8.0Hz	4.50, d, <i>J</i> =7.9Hz	4.47, d, <i>J</i> =8.1Hz
2'.	4.95, dd, <i>J</i> =8.0, 9.7Hz	4.93, dd, <i>J</i> =7.1, 9.3Hz	4.99-4.87, m
3'.	5.17, brt, <i>J</i> =9.7Hz	5.25, t, <i>J</i> =9.3Hz	5.30, t, <i>J</i> =9.5Hz
4'.	4.91, brt, <i>J</i> =9.4Hz	4.89, brt, <i>J</i> =9.3Hz	4.99-4.87, m
5'.	3.70-3.55, m	3.59, m	3.8-3.5, m
6a'.	3.70-3.55, m	3.80, dd, <i>J</i> =11.5, 4.6Hz	3.8-3.5, m
6b'.	3.70-3.55, m	3.70, brd, <i>J</i> =11.5Hz	3.8-3.5, m
1''.	5.01, brs, <i>J</i> <1	5.01, s	5.01, s
2''.	5.32, brs, <i>J</i> <1	5.13, brs	5.43, s
3''.		obsc.	
4a''.	3.90, d, <i>J</i> =11.5Hz	3.50-3.60, m	3.95, d, <i>J</i> =11.4Hz
4b''.	4.25, d, <i>J</i> =11.5Hz	obsc.	4.15, d, <i>J</i> =11.4Hz
5a''.	4.55, d, <i>J</i> =11.9Hz	4.15-4.20, m	4.54, d, <i>J</i> =12.5Hz
5b''.	4.70, d, <i>J</i> =11.9Hz	4.15-4.20, m	4.74, d, <i>J</i> =12.5Hz
Acetates	1.97 (1s), 2.00 (1s), 2.01 (1s), 2.03 (1s), 2.04 (1s), 2.06 (1s), 2.08 (1s)	1.90-2.10, 6xAcO-	1.97 (1s), 2.00 (1s), 2.01 (2s), 2.06 (1s), 2.09 (1s)

Chapter 5

VERY EARLY-ELUTING GLYCOCONJUGATES

5.1 Introduction

In a two-dimensional GC-DCCC analysis of the conjugates of volatile aroma constituents from Riesling wine, the presence of conjugates more polar than disaccharides was suggested (Winterhalter *et al.* 1990b). It had been considered that the added polarity of these constituents which eluted early off the DCCC was due to mixed conjugation (Winterhalter *et al.* 1990b). Thus, glycoconjugates in which the sugar moiety was further substituted with a malonic, tartaric, maleic, cinnamic, or monoterpene acid residue may have been involved. Such mixed conjugates have been recently identified in glycosidic fractions isolated from natural sources other than wine (Schwab and Schreier 1988, Shimomura *et al.* 1987, Gering-Ward 1989). This chapter describes the structural elucidation of some of the very polar wine constituents.

5.2 Results and discussion

Preliminary examination of material contained in early-eluting DCCC fractions (i.e. frs. 60-75) by paper electrophoresis using different pH buffer solutions, indicated the presence of two major groups of compounds in these fractions. The first group under all conditions employed (i.e. HCOOH/AcOH buffer pH 1.75, citrate buffer pH 5.0, NH₄OH/NH₄HCO₃ buffer pH 9.2) showed no mobility towards either the anode or the cathode and had a mobility value of zero ($M_{O,G}=0.0$) relative to the marker dyestuff orange G and equal to that of the zero mobility marker (fructose). Thus, it was deduced that compounds in this first group were neutral species. The second group exhibited medium to high mobility towards the anode at pH 5.0. This suggested the presence of negatively charged ionic compounds. The bulk of this ionic material at pH 1.75 exhibited minimal mobility ($M_{O,G}=0.09$) towards the anode, indicating the presence of carboxylic acids with pK_a values near 5. The observation that a minor proportion of this material had a medium mobility under these conditions suggested the presence of very strong acids such as glycosides of α -keto acids, phosphoric or sulphonic acid esters. Finally, the observation that some of the UV absorbing ionic material showed a substantial increase in mobility at

pH 9.2 suggested that in addition to the presence of carboxylic acids, phenolic constituents may also have been present.

Pooled fractions 60-75, after preliminary examination by paper electrophoresis, were extracted with CH_2Cl_2 to remove any volatile constituents that may have resulted from hydrolysis during storage of the fractions. The volatile-free fraction was adsorbed on C18 reversed-phase silica, washed thoroughly with water to remove any unconjugated carbohydrates resulting from hydrolysis during storage of the fractions and the adsorbed material was eluted from the C18 reversed-phase with methanol. This material was separated by anion ion exchange chromatography (Diaion column) into two broad fractions. One contained the neutral material and the other fraction contained the ionic material. A portion of each fraction from the ion exchange chromatography was trimethylsilylated and examined by GC-MS. None of the compounds present in both the neutral and the ionic fraction showed peaks exhibiting EIMS fragment ions characteristic for a trimethylsilylated glycoside. This suggested that the isolated fractions were consisted of compounds which either were not glycosidic, or that the glycoconjugates contained in the ion exchange chromatography fractions were too polar to chromatograph under GC conditions. In the case of the ion exchange chromatography fraction which contained the neutral species, the GC trace showed only peaks corresponding to some residual, free sugars which had not been removed from the original pooled fractions 60-75 when the latter were firstly adsorbed on C18 reversed-phase silica and then washed with H_2O . The GC trace of the fraction which contained ionic material was dominated by peaks exhibiting EIMS fragment ions indicating the presence of TMSi derivatives of carboxylic and phenolic acids (Horman and Viani 1971, Baranowski and Nagel 1981). Two of these peaks were tentatively identified from their mass spectral characteristics as isomeric forms of the tartaric acid ester of caffeic acid, i.e. tartaryl-cafate or caftaric acid (Baranowski and Nagel 1981). The longer retention time isomer when examined under GC-MS conditions in parallel with a reference sample of trimethylsilylated *trans*-caftaric acid, was found to be indistinguishable from that reference sample. Thus, it was concluded that the longer retention time isomer was the *trans*-caftaric acid. The shorter retention time isomer which showed EIMS fragment ions similar to those of the *trans*-isomer, was tentatively assigned as the *cis*-caftaric acid. Electrophoresis of the reference sample of *trans*-caftaric acid at pH 1.75, 5.0 and 9.2 showed it to have the same migration behaviour as the major, UV absorbing constituent in the isolated ionic material. This provided independent confirmation of the assignment of the major, UV absorbing constituent of the isolated ionic material as *trans*-caftaric acid.

Acid hydrolysis of both the neutral and the ionic material, extraction of the hydrolysates with CH_2Cl_2 and GC-MS examination of the resulted organic phases revealed that only the neutral fraction liberated volatile aroma constituents, and hence, our interest was focused on this material.

The ^{13}C NMR spectrum of the neutral fraction not retained by the ion exchange column provided significant structural information about these flavour precursors. The presence of a series of signal at a chemical shift between δ 100-105, attributed to anomeric carbons, verified the presence of glycoconjugates in this fraction. Intense signals at δ 77.5 (C-3, C-5), 76.3 (C-2), 70.2 (C-4), and 61.3 (C-6) could be attributed to glucose suggesting that this was the dominant sugar in these glycoconjugates. A signal at a chemical shift of δ 61.3 and attributed to C-6 of glucose suggested that most of the glucose was not substituted at position C-6 (Bock and Pedersen 1983, Bock *et al.* 1984). Minor signals attributed to other sugar moieties were also observed including a signal at δ 64 which could reasonably be attributed to the C-6 carbon of a hexose unit substituted at position C-6. The intensity ratio (7:1) between the signals attributed to the C-6 carbon of a terminal, non-substituted at that position glucose unit (δ 61.3) and a further substituted at position C-6 glucose (δ 64) implied that glucose was involved in conjugation mainly as a terminal unit. The ^{13}C spectrometric data for these very polar constituents were consistent with their being a mixture of polar monoglucosides, such as the lignan glycosides reported in chapter 3, and/or diglucosides (e.g. gentiobiosides), and/or other oligosaccharide conjugates having glucose both as an intermediate and a terminal sugar. Other signals observed in the ^{13}C NMR spectrum of the neutral material, were attributed to paraffinic carbons (δ 15-40), olefinic carbons (δ 127-131), and methoxyls (δ 55).

Flash chromatography of the isolated neutral material and monitoring of the resultant flash fractions by positive and negative FAB-MS indicated the presence of three glycoconjugates with apparent M_r s of 486, 508 and 626. The molecular mass of each of these glycoconjugates was deduced from the presence of protonated and pseudo-molecular ions in the positive ion mode at m/z 487 ($\text{M}+\text{H}$)⁺, 509 ($\text{M}+\text{Na}$)⁺, 509 ($\text{M}+\text{H}$)⁺, 531 ($\text{M}+\text{Na}$)⁺, 627 ($\text{M}+\text{H}$)⁺, 649 ($\text{M}+\text{Na}$)⁺, and deprotonated molecular ions ($\text{M}-\text{H}$)⁻ in the negative ion mode at m/z 485, 507 and 625, respectively.

FAB-MS/MS experiments on these protonated and deprotonated molecular ions provided substantial structural information for the sugar moieties. For the glycoside with apparent M_r 486, MS/MS data suggested that it was a monohexoside which, in both positive and

negative ion modes, showed fragment ions at m/z 325, and 323 resulted from the elimination of an anhydrohexose unit from the protonated and the deprotonated molecular ions, respectively. Furthermore, in the negative mode spectrum fragment ions for only a hexose ring at m/z 161 [(hexose-H)-H₂O]⁻, 149, 119 [(hexose-H-H₂O)-CH₂=C=O]⁻, 101, 89, were also observed (Pare *et al.* 1988, Domon and Costello 1988b). For the glycoside with apparent M_r 508, MS/MS data again suggested that it was a monohexoside which showed daughter ions at m/z 347 in the positive mode, and m/z 345 and 327 in the negative mode, formed after elimination of an anhydrohexose unit from the protonated molecular ion, and of anhydrohexose and hexose units from the deprotonated molecular ion, respectively. The fragmentation pattern of the glycoside with apparent M_r 626 in the positive ion mode showed subsequent losses from the protonated molecular ion of 162 (loss of anhydrohexose), 132 (loss of anhydropentose) and another 162 (anhydrohexose) leading to a protonated ion at m/z 171. Alternatively, elimination of the aglycone moiety from the protonated molecular ion, followed by subsequent eliminations of anhydrohexose and anhydropentose segments leading to a terminal unit of a protonated anhydrohexose, were also observed. Furthermore, the spectrum showed fragment ions for the individual sugar rings at m/z 163 (hexose+H-H₂O)⁺, 145 (hexose+H-2H₂O)⁺, 127 (hexose+H-3H₂O)⁺, 133 (pentose+H-H₂O)⁺. In the negative mode losses were observed from the deprotonated molecular ion of 162 (anhydrohexose) and 132 (anhydropentose), followed by loss of 170 leading to a deprotonated ion of anhydrohexose at m/z 161. Fragment ions characteristic for the individual sugar rings were also observed at m/z 179, 161, 119, 113, 101, 88 (hexose) and 149, 131 (pentose) (Domon and Costello 1988b, Domon and Costello 1988a). These fragmentation patterns are consistent with a molecule comprising four individual parts, i.e. a hexose unit, a pentose unit, another hexose unit and a moiety of 170 mass units, presumably a monoterpene at the oxidation state of the linalool oxides. The presence of fragment ions at m/z 465 and m/z 457 corresponding to eliminations from the protonated molecular ion of an anhydrohexose unit or the aglycone moiety, respectively, indicated that one of the hexose units and the aglycone were terminal units in the sequence of the molecule (Domon and Hostettmann 1985). The absence in the positive MS/MS spectrum of the compound with M_r 626 of a fragment ion at m/z 495, indicating the elimination from the protonated molecular ion of an anhydropentose unit, suggested that the pentose was an intermediate unit in the sequence of the molecule (Schulten 1979, Domon and Hostettmann 1985). These MS/MS data were not consistent with the presence of a branched sugar sequence in the molecule (i.e. presence of a tri-substituted hexose unit in the molecule). They suggested a linear pattern of substitution where the sequence in the

sugar moiety was hexose-pentose-hexose with one hexose being a terminal unit and the other being conjugated to the aglycone moiety.

After the preliminary FAB-MS/MS examination, the flash fractions under investigation were further separated by chromatography on HPLC. The sub-fractions so obtained were monitored by positive-negative FAB-MS and those which contained glycoconjugates with the previously mentioned spectrometric characteristics were derivatised (i.e. per-O-acetylated by pyridine/acetic anhydride) and further purified by flash chromatography on silica gel. The procedure gave three per-O-acetylated derivatives, i.e. **99a**, **100a** and **101a** of the putative M_r 508 monosaccharide (**99**), the M_r 626 trisaccharide (**100**) and the M_r 486 monosaccharide (**101**), respectively.

FAB-MS examination of glycoside **99a** suggested a heptaacetate [$(M+H)^+=803$, $(M+H+glycerol)^+=895$]. The observation in the MS/MS spectrum of fragment ions at m/z 331, 271, 211, 169 indicated the presence of a monosubstituted tetra-O-acetylated hexopyranoside (Radford and Dejongh 1972). The 1H NMR spectrum (table 8), showed signals for five aromatic protons with only three of them showing a coupling relation ($J=8.4$ Hz, *o*-coupling; $J=2.0$ Hz, *m*-coupling; $J=1.7$ Hz, *p*-coupling), indicating the presence of two aromatic benzene ring systems with one ring being tri- and the other tetrasubstituted. Additionally, two unequal doublets for a low field proton (δ 5.48, 5.47) on a benzylic carbon also bearing oxygen, two singlets for an arylmethoxyl group (δ 3.82, 3.80), signals for the presence of a phenylpropanol side chain (δ 1.68, 2.58, 4.15-4.0), and one anomeric proton with a β -configuration (δ 5.10, $J=8.0$ Hz) were observed. The data support the assignment of **99a** as a diastereoisomeric mixture of 2,3-dihydro-7-hydroxy-2-(4'-hydroxy-3'-methoxyphenyl)-3-hydroxymethyl-5-benzofuranpropanol- β -glucoside, (i.e. a β -glucopyranoside of cedrusin). The chemical shift observed for the anomeric proton (δ 5.10) suggested that **99a** was a phenolic glycoside. This was further supported by the observation in the 1H NMR spectrum of only one phenolic acetate (δ 2.29). The observed deshielding effect on the aromatic proton on C-6 as a consequence of acetylation, i.e. presence of a chemical shift at δ 6.96 instead of a value between δ 6.70-6.50 which is the reported resonance of the proton on C-6 in the cases of the anacetylated glucoside and the aglycone of cedrusin (Agrawal *et al.* 1980, Strack *et al.* 1989), suggested that **99a** was glycosylated via the hydroxyl located on C-4 of the trisubstituted aromatic ring. This data is consistent with those previously published for 2,3-dihydro-7-hydroxy-2-(4'-O- β -glucopyranosyl-3'-methoxyphenyl)-3-hydroxymethyl-5-benzofuranpropanol, i.e. cedrusin-

β -glucopyranoside (Popoff and Theander 1975, Higuchi *et al.* 1977, Miki and Sasaya 1979b).

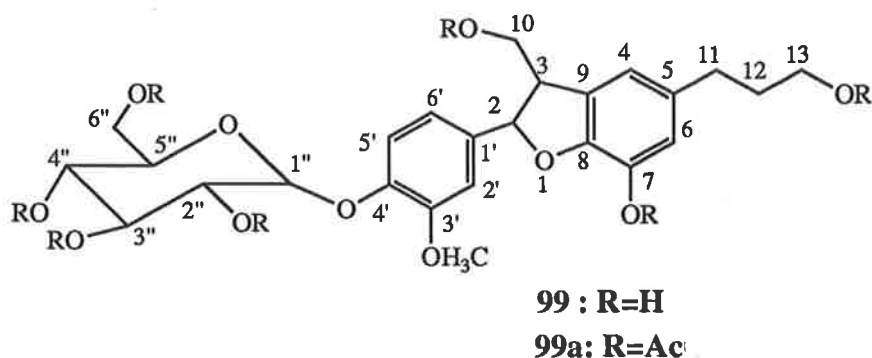


Figure 27: 2,3-Dihydro-7-hydroxy-2-(4'-O- β -glucopyranosyl-3'-methoxyphenyl)-3-hydroxymethyl-5-benzofuranpropanol, a novel Riesling wine constituent and its per-O-acetylated derivative.

Enzymic hydrolysis of **99** with a β -D-glucosidase liberated an aglycone (**99b**) with an apparent molecular weight 346 (probe EIMS), the mass spectrum of which exhibited similarities as well as differences to the mass spectral data previously published for 2,3-dihydro-7-hydroxy-2-(4'-hydroxy-3'-methoxybenzyl)-3-hydroxymethyl-5-benzofuranpropanol, i.e. cedrusin (**99b**), (Popoff and Theander 1975, Miki and Sasaya 1979b, Agrawal *et al.* 1980). The observation of additional fragment ions at m/z 162, 180 was attributed to the presence of diastereoisomeric forms of **99b**. The same fragment ions were also observed in the MS/MS spectrum of **99b**. Finally, acid hydrolysis of **99**, followed by GC-MS examination of the acid hydrolysis products as their TMSi derivatives, independently confirmed the sugar part as glucose. Thus, it was concluded that **99** is a diastereoisomeric mixture of 2,3-dihydro-7-hydroxy-2-(4'-O- β -glucopyranosyl-3'-methoxyphenyl)-3-hydroxymethyl-5-benzofuranpropanol (cedrusin-4'- β -D-glucopyranoside).

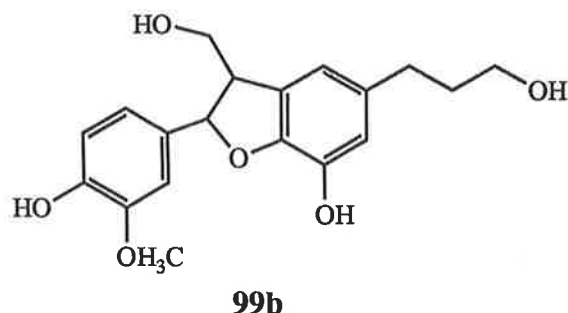


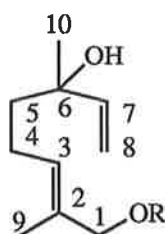
Figure 28: 2,3-Dihydro-7-hydroxy-2-(4'-hydroxy-3'-methoxybenzyl)-3-hydroxymethyl-5-benzofuranpropanol.

Cedrusin glycosides have previously been isolated from the needles of *Pinus sylvestris* (Popoff and Theander 1975), from the leaves of *Pinus contorta* (Higuchi *et al.* 1977), from the inner bark of *Larix leptolepis* Gord (Miki and Sasaya 1979b), from the *Cedrus deodara* plant (Agrawal *et al.* 1980), and from the needles of the Norway spruce (Strack *et al.* 1989). In all cases reported, cedrusin was glycosylated at position C-4'. Cedrusin-4'- β -D-glucoside (**99**) is the first neolignan glycoside identified in wine.

Acetylation of **100** produced a nonacetate **100a**. This was deduced from the observation of pseudomolecular ions at m/z 1027=(M+Na)⁺, and 1022=(M+NH₄)⁺, in the FAB-MS and the probe CI-MS spectra, respectively. The presence in the CI-MS/MS spectrum of **100a** of fragment ions at m/z 331, 271, 211 suggested the presence of a monosubstituted tetra-O-acetylated hexopyranose in the molecule (Harrison 1983). Other fragment ions observed at m/z 547, 259 were attributed to a monosubstituted per-O-acetylated hexopyranosyl-pentofuranoside. These data again implied the presence in the molecule of a tetra-O-acetylated hexopyranose as a terminal unit (i.e. mono-substituted). The ¹H NMR spectrum of **100a** (see table 8) showed signals for the aglycone moiety at δ 1.27 due to a tertiary methyl group on a carbon bearing oxygen, an ABX system for a mono-substituted double bond (δ 5.90, 5.20, 5.05) and an olefinic proton for a tri-substituted double bond (δ 5.45). These data were consistent with the presence of 2,6-dimethylocta-2,7-diene-1,6-diol as the aglycone moiety. The coupling constants observed for the two anomeric protons of the glucose units (δ 4.43, $J=7.8$ Hz and 4.52, $J=7.3$ Hz) indicated that both have the β -configuration. The absence from the ¹H NMR spectrum of a broad doublet at around δ 4.6 for the two methylene protons on C-1 (the expected chemical shift if the hydroxyl on C-1 was acetylated, see tables 6 and 7 for analogue molecules **94a**, **96a**), again suggested that the aglycone moiety was conjugated via the hydroxyl on C-1.

De-acetylation of **100a** produced a trisaccharide glycoside of apparent M_r 626 [(M+H)⁺=627, (M+Na)⁺=649] with the same MS/MS characteristics as those described above for glycosides with apparent M_r 626 in the original flash chromatography fractions. Enzymic hydrolysis of **100** with a fungal enzyme (*Novoferm* 12) liberated an aglycone **94b** which on GC-EIMS conditions co-chromatographed with and gave a spectrum identical to, a reference sample of (E)-2,6-dimethylocta-2,7-diene-1,6-diol (**94b**). Finally, acid hydrolysis of **100** liberated only two sugars which were identified by GC-EIMS (TMSi derivatives) as arabinose and glucose. Thus, the glycoside **100** was assigned as a (E)-2,6-dimethylocta-2,7-diene-1,6-diol-1-O- β -glucopyranosyl-O-arabinofuranosyl-O- β -glucopyranoside.

Glycoconjugate **100** was present in the Riesling wine in minute amounts, and thus it was not possible to isolate sufficient material for a better proton and for a carbon thirteen NMR study to positively characterize the position of conjugation between the sugar units. However, because in all cases that arabinoglucosides were identified in wine the conjugation was (1→6), it would be reasonable to suggest that in case of **100**, the arabinose unit is linked to the intermediate glucose unit via the hydroxyl on C-6 of that sugar.



100: R=Glucose-O-arabinose-O-glucose

100a: R=Glucose-O-arabinose-O-glucose (9xAc)

Figure 29: (E)-2,6-Dimethylocta-2,7-diene-1,6-diol-1-O-β-glucopyranosyl-O-arabinofuranosyl-O-β-glucopyranoside and its per-O-acetylated derivative.

This is the first time that a trisaccharide glycoside has been detected, isolated and partially characterized by spectrometric techniques, in wine. Glycoside **100**, thus, represents a new class of flavour precursors. Another terpenoid trisaccharide has recently been identified in the glycosidic fraction of apple fruits (Herderich *et al.* 1992).

Examination by FAB-MS of glycoside **101a** suggested a heptaacetate [(M+H)⁺=781, (M+Na)⁺=803]. The observation in the FAB-MS/MS spectrum of fragment ions at *m/z* 331, 271, 211, 169 indicated the presence of a monosubstituted tetra-O-acetylated hexopyranoside. The signals observed for the sugar moiety in the ¹H NMR spectrum of **101a** were in agreement with data previously published for tetra-O-acetylglucopyranosides (Schwab and Schreier 1988). The splitting (*J*=8.0 Hz) of the signal for the anomeric proton suggested a β-configuration for the glucoside. Other signals for the aglycone moiety were also observed but the information obtained was not enough to allow an assignment of the aglycone of **101a**. All ¹H NMR data obtained for **101a** are presented in the experimental part. De-acetylation of **101a**, acid hydrolysis of the resulted glucoside and GC-MS examination of the resulted hydrolysates as TMSi derivatives, independently

proved the sugar moiety being a glucose. The data on hand do not permit a structure elucidation of the aglycone moiety of glucoside **101**. However, the UV absorption spectrum of **101** (λ_{max} =232, 241 (sh), 265, 323, 339 nm) suggested that the aglycone moiety was not belonging to any of the aglycone classes observed in the wine so far (i.e. terpenoids, phenylpropanoids, lignans or neolignans).

The observation of the β -glucoside **101** along with the identification of cedrusin-4'- β -glucoside (**99**) as constituents of the neutral species, is in agreement with the initial interpretation of the ^{13}C NMR data of the crude neutral fraction from ion exchange chromatography as being a mixture of, mainly, polar monoglucosides. The presence in the original ^{13}C NMR data of minor signals attributed to sugars other than glucose, and to glucose units being conjugated both as intermediate and terminal sugars, is explained by the isolation and structurally characterization of the monoterpenyl-O-glucopyranosyl-O-arabinofuranosyl-glucopyranoside **100**. Thus, it was concluded that the ion exchange chromatography fraction containing neutral species consisted, at least in part, of monoglucosides of polar aglycones such as the neolignan cedrusin (**99b**) and also trisaccharide glycosides of volatile monoterpenes such as the (E)-2,6-dimethylocta-2,7-diene-1,6-diol. It is proposed that the latter conjugates constitute the very polar flavour precursors referred to by Winterhalter *et al.* (1990b).

Table 8 : ¹H NMR data of compounds **99a**, **100a** (300MHz, δ ppm).

H	99a (CDCl ₃)	H	100a (CDCl ₃)
Aglycone moiety		Aglycone moiety	
2.	5.47, d, <i>J</i> =6.3Hz 5.48, d, <i>J</i> =6.3Hz	1.	4.05-4.22, m
3.	3.78, m	3.	5.45, m
4.	6.75, brs	4.	obsc.
6.	6.96, brs	5.	obsc.
10a.	4.25, dd, <i>J</i> =4.3, 11.0Hz	7.	5.90, dd, <i>J</i> =10.6, 17.2Hz
10b.	4.45, dd, <i>J</i> =1.9, 10.6Hz		
11.	2.58, brt, <i>J</i> =7.8Hz	8a.	5.05, d, <i>J</i> =10.6Hz
12.	1.68, m	8b.	5.20, d, <i>J</i> =17.2Hz
13.	4.0-4.15, m	9.	obsc.
2'.	6.83, brs	10.	1.27, s
5'.	6.99, dd, <i>J</i> =8.4, 1.7Hz		
6'.	6.94, dd, <i>J</i> =8.4, 2.0Hz		
CH ₃ O-	3.80, s 3.82, s		
Sugar moiety		Sugar moiety	
1'.	5.10, d, <i>J</i> =8.0Hz	1'.	4.43, d, <i>J</i> =7.8Hz
2'.	5.15-5.25, m	2'.	4.90-5.10, m
3'.	5.15-5.25, m	3'.	5.15, brt, <i>J</i> =9.5Hz
4'.	5.05, brt, <i>J</i> =10Hz	4'.	4.90-5.10, m
5'.	3.60-3.70, m	5', 6a', 6b'.	3.47-3.66, m
6a'.	4.27, dd, <i>J</i> =3.6, 11.2Hz		
6b'.	4.0-4.15, m	1''-3''	4.90-5.10, m
		4''	4.05-4.22, m
		5a'', b''	3.47-3.66, m
		1'''	4.53, d, <i>J</i> =7.3Hz
		2'''	4.90-5.10, m
		3'''	5.18, brt, <i>J</i> =9.4Hz
		4'''	4.90-5.10, m
		5'''	3.47-3.66, m
		6a''', b'''	4.05-4.22, m
Acetates:	1.99-2.05, (5xAcO-) 2.29 (1s)	Acetates:	1.95-2.10 (9xAcO-)

Chapter 6

β -CARBOLINES: POLAR, NON-VOLATILE MATERIAL IN RIESLING WINE.

6.1 Introduction

Fractions of polar, non-volatile material obtained from the same Riesling wine by C18 reversed-phase column chromatography followed by DCCC were screened by TLC for the presence of compounds containing the indole nucleus. The monitoring revealed the presence of two major spots with TLC chromatographic retention indices of $R_f=0.25$ (in DCCC frs. 100-130) and $R_f=0.48$ (in DCCC frs. 190-220) which responded intensely to the Van Urk-Salkowski indole reagent (Ehmann 1977). Application of FAB-MS/MS and other spectrometric techniques (e.g. ^1H NMR) identified the material with $R_f=0.48$ as the ethyl indole-lactate- β -D-glucoside (see chapter 2). The same approach was employed to identify the presence of β -carbolines in the second fraction with $R_f=0.25$. The results of this analytical work are reported below.

6.2 Results and discussion

Flash chromatography of the relatively polar DCCC group of fractions (i.e. frs. 100-130) followed by semi-preparative HPLC resolution of the individual flash fractions, enabled the isolation of a sub-fraction which fluoresced under UV light. This sub-fraction also responded positively to the indole TLC spray reagent (Ehmann 1977) and exhibited a characteristic indole UV spectrum, λ_{max} 220, 272, 280, 289 (sh) nm (D. H. Williams and Fleming 1987).

Positive and negative FAB-MS examination indicated that the isolate was a mixture of more than one compound with the major component or components being of M_r 230 [$(\text{M}+\text{H})^+=231$, $(\text{M}-\text{H})^-=229$]. The minor components exhibited protonated molecular ions $(\text{M}+\text{H})^+$ at m/z 344 and 358, and deprotonated molecular ions $(\text{M}-\text{H})^-$ at m/z 342, 356, respectively. MS/MS examination of the molecular ions in both positive and negative ion modes suggested that none of the above mentioned components were glycosylated. This was inferred from the absence, in both positive and negative ion mode spectra, of fragment

ions generated from the elimination of an anhydro-sugar from the molecular ions and also the absence of ring fragment ions.

Signals from the ^1H NMR examination of the isolate are presented in table 9; these data are consistent with those previously reported for 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (**102**) (Brossi *et al.* 1973). In particular, the absence of two high field aromatic signals near δ 7.0 and 6.4 indicated that the indole nucleus was substituted at positions 2 and 3 (Hiremath and Hosmane 1973). The presence of two doublets at δ 1.68 and δ 1.70 due to the protons of the methyl group on C-13 and integrating for three protons, suggested that the sample was a mixture of diastereoisomers. This was further supported by the presence in the spectrum of two unequal doublet of doublets (δ 3.91, δ 4.22) which integrated for one proton, due to the methine proton adjacent to a carbon bearing a strong electron drawing group, i.e. C-3 (Brossi *et al.* 1973). Thus, the major components of the isolate were assigned as a diastereoisomeric mixture of (1S, 3S) and (1R, 3S) 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (**102**) (Brossi *et al.* 1973). The ratio of the diastereoisomers was approximately 1:2 in favour of the (1S, 3S) diastereoisomer. This was deduced from the relative intensities of the signals discussed above. The presence in the ^1H NMR spectrum of other minor signals was attributed to compounds giving rise in the FAB-mass spectra to the protonated molecular ions $(\text{M}+\text{H})^+=344, 358$ and deprotonated molecular ions $(\text{M}-\text{H})^-=342, 356$, respectively. However, the intensity of the minor signals did not permit a structural assignment for these molecules. ^{13}C NMR data for the isolate are given in table 10. They were, again, consistent with the presence of both diastereoisomers of **102** as the major constituents in the isolate. Minor signals for the other constituents were also observed.

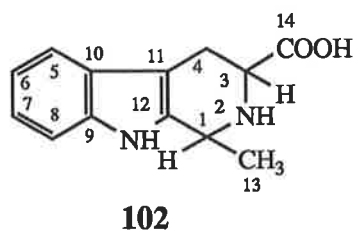


Figure 30: 1-Methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid: a novel constituent of Riesling wine.

Table 9: ¹H-NMR data of naturally occurring and synthetic diastereoisomers of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (CD₃OD + 1.5% HCL, 300 MHz, δ ppm).

H	Naturally occurring isomers		Synthetic isomers	
	102	(1S, 3S) 295-297°C	(1S, 3S) 275-277°C	(1R, 3S) 242-244°C
1.	4.62, dq, <i>J</i> =6.2, 1.0 Hz	4.63, dq, <i>J</i> =6.8, 0.8 Hz	4.40, dq, <i>J</i> =6.7, 0.9 Hz	4.23, dq, <i>J</i> =6.9, 1.0 Hz
3.	3.91, dd, <i>J</i> =5.2, 12.0 Hz (1S, 3S)	4.20, dd, <i>J</i> =5.4, 12.1 Hz	3.85, dd, <i>J</i> =5.4, 12.1 Hz	
	4.22, dd, <i>J</i> =5.5, 9.2 Hz (1R, 3S)			4.25, dd, <i>J</i> =5.8, 8.8 Hz
4a.	2.99, ddd, <i>J</i> =1.9, 12.6, 15.9 Hz	3.03, ddd, <i>J</i> =2.2, 12.2, 16.4 Hz	2.95, ddd, <i>J</i> =2.5, 12.1, 16.4 Hz	2.90, ddd, <i>J</i> =0.9, 8.1, 16.6 Hz
4b.	3.37, brdd, <i>J</i> =1.4, 4.6, 15.9 Hz	3.37, ddd, <i>J</i> =1.4, 5.3, 16.6 Hz	3.22, ddd, <i>J</i> =1.4, 5.4, 16.5 Hz	2.97, ddd, <i>J</i> =1.4, 5.7, 16.6 Hz
5.	7.59, d, <i>J</i> =7.8 Hz	7.50, brd, <i>J</i> =7.8 Hz	7.46, dd, <i>J</i> =7.9, 1.0 Hz	7.23, brd, <i>J</i> =7.8 Hz
6.	7.26, brt, <i>J</i> =7.5 Hz	7.19, ddd, <i>J</i> =8.2, 1.1, 1.3 Hz	7.18, ddd, <i>J</i> =8.2, 0.8, 1.2 Hz	6.96, dt, <i>J</i> =1.2, 8.0 Hz
7.	7.17, brt, <i>J</i> =7.3 Hz	7.09, ddd, <i>J</i> =8.2, 1.1, 1.3 Hz	7.08, ddd, <i>J</i> =7.9, 0.9, 1.1 Hz	6.88, dt, <i>J</i> =0.8, 7.0 Hz
8.	7.46, d, <i>J</i> =8.0 Hz			7.18, brd, <i>J</i> =8.1 Hz
	7.45, d, <i>J</i> =7.9 Hz	7.39, brd, <i>J</i> =8.1 Hz	7.36, dd, <i>J</i> =7.9, 1.1 Hz	
13.	1.70, d, <i>J</i> =6.3 Hz (1S, 3S)			1.42, d, <i>J</i> =6.8 Hz
	1.68, d, <i>J</i> =6.3 Hz (1R, 3S)	1.64, d, <i>J</i> =6.8 Hz	1.59, d, <i>J</i> =6.8 Hz	

Synthesis of 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (**102**) according to Brossi *et al.* (1973) from the condensation of L-tryptophan with acetaldehyde, followed by fractional crystallization, yielded 3 crops of crystals with mp 295-297°C, 275-277°C and 242-244°C, respectively. Crystalline fractions with mp 295-297°C and 242-244°C were previously reported by Brossi *et al.* (1973) as the only isomers obtained from the condensation of L-tryptophan with acetaldehyde. These authors identified the diastereoisomers with mp 295-297°C and 242-244°C from spectrometric data (i.e. ^1H NMR) and optical rotation experiments as the (1S, 3S) and (1R, 3S) isomers, respectively. The crystalline fraction with mp 275-277°C was not previously reported. ^1H NMR and ^{13}C NMR studies on the three crystalline fractions synthesised during the course of this study, suggested that both fractions with mp 295-297°C and 275-277 °C were the (1S, 3S) isomer and that crystalline fraction with mp 242-244°C was the (1R, 3S) isomer. This was further supported from the chromatographic properties of the various crystalline fractions under HPLC conditions (i.e. analytical gradient, see experimental part). The higher melting point fractions were indistinguishable from one another by HPLC but were clearly resolved from the more slowly eluting, lower melting point fraction. It is probable that the two higher melting point forms are probably crystalline allotropes.

FAB-MS and MS/MS examination of all crystalline fractions, in both positive and negative ion modes, resulted similar spectrometric data to those obtained for the naturally occurring **102**. The agreement between the spectrometric data (i.e. UV, ^1H and ^{13}C NMR, FAB-MS/MS) for the synthetic and isolated material confirmed that the major and minor constituents of the isolated material were, respectively, the (1S, 3S) and (1R, 3S) diastereoisomers of 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid.

Co-chromatography on HPLC (i.e. analytical gradient) of the isolated material with the synthetic diastereoisomers of 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid independently established the presence of both (1S, 3S) and (1R, 3S) diastereoisomers in the isolate.

Table 10: ^{13}C NMR data of naturally occurring and synthetic diastereoisomers of 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (300 MHz, δ ppm).

C	Naturally occurring isomers		Synthetic isomers		
	102 (1S, 3S)	102 (1R, 3S)	(1S, 3S) 297-295°C	(1S, 3S) 275-277°C	(1R, 3S) 242-244°C
1	52.8	50.9	52.8	52.8	50.7
3	60.9	56.3	58.3	58.3	54.3
4	25.6	25.3	24.6	24.6	24.1
5	121.4	--- ^a	121.0	121.0	120.9
6	122.9	--- ^a	122.7	122.7	122.5
7	125.7	--- ^a	125.5	125.5	125.4
8	114.7	--- ^a	114.5	114.5	114.2
9	139.5	--- ^a	139.5	139.4	139.1
10	133.5	--- ^a	132.7	132.7	132.5
11	106.9	--- ^a	107.5	107.6	106.4
12	128.6	133.7	128.1	128.1	132.5
13	19.3	20.7	18.9	18.9	20.2
14	176.9	--- ^a	173.6	173.8	176.9

^a: Signals obscured by those of the (1S,3S) isomer.

For the minor constituents present in the isolated material (i.e. compounds with M_r 343, 357), the UV spectra obtained by diode array detection after chromatographic resolution via HPLC, again suggested that they were β -carboline derivatives. The odd mass numbers indicated that the additional segments in these molecules also contained an odd number of nitrogen atoms. The fragmentation pattern of the carboline derivative with M_r 343 (**103**) under MS/MS conditions in the positive ion mode showed subsequent losses from the molecular ion of a segment of 131 mass units followed by another of 28 mass units leading to a fragment ion at m/z 185. Losses of 131 and 28 mass units fragments could be attributed to the elimination of a moiety linked to the 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid skeleton followed by elimination of carbon monoxide. Alternatively, elimination from the protonated molecular ion of a segment of 113 mass units followed by elimination of 46 mass units (HCOOH) leading again to a fragment ion at m/z 185, were also observed. The ion at m/z 185 could be attributed to a 1-methyl-1,2,3,4-tetrahydro- β -carboline positively charged species. These patterns were consistent with the presence of a 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid conjugated with a moiety of 131 mass units through an ester linkage. In the case of the constituent with M_r 357 (**104**), the observation of a similar fragmentation pathway in the

positive ion mode, again suggested the presence of a β -carboline derivative. The increased molecular weight by 14 mass units was consistent with the presence of an additional methyl or methylene group in the molecule. The observation of subsequent fragment ions analogues to those observed in the case of **103** but increased by 14 mass units, implied the presence of an additional methyl group on the skeleton of the β -carboline. A possible interpretation is that **104** is a derivative of 1,1-dimethyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid. In both cases of compounds with M_r 343 and 357, the extra moiety coupled through the ester linkage appears to be the same or isomeric compounds.

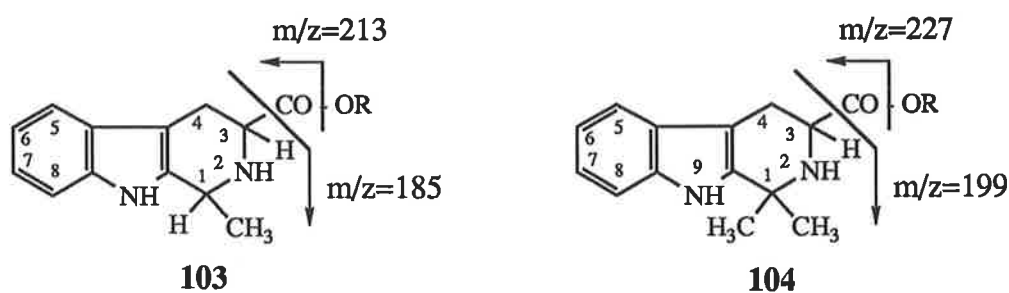


Figure 31: β -Carboline derivatives in Riesling wine and some of their mass spectral fragmentations.

Of the candidate molecules having a molecular weight of 131 and odd number of nitrogen atoms, hydroxyproline is one possibility. Unfortunately, insufficient amounts of these minor putative β -carboline derivatives were available for further spectral studies.

β -Carbolines have been identified in mammalian tissues, foods and beverages (Rommelspacher *et al.* 1991, Sato *et al.* 1975, Beck *et al.* 1983, Bosin *et al.* 1983, Adachi *et al.* 1991). Although the origin of these compounds is unknown, they are thought to be formed by a Pictet-Spengler condensation of biogenic tryptamines and a carbonyl compound (Beck *et al.* 1983, Rommelspacher *et al.* 1991).

Tetrahydro- β -carbolines (THBC's) exhibit several physiological effects, including the voluntary intake of ethanol in rats after intraventricular administration (Melchior and Myers 1977). Evidence for the *in vivo* synthesis of THBC in mammalian tissues implicated these compounds in the alcoholic syndrome (Barker 1982) because of their ability to function as neurotransmitters or neuromodulators (Buckholtz 1980, Rommelspacher *et al.* 1991). However, the evidence that has been presented up to date is inconclusive and it is still unresolved as to whether THBC's are formed *in vivo* as a result

of alcohol consumption, or THBC's are mainly introduced into the body via dietary sources (Barker 1982).

In addition to the possible biological role of 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (Bosin *et al.* 1986), it is also a possible off-flavour factor because the compound has been identified as a bitter principal produced in sake as a result of heat processing (Sato *et al.* 1975). The presence of **102** in wine was first reported by Bosin *et al.* (1986) who identified both (1S, 3S) and (1R, 3S) diastereoisomers of **102** in a ratio of approximately 2:1 and in high concentration. The results obtained during the course of this project are in agreement with the observations of Bosin *et al.* (1986). Those workers ascribed the origin of **102** in wine as the result of a Pictet-Spengler condensation of L-tryptophan with acetaldehyde and/or as naturally occurring in grape sources. The detection during the course of this project, of 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid in the C₁₈ reversed-phase isolate of a Semillon grape juice (Marinos and Francis, unpublished data), supports the suggestion of Bosin *et al.* (1986) that 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid and other β -carbolines may naturally occur in grapes. The detection in the same C₁₈ reversed-phase isolate from Semillon grape juice of the possible 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid derivatives with M_r 343, 357 indicates that these compounds may also occur naturally in grapes. If so, it would be reasonable to suggest that 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid could also arise in wine from a precursor, i.e. **103**, which is naturally present. The possible importance of THBC's both as biological active compounds and, in the case of 1-methyl-1,2,3,4-THBC-3-carboxylic acid, as an off-flavour principal, suggests that it may be profitable to examine these grape and wine compounds in more detail in the future.

CONCLUSIONS

Fast atom bombardment mass spectrometry (FAB-MS) combined with tandem mass spectrometry (MS/MS) constitute an advance for the analysis of flavour precursors and other glycosidically bound secondary metabolites. A protocol for the direct analysis of grape juice and wine glycoconjugates without prior derivatization of these compounds has been developed. This protocol combines partition liquid chromatographic techniques (e.g. DCCC, HPLC) with soft ionization mass spectrometric techniques (i.e. FAB-MS and MS/MS). The application of the protocol to the examination of a glycosidic mixture isolated from a Riesling wine, initially resulted a series of sub-fractions of various polarities, each one containing a relatively small number of constituents. Monitoring the so obtained sub-fractions by FAB-MS and FAB-MS/MS demonstrated that these spectrometric techniques can be used in the analysis of polar, non-volatile and thermally labile material from wine in two dimensions. Firstly, positive and negative FAB-MS experiments can be used as a detection method to target individual constituents in mixtures by determining the corresponding molecular weights of the constituents. Secondly, combinations of parent and daughter ion experiments in both positive and negative ion modes can be utilised as a further analytical method to obtain structural information for individual constituents in mixtures.

In many cases applied during the course of this study, FAB-MS and MS/MS experiments provided reliable structural information for the molecular weight of glycosidic derivatives, the number of sugars involved in conjugation, the sequence in which the sugars were attached to each other, as well as information about the aglycone moieties. This reliability was checked by isolating individual wine glycoconjugates that had initially been tentatively assigned by FAB-MS/MS, and then structurally characterising them positively by further spectrometric means (e.g. UV, ^1H NMR). Although the information provided by FAB-MS and MS/MS experiments is sufficient for only tentative structural assignments, it can be obtained under experimental circumstances that: a) require a minimal amount of sample; b) minimise sample preparation time by avoiding the necessity of laborious and often only partially effective chemical derivatizations; c) provide time efficiency as the analysis, in certain cases, can be performed even from mixtures, thus, avoiding additional steps of time consuming and occasionally partially effective chromatographic separations. Therefore, it is suggested that the protocol developed during the course of this study can be used for rapidly assessing the flavour status of wines by screening flavour precursors in partially fractionated glycosidic isolates.

However, there are certain limitations to the protocol which became apparent during its development. The most serious of these was that the number of molecular ions observed in the examination of a mixture does not necessarily represent the qualitative composition of that mixture. The presence of more than one isomeric compound in the mixture will result in the observation of only a common molecular ion. Additionally, the intensity of the individual pseudo-molecular ions observed in a FAB mass spectrum of a mixture is very much dependent on the solubility of each compound in the liquid matrix employed, and on the presence of other competing solutes in the matrix. Thus, the observation of individual components may be obscured by the presence of other constituents. Furthermore, when analysing mixtures, it was observed that sometimes pseudo-molecular ions happen to coincide with fragment ions of higher molecular weight constituents. This resulted in MS/MS spectra that were difficult to analyse because they were the summation of fragment ions arising from more than one constituent. Lastly, it was observed that structurally isomeric compounds, when present as a mixture, are also difficult to analyse, simply by FAB-MS/MS experiments, because they give rise to a common pseudo-molecular ion in the FAB spectrum, and therefore, the obtained MS/MS spectra are again the summation of fragment ions arising from more than one constituent.

The above mentioned points constitute deficiencies of the proposed analytical protocol. To overcome these difficulties, further purification of the analytes with conventional chromatographic techniques was found necessary. This highlighted the necessity of employing conventional chromatographic techniques as pre-analysis steps, especially in the cases where very complex mixtures of unknown constituents are under examination. However, in the past, mixtures of two or three constituents were very difficult to analyse by spectrometric techniques (e.g. ^1H NMR) and mixtures of more than three constituents were intractable. Only in cases of relatively non-polar conjugates, such as the glycosides discussed in chapter 2, chemical derivatisation together with GC-MS examination would often permit an analysis of pre-fractionated glycosidic mixtures to be performed. Now, with the introduction of FAB-MS and MS/MS to the analysis of high-polarity material, such as the glycoconjugates discussed in chapters 3-5, mixtures of three or even more constituents can be processed without the necessity of any prior chemical transformation.

Alongside with the examination of the applicability and the limitations of FAB-MS/MS to the analysis of material in grape juice and wine, this research study has established the presence in Riesling wine of a number of new glycosidic constituents. In a relatively non-polar, late eluting DCCC group of fractions, i.e. frs. 190-220, β -D-glucopyranosides of

monoterpene diols at the oxidation state of linalool oxide, i.e. uroterpenol and cis-pyran linalool oxide, were identified. In the same glycosidic fraction, unspecified β -hexopyranosides of the possible aroma contributing norisoprenoids, 3-hydroxy-5,6-epoxymegastigm-7-ene-9-one and diastereoisomeric forms of 3,4-dihydroxy-7,8-dihydro- β -ionone were also detected. The β -D-glucopyranoside of the ethyl ester of indole lactic acid was also identified and its presence in wine was rationalised.

In the relatively non-polar, late-eluting DCCC group of fractions, i.e. frs. 90-100, where disaccharide glycosides of monoterpenes and norisoprenoids are to be expected (Strauss *et al.* 1987a, Winterhalter *et al.* 1990b), not surprisingly the α -arabinofuranosyl-(1 \rightarrow 6)- β -glucopyranoside of (E)-3,7-dimethylocta-2-ene-1,7-diol, a monoterpene of the oxidation state of linalool oxide, was identified together with two other known flavour precursors. In addition, two new classes of wine glycoconjugates were identified. The first class involves the recently observed in grape juice apiosylglucosidic flavour precursors. The β -D-apiofuranosyl-(1 \rightarrow 6)- β -D-glucopyranosides of (E)-2,6-dimethylocta-2,7-diene-1,6-diol, (E,E)-2,6-dimethylocta-2,6-diene-1,8-diol and *p*-menth-1-ene-7,8-diol were identified. The second class of the newly identified glycoconjugates is constituted, surprisingly, of monoglucosides. However, the presence of polyhydroxylated compounds as the aglycone moiety of the identified glycoconjugates explains the observed polarity of these constituents. This class of compound is constituted of the 1-*O*-(β -D-glucopyranosyl)-2-[2-methoxy-4-(ω -hydroxypropyl)-phenoxy]-propan-3-ol and the phenolic derivatives isolariciresinol- β -4'-*O*-glucopyranoside and *seco*-isolariciresinol- β -D-glucopyranoside. These compounds were observed in wine for the first time and the isolariciresinol- β -4'-*O*-glucopyranoside, to the best of our knowledge, is observed in nature for the first time. These compounds, although they have no apparent flavour precursor role, contain individuals which have been suggested as precursors in the formation of biologically active compounds (i.e. *seco*-isolariciresinol- β -D-glucopyranoside). The presence in grape juice and wine of glycoconjugates with a potential pharmacological or generally biologically active role, constitutes a research area that should attract more attention in the near future.

The most polar glycosidic constituents of the wine flavour precursors, i.e. DCCC frs. 60-75, have been investigated in terms of their chemical nature. Spectrometric, ion-exchange chromatographic and electrophoretic studies on these fractions revealed that they were composed of two major groups of compounds. The first group consisted of ionic species such as the UV absorbing caftaric acid. Compounds comprising this first group appear to have no flavour precursor role. The second group is composed of neutral glycosidic

material which under acid hydrolysis conditions liberated monoterpene and norisoprenoid aroma constituents. However, the bulk of the material appeared to be made up of monoglucosidic derivatives with no apparent flavour precursor role, such as the neolignan 2,3-dihydro-7-hydroxy-2-(4'-hydroxy-3'-methoxybenzyl)-3-hydroxymethyl-5-benzofuranpropanol (i.e. cedrusin) the 4'-O- β -D-glucopyranoside of which was isolated and subsequently structurally characterized by spectrometric techniques. Glycoconjugates of monoterpene and norisoprenoid volatile aroma compounds were relatively minor constituents of these very polar, early-eluting DCCC fractions. The isolation and structural characterization of a glucopyranosyl-O-arabinofuranosyl-O-glucopyranoside of (E)-2,6-dimethylocta-2,7-diene-1,6-diol, indicated that these very polar flavour precursors are consisted of the already recognised monoterpene and norisoprenoid disaccharide glycosides but having an extra glucose unit in their sugar moiety.

The above observations of diverse glycoconjugates with different glycosidic moieties co-eluting under DCCC conditions, indicated that wine isolates, when resolved by DCCC, chromatograph according to their overall polarity (i.e. the summation of the polarity exhibited by both the aglycone and the sugar moiety). Thus, disaccharide glycosides of relatively non-polar aglycones co-elute on DCCC with monoglucosides of very polar aglycones such as the co-occurrence of the monoterpenyl-apiosylglucosides together with the glyceride and the lignan glucosides.

Lastly, the presence in a Riesling wine and in a Semillon juice of polar, non-volatile, thermally labile, but non-glycosidically bound β -carboline derivatives, was also observed. The presence of a diastereoisomeric mixture of the possible off-flavour principal, 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid in a Riesling wine was demonstrated by isolating the components from wine and structurally characterising them by a number of spectrometric and spectroscopic techniques. The formation of this heterocyclic compound, which has been implicated in alcoholism, from a series of higher molecular weight β -carboline derivatives has been rationalised. The latter compounds have been detected in both wine and grape juice by FAB-MS and subsequent MS/MS experiments provided some initial structural evidence.

Details of the C₁₈ columns used for HPLC are as follows. For the analytical separations a Merck Hibar LiChrosorb RP-18 column, 25 cm x 4 mm, containing 5 μm packing was used. For the semi-preparative work an Alltech Econosil column, 25 cm x 6.5 mm, containing 10 μm packing was used. The precolumn was an Alltech Adsorbosphere Guard Column Cartridge C₁₈, 10 mm x 4.6 mm, with a 5 μm packing.

EXPERIMENTAL

Chapter 1. General

Preparation of concentrates from 60 litres of an Australian Riesling wine and DCCC resolution of these concentrates has been previously described (Winterhalter *et al.* 1990b).

Flash chromatography

a) Underivatised glycosides. Flash chromatography was carried out as previously described (Still *et al.* 1978) employing the bottom phase of a $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$: 7/13/8 solvent system as eluent.

b) Per-O-acetylated glycosides. Flash chromatography was carried out as described above, employing diethyl ether or ethyl acetate/chloroform (50/50) as eluents. Ethyl acetate/chloroform (50/50) solvent system was employed in some cases of very polar per-O-acetylated glycosides (i.e. acetates of DCCC fractions frs. 60-75).

Thin layer chromatography

TLC of underivatized glycosides was carried out as previously described (Strauss *et al.* 1987a), and this system was also employed to monitor the progress of enzymic hydrolyses. For per-O-acetates, diethylether and chloroform/ethyl acetate (50/50) were used as eluents in cases of low and medium polarity glycosides (i.e. DCCC frs. 190-220) and very polar glycosides (i.e. DCCC frs. 60-75), respectively. Visualising agents were: a) 0.5% w/v vanillin in ethanol containing 0.5% v/v concentrated sulphuric acid and a few drops of glacial acetic acid; b) the Van Urk-Salkowski reagent (Ehmann 1977).

HPLC resolution of glycosidic fractions

The analytical and semi-preparative work was done at 35°C with reversed phase C_{18} columns. Both analytical (25 cm, 4 mm i.d.) and semi-preparative (25cm, 6.5 mm i.d.) columns were protected by a C_{18} precolumn. ^(For column details see opposite) All solvents were HPLC grade, filtered through 0.45 mm membrane and ultrasonically degassed before use. Samples were membrane filtered (0.45 mm) before analysis. Elutions were conducted at 1ml/min (analytical) and 2ml/min (semi-preparative) with linear gradients of CH_3CN (A) and H_2O (B). Gradients were varied according to the polarity of the analysed glycosides. Peak

spectra were recorded at 210-400 nm with a diode array detector and detection was at 220 and/or 280 nm.

Analytical gas chromatography (GC)

Analytical GC was carried out on a Varian 3300 instrument or a Perkin Elmer Sigma 2 instrument both fitted with capillary columns. Chromatograms were recorded with a Milton Roy CI-10B or a Hewlett-Packard 3380S integrator. For aglycones analyses, the chromatographs were equipped with either a 15 m or a 30m J&W DB 1701 fused silica column, 0.25 mm i.d. and 0.25 μ m film thickness. For analyses of per-O-acetylated glycosides, a J&W DB5, fused silica column, 0.25 mm i.d., and 0.25 μ m film thickness or a 15 m J&W DB 1701 fused silica column, 0.25 mm i.d. and 0.25 μ m film thickness, were used. Trimethylsilylated sugar derivatives were analysed with a SGE 25 m BP1 fused silica column, 0.33 mm i.d. and 0.5 μ m film thickness. In all cases, helium was used as carrier gas at a linear velocity of 40 cm/sec. Injections were made by using a split/splitless mode (1:10) at 220°C (for aglycones analyses), or at 280°C (for per-O-acetyl-, and trimethylsilyl-derivatives). The temperature program for aglycones analyses (on DB 1701) was 1min isothermal at 60°C followed by a gradient from 60°C to 250°C at 4°C/min. For analyses of the peracetylated glycosides, the DB 1701 column was held isothermal (1min) at 100°C followed by a gradient from 100°C to 300°C at 5°C/min. In the case of DB 5, the column was 5 min isothermal at 100°C and then the temperature was programmed to 320°C at 5 C/min. For analysis of the TMSi derivatives of sugars on the BP1 column, the protocol was 1min isothermal at 120°C followed by a gradient from 120°C to 180°C at 2°C/min, 1min isothermally at 180°C and finally from 180°C to 300°C at 7°C/min. The temperature of the flame ionization detector (FID) was in all cases kept at 280°C.

Gas chromatography-EI mass spectrometry (GC-MS)

Analyses were made with a Varian 3400 chromatograph conjugated to a Finnigan Mat TSQ 70 triple stage quadrupole mass spectrometer at an electron beam energy of 70 eV. In all cases analyses were made as described above.

Probe-EI mass spectrometry (EI-MS)

In all cases examined, the probe temperature was programmed from 100°C to 350°C at 100°C/min.

Probe-CI mass spectrometry (CI-MS)

The probe temperature was programmed from 100°C to 350°C at 100°C/min, the source temperature was 160°C, and ammonia was used as reagent gas.

Fast atom bombardment-mass spectrometry (FAB-MS)

Analyses were performed with a Finnigan Mat TSQ 70 triple stage quadrupole mass spectrometer tuned for operation under FAB mode, and using Xe as bombardment gas. The established voltage was 7-10 KeV, and the ion current <0.5 mA. Glycerol or acidified glycerol (5% CH₃COOH) were used as liquid matrix in the positive ion mode and triethanolamine or glycerol in the negative ion mode. Typical sample concentration was 1mg/ml. During MS/MS experiments the collision offset (COFF) varied between -15 to -30 eV (optimum conditions), the collision cell pressure was approximately 19 Pa, and the source temperature was 70°C.

Melting points

Melting points were determined using a Kofler hot-stage melting point apparatus and are uncorrected.

Infrared spectra (IR)

Infrared spectra were recorded on a Perkin Elmer 983G infrared spectrometer, using the 1603 cm⁻¹ absorption band of polystyrene for calibration.

Ultraviolet-visible (UV-Vis)

UV spectra were recorded on a Varian DMS 200 UV-Vis spectrometer.

Nuclear magnetic resonance spectroscopy (NMR)

¹H NMR spectra were recorded on either a Jeol FX 90Q spectrometer operating at 89.55 MHz or a Bruker HR ACP 300 spectrometer operating at 300 MHz. ¹³C NMR were recorded on the Jeol FX 90Q at 22.49 MHz or on the Bruker ACP 300 instrument operating at 75.47 MHz. Chemical shifts have been quoted in parts per million (ppm) downfield from tetramethylsilane. Multiplicities have been abbreviated to s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet; br, broad; obsc, obscure.

Solvents

All solvents used were of analytical grade at purchase and were redistilled before use.

Reference monoterpene, C₁₃ norisoprenoid, lignan and hydroxycinnamate compounds.

Reference materials used during the course of this study were obtained commercially or by donation. For work presented in subsequent chapters:

A mixture of diastereoisomeric *p*-menth-1-ene-8,9-diols (**30a,b**) was donated by Dr R. M. Carman (Carman *et al.* 1986). The mixture of *cis*- and *trans*- isomers of tetra-O-acetylated- β -D-glucopyranosides of 4-hydroxy-2,2,6-trimethyl-2-vinyltetrahydropyran was obtained from the collection of authentic monoterpene glycoside samples of The Australian Wine Research Institute, and it had been synthesised by Dr C. R. Strauss (Strauss 1983). 3-Hydroxy-5,6-epoxymegastigm-7-ene-9-one (**73**) and diastereoisomeric 3,4-dihydroxy-7,8-dihydro- β -ionones were donated by Dr P. Winterhalter (Winterhalter and Schreier 1988, Winterhalter 1991).

(2R, 3R) *Seco*-isolariciresinol (**92a**) was donated by Dr R. Cambie (Cambie *et al.* 1985)

(E)-2,6-dimethylocta-2,7-diene-1,6-diol (**94b**), (E,E)-2,6-dimethylocta-2,6-diene-1,8-diol (**95b**), (ξ)-3,7-dimethylocta-2-ene-1,7-diol (**96b**) were obtained from the collection of authentic monoterpene samples of The Australian Wine Research Institute, and they had been synthesised by Dr C. R. Strauss (Strauss *et al.* 1987a, Strauss *et al.* 1988). 4',5,7-trihydroxyflavone-7-O- β -D-apiofuranosyl- β -D-glucopyranoside (i.e. apiin) was purchased from Carl Roth GmbH, Co.

A reference sample of *trans*-caftaric acid was available at The Australian Wine Research Institute (Vérette *et al.* 1988).

Preparation of acetyl esters (per-O-acetates)

Per-O-acetylated derivatives were prepared with pyridine/acetic anhydride (1:1) as reagent, under anhydrous conditions at room temperature overnight.

Preparation of trimethylsilyl ethers (TMSi derivatives)

TMSi derivatives were prepared with Tri-Sil reagent (Pierce Cat. No. 48999), under anhydrous conditions by heating the reaction mixture at 120°C for 15 min.

Hydrolyses of glycosides

a) Enzymic hydrolyses. The enzymic hydrolyses of glycoconjugates were carried out by incubation of the solvent-free isolates dissolved in phosphate buffer pH 5.0 using the commercial pectinase *Rohapect C* (Winterhalter *et al.* 1990b), sweet almond β -D-

glucosidase (Serva, E.C. 3. 2. 1. 21), and the fungal enzyme preparation *Novoferm 12* (Novo Nordisk). Hydrolysis solutions were incubated for 24 hours at 37°C, in cases that enzymic preparations of *Rohapect C* and *Novoferm* were used, and for 48 hours at 37°C, in cases that the enzymic preparation from sweet almond was used. Then, the hydrolysates were extracted with CH₂Cl₂ (3x2ml), the solvent extracts were concentrated by distillation through a column of Fenske's helices and the liberated aglycones were analysed by GC-MS, or EI-MS/MS and/or FAB-MS/MS.

b) Acid hydrolyses. Acidic solutions (0.035% perchloric acid aqueous solution, pH=2.5) of the glycosides were heated at 100°C for 45 min. The solutions were neutralized (equivalent molarity KOH), dried (stream of N₂), dessicated overnight, and after preparation of the TMSi derivatives, the samples were examined by GC or GC-MS as described above.

EXPERIMENTAL

Chapter 2. Late-eluting, relatively non-polar wine glucosides

Flash chromatography

Flash chromatography was carried out as described above. Using the solvent system for underivatized glycosides, late eluting material off the DCCC (i.e. frs. 190-220, 34mg) was separated into thirty fractions from which frs. 7-12 were recombined and prepared for further purification by HPLC.

HPLC resolution of glycosidic fractions

In the case of combined glycosidic fractions frs. 7-12, both the analytical and semi-preparative work was done with the analytical C₁₈ reversed phase column. Elution was conducted at 1ml/min. Gradients of CH₃CN (A) and H₂O (B) were from 20% (A) to 35% (A) over 15 min and from 35% (A) to 20% (A) over the next 3 min, and then held at that gradient for 5 min. The combined flash chromatography fractions frs. 7-12 (13.8 mg) were separated into 14 new fractions with fr. 5 (0.2 mg, eluted at a H₂O/CH₃CN gradient of 75/25 and a RT between 5.70-6.20 min), fr. 9 (eluted at a H₂O/CH₃CN gradient of 71/29 and a RT between 8.60-9.0 min), fr. 10 (0.3 mg, eluted at a H₂O/CH₃CN gradient of 70/30 and a RT between 9.40-9.80 min) and fr. 11 (eluted at a H₂O/CH₃CN gradient of 69/31 and a RT between 10.50-11.50 min) being those of interest.

Ethyl indole-3-lactate-O-β-D-glucopyranoside (85)

0.57 mg (amorphous solid); TLC R_f=0.42; UV λ_{max}=220, 280, 289 (sh) nm; FAB-MS (positive ion mode): (M+H)⁺=396, (M+Na)⁺=418, (M+K)⁺=434; (negative ion mode): (M-H)⁻=394; FAB-MS/MS (positive ion mode): *m/z* 396=[M+H]⁺, 234=[(M+H)-anhydroglucose]⁺, 216=[(aglycone+H)-H₂O]⁺, 160=[(aglycone+H)-HCOOCH₂CH₃]⁺, 130=[(aglycone+H)-HOCH₂COOCH₂CH₃]⁺; (negative ion mode): *m/z* 394=[M-H]⁻, 232=[(M-H)-162]⁻, 214=[(aglycone-H)-H₂O]⁻, 204=[(aglycone-H)-CH₂CH₃]⁻, 185=[(aglycone-H)-HOCCH₂CH₃]⁻, 179=[glucose-H]⁻, 161=[(glucose-H)-H₂O]⁻, 119=[C₄H₇O₄]⁻, 113, 101=[C₄H₅O₃]⁻; 89=[C₃H₅O₃]⁻; note, assignments are based on likely fragment ion composition). **85a**: TLC R_f=0.46; EIMS: *m/z* (rel. intens.%) = 563 (10), 331 (3), 281 (3), 216 (15), 215 (35), 208 (8), 169 (10), 143 (9), 130 (100), 117 (9), 43 (25); FAB-MS: (M+H)⁺=564; ¹H NMR of the acetates: see table 2.

Synthesis of D, L-ethyl indole-3-lactate (85b)

The esterification reaction was made in a slightly modified soxhlett apparatus (sintered glass bottom instead of thimble). Racemic indole lactic acid (102 mg) was refluxed at 100°C, under nitrogen, with absolute ethanol (3.05 ml) containing a mixture of concentrated H₂SO₄ (0.1 ml) and benzene (10ml). The reaction was monitored by TLC employing ILA as reference, and when it reached completion the mixture was concentrated in *vacuo* to yield a brown liquid. The reaction mixture was dissolved in CH₂Cl₂, washed with saturated sodium bicarbonate (3x15 ml) and water (15ml). The organic phase was dried overnight (magnesium sulphate), filtered and concentrated to yield a brown-yellow oil (61.1 mg, 49.27%, R_f=0.52). Further purification of the crude reaction mixture by flash chromatography gave a yellow oil (40 mg) which was sensitive to day-light and air. ¹H NMR data : see table 2; EIMS: *m/z* (rel. intens.%) =233 (7.1), 160 (28), 131 (8.1), 130 (100), 117 (5.7), 103 (2.8), 77 (5.7).

p-Menth-1-ene-9-(β-D-glucopyranosyl)-8-ol (86)

0.4mg (hygroscopic solid); TLC R_f=0.38 ; UV λ_{max}=220 nm (end absorption); FAB-MS (positive ion mode) : (M+H)⁺=333, (M+Na)⁺=355; (negative ion mode) : (M-H)⁻=331; FAB-MS/MS (positive ion mode) : *m/z* 333=[M+H]⁺, 315=[(M+H)-H₂O]⁺, 171=[aglycone+H]⁺=[(M+H)-anhydroglucose]⁺, 163=[(glucose+H)-H₂O]⁺, 153=[(aglycone+H)-H₂O]⁺, 145=[(glucose+H)-2H₂O]⁺, 135=[(aglycone+H)-2H₂O]⁺, 127=[(glucose+H)-3H₂O]⁺; (negative ion mode) : *m/z* 331=[M-H]⁻, 179=[glucose-H]⁻, 161=[(glucose-H)-H₂O]⁻, 119=[C₄H₇O₄]⁻, 101=[(glucose-H)-C₂H₄O₂]⁻=[C₄H₅O₃]⁻; (note, assignments are based on likely fragment ion composition). **86a**: FAB-MS : (M+H)⁺=501, (M+Na)⁺=523; FAB-MS/MS : *m/z* 501=[M+H]⁺, 331=[(M+H)-aglycone]⁺, 271=[(M+H)-aglycone-CH₃COOH]⁺, 211=[(M+H)-aglycone-2CH₃COOH]⁺, 169=[C₈H₉O₄]⁺, 135=[(aglycone+H)-2H₂O]⁺, 127=[C₆H₇O₂]⁺, 109=[C₆H₅O₂]⁺; (note, assignments are based on likely fragment ion composition). EIMS : *m/z* (rel. intens. %)=331 (30), 271 (10), 211 (7), 181 (10.5), 170 (6.9), 169 (100), 152 (8.5), 139 (30), 135 (20), 127 (9), 115 (6.5), 109 (5), 97 (11), 95 (16.5), 94 (21), 93 (11), 81 (11), 67 (7), 57 (5); ¹H NMR data : see table 3.

cis-2,2,6-Trimethyl-2-vinyl-4-(β-D-glucopyranosyl)-tetrahydropyran (87)

TLC R_f=0.39; λ_{max}=220 nm (end absorption); FAB-MS (positive ion mode) : (M+H)⁺=333, (M+Na)⁺=355, (M+Glycerol+H)⁺=425; (negative ion mode) : (M-H)⁻=331; FAB-MS/MS (positive ion mode) : *m/z* 333=[M+H]⁺, 315=[(M+H)-H₂O]⁺, 171=[aglycone+H]⁺=[(M+H)-anhydroglucose]⁺, 163=[(glucose+H)-H₂O]⁺,

153=[(aglycone+H)-H₂O]⁺, 145=[(glucose+H)-2H₂O]⁺, 135=[(aglycone+H)-2H₂O]⁺, 127=[(glucose+H)-3H₂O]⁺, 107, 93, 71; (negative ion mode) : *m/z* 331=[M-H]⁻, 161=[(glucose-H)-H₂O]⁻, 119=[C₄H₇O₄]⁻, 113, 101=[C₄H₅O₃]⁻; 89=[C₃H₅O₃]⁻, 71; (note, assignments are based on likely fragment ion composition). **87a**: TLC R_f=0.36; FAB-MS : (M+H)⁺=501, (M+Na)⁺=523; FAB-MS/MS : *m/z* 501=[M+H]⁺, 331=[(M+H)-aglycone]⁺, 271=[(M+H)-aglycone-CH₃COOH]⁺, 211=[(M+H)-aglycone-2CH₃COOH]⁺, 169=[C₈H₉O₄]⁺, 153=[(aglycone+H)-H₂O]⁺, 135=[(aglycone+H)-2H₂O]⁺, 127=[C₆H₇O₂]⁺, 109=[C₆H₅O₂]⁺; (note, assignments are based on likely fragment ion composition). EIMS : *m/z* (rel. intens. %) = 485 (1.5), 473 (0.5), 331 (37), 271 (8), 211 (1), 169 (80), 153 (23), 145 (5), 139 (8), 135 (10), 127 (25), 115 (8), 109 (100), 97 (9), 94 (31), 93 (25), 81 (30), 71 (15), 67 (14).

3-(β-D-Hexopyranosyl)-5,6-epoxymegastigm-7-ene-9-one (88)

FAB-MS (positive ion mode) : (M+H)⁺=387, (M+Na)⁺=409; (negative ion mode) : (M-H)⁻=385; FAB-MS/MS (positive ion mode) : *m/z* 387=[M+H]⁺, 225=[aglycone+H]⁺=[(M+H)-anhydrohexose]⁺, 207=[(aglycone+H)-H₂O]⁺, 205, 189=[(aglycone+H)-2H₂O]⁺, 181=[hexose+H]⁺, 163=[(hexose+H)-H₂O]⁺, 151, 149, 145=[(hexose+H)-2H₂O]⁺, 127=[(hexose+H)-3H₂O]⁺, 123, 97, 85; (negative ion mode) : *m/z* 385=[M-H]⁻, 223=[aglycone-H]⁻=[(M-H)-anhydrohexose]⁻, 205=[(aglycone-H)-H₂O]⁻, 179=[hexose-H]⁻, 161=[(hexose-H)-H₂O]⁻, 119=[C₄H₇O₄]⁻, 113, 101=[C₄H₅O₃]⁻; 89=[C₃H₅O₃]⁻, 71; (note, assignments are based on likely fragment ion composition). **88a**: FAB-MS: (M+H)⁺=555; CI-MS: (M+NH₄)⁺=572; EIMS : *m/z* (rel. intens. %) = 331 (5), 271 (1), 211 (1.5), 207 (20), 191 (3), 169 (43), 151 (15), 123 (100), 109 (30), 97 (2), 73 (13).

3,4-Dihydroxy-7,8-dihydro-β-ionone-β-D-hexopyranoside (89)

FAB-MS (positive ion mode) : (M+H)⁺=389, (M+Na)⁺=411; (negative ion mode) : (M-H)⁻=387; FAB-MS/MS (positive ion mode) : *m/z* 389=[M+H]⁺, 371=[(M+H)-H₂O]⁺, 227=[aglycone+H]⁺=[(M+H)-anhydrohexose]⁺, 209=[(aglycone+H)-H₂O]⁺, 205, 191=[(aglycone+H)-2H₂O]⁺, 173=[(aglycone+H)-3H₂O]⁺, 163=[(hexose+H)-H₂O]⁺, 151, 149, 145=[(hexose+H)-2H₂O]⁺, 133, 127=[(hexose+H)-3H₂O]⁺, 121 105, 95; (negative ion mode) : *m/z* 387=[M-H]⁻, 225=[aglycone-H]⁻=[(M-H)-anhydrohexose]⁻, 205=[(aglycone-H)-H₂O]⁻, 179=[hexose-H]⁻, 161=[(hexose-H)-H₂O]⁻, 119=[C₄H₇O₄]⁻, 113, 101=[C₄H₅O₃]⁻; 89=[C₃H₅O₃]⁻, 71; (note, assignments are based on likely fragment ion composition). **89a**: FAB-MS: (M+H)⁺=599, (M+Na)⁺=621; CI-MS:

(M+NH₄)⁺=616; EIMS : *m/z* (rel. intens. %) = 331 (20), 271 (5), 250 (17), 208 (25), 191 (10), 190 (35), 173 (13), 169 (100), 151 (17), 133 (19), 121 (10), 109 (52), 97 (6), 69 (10).

Hydrolyses of isolates

a) Enzymic hydrolyses. The enzymic hydrolyses were carried out as described above. Sweet almond β -D-glucosidase and the commercial pectinase *Rohapect C* were used to hydrolyse two different aliquots of glucoside **85**, respectively. Glycosides **86-89** were hydrolysed using the β -D-glucosidase. The liberated aglycones were examined by GC-MS and showed the following characteristics. **85c**) EIMS : *m/z* (rel. intens.%) = 233 (7.1), 160 (28), 131 (8.6), 130 (100), 103 (5.7), 77 (5.7). In the case of enzymic hydrolysis of **85** with the non-specific glucosidase (*Rohapect C*) the absence of a peak corresponding to EILA from the GC trace of the extracted organic phase suggested the further degradation of **85** by the enzyme to ILA. This was confirmed by monitoring the hydrolysis reaction by TLC using ILA and EILA as references. **30**) EIMS data similar to those published by Winterhalter *et al.* (1990b). **6**) EIMS data similar to those published previously by Felix *et al.* (1963). **73**) EIMS data similar to those published by Winterhalter and Schreier (1988). **89b**) early eluting isomer : EIMS : *m/z* (rel. intens.%) = 208 (5), 193 (2), 190 (4), 175 (5), 168 (26), 152 (57), 150 (17), 135 (30), 121 (22), 109 (65), 107 (35), 95 (18), 91 (12), 81 (10), 67 (17), 55 (12), 43 (100), 41 (25); late eluting isomer : EIMS : *m/z* (rel. intens.%) = 208 (17), 193 (10), 190 (5), 175 (9), 168 (42), 152 (8), 150 (35), 135 (45), 123 (16), 121 (16), 109 (57), 107 (36), 95 (15), 91 (10), 81 (10), 67 (12), 55 (18), 43 (100), 41 (24). These data are similar to those published by Winterhalter (1991) for undefined diastereoisomer or mixture of diastereoisomers of **89b**.

b) Acid hydrolyses. They were performed as described above. The obtained mass spectrometric data for the TMSi derivatives of the liberated sugar (i.e. glucose), were similar to those reported by Radford and Dejongh (1972).

c) Enzymic assay for β -D-glucose determination. For the specific measurement of β -D-glucose a coupled enzyme kit was employed (Sigma Chemical Co, cat N^o 510). The steps involved: i) hydrolysis of the isolates (c=0.55 μ mol/mL) by incubation with β -D-glucosidase in a phosphate buffer pH=5.0, at 37°C, for 24 hrs, (the ratio of enzyme to substrate was 1:10 in a total volume of 2.0 ml solution). ii) An aliquot (0.1 ml) was removed and the glucosidase enzyme inactivated by heating, followed by the addition of 1 ml of colour reagent (peroxidase/o-dianisidine) and the mixture incubated at 37°C for 30

min. Colour intensity was read at 470 nm. Calibration with D-glucose solutions confirmed that there was a linear relation between absorbance (A) and glucose concentration (C) in the range of 20-100 μg glucose/ml (0.11-0.55 $\mu\text{mol/ml}$). Positive and negative control hydrolyses with p-nitrophenyl- β -D-glucoside, arbutin, salicin, octyl- β -D-glucoside and p-nitrophenyl- β -D-galactoside, and the disaccharide rutin, confirmed the specificity of the method for the detection of β -D-glucopyranosides.

EXPERIMENTAL

Chapter 3. Early-eluting polar monoglucosides

Flash chromatography

Flash chromatography was carried out as described above. Using the solvent system for underivatized glycosides, early eluting material off the DCCC (i.e. frs. 90-100, 65 mg) was separated into fifteen fractions from which combined frs. 6-7 (3.0 mg) and combined frs. 10-12 (19.7 mg) were prepared for further purification by HPLC.

HPLC resolution of glycosidic fractions

For the analytical work, gradients of CH₃CN (A) and H₂O (B) were from 10% (A) to 20% (A) over 10 min and then held at that gradient for 5 min. For the semi-preparative work, gradients were from 10% (A) to 20% (A) over 15 min and from 20% (A) to 100% (A) over the next 5 min, and then held at that gradient for 8 min. HPLC separation of the combined flash chromatography fractions frs. 6-7 gave a fraction containing **92** which eluted at a H₂O / CH₃CN gradient of 65/35 and a RT between 16.60-17.00 min. Flash chromatography fractions frs. 10-12 were separated into six new fractions with fr. 1 (eluted at a H₂O / CH₃CN gradient of 83/17 and a RT between 10.00 -10.50 min) containing glucoside **90** (0.4 mg), and fr. 2 (eluted at a H₂O / CH₃CN gradient of 81/19 and a RT between 123.70-13.20 min) containing glucoside **91** (0.5 mg).

1-O-(β-D-glucopyranosyl)-2-[2-methoxy-4-(ω-hydroxypropyl)-phenoxy]-propan-3-ol (90)
0.4 mg; TLC R_f=0.43; UV λ_{max}=280 nm; FAB-MS (positive ion mode): (M+H)⁺=419, (M+Na)⁺=441; (negative ion mode): (M-H)⁻=417; FAB-MS/MS (positive ion mode): *m/z* 419=[M+H]⁺, 257=[(M+H)-anhydroglucose, i.e. aglycone+H]⁺, 239=[(M+H)-anhydroglucose-H₂O]⁺, 221=[(M+H)-anhydroglucose-2H₂O]⁺, 183=[(aglycone+H)-anhydroglycerol]⁺, 165=[(aglycone+H)-anhydroglycerol-H₂O]⁺, 163=[(glucose+H)-H₂O]⁺, 145=[(glucose+H)-2H₂O]⁺, 137=[C₈H₉O, i.e. hydroxy-methoxy-benzylum]⁺, 127=[(glucose+H)-3H₂O]⁺; (negative ion mode) : *m/z* 417=[M-H]⁻, 235=[(M-H)-C₁₀H₁₄O₃]⁻, 181=[(aglycone-H)-anhydroglycerol]⁻, 161=[(glucose-H)-H₂O]⁻; (note, assignments are based on likely fragment ion compositions). **90a**: TLC R_f=0.25; EIMS : *m/z* (rel. intens.%) = 670 (3), 448 (89), 331 (3), 323 (4), 223 (5), 211 (2), 203 (5), 175 (9), 169 (100), 164 (13), 159 (23), 145 (19), 127 (17), 117 (14), 115 (14), 109 (54), 103 (14), 81 (13), 57 (7); FAB-MS : (M+H)⁺=671; FAB-MS/MS : *m/z* 671=[M+H]⁺, 448=[(M+H)-

$C_{12}H_{19}O_9]^+$, $331=[C_{14}H_{19}O_9]^+$, $271=[C_{12}H_{15}O_7]^+$, $211=[C_{10}H_{11}O_5]^+$, $169[C_8H_9O_4]^+$, $109=[C_6H_5O_2]^+$ (note, assignments are based on likely fragment ion compositions); 1H NMR of **90** and **90a** : see table 4.

1,2,3,4-Tetrahydro-7-hydroxy-1-(4'- β -glucopyranosyl-3'-methoxyphenyl)-6-methoxy-2,3-naphthalenedimethanol (91)

0.5 mg (amorphous solid); TLC $R_f=0.51$; UV $\lambda_{max}=280$ nm; FAB-MS (positive ion mode) : $(M+H)^+=523$, $(M+Na)^+=545$; (negative ion mode) : $(M-H)^-=521$; FAB-MS/MS (positive ion mode) : m/z $523=[M+H]^+$, $505=[(M+H)-H_2O]^+$, $361=[(M+H)-anhydroglucose]^+$, $343=[(aglycone+H)-H_2O]^+$, $311=[(aglycone+H)-H_2O-MeOH]^+$, $237=[(M+H)-anhydroglucose-C_7H_8O_2]^+$, $219=[(M+H)-anhydroglucose-C_7H_8O_2-H_2O]^+$, $163=[(glucose+H)-H_2O]^+$, $145=[(glucose+H)-2H_2O]^+$, $137=[C_8H_9O_2]^+$; (negative ion mode) : m/z $521=[M-H]^-$, $359=[aglycone-H]^-$, $161=[(glucose-H)-H_2O]^-$ (note, assignments are based on likely fragment ion compositions); **91a**: TLC $R_f=0.17$; NH_4 -CIMS : $(M+NH_4)^+=834$, $(M-H)^-=815$; 1H NMR data of **91** and **91a** : see table 5.

2,3-Bis(4'-hydroxy-3'-methoxybenzyl)butane-1,4-diol- β -D-glucopyranoside (92)

0.12 mg; UV $\lambda_{max}=210$ (sh), 280 nm; FAB-MS (positive ion mode) : $(M+H)^+=525$, (negative ion mode) : $(M-H)^-=523$; FAB-MS/MS (positive ion mode) : m/z $525=[M+H]^+$, $363=[(M+H)-anhydroglucose]^+$, $345=[(aglycone+H)-H_2O]^+$, $331=[(M+H)-C_7H_{14}O_6$ (i.e. methylhexose)] $^+$, $313=[(aglycone+H)-MeOH-H_2O]^+$, 287, 222, 178, 146, (aglycone fragments); (negative ion mode) : m/z $523=[M-H]^-$, $343=[aglycone-H]^-$ (note, assignments are based on likely fragment ion compositions).

Hydrolyses of glycosides:

a) Enzymic hydrolyses. The enzymic hydrolyses of glycoconjugates **90** and **92** were carried out as described above using β -D-glucosidase (Serva, E.C. 3. 2. 1. 21). Glucoside **91** was hydrolysed with the fungal enzyme preparation *Novoferm 12* (Novo Nordisk). The liberated aglycones were examined by probe EI-MS/MS and FAB-MS/MS (i.e. **90b**), by EI-MS/MS (i.e. **92a**) and by GC-MS as their TMSi derivatives (i.e. **91b**, **92a**) and showed the following previously unpublished spectral data. **90b**) EIMS (probe) : $M^+=256$; FAB-MS : $(M+H)^+=257$, $(M+Na)^+=279$, $(M-H)^-=255$; FAB-MS/MS : m/z $257=[aglycone+H]^+$, $183=[(aglycone+H)-anhydroglycerol]^+$, $165=[(aglycone+H)-anhydroglycerol-H_2O]^+$, $137=[C_8H_9O$, i.e. hydroxy-methoxy-benzylum] $^+$, $133=[C_8H_9O-CH_3OH]^+$ (note, assignments are based on likely fragment ion compositions). **91b**) EIMS

data (as TMSi derivatives) essentially identical to those published previously by Kraus and Spiteller (1990) and Ekman (1976). **92a**) EIMS (as TMSi derivatives) were similar to those published previously by Ekman (1976): m/z (rel. intens.%) = 650 (1), 560 (1), 470 (2), 439 (2), 424 (1), 274 (2), 261 (35), 248 (13), 209 (100), 179 (22), 147 (9), 103 (8), 73(9).

b) Acid hydrolyses. They were performed as described above. The obtained mass spectrometric data for the TMSi derivatives of the liberated sugar (i.e. glucose), were similar to those reported by Radford and Dejongh (1972).

EXPERIMENTAL

Chapter 4. Early-eluting disaccharide glycosides

Flash chromatography

a) Underivatized glycosides. Flash chromatography was carried out as described above. A second lot of material eluting early off DCCC (i.e. frs. 90-100, 107 mg) was separated into twenty five fractions from which frs. 6-15 (35.0 mg) were recombined and prepared for further purification by HPLC.

b) Per-O-acetylated glycosides. Using the solvent system for per-O-acetylated glycosides, HPLC fr. 3 (2.4 mg) was separated into thirty fractions (30 x 1 ml fractions) from which fl. frs. 3-5, and fl. frs. 22-27 were found (by positive FAB-MS examination) to contain glycosides of interest with MW=416 (**93**), and 464 (**97**), 518 (**98**) respectively. Derivatized HPLC fr. 6 (7.3 mg), separated into 32 fractions (70 ml diethyl ether) from which fl. frs. 12-13, fl. frs. 16-20, and fl. frs. 22-25 were found to contain glycosides with MW=464 (**94**), 464 (**95**), and 466 (**96**), respectively.

HPLC resolution of glycosidic fractions

For the analytical work, gradients were from 10% (A) to 20% (A) over 10 min and then held at that gradient for 5 min. For the semi-preparative work, gradients were from 10% (A) to 20% (A) over 15 min and from 20% (A) to 100% (A) over the next 5 min, and then held at that gradient for 5 min. The combined flash chromatography fractions frs. 6-15 were separated into 8 new fractions with fr. 3 (2.0 mg, eluted at a H₂O/CH₃CN gradient of 81/19 and a RT between 13.5-15.0 min) containing glycosides **93**, **97**, **98**, and fr. 6 (4.9 mg, eluted at a H₂O/CH₃CN gradient of 49/51 and a RT between 18.0-19.0 min) containing glycosides **94**, **95**, **96**.

Benzylalcohol-O-β-rutinoside (93)

0.2 mg; FAB-MS (positive ion mode) : (M+H)⁺=417, (M+Na)⁺=439; (negative ion mode) : (M-H)⁻=415; FAB-MS/MS (positive ion mode) : m/z 417=[M+H]⁺, 309=[C₁₂H₂₁O₉]⁺, 163=[(glucose+H)-H₂O]⁺, 147=[(rhamnose+H)-H₂O]⁺, 145=[(glucose+H)-2H₂O]⁺, 129=[(rhamnose+H)-2H₂O]⁺, 91=[tropylium ion]⁺; (negative ion mode) : m/z 415=[M-H]⁻, 269=[(M-H)-anhydroramnose]⁻, 163=[(rhamnose-H)-H₂O]⁻, 161=[(glucose-H)-H₂O]⁻

, 125, 119=[C₄H₇O₄]⁻, 113, 101=[C₄H₅O₃]⁻, 89=[C₃H₅O₃]⁻; (note, assignments are based on likely fragment ion compositions). **93a**: TLC R_f=0.45; FAB-MS : *m/z* 761=[(M+H)+glycerol]⁺, 669=[M+H]⁺, 609=[(M+H)-CH₃COOH]⁺, 561=[C₂₄H₃₃O₁₅]⁺, 331=[C₁₄H₁₉O₉]⁺, 273=[C₁₂H₁₇O₇]⁺ (note, assignments are based on likely fragment ion composition). EIMS characteristics of **93a** were similar to those published elsewhere (Williams *et al.* 1983).

(E)-2,6-dimethylocta-2,7-diene-1-(β-D-apiofuranosyl-(1-6)-β-D-glucopyranosyl)-6-ol

(94)

1.5 mg (hygroscopic solid); FAB-MS (positive ion mode) : (M+H)⁺=465, (M+Na)⁺=487, (M+K)⁺=503; (negative ion mode) : (M-H)⁻=463; FAB-MS/MS (positive ion mode) : *m/z* 465=[M+H]⁺, 333=[(M+H)-anhydroapiose]⁺, 315=[(M+H)-apiose]⁺, 295=[C₁₁H₁₉O₉]⁺, 267=[C₁₀H₁₉O₈]⁺, 163=[(glucose+H)-H₂O]⁺, 153=[(aglycone+H)-H₂O]⁺, 145=[(glucose+H)-2H₂O]⁺, 135=[(aglycone+H)-2H₂O]⁺, 133=[(apiose+H)-H₂O]⁺, 127=[(glucose+H)-3H₂O]⁺, 115=[(apiose+H)-2H₂O]⁺; (negative ion mode) : *m/z* 463=[M-H]⁻, 331=[(M-H)-anhydroapiose]⁻, 251=[C₉H₁₅O₈]⁻, 221=[C₈H₁₃O₇]⁻, 191=[C₇H₁₁O₆]⁻, 179=[glucose-H]⁻, 161=[(glucose-H)-H₂O]⁻, 149=[apiose-H]⁻, 131=[(apiose-H)-H₂O]⁻, 119=[C₄H₇O₄]⁻, 113, 101=[C₄H₅O₃]⁻, 89=[C₃H₅O₃]⁻; (note, assignments are based on likely fragment ion compositions). **94a**: TLC R_f=0.38; FAB-MS : *m/z* 717=[M+H]⁺, 739=[M+Na]⁺, 755=[M+K]⁺, 547=[C₁₃H₃₁O₁₅]⁺, 331=[C₁₄H₁₉O₉]⁺, 259=[C₁₁H₁₅O₇]⁺, 139=[C₇H₇O₃]⁺; FAB-MS/MS : *m/z* 717=[M+H]⁺, 657=[(M+H)-CH₃COOH]⁺, 531, 259=[C₁₁H₁₅O₇]⁺, 139=[C₇H₇O₃]⁺; (note, assignments are based on likely fragment ion compositions). ¹H NMR data of the anacetylated material and the acetates : see table 6.

(E,E)-2,6-Dimethylocta-2,6-diene-1-(β-D-apiofuranosyl-(1-6)-β-D-glucopyranosyl)-8-ol

(95)

0.5 mg (hygroscopic solid); FAB-MS (positive ion mode) : (M+H)⁺=465, (M+Na)⁺=487; (negative ion mode) : (M-H)⁻=463; FAB-MS/MS (positive ion mode) : *m/z* 465=[M+H]⁺, 333=[(M+H)-anhydroapiose]⁺, 315=[(M+H)-apiose]⁺, 295=[C₁₁H₁₉O₉]⁺, 267=[C₁₀H₁₉O₈]⁺, 163=[(glucose+H)-H₂O]⁺, 153=[(aglycone+H)-H₂O]⁺, 145=[(glucose+H)-2H₂O]⁺, 135=[(aglycone+H)-2H₂O]⁺, 133=[(apiose+H)-H₂O]⁺, 127=[(glucose+H)-3H₂O]⁺, 115=[(apiose+H)-2H₂O]⁺; (negative ion mode) : *m/z* 463=[M-H]⁻, 331=[(M-H)-anhydroapiose]⁻, 251=[C₉H₁₅O₈]⁻, 221=[C₈H₁₃O₇]⁻, 191=[C₇H₁₁O₆]⁻, 179=[glucose-H]⁻, 161=[(glucose-H)-H₂O]⁻, 149=[apiose-H]⁻, 131=[(apiose-H)-H₂O]⁻, 119=[C₄H₇O₄]⁻, 113, 101=[C₄H₅O₃]⁻, 89=[C₃H₅O₃]⁻; (note,

assignments are based on likely fragment ion compositions). **95a**: TLC R_f = 0.53; FAB-MS : m/z 759=[M+H]⁺, 781=[M+Na]⁺, 699=[(M+H)-CH₃COOH]⁺, 547=[C₁₃H₃₁O₁₅]⁺, 331=[C₁₄H₁₉O₉]⁺, 259=[C₁₁H₁₅O₇]⁺; FAB-MS/MS : m/z 759=[M+H]⁺, 699=[(M+H)-CH₃COOH]⁺, 259=[C₁₁H₁₅O₇]⁺, 139=[C₇H₇O₃]⁺; (note, assignments are based on likely fragment ion compositions). ¹H NMR data of the acetates **95a** : see table 7.

(E)-3,7-Dimethylocta-3-ene-1-(α -arabinofuranosyl-(1-6)- β -glucopyranosyl)-7-ol (96)

0.8 mg; FAB-MS (positive ion mode) : (M+H)⁺=467, (M+Na)⁺=489, (M+K)⁺=505; (negative ion mode) : (M-H)⁻=465; FAB-MS/MS (positive ion mode) : m/z 467=[M+H]⁺, 335=[(M+H)-anhydroapiose]⁺, 317=[(M+H)-apiose]⁺, 295=[C₁₁H₁₉O₉]⁺, 267=[C₁₀H₁₉O₈]⁺, 259=[C₁₃H₂₃O₅]⁺, 181=[glucose+H]⁺, 173=[aglycone+H]⁺, 163=[(glucose+H)-H₂O]⁺, 155=[(aglycone+H)-H₂O]⁺, 145=[(glucose+H)-2H₂O]⁺, 137=[(aglycone+H)-2H₂O]⁺, 133=[(apiose+H)-H₂O]⁺, 127=[(glucose+H)-3H₂O]⁺, 115=[(apiose+H)-2H₂O]⁺; (negative ion mode) : m/z 465=[M-H]⁻, 333=[(M-H)-anhydroapiose]⁻, 251=[C₉H₁₅O₈]⁻, 221=[C₈H₁₃O₇]⁻, 191=[C₇H₁₁O₆]⁻, 179=[glucose-H]⁻, 161=[(glucose-H)-H₂O]⁻, 149=[apiose-H]⁻, 131=[(apiose-H)-H₂O]⁻, 119=[C₄H₇O₄]⁻, 113, 101=[C₄H₅O₃]⁻, 89=[C₃H₅O₃]⁻; (note, assignments are based on likely fragment ion compositions). **96a**: TLC R_f =0.46; FAB-MS : m/z 719=[M+H]⁺, 659=[(M+H)-CH₃COOH]⁺, 565=[C₁₃H₃₃C₁₆]⁺, 547=[C₁₃H₃₁O₁₅]⁺, 331=[C₁₄H₁₉O₉]⁺, 259=[C₁₁H₁₅O₇]⁺, 139=[C₇H₇O₃]⁺; FAB-MS/MS : m/z 719=[M+H]⁺, 701=[(M+H)-H₂O]⁺, 565=[C₁₃H₃₃C₁₆]⁺, 547=[C₁₃H₃₁O₁₅]⁺, 259=[C₁₁H₁₅O₇]⁺, 139=[C₇H₇O₃]⁺; (note, assignments are based on likely fragment ion compositions). ¹H NMR data of the acetates **96a** : see table 7.

p-Menth-1-ene-7-(β -apiofuranosyl-(1-6)- β -glucopyranosyl)-8-ol (97)

0.4 mg; FAB-MS (positive ion mode) : (M+H)⁺=465, (M+Na)⁺=487; (negative ion mode) : (M-H)⁻=463; FAB-MS/MS (positive ion mode) : m/z 465=[M+H]⁺, 333=[(M+H)-anhydroapiose]⁺, 315=[(M+H)-apiose]⁺, 295=[C₁₁H₁₉O₉]⁺, 267=[C₁₀H₁₉O₈]⁺, 259=[C₁₃H₂₃O₅]⁺, 189, 171=[aglycone+H]⁺, 163=[(glucose+H)-H₂O]⁺, 153=[(aglycone+H)-H₂O]⁺, 145=[(glucose+H)-2H₂O]⁺, 135=[(aglycone+H)-2H₂O]⁺, 133=[(apiose+H)-H₂O]⁺, 127=[(glucose+H)-3H₂O]⁺, 115=[(apiose+H)-2H₂O]⁺; (negative ion mode) : m/z 463=[M-H]⁻, 331=[(M-H)-anhydroapiose]⁻, 279, 251=[C₉H₁₅O₈]⁻, 221=[C₈H₁₃O₇]⁻, 191=[C₇H₁₁O₆]⁻, 179=[glucose-H]⁻, 161=[(glucose-H)-H₂O]⁻, 149=[apiose-H]⁻, 131=[(apiose-H)-H₂O]⁻, 119=[C₄H₇O₄]⁻, 113, 101=[C₄H₅O₃]⁻, 89=[C₃H₅O₃]⁻; (note, assignments are based on likely fragment ion compositions). **97a**: TLC R_f = 0.28; FAB-MS : m/z 717=[M+H]⁺, 565=[C₁₄H₃₃O₁₅]⁺,

547=[C₁₃H₃₁O₁₅]⁺, 331=[C₁₄H₁₉O₉]⁺, 259=[C₁₁H₁₅O₇]⁺; FAB-MS/MS : *m/z* 759=[M+H]⁺, 699=[(M+H)-CH₃COOH]⁺, 259=[C₁₁H₁₅O₇]⁺, 139=[C₇H₇O₃]⁺; (note, assignments are based on likely fragment ion compositions). ¹H NMR data of the acetates **97a** : see table 7.

Vomifoliol-9-O-arabinofuranosyl-O-glucopyranoside (98)

FAB-MS (positive ion mode) : (M+H)⁺=519, (M+Na)⁺=541; (negative ion mode) : (M-H)⁻=517; FAB-MS/MS (positive ion mode) : *m/z* 519=[M+H]⁺, 387=[(M+H)-anhydroarabinose]⁺, 369=[(M+H)-arabinose]⁺, 295=[C₁₁H₁₉O₉]⁺, 225=[aglycone+H]⁺, 207=[(aglycone+H)-H₂O]⁺, 189=[(aglycone+H)-2H₂O]⁺, 163=[(glucose+H)-H₂O]⁺, 161=[(aglycone+H)-2H₂O-CO]⁺, 149=[C₁₀H₁₃O]⁺, 133=[Apiose+H-H₂O]⁺, 123, 113, 95; (negative ion mode) : *m/z* 517=[M-H]⁻, 385=[(M-H)-anhydroarabinose]⁻, 311=[C₁₁H₁₉O₁₀]⁻, 293=[C₁₁H₁₇O₉]⁻, 251=[C₉H₁₅O₈]⁻, 233, 223=[aglycone-H]⁻, 221=[C₈H₁₃O₇]⁻, 205=[(aglycone-H)-H₂O]⁻, 191=[C₇H₁₁O₆]⁻, 179=[glucose-H]⁻, 161=[(glucose-H)-H₂O]⁻, 149=[arabinose-H]⁻, 131=[(arabinose-H)-H₂O]⁻, 125, 119=[C₄H₇O₄]⁻, 113, 101=[C₄H₅O₃]⁻, 89=[C₃H₅O₃]⁻; (note, assignments are based on likely fragment ion compositions). **98a**: TLC R_f= 0.29; FAB-MS : *m/z* 771=[M+H]⁺, 547=[C₁₃H₃₁O₁₅]⁺, 331=[C₁₄H₁₉O₉]⁺, 259=[C₁₁H₁₅O₇]⁺ ; FAB-MS/MS : *m/z* 771=[M+H]⁺, 547=[C₁₃H₃₁O₁₅]⁺, 259=[C₁₁H₁₅O₇]⁺; (note, assignments are based on likely fragment ion compositions).

Hydrolyses of isolates :

a) Enzymic hydrolyses. The enzymic hydrolyses were carried out as described above using the fungal enzyme preparation *Novoferm 12* (Novo Nordisk). The liberated aglycones were examined by GC-MS and showed mass spectrometric characteristics similar to those published elsewhere for (E)-2,6-dimethylocta-2,7-diene-1,6-diol (**94b**) (Strauss *et al.* 1988), (E,E)-2,6-dimethylocta-2,6-diene-1,8-diol (**95b**) (Strauss *et al.* 1988), (E)-3,7-dimethylocta-2-ene-1,7-diol (**96b**) (Mousseron-Canet *et al.* 1964), p-menth-1-ene-7,8-diol (**32**) (Versini *et al.* 1991), and 6,9-dihydroxymegastigma-4,7-dien-3-one, i.e. vomifoliol, (**63**) (Strauss *et al.* 1987c).

b) Acid hydrolyses. In all cases, acid hydrolyses were performed as described above. In the case of glycoside **94**, the acid hydrolysates were first extracted with CH₂Cl₂ (3x1 mL) and then the extract was analysed by GC-MS. Diastereoisomeric p-menth-1-en-9-als, uroterpenol (**30**) and (E)-2,6-dimethylocta-2,7-diene-1,6-diol (**94b**) were observed *inter alia* with other not-recognised terpenoids. The assignments of the above mentioned

monoterpenes was based on their gas chromatographic and mass spectrometric characteristics (Strauss *et al.* 1988, Carman *et al.* 1986). In the other cases (i.e. glycosides **95-98**), only the water-soluble fractions of the hydrolysates were analysed by GC and/or GC-MS. The obtained mass spectrometric data for the TMSi derivatives of the liberated sugars were similar to those reported by Radford and Dejongh (1972) for trimethylsilylated glucose. The arabinose trimethylsilyl derivatives showed the following EIMS characteristics. 1) α -Anomer: m/z (rel. intens.%)=333 (2), 305 (3), 291 (1), 279 (2), 259 (3), 218 (17), 217 (95), 205 (11), 204 (67), 191 (75), 189 (15), 147 (30), 133 (10), 101 (5), 73 (100). 2) β -Anomer: m/z (rel. intens.%)=333 (2), 305 (6), 291 (1), 279 (3), 259 (3), 218 (17), 217 (95), 205 (11), 204 (67), 191 (75), 189 (13), 147 (30), 133 (10), 101 (5), 73 (100). The TMSi derivatives of all four isomers of apiose showed the following EIMS characteristics. 1) First eluting isomer: m/z (rel. intens.%)=333 (1), 319 (0.5), 305 (1), 291 (0.2), 279 (2), 259 (2), 218 (5), 217 (22), 204 (3), 191 (100), 189 (3), 147 (15), 133 (5), 129 (7), 103 (5), 73 (60). 2) Second eluting isomer: m/z (rel. intens.%)=423 (0.1), 393 (0.3), 333 (0.5), 319 (0.1), 305 (0.3), 259 (1), 230 (8), 218 (2), 217 (20), 216 (5), 204 (3), 191 (100), 189 (5), 147 (17), 133 (5), 129 (6), 103 (5), 73 (58). Third eluting isomer: m/z (rel. intens.%)=423 (0.1), 393 (0.5), 367 (0.2), 333 (0.5), 319 (0.3), 305 (0.4), 259 (1), 230 (5), 218 (2), 217 (25), 216 (5), 204 (3), 191 (100), 189 (2), 147 (13), 133 (5), 129 (8), 103 (5), 73 (65). 4) Fourth eluting isomer: m/z (rel. intens.%)=423 (0.1), 393 (0.2), 333 (1), 319 (0.4), 305 (0.4), 259 (1), 230 (5), 218 (2), 217 (21), 216 (5), 204 (3), 191 (100), 189 (2), 147 (13), 133 (5), 129 (8), 103 (8), 73 (63).

Acetolysis of per-O-acetylated glycosides. A portion of **94a**, ^{was} **95a** dried for 15 min at high vacuum and then, along with reference Apiin (Carl Roth GmbH, Co), were dissolved in 5 drops of a 0.1% perchloric acid solution in acetic anhydride. The reaction mixtures were left at room temperature for thirty hours. The reactions were monitored by TLC (Tate and Bishop 1962). When acetolyses were complete, the reaction mixtures were neutralized with CH₃COOK, excess acetic anhydride was destroyed by addition of methanol, and samples were dried (stream of N₂). Then, acetolyses products were redissolved in CH₂Cl₂, filtered and examined by GC-MS. They showed the following characteristics. 1) tetra-O-acetylated- α -D-apiofuranose: m/z (rel. intens.%)= 259 (14.2), 219 (2.5), 216 (6.5), 207 (9.4), 187 (3.0), 170 (41.5), 156 (38.0), 145 (24.5), 143 (26.7), 139 (58.2), 128 (49.8), 110 (100), 103 (47.0), 101 (38.0), 97 (36.5), 85 (46.0), 68 (50.0); 2) tetra-O-acetylated- α -L-apiofuranose: m/z (rel. intens.%)= 259 (14.2), 219 (2.5), 216 (6.5), 207 (9.4), 187 (3.0), 170 (39.0), 156 (36.5), 145 (24.5), 143 (26.7), 139 (58.2), 128 (51.0), 110 (100), 103 (47.0), 101 (36.0), 97 (34.5), 85 (44.0), 68 (48.0); 3) tetra-O-acetylated- β -L-apiofuranose:

m/z (rel. intens.%)= 259 (13.5), 219 (5.0), 216 (1.5), 207 (14.8), 187 (1.5), 170 (37.5), 156 (22.3), 145 (19.7), 143 (9.5), 139 (65.0), 128 (50.0), 110 (100), 103 (42.5), 101 (17.0), 97 (32.5), 85 (36.0), 68 (53.0); 4) tetra-O-acetylated- β -D-apiofuranose: *m/z* (rel. intens.%)= 259 (14.0), 219 (2.5), 216 (3.0), 207 (9.4), 187 (3.0), 170 (36.5), 156 (42.2), 145 (24.9), 143 (11.5), 139 (76.0), 128 (50.5), 110 (100), 103 (45.1), 101 (20.1), 97 (39.2), 85 (42.4), 68 (53.5); 5) penta-O-acetylated- α,β -D-glucopyranose: *m/z* (rel. intens.%)= 331 (4.0), 242 (20.1), 211 (3.0), 200 (19.5), 182 (7.0), 169 (19.2), 157 (40.0), 145 (16.5), 140 (23.0), 127 (7.0), 115 (100), 109 (21.0), 103 (26.6), 98 (62.5), 73 (16.5).

EXPERIMENTAL

Chapter 5. Very early-eluting glycoconjugates

Paper electrophoresis

Paper electrophoresis experiments were performed using the apparatus previously described by Tate (1968). As buffer solutions were used 0.1M citric acid solution adjusted to pH 5.0, 1M AcOH/0.75 M, HCOOH buffer pH 1.75, and 0.1M NH₄HCO₃ buffer pH 9.2. Electrophoretograms were developed for 30 min at a power of 1800 V and a current of 55 mA, and examined by UV light at 254 nm and 310 nm and silver nitrate reagent for vicinal glycols.

Ion exchange chromatography

Early eluting glycosidic material (i.e. DCCC frs. 60-75), was first extracted with 12 mls CH₂Cl₂ (3x4) and treated with sep-pak to remove any hydrolysis products which may formed during storage. Then pooled fractions 60-75 (180 mg) were chromatographed on an ion exchange Diaion column (3 gr, Nippon Rensui Co.). The column was preconditioned by washing with 50 ml H₂O-50 ml HCl (0.5 M)-70 ml H₂O. Neutral fraction was collected immediately (unretained material, 120 mg). Retained (i.e. ionic) material was eluted with 20 ml HCOOH (1M), 15 ml NH₄OH (15% v/v), 10 ml HCl (2M). The resultant ionic fractions were examined by paper electrophoresis and then combined to yield a fraction of the whole ionic material (60 mg).

Flash chromatography

a) Underivatised glycosides. Flash chromatography was carried out as described above. Unretained, non ionic material from ion exchange chromatography (120 mg) loaded on a silica gel column and was separated into thirty two fractions from which flash chromatography fractions frs. 8-9 (7.4 mg), frs. 13-17 (23.5 mg), and frs. 18-19 (6.0 mg) were further purified by HPLC.

b) Per-O-acetylated glycosides. Using ethyl acetate/chloroform : 50/50 as eluent, per-O-acetylated HPLC fr. 3, ex fl. frs. 8-9, was separated into thirty fractions (35 ml eluent) from which fl. frs. 9-11 (0.9 mg) were found (positive FAB-MS examination) to contain glycoside **99a**. Per-O-acetylated HPLC fr. 10, ex fl. frs. 13-17, was separated to thirty fractions (30 ml eluent, acetate/chloroform : 50/50) from which fl. fr. 6 (0.5 mg) was

found (positive FAB-MS, NH₄ CIMS examination) to contain glycoside **100a**. Per-O-acetylated HPLC fr. 2, ex fl. frs. 18-19, was separated to 30 fractions using as eluent 40 ml acetate/chloroform : 60:40 and to further five fractions using as eluent 20 ml of the bottom phase of a CHCl₃/MeOH/H₂O : 7/13/8 solvent system. **101a** (1.1 mg) was detected by FAB-MS in fl. frs. 32-34.

HPLC resolution of glycosidic (neutral) fractions

Both for the analytical and semi-preparative work, gradients of CH₃CN (A) and H₂O (B) were from 10% (A) to 20% (A) over 15 min and from 20% (A) to 100% (A) over the next 5 min, and then held at that gradient for 5 min. The combined flash chromatography fractions frs. 8-9 were separated into 6 new fractions with HPLC fr. 3 (0.7 mg, eluted at a H₂O/CH₃CN gradient of 80.5/19.5 and a RT between 14.20-14.60 min) containing glycoside **99**. Flash chromatography fractions frs. 13-17 were separated into 16 new fractions with HPLC fr. 10 (1.4 mg, eluted at a H₂O/CH₃CN gradient of 80.9/19.1 and a RT between 13.30-14.10 min) containing glycoside **100**. Flash chromatography fractions frs. 18-19 were separated into 5 new fractions with HPLC fr. 2 (1.2 mg, eluted at a H₂O/CH₃CN gradient of 82.2/17.8 and a RT between 11.70-12.10 min) containing the unknown glucoside.

2,3-dihydro-7-hydroxy-2-(4'-O-β-glucopyranosyl-3'-methoxyphenyl)-3-hydroxymethyl-5-benzofuranpropanol (99):

TLC R_f=0.16; UV λ_{max}=230, 280 nm; FAB-MS (positive ion mode): (M+H)⁺=509, (M+Na)⁺=531; (negative ion mode) : (M-H)⁻=507; FAB-MS/MS (positive ion mode) : *m/z* 509=[M+H]⁺, 347=[(M+H)-anhydroglucose]⁺, 329=[(M+H)-glucose]⁺, 317=[(M+H)-anhydroglucose-CH₂=O]⁺, 193, 163, 137 [tropylium ion]⁺; (negative ion mode) : *m/z* 507=[M-H]⁻, 489=[(M-H)-H₂O]⁻, 477=[(M-H)-CH₂=O]⁻, 371=[(M-H)-136]⁻, 345=[(M-H)-162]⁻, 327=[(M-H)-162-H₂O]⁻, 315=[(M-H)-162-H₂O-CH₂=O]⁻, 205; (note, assignments are based on likely fragment ion composition). **99a**: 0.9 mg; TLC R_f=0.55 (eluent ethylacetate/chloroform : 50/50); FAB-MS : (M+H)⁺=803, ([M+glycerol]+H)⁺=895; FAB-MS/MS : *m/z* 803=[M+H]⁺, 331=[C₁₄H₁₉O₉]⁺, 271=[C₁₂H₁₅O₇]⁺, 211=[C₁₀H₁₁O₅]⁺, 169=[C₈H₉O₄]⁺, (note, assignments are based on likely fragment ion compositions); ¹H NMR of the acetates : see table 8.

(E)-2,6-dimethylocta-2,7-diene-1,6-diol-1-O-β-glucopyranosyl-O-arabinofuranosyl-O-β-glucopyranoside (100):

TLC $R_f=0.12$; UV $\lambda_{max}=225$ nm, end absorption; FAB-MS (positive ion mode): $(M+H)^+=627$, $(M+Na)^+=649$; (negative ion mode) : $(M-H)^-=625$; FAB-MS/MS (positive ion mode) : m/z $627=[M+H]^+$, $609=[(M+H)-H_2O]^+$, $465=[(M+H)-anhydroglucose]^+$, $457=[(M+H)-aglycone]^+$, $333=[(M+H)-anhydroglucose-anhydropentose]^+$, $295=[(M+H)-aglycone-anhydroglucose]^+$, $171=[aglycone+H]^+$, $163=[anhydroglucose+H]^+$, $153=[(aglycone+H)-H_2O]^+$, $135=[(aglycone+H)-2H_2O]^+$; (negative ion mode) : m/z $625=[M-H]^-$, $463=[(M-H)-anhydroglucose]^-$, $445=[(M-H)-glucose]^-$, $331=[(M-H)-anhydroglucose-anhydropentose]^-$, $293=[(M-H)-anhydroglucose-aglycone]^-$, $179=[Glucose-H]^-$, $161=[(Glucose-H)-H_2O]^-$, $149=[arabinose-H]^-$, $131=[(arabinose-H)-H_2O]^-$, $119=[C_4H_7O_4]^-$, 113 , $101=[C_4H_5O_3]^-$, $89=[C_3H_5O_3]^-$; (note, assignments are based on likely fragment ion composition). **100a**: 0.5 mg; TLC $R_f=0.32$ (eluent ethylacetate/chloroform : 50/50); FAB-MS : $(M+Na)^+=1027$, $(M+Na-CH_2=C=O)^+=985$; probe NH_4 -CI-MS : $(M+NH_4)^+=1022$, $(M+NH_4-CH_2=C=O)^+=980$; probe NH_4 -CI-MS/MS : m/z $1022=[M+NH_4]^+$, 657 , $547=[C_{13}H_{31}O_{15}]^+$, 427 , 371 , $331=[C_{14}H_{19}O_9]^+$, $271=[C_{12}H_{15}O_7]^+$, $259=[C_{11}H_{15}O_7]^+$ (note, assignments are based on likely fragment ion compositions); 1H NMR of the acetates : see table 8.

Unknown-glucopyranoside (101)

TLC $R_f=0.08$; UV $\lambda_{max}=232, 241(sh), 265, 323, 339$ nm; FAB-MS (positive ion Mode) : $(M+H)^+=487$, $(M+Na)^+=509$; (negative ion mode) : $(M-H)^-=485$; FAB-MS/MS (positive ion mode) : m/z $487=[M+H]^+$, $325=[(M+H)-anhydroglucose]^+$, $307=[(M+H)-glucose]^+$, $191=[(M+H)-anhydroglucose-134]^+$; (negative ion mode) : m/z $485=[M-H]^-$, $323=[(M-H)-anhydroglucose]^-$, 203 , $189=[(M-H)-anhydroglucose-134]^-$, $161=[(glucose-H)-H_2O]^-$, $119=[C_4H_7O_4]^-$, $101=[C_4H_5O_3]^-$, $89=[C_3H_5O_3]^-$; (note, assignments are based on likely fragment ion composition). **101a**: 1.1 mg; TLC $R_f=0.08$ (eluent ethylacetate/chloroform : 50/50); FAB-MS : $(M+H)^+=781$, $(M+Na)^+=803$; FAB-MS/MS : m/z $781=[M+H]^+$, 619 , 559 , $451=[(M+H)-C_{14}H_{18}O_9]^+=[aglycone\ tri-O-acetate]^+$, $433=[(M+H)-C_{14}H_{18}O_9-H_2O]^+=[(aglycone\ tri-O-acetate)-H_2O]^+$, $409=[(aglycone\ tri-O-acetate)-CH_2=C=O]^+$, $391=[(aglycone\ tri-O-acetate)-CH_3COOH]^+$, $331=[C_{14}H_{19}O_9]^+$, $271=[C_{12}H_{15}O_7]^+$, $211=[C_{10}H_{11}H_5]^+$, $169=[C_8H_9O_4]^+$ (note, assignments are based on likely fragment ion compositions); 1H NMR of the acetates : (300 MHz, δ 1.19 [s, 3H, CH_3-C-O], 1.23 [s, 3H, CH_3-C-O], 1.97-2.08 [15xs, 15H, 5xAcO-], 2.24 [s, 3H, AcO-], 2.32 [s, 3H, AcO-], 3.76-3.62 [m, 3H, GH-5, else], 4,17 [brs, 1H], 4.30 [brs, 1H], 4.39 [m, 1H, GH-6a], 4.74 [d, 1H, $J=8.0$ Hz, GH-1], 4.93 [dd, 1H, $J=8.0, 9.5$ Hz, GH-2], 5.06 [t, 1H, $J=9.6$ Hz, GH-

4], 5.21 [t, 1H, $J=9.4$ Hz, GH-3], 5.5 [m, 1H, proton of a trisubstituted double bond system], 7.13 [brs, 1H, aromatic proton].

Hydrolyses of isolates:

a) Enzymic hydrolyses. The enzymic hydrolyses were carried out as described above. In the case of glycoside **99** sweet almond β -D-glucosidase was used. The liberated aglycone moiety was examined by probe EIMS and MS/MS and showed the following characteristics. **99b**) probe EIMS : m/z (rel. intens%)=55 (57), 69 (23), 77 (15), 91 (28), 109 (15), 119 (18), 124 (65), 137 (100), 151 (10), 152 (23), 162 (58), 167 (5), 178 (3), 180 (65), 237 (7), 241 (8), 257 (5), 269 (7), 313 (6), 328 (22), 346 (62); probe MS/MS : rel. intens%)=346 (22), 328 (100), 316 (3), 313 (6), 297 (5), 289 (6), 274 (21), 269 (18), 241 (15), 237 (19), 209 (13), 180 (10), 179 (12), 162 (81), 152 (3), 149 (4), 147 (18), 137 (5), 130 (9), 119 (3). For glycoside **100** enzymic hydrolysis was carried out using the fungal enzyme preparation *Novoferm 12* (Novo Nordisk). The liberated aglycone was examined by GC-MS and showed mass spectrometric characteristics similar to those published elsewhere for (E)-2,6-dimethylocta-2,7-diene-1,6-diol (Strauss *et al.* 1988).

b) Acid hydrolyses. They were performed as described above. The obtained mass spectrometric data for the TMSi derivatives of the liberated glucose were similar to those reported by Radford and Dejongh (1972). For the liberated arabinose the spectrometric data were similar to those described previously for trimethylsilylated arabinose (experimental-chapter 4).

EXPERIMENTAL

Chapter 6. β -Carbolines: polar, non-volatile material in Riesling wine

Flash chromatography

Flash chromatography was carried out as described above. Using the solvent system for underivatized glycosides, medium polarity eluting material off the DCCC (i.e. frs. 100-130, 120mg) was separated into thirty three fractions from which frs. 10-21 were recombined and prepared for further purification by HPLC.

HPLC resolution of glycosidic fractions

For the both the analytical and semi-preparative work (i.e. fractionation) of the flash chromatography fractions frs. 10-21, gradients were from 20% (A) to 43% (A) over 15 min and from 43% (A) to 100% (A) over the next 3 min, and then held at that gradient for 2 min. The combined flash chromatography fractions frs. 10-21 (25 mg) were separated into 8 new fractions with fr. 3 (3.2 mg, eluted at a H₂O/CH₃CN gradient of 66/34 and a RT between 8.10-9.40 min) and fr. 4 (1.5 mg, eluted at a H₂O/CH₃CN gradient of 64.5/35.5 and a RT between 9.50-10.20 min) being those of interest. Analytical work for both the naturally occurring (i.e. combined fractions frs. 3-4) and the synthetic 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid diastereoisomers was conducted at an elution of 0.8 ml/min with a linear gradient of CH₃CN (A) and 0.1% perchloric acid (B). Gradients were from 10% (A) to 55% (A) over 32 min and from 55% (A) to 100% (A) over the next 3 min, and then held at that gradient for other 3 min.

Naturally occurring 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid diastereoisomers (102)

4.7 mg; TLC R_f=0.29 (eluent for underivatized glycosides); UV λ_{max} =220, 272, 280, 289 (sh) nm; FAB-MS (pos. ion mode): (M+H)⁺=231, (neg. ion mode): (M-H)⁻=229; FAB-MS/MS (pos. ion mode): *m/z* 231=[M+H]⁺, 216=[M+H-CH₃]⁺, 214=[M+H-OH]⁺, 197, 188, 168, 158, 155, 146, 143, 130, 123, 118, 105, 102, 90, 74, 61, 57; (neg. ion mode) *m/z* 229=[M-H]⁻, 185=[M-H-CO₂]⁻, 183, 168, 156, 142=[M-H-CO₂-(CH₂=CH-NH₂)]⁻, 116, 100, 92; (note, assignments are based on likely fragment ion composition). ¹H NMR data: see table 9; ¹³C NMR data: see table 10.

Synthesis of 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (102)

1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**102**) was synthesised according to Brossi *et al.* (1973). a) (1S, 3S) 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid: i) mp 295-297°C; TLC R_f=0.29 (eluent for underivatised glycosides); UV λ_{max}=220, 272, 280, 289 (sh) nm; FAB-MS (pos. ion mode): (M+H)⁺=231, (neg. ion mode): (M-H)⁻=229; FAB-MS/MS (pos. ion mode): *m/z* 231=[M+H]⁺, 216=[M+H-CH₃]⁺, 214=[M+H-OH]⁺, 197, 188, 168, 158, 155, 146, 143, 130, 123, 118, 105, 102, 90, 74, 61, 57; (neg. ion mode) *m/z* 229=[M-H]⁻, 185=[M-H-CO₂]⁻, 183, 168, 156, 142=[M-H-CO₂-(CH₂=CH-NH₂)]⁻, 137, 116, 100, 92 (note, assignments are based on likely fragment ion composition); IR: ν_{max} 3292, 2981, 2919, 2763, 2671, 2493, 1640, 1574, 1383, 1312cm⁻¹; ¹H NMR data: see table 9; ¹³C NMR data: see table 10; ii) mp 275-277°C; TLC R_f=0.29 (eluent for underivatised glycosides); UV λ_{max}=220, 272, 280, 289 (sh) nm; FAB-MS (pos. ion mode): (M+H)⁺=231, (neg. ion mode): (M-H)⁻=229; FAB-MS/MS (pos. ion mode): *m/z* 231=[M+H]⁺, 216=[M+H-CH₃]⁺, 214=[M+H-OH]⁺, 197, 188, 168, 158, 155, 146, 143, 130, 123, 118, 105, 102, 90, 74, 61, 57; (neg. ion mode) *m/z* 229=[M-H]⁻, 185=[M-H-CO₂]⁻, 183, 168, 156, 142=[M-H-CO₂-(CH₂=CH-NH₂)]⁻, 137, 116, 100, 92 (note, assignments are based on likely fragment ion composition); IR: ν_{max} 3229, 2939, 2787, 2494, 1639, 1580, 1377, 1314 cm⁻¹; ¹H NMR data: see table 9; ¹³C NMR data: see table 10; iii) mp 242-244°C; TLC R_f=0.29 (eluent for underivatised glycosides); UV λ_{max}=220, 272, 280, 289 (sh) nm; FAB-MS (pos. ion mode): (M+H)⁺=231, (neg. ion mode): (M-H)⁻=229; FAB-MS/MS (pos. ion mode): *m/z* 231=[M+H]⁺, 216=[M+H-CH₃]⁺, 214=[M+H-OH]⁺, 197, 188, 168, 158, 155, 146, 143, 130, 123, 118, 105, 102, 90, 74, 61, 57; (neg. ion mode) *m/z* 229=[M-H]⁻, 185=[M-H-CO₂]⁻, 183, 168, 156, 142=[M-H-CO₂-(CH₂=CH-NH₂)]⁻, 137, 116, 100, 92 (note, assignments are based on likely fragment ion composition); IR: ν_{max} 3394, 3270, 2921, 2853, 2496, 1397, 1321 cm⁻¹; ¹H NMR data: see table 9; ¹³C NMR data: see table 10.

1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid derivative (103)

UV λ_{max}=220, 272, 280, 289 (sh) nm; FAB-MS (pos. ion mode): (M+H)⁺=344, (neg. ion mode): (M-H)⁻=342; FAB-MS/MS (pos. ion mode): *m/z* 344=[M+H]⁺, 231, 213, 199, 185, 171, 158, 157, 146, 132, 118, 86, 76; (neg. ion mode) *m/z* 342=[M-H]⁻, 298=[M-H-CO₂]⁻, 211, 185, 167, 156, 137, 130, 116; (note, assignments are based on likely fragment ion composition).

Putative 1,1-dimethyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid derivative (104)

UV λ_{max} =220, 272, 280, 289 (sh) nm; FAB-MS (pos. ion mode): (M+H)⁺=358, (neg. ion mode): (M-H)⁻=356; FAB-MS/MS (pos. ion mode): m/z 358=[M+H]⁺, 245, 227, 132, 86; (neg. ion mode) m/z 342=[M-H]⁻, 312=[M-H-CO₂]⁻, 225, 199, 130; (note, assignments are based on likely fragment ion composition).

REFERENCES

- Abbott, N. A.; Coombe, B. G.; Williams, P. J. The contribution of hydrolyzed flavor precursors to quality differences in Shiraz juice and wines: an investigation by sensory descriptive analysis. *Am. J. Enol. Vitic.* **1991**, 42, 167-174.
- Ackermann, I. E.; Banthorpe, D. V.; Fordham, W. D.; Kinder, J. P.; Poots, I. β -glucosides of aroma components from petals of *Rosa* species: assay, occurrence, and biosynthetic implications. *J. Plant Physiol.* **1989**, 134, 567-572.
- Adachi, J.; Mizoi, Y.; Naito, T.; Yamamoto, K.; Fujiwara, S.; Ninomiya, I. Determination of β -carbolines in foodstuffs by high-performance liquid chromatography and high-performance liquid chromatography-mass spectrometry. *J. Chromatogr.* **1991**, 538, 331-339.
- Adlercreutz, H. Does fiber-rich food containing animal lignan precursors protect against both colon and breast cancer? An extension of the "fiber hypothesis". *Gastroenterology*, **1984**, 86, 761-766.
- Agrawal, P. K.; Agarwal, S. K.; Rastogi, R. P. A new neolignan and other phenolic constituents from *Cedrus deodara*. *Phytochemistry* **1980**, 19, 1260-1261.
- Allen, M. S.; Lacey, M. J.; Brown, W. V.; Harris, R. L. N. Contribution of methoxypyrazines to the flavour of Cabernet Sauvignon and Sauvignon Blanc grapes and wines. In *Proceedings, 7th Australian Wine Industry Technical Conference; 13-17 August 1989*; Williams, P. J.; Davidson, D.; Lee, T. H., Eds.; Australian Industrial: Adelaide, SA, 1990: pp 113-116.
- Amerine, A. M.; Roessler, E. B. *Wines. Their sensory evaluation*; W. H. Freeman and company, 1983; preface p xi.
- Amerine, A. M.; Singleton, V. L. *Wine*; University of California press Ltd, Los Angeles, California, 1977, pp 298-323.

Angel, A-S.; Lindh, F.; Nilsson, B. Determination of binding positions in oligosaccharides and glycosphingolipids by fast-atom-bombardment mass spectrometry. *Carbohydrate Res.* **1987**, 168, 15-31.

Aubagnac, J-L.; Devienne, F. M.; Combabieu, R. A new mass spectrometer equipped with fast atom bombardment for analysis of polar compounds. Study of oligosaccharides. *Org. Mass Spectrom.* **1983**, 18, 173-175,

Austerweil, A. G. Quelques observations sur les parfumes des vins. *Ind. Parfum.* **1946**, 1, 195-199.

Axelson, M.; Sjövall, J.; Gustafsson, B. E.; Setchell, K. D. R. Origin of lignans in mammals and identification of a precursor from plants. *Nature*, **1982**, 298, 659-660.

Baranowski, J. D.; Nagel, C. W. Isolation and identification of the hydroxycinnamic acid derivatives in white Riesling wine. *Am. J. Enol. Vitic.* **1981**, 32, 5-13.

Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tyler, A. N. Fast atom bombardment of solids as an ion source in mass spectrometry. *Nature* **1981a**, 293, 270-275.

Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tyler, A. N. Fast atom bombardment of solids (FAB): a new ion source for mass spectrometry. *J. Chem. Soc., Chem. Commun.* **1981b**, 7, 325-327.

Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tetler, L. W. Fast atom bombardment mass spectrometry of two isomeric tripeptides. *Org. Mass Spectrom.* **1981c**, 16, 256-260.

Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tyler, A. N. Fast atom bombardment mass spectrometry of cobalamines. *Biomed. Mass Spectrom.* **1981d**, 8, 492-495.

Barber, M.; Bordoli, R. S.; Elliott, G. J.; Sedgwick, R. D.; Tyler, A. N. Fast atom bombardment mass spectrometry. *Anal. Chem.* **1982**, 54, 645A-657A.

Barber, M.; Bordoli, R. S.; Sedgwick, R. D.; Tyler, A. N. Fast atom bombardment mass spectrometry. In *Recent developments in mass spectrometry in biochemistry, medicine and*

environmental research, 8; Frigerio, A., Ed.; ACS Symposium Ser. 12; Elsevier Scientific: Amsterdam, 1983: pp 177-183.

Bare, K. J.; Read, H. Use of fast atom bombardment mass spectrometry to identify materials separated on high-performance thin-layer chromatography plates. *Analyst* **1987**, 112, 433-436.

Barker, S. A. GC/MS quantification and identification of endogenous tetrahydro- β -carbolines in rat brain and adrenal. In *Beta-Carbolines and tetrahydroisoquinolines*; Bloom, F.; Barchas, J.; Sandler, M.; Usdin, E., Eds.; Liss, A. R., Inc., New York, 1982: pp 113-124.

Barz, W.; Köster, J. Turnover and degradation of secondary (natural) products. In *The biochemistry of plants. Secondary plant products*. Conn, E. E., Ed.; Academic: London, 1981: vol. 7, pp 35-84.

Baumes, R.; Cordonnier, R.; Nitz, S.; Drawert, F. Identification and determination of volatile constituents in wines from different vine cultivars. *J. Sci. Food Agric.* **1986**, 37, 927-943.

Bayonove, C.; Cordonnier, R. Reserches sur l' arôme du muscat. II.-Profils aromatiques de cépages muscat et non muscat. Importance du linalool chez les muscats. *Ann. Technol. Agric.* **1970**, 19, 95-105.

Bayonove, C.; Cordonnier, R. Reserches sur l' arôme du muscat. III.-Etude de la fraction terpénique. *Ann. Technol. Agric.* **1971**, 20, 347-355.

Bayonove, C.; Cordonnier, R.; Dubois, P. Étude d' une fraction caracteristique de l' arome du raisin de la variété Cabernet sauvignon: mise en évidence de la 2-methoxy-3-isobutylpyrazine. *C. R. Acad. Sci. Paris* **1975**, t 281, Ser. D, 75-78.

Bayonove, C.; Richard, H.; Cordonnier, R. Isolement et identification du diméthyl-3.7 octatriène-1.5.7 ol-3 outrans hotrienol, constituant à odeur de Tilleul de l' huile essentielle de Muscat de Frontignan. *C. R. Acad. Sc. Paris* **1976**, t 283, Ser. C, 549-551.

- Beck, O.; Bosin, T. R.; Lundman, A. Analysis of 6-hydroxy-1-methyl-1,2,3,4-tetrahydro- β -carboline in alcoholic beverages and food. *J. Agric. Food Chem.* **1983**, 31, 288-292.
- Beck, E.; Hopf, H. Branched-chain sugars and sugar alcohols. In *Methods in plant biochemistry. Volume 2. Carbohydrates*. Dey, P. M., Ed.; Academic press: 1990: pp. 235-289.
- Benecke, R.; Nishibe, S.; Kitagawa, S.; Hanschmann, H. Structure of (+)-isolariciresinol-monoglucoside from *Populus nigra* L. *Pharmazie*, **1989**, 44, 357-358.
- Bertuccioli, M.; Viani, R. Red wine aroma: identification of headspace constituents *J. Sci. Food Agric.* **1976**, 27, 1035-1038.
- Bitteur, S. M.; Baumes, R. L.; Bayonove, C. L.; Versini, G.; Martin, C. A.; Dalla Serra, A. 2-exo-Hydroxy-1,8-cineole: a new component from grape var. Sauvignon. *J. Agric. Food Chem.* **1990**, 38, 1210-1213.
- Bock, K.; Pedersen, C. Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides. *Adv. Carbohydrate Chem. Biochem.* **1983**, 41, 27-66.
- Bock, K.; Pedersen, C.; Pedersen, H. Carbon-13 nuclear magnetic resonance data for oligosaccharides. *Adv. Carbohydrate Chem. Biochem.* **1984**, 42, 193-225.
- Bosin, T. R.; Lundman, A.; Beck, O. Identification and quantitation of 6-hydroxy-1,2,3,4-tetrahydro- β -carboline in alcoholic beverages. *J. Agric. Food Chem.* **1983**, 31, 444-446.
- Bosin, T. R.; Krogh, S.; Mais, D. Identification and quantitation of 1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid and 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid in beer and wine. *J. Agric. Food Chem.* **1986**, 34, 843-847.
- Brossi, A.; Focella, A.; Teitel, S. Alkaloids in mammalian tissues. 3. Condensation of L-tryptophan and L-5-hydroxytryptophan with formaldehyde and acetaldehyde. *J. Medicinal Chem.* **1973**, 16, 418-420.
- Bredenkamp, M. W.; Drewes, S. E.; Wentler, G. L. A geraniol glycoside from *Hypoxis acuminata*. *Phytochemistry* **1989**, 28, 263-265.

Brillouet, J.-M.; Gunata, Z.; Bitteur, S.; Cordonnier, R.; Bosso, C. Terminal apiose: a new sugar constituent of grape juice glycosides. *J. Agric. Food Chem.* **1989**, *37*, 910-912.

Buckholtz, N. S. Neurobiology and tetrahydro- β -carbolines. *Life Sci.* **1980**, *27*, 893-903.

Busch, K. L.; Cooks, G. R. Mass spectrometry of large, fragile and involatile molecules. *Science* **1982**, *218*, 247-253.

Busch, K. L.; Unger, S. E.; Vincze, A.; Cooks, R. G.; Keough, T. Desorption ionization mass spectrometry: sample preparation for secondary ion mass spectrometry, laser desorption, and field desorption. *J. Am. Chem. Soc.* **1982**, *104*, 1507-1511.

Cambie, R. C.; Clark, G. R.; Craw, P. A.; Jones, T. C.; Rutledge, P. S.; Woodgate, P. D. Chemistry of the Podocarpaceae. LXIX. Further lignans from the wood of *Dacrydium intermedium*. *Aust. J. Chem.* **1985**, *38*, 1631-1645.

Caprioli, R. M.; DaGue, B. B.; Wilson, K. Optimization of chromatographic conditions for combined microbore HPLC/continuous-flow fast-atom bombardment mass spectrometry. *J. Chromatogr. Sci.* **1988**, *26*, 640-644.

Carman, R. M.; Greenfield, K. L.; Robinson, W. T. Halogenated terpenoids. XXII. Uroterpenol. The C₈ stereochemistry. *Aust. J. Chem.* **1986**, *39*, 21-30.

Castaing, R.; Slodzian, G. Premiers essais de microanalyse par émission ionique secondaire. *C. R. Hebd. Seances Acad. Sci.* **1962**, *255*, 1893-1895.

Chang, T. T.; Lay, J. O.; Francel, R. J. Direct analysis of thin-layer chromatography spots by fast atom bombardment mass spectrometry. *Anal. Chem.* **1984**, *56*, 109-111.

Chatonnet, P.; Boidron, J. N.; Pons, M. Maturation of red wines in oak barrels: evolution of some volatile compounds and their aromatic impact. *Sci. Aliments* **1990**, *10*, 565-587.

Clayton, E.; Wakefield, A. Fast atom bombardment (FAB) mass spectrometry: mechanism of ionization. *J. Chem. Soc., Chem. Commun.* **1984**, *15*, 969-970.

Clifford, A. J.; DeLuca, L. M. Role of the matrix in the FAB MS analysis of monosaccharides. *Org. Mass Spectrom.* **1985**, *20*, 157-159.

Cohen, J. D.; Bialek, K. The biosynthesis of indole-3-acetic acid in higher plants. In *The biosynthesis and metabolism of plant hormones*; Crozier, A.; Millman, J. R., Eds.; Society for Experimental Biology: Seminar Series **23**, Cambridge University Press, Cambridge, 1984: pp 165-181.

Connell, D. W. The chemistry of the essential oil and oleoresin of ginger (*Zingiber officinale* Roscoe). *Flavour Ind.* **1970**, *1*, 677-693.

Cooks, R. G.; Busch, K. L. Matrix effects, internal energies and ms/ms spectra of molecular ions sputtered from surfaces. *Intern. J. Mass Spectrom. Ion Phys.* **1983**, *53*, 111-124.

Cooks, R. G.; Glish, G. L. Mass spectrometry/mass spectrometry. *Chem. Eng. News* **1981** *54*, 40-52.

Cordonnier, R. Recherches sur l'aromatisation et le parfum des vins doux naturels et des vins de liqueur. *Ann. Technol. Agric.* **1956**, *5*, 75-110.

Cordonnier, R.; Bayonove, C. Mise en évidence dans la baie de raisin, variété muscat d'Alexandrie, de monoterpènes liés révélables par une ou plusieurs enzymes du fruit. *C. R. Acad. Sc. Paris* **1974**, t 278, Ser. D, 3387-3390.

Croteau, R. Biosynthesis and catabolism of monoterpenes. In *Isopentenoids in plants*; Nes, D. W.; Fuller, G.; Tsai, L-S., Eds.; Marcel Dekker, New York, Basil, 1984: pp 31-64.

Croteau, R.; Martinkus, C. Demonstration of (+)-neomenthyl- β -D-glucoside as a major metabolite of (-)-menthone in peppermint (*Mentha piperita*). *Plant Physiol.* **1979**, *64*, 169-175.

Crow, F. W.; Tomer, K. B.; Looker, J. H.; Gross, M. L. Fast atom bombardment and tandem mass spectrometry for structure determination of steroid and flavanoid glycosides. *Anal. Biochem.* **1986**, *155*, 286-307.

Dell, A.; Taylor, G. W. High-field-magnet mass spectrometry of biological molecules. *Mass Spectrom. Rev.* **1984**, 3, 357-394.

Dell, A. Fast atom bombardment mass spectrometry of carbohydrates. *Adv. Carbohydrate Chem. Biochem.* **1987**, 45, 20-72.

DePauw, E. Ion promotion in fast-atom bombardment mass spectrometry by charge transfer complexation. *Anal. Chem.* **1983**, 55, 2195-2196.

Devienne, F. M.; Grandclément, G. Étude des facteurs influençant l'éjection d'atomes d'une lame mince d'antimoine soumise à l'impact d'un jet moléculaire d'argon de grande énergie. *C. R. Acad. Sci. Paris* **1966**, t 262, Ser. B, 696-699.

Devienne, F. M. Différentes applications des jets moléculaires de haute énergie. *Entropie* **1967**, 18, 61-67.

Devienne, F. M.; Roustan, J-C. Nouvelle méthode de détermination de la composition chimique d'un corps solide quelconque, en surface ou en profondeur. Application à l'analyse de la couche adsorbée. *C. R. Acad. Sci. Paris* **1973**, t 276, Ser. C, 923-925.

Devienne, F. M. Nouvelle méthode de détermination de la composition des ions. Application à la mesure précise des masses moléculaires et à la séparation des isotopes et des isobares. *C. R. Acad. Sci. Paris* **1974**, t 278, Ser. B, 165-166.

Devienne, F. M.; Roustan, J-C. "Fast atom bombardment"-a rediscovered method for mass spectrometry. *Org. Mass Spectrom.* **1982**, 17, 173-181.

Dimitriadis, E.; Williams, P. J. The development and use of a rapid analytical technique for estimation of free and potentially volatile monoterpene flavorants of grapes. *Am. J. Enol. Vitic.* **1984**, 35, 66-71.

Dimitriadis, E.; Strauss, C. R.; Wilson, B.; Williams, P. J. The actinidols: nor-isoprenoid compounds in grapes, wines and spirits. *Phytochemistry* **1985**, 24, 767-770.

Domon, B.; Hostettmann, K. Mass spectrometric studies of underivatized polyphenolic glycosides. *Phytochemistry* **1985**, 24, 575-580.

- Domon, B.; Costello, C. E. A systematic nomenclature for carbohydrate fragmentations in fab-ms/ms spectra of glycoconjugates. *Glycoconjugate J.* **1988a**, *5*, 397-409.
- Domon, B.; Costello, C. E. Structure elucidation of glycosphingolipids and gangliosides using high-performance tandem mass spectrometry. *Biochem.* **1988b**, *27*, 1534-1543.
- Drawert, F.; Rapp, A. Gas-chromatographic analysis of plant aromas. I. The enrichment separation and identification of volatile aroma substances in grape musts and wines. *Chromatographia* **1968**, *1*, 446-457.
- Dube, G. The behaviour of aromatic hydrocarbons under fast atom bombardment. *Org. Mass Spectrom.* **1984**, *19*, 242-243.
- Dubois, P. Volatile phenols in wines. In *Flavour of distilled beverages. Origin and development*. Piggott, J. R., Ed.; Society of Chemical Industry: London, 1983; pp 110-119.
- Dzhakhua, M. J.; Drboglav, E. S.; Dzhaparidze, M. A. Study of the volatile phenol compounds in white wines. *Prikl. Biokhim. Mikrobiol.* **1978**, *14*, 156-158.
- Egge, H.; Katalinic, J. P. Fast atom bombardment mass spectrometry for structural elucidation of glycoconjugates. *Mass Spectrom. Rev.* **1987**, *6*, 331-393.
- Ehmann, A. Indoles in wine. I. Chloroform soluble nonvolatile indoles. *Plant Physiology (suppl.)* **1976**, *57*, 30.
- Ehmann, A. The Van Urk-Salkowski reagent-a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. *J. Chromatogr.* **1977**, *132*, 267-276.
- Ekman, R. Analysis of lignans in Norway spruce by combined gas chromatography-mass spectrometry. *Holzforschung* **1976**, *30*, 79-85.
- Enzell, C. R. Influence of curing on the formation of tobacco flavour. In *Flavour '81*. Schreier, P., Ed.; Walter de Gruyter & Co: Berlin, New York, 1981; pp 449-478.

Enzell, C. Biodegradation of carotenoids-an important route to aroma compounds. *Pure & Appl. Chem.* **1985**, *57*, 693-700.

Etiévant, P. X. Volatile phenol determination in wine. *J. Agric. Food Chem.* **1981**, *29*, 65-67.

Etiévant, P. X.; Issanchou, S. N.; Marie, S.; Ducruet, V.; Flanzy, C. Sensory impact of volatile phenols on red wine aroma: influence of carbonic maceration and time of storage. *Sci. Aliments* **1989**, *9*, 19-33.

Etiévant, P. X. Wine. In *Volatile compounds in foods and beverages*. Maarse, H., Ed.; Marcel Dekker, New York, 1991; pp 483-546.

Eugster, C. H.; Märki-Fischer, E. The chemistry of rose pigments. *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 654-672.

Felix, D.; Melera, A.; Seibl, M. J.; Kováts, E. Die struktur der sogenannten "Linalooloxide". *Helv. Chim. Acta* **1963**, *46*, 1513-1536.

Fenselau, C. Fast atom bombardment (review). *Springer Ser. Chem. Phys.* **1983**, *25*, 90-100.

Fischer, E.; Helferich, B. Über neue synthetische glucoside. *Ann.* **1911**, *383*, 68-79.

Fong, C. H.; Hasegawa, S.; Herman, Z.; Ou, P. Biosynthesis of limonoid glucosides in lemon (*Citrus limon*). *J. Sci. Food Agric.* **1991**, *54*, 393-398.

Gao, F.; Wang, H.; Mabry, T. J.; Jakupovic, J. Monoterpene glycosides, sesquiterpene lactone glycoside and sesquiterpene lactone aglycones from *Hymenoxys ivesiana*. *Phytochemistry* **1991**, *30*, 553-562.

Garler, G.; Colligon, J. *Ion bombardment of solids*; Heinenmann, London, 1968.

- Garozzo, D.; Giuffrida, M.; Impallomeni, G.; Ballistreri, A.; Montaudo, G. Determination of linkage position and identification of the reducing end in linear oligosaccharides by negative ion fast atom bombardment mass spectrometry. *Anal. Chem.* **1990**, *62*, 279-286.
- Gallegos, E. J. Gas chromatography/fast-atom bombardment mass spectrometry of carboxylic acids. *J. Chromatogr. Sci.* **1987**, *25*, 296-301.
- Gering-Ward, B. 2'-O-(8-Hydroxy-2,6-dimethyl-2(E), 6(E)-octadienoyl)-dihydropenstemide, a new iridoid glycoside from *Penstemon confertus*. *Planta Med.* **1989**, *55*, 79-80.
- Goodwin, T. W.; Mercer, E. I. *Introduction to plant biochemistry*, Pergamon: Oxford, 1983, p 227.
- Gower, J. L. Fast atom bombardment mass spectrometry of β -lactam antibiotics. *Intern. J. Mass Spectrom. Ion phys.* **1983**, *46*, 431-434.
- Gower, J. L. Matrix compounds for fast atom bombardment mass spectrometry. *Biomed. Mass Spectrom.* **1985**, *12*, 191-196.
- Gross, G. G. Phenolic Acids. In *The biochemistry of plants. Secondary plant products*, Conn, E. E., Ed.; Academic: London, 1981; vol. 7, p 301-316.
- Güldner, A.; Winterhalter, P. Structures of two new ionone glycosides from Quince fruit (*Cydonia oblonga* Mill.). *J. Agric. Food Chem.* **1991**, *39*, 2142-2146.
- Günata, Y. Z.; Bayonove, C. L.; Baumes, R. L.; Cordonnier, R. E. The aroma of grapes. 1. Extraction and determination of free and glycosidically bound fractions of some grape aroma components. *J. Chromatogr.* **1985**, *331*, 83-90.
- Güntert, M.; Rapp, A.; Takeoka, G.; Jennings, W. HRGC and HRGC-MS applied to wine constituents of lower volatility. *Z. Lebensmittel.- Unters. Forsch.* **1986**, *182*, 200-204.
- Harrison, A. G. *Chemical ionization mass spectrometry*; CRC Press, Boca Raton, Florida, 1983; pp 119-120.

Hase, T.; Iwagawa, T.; Munesada, K. A bitter monoterpene glucoside from *Viburnum phlebotrichum*. *Phytochemistry* **1982**, 21, 1435-1437.

Hattori, M.; Kawata, Y.; Kakiuchi, N.; Matsuura, K.; Namba, T. Application of liquid chromatography-mass spectrometry to the qualitative analysis of saponins (I). *Shoyakugaku Zasshi* **1988**, 42, 228-235.

Herderich, M.; Feser, W.; Schreier, P. Vomifoliol 9-O- β -D-glucopyranosyl-4-O- β -D-xylopyranosyl-6-O- β -D-glucopyranoside: a trisaccharide glycoside from apple fruit. *Phytochemistry* **1992**, 31, 895-897.

Heyes, D. M.; Barber, M.; Clarke, J. H. R. The use of sputtering as a method for analysing surface chemical composition: a molecular dynamics study. *Surf. Sci.* **1981**, 105, 225-239.

Higuchi, R.; Aritomi, M.; Donnelly, D. M. X. Monolignol and dilignol glycosides from *Pinus conorta* leaves. *Phytochemistry* **1977**, 16, 1007-1011.

Hiremath, S.P.; Hosmane, R.S. Applications of nuclear magnetic resonance spectroscopy to heterocyclic chemistry: indole and its derivatives. *Adv. Heterocyclic Chem.* **1973**, 15, 277-324.

Horman, I.; Viani, R. Plant-polyphenols and related compounds. A mass-spectrometric study of the trimethylsilyl derivatives of hydroxy- and/or methoxy-substituted cinnamic acids and of their methyl esters. *Org. Mass Spectrom.* **1971**, 5, 203-219.

Hösel, W. Glycosylation and glycosidases. In *The biochemistry of plants. Secondary plant products*. Conn, E. E., Ed.; Academic: London, 1981: vol. 7, pp 725-753.

Hunt, D. F.; Crow, F. W. Electron capture negative ion chemical ionization mass spectrometry. *Anal. Chem.* **1978**, 50, 1781-1784.

Hunt, D. F.; Bone, W. M.; Shabanowitz, J.; Rhodes, J.; Ballard, J. M. Sequence analysis of oligopeptides by secondary ion/collision activated dissociation mass spectrometry. *Anal. Chem.* **1981**, 53, 1704-1706.

- Inoshiri, S.; Sasaki, M.; Kohda, H.; Otsuka, H.; Yamasaki, K. Aromatic glycosides from *Berchemia racemosa*. *Phytochemistry*, **1987**, *26*, 2811-2814.
- Isoe, S.; Katsumura, S.; Sakan, T. The synthesis of damascenone and β -damascone and the possible mechanism of their formation from carotenoids. *Helv. Chim. Acta* **1973**, *56*, 1514-1516.
- Johnson, G. S.; Ruliffson, W. S.; Cooks, R. G. Peracetylated 1-phenylflavazoles as carbohydrate derivatives for mass spectrometry. *Carbohydrate Res.* **1971**, *18*, 233-242.
- Kanasawud, P.; Crouzet, J. C. Mechanism of formation of volatile compounds by thermal degradation of carotenoids in aqueous medium. 1. β -carotene degradation. *J. Agric. Food Chem.* **1990**, *38*, 237-243.
- Keough, T. Cationization of organic molecules using fast atom bombardment mass spectrometry. *Anal. Chem.* **1985**, *57*, 2027-2034.
- Kralj, B.; Kramer, V.; Vrscaj, V. Fast atom bombardment of molecules in the gaseous state. *Intern. J. Mass Spectrom. Ion Phys.* **1983**, *46*, 399-402.
- Kraus, R.; Spiteller, G. Phenolic compounds from roots of *Urtica dioica*. *Phytochemistry*, **1990**, *29*, 1653-1659.
- Leffingwell, J. C.; Leffingwell, D. GRAS flavor chemicals - detection thresholds. *Perfumer & Flavorist* **1991**, *16*, 1-19.
- Lehmann, W. D.; Kessler, M.; König, W. A. Investigations on basic aspects of fast atom bombardment mass spectrometry: matrix effects, sample effects, sensitivity and quantification. *Biomed. Mass Spectrom.* **1984**, *11*, 217-222.
- Lew, H. *Methods of experimental physics*; Hughes, V. W.; Schultz, H. L., Eds.; Acad. Press, New York, 1967.
- Ligon, W. V. Studies of the mixing of glycerol in glycerol at the glycerol-vacuum interface using secondary ion mass spectrometry. *Intern. J. Mass Spectrom. Ion phys.* **1983**, *52*, 183-187.

- Lundgren, L. N.; Popoff, T.; Theander, O. Dilignol glycosides from needles of *Picea abies*. *Phytochemistry*, **1981**, 20, 1967-1969.
- Lundgren, L. N.; Shen, Z.; Theander, O. The constituents of conifer needles. Dilignol glycosides from *Pinus massoniana* Lamb. *Acta Chem. Scandinavica*, **1985**, B 39, 241-248.
- Magee, C. W. Sputtering of organic molecules. *Intern. J. Mass Spectrom. Ion Phys.* **1983**, 49, 211-221.
- Magnus, V. Tryptophyl β -D-glucopyranoside: chemical synthesis, metabolism, and growth-promoting activity. *Carbohydrate Res.* **1979**, 76, 261-264.
- Mandery, H. Einfluß von *Botrytis cinerea* auf die monoterpenfraktion des weinaromas. Diplomarbeit, University Karlsruhe, 1986.
- Mann, J. *Secondary Metabolism*; Oxford chemistry series, 1980a; pp 6-8.
- Mann, J. *Secondary Metabolism*; Oxford chemistry series, 1980b; pp 153-169.
- Marais, J. Terpenes in the aroma of grapes and wines: A review. *S. Afr. J. Enol. Vitic.* **1983**, 4, 49-60.
- Marais, J.; Rapp, A. The selection of aroma-rich clones of *Vitis vinifera* L. cv. Gewürztraminer and Weisser Riesling by means of terpene analyses. *S. Afr. J. Enol. Vitic.* **1991**, 12, 51-56.
- Martin, S. A.; Costello, C. E.; Biemann, K. Optimization of experimental procedures for fast atom bombardment mass spectrometry. *Anal. Chem.* **1982**, 54, 2362-2368.
- Masuda, M.; Nishimura, K. Occurrence and formation of damascenone, *trans*-2,6,6-trimethyl-1-crotonyl-cyclohexa-1,3-diene, in alcoholic beverages. *J. Food Sci.* **1980**, 45, 396-397.
- Meili, J.; Seibl, J. Matrix effects in fast atom bombardment (FAB) mass spectrometry. *Intern. J. Mass Spectrom. Ion Phys.* **1983**, 46, 367-370.

- Meili, J.; Seibl, J. A new versatile matrix for fast atom bombardment analysis. *Org. Mass Spectrom.* **1984**, *19*, 581-582.
- Melchior, C. L.; Myers, R. D. In *Alcoholic and aldehyde metabolizing systems*; Turman, R. G.; Williamson, J. R.; Drott, H. R.; Chance, B., Eds.; Academic Press: New York, 1977: vol. III, pp 545-554.
- Miki, K.; Sasaya, T. Glycerol derivatives in the inner bark of *Larix leptolepis* Gord. *Mokuzai Gakkaishi*, **1979a**, *25*, 361-366.
- Miki, K.; Sasaya, T. Dihydrobenzofuran derivatives from inner bark of *Larix leptolepis* Gord. *Mokuzai Gakkaishi* **1979b**, *25*, 437-441.
- Mizukami, H.; Shiiba, K.; Ohashi, H. Enzymatic determination of stevioside in *Stevia rebaudiana*. *Phytochemistry* **1982**, *21*, 1927-1930.
- Monaghan, J. J.; Barber, M.; Bordoli, R. S.; Sedgwick, D.; Tyler, A. N. Fast atom bombardment mass spectra of involatile naphthalene sulfonic acids and sulphonate salts. *Org. Mass spectrom.* **1982**, *17*, 529-533.
- Mousseron-Canet, M.; Mousseron, M.; Levallois, C. Epoxide of geranyl acetate and of the pyranyl derivative of geraniol. *Bull. Soc. Chim. France* **1964**, *2*, 297-300.
- Nishimura, K.; Ohnishi, M.; Masuda, M.; Koga, K.; Matsuyama, R. Reactions of wood components during maturation. In *Flavour of distilled beverage: origin and development*; Piggott, J. R., Ed.; Society of Chemical Industry: London, 1983: pp 241-255.
- Nishimura, H.; Sasaki, H.; Morota, T.; Chin, M.; Mitsuhashi, H. Six glycosides from *Rehmannia glutinosa* var. *purpurea*. *Phytochemistry* **1990**, *29*, 3303-3306.
- Noble, A. C.; Strauss, C. R.; Williams, P. J.; Wilson, B. Sensory evaluation of non-volatile flavour precursors in wine. In *Flavour science and technology*; Martens, M.; Dalen, G. A.; Russwurm, H. Jr., Eds; J. Wiley & Sons: New York, 1987: pp 383-390.

Noble, A. C.; Strauss, C. R.; Williams, P. J.; Wilson, B. Contribution of terpene glycosides to bitterness in Muscat wines. *Am. J. Enol. Vitic.* **1988**, 39, 129-131.

Nykänen, L.; Suomalainen, H. *Aroma of beer, wine and distilled alcoholic beverages*; Reidel, D., Dordrecht, 1983; pp 263-272.

Ohloff, G.; Rautenstrauch, V.; Schulte-Elte, K. H. Modellreaktionen zur biosynthese von verbindungen der damascon-reihe und ihre präparative anwendung. *Helv. Chim. Acta* **1973**, 56, 1503-1513.

Ohloff, G. Importance of minor components in flavors and fragrances. *Perfumer & Flavorist* **1978**, 3, 11-23.

Oka, H.; Ikai, Y.; Kawamura, N.; Hayakawa, J.; Harada, K-I.; Murata, H.; Suzuki, M.; Ito, Y. Direct interfacing of high-speed countercurrent chromatography to frit electron ionization, chemical ionization, and fast atom bombardment mass spectrometry. *Anal. Chem.* **1991**, 63, 2861-2865.

Paré, J. R. J.; Jankowski, K.; ApSimon, J. W. Mass spectral techniques in heterocyclic chemistry: applications and stereochemical considerations in carbohydrates and other oxygen heterocycles. *Adv. Heterocyclic Chem.* **1988**, 42, 335-410.

Popoff, T.; Theander, O. Two glycosides of a new dilignol from *Pinus silvestris*. *Phytochemistry* **1975**, 14, 2065-2066.

Popoff, T.; Theander, O. The constituents of conifer needles. VI. Phenolic glycosides from *Pinus sylvestris*. *Acta Chem. Scandinavica*, **1977**, B 31, 329-337.

Porter, Q.N. *Mass spectrometry of heterocyclic compounds*; J. Wiley & Sons: New York, 1985; p. 555.

Pravdic, N.; Keglevic, D. N.M.R. spectra of acetylated 1-O-acyl-D-glucopyranoses and acetylated methyl 1-O-acyl-D-glucopyranuronates. Shielding effect of the aromatic substituent on the 2-acetoxy group. *Carbohydrate Res.* **1970**, 12, 193-199.

Radford, T.; Dejongh, D.C. Carbohydrates. In *Biochemical applications of mass spectrometry*; Waller, G. R., Ed.; Wiley-Interscience: New York, 1972: pp 313-350.

Rapp, A.; Knipser, W. 3,7-Dimethyl-octa-1,5-dien-3,7-diol - a new terpenoid component of grape and wine aroma. *Vitis*, **1979**, 18, 229-233.

Rapp, A.; Knipser, W.; Engel, L. Identification of 3,7-dimethyl-octa-1,7-dien-3,6-diol in grape and wine aroma of muscat varieties. *Vitis*, **1980**, 19, 226-229.

Rapp, A.; Knipser, W.; Hastrich, H.; Engel, L. Possibilities of characterizing wine quality and vine varieties by means of capillary chromatography. In *Grape and wine centennial symposium proceedings; 18-21 June 1980; Webb, A. D., Ed.*; University of California: Davis, CA, 1982: pp 304-316.

Rapp, A.; Mandery, H.; Ullemeyer, H. 3,7-Dimethyl-1,7-octandiol - a new terpene compound of the grape and wine volatiles. *Vitis* **1983**, 22, 225-230.

Rapp, A.; Mandery, H.; Ullemeyer, H. New monoterpenic alcohols in grape must and wine and their significance for the biogenesis of some cyclic monoterpene ethers. *Vitis* **1984a**, 23, 84-92.

Rapp, A.; Mandery, H.; Güntert, M. Terpene compounds in wine. In *Proceedings, Alko Symposium on flavour research of alcoholic beverages*; Helsinki 1984; Nykänen, L.; Lehtonen, P., Eds.; Foundation for Biotechnical and Industrial Fermentation Research 3, 1984b: pp 255-274.

Rapp, A.; Mandery, H. Wine aroma. *Experientia* **1986**, 42, 873-884.

Rapp, A. Wine aroma substances from gas chromatographic analysis. In *Modern methods of plant analysis*. Vol. 6, Wine Analysis; Linskens, H. F.; Jackson, J. F., Eds.; Springer Verlag, Berlin, 1988: pp 29-66.

Rapp, A.; Mandery, H. Influence of *Botrytis cinerea* on the monoterpene fraction of wine aroma. In *Bioflavour '87*, Schreier, P., Ed.; Walter de Gruyter & Co: Berlin, New York, 1988: pp 445-452.

- Rapp, A.; Ringlage, S. Gaschromatographic investigations comparing the volatiles of wines of *Vitis vinifera* and fungus resistant grapevine varieties. *Vitis* **1989**, 28, 21-29.
- Razungles, A.; Bayonove, C. L.; Cordonnier, R. E.; Sapis, J. C. Grape carotenoids: changes during the maturation period and localization in mature berries. *Am. J. Enol. Vitic.* **1988**, 39, 44-48.
- Rinehart, K. L. Jr. Fast atom bombardment mass spectrometry. *Science* **1982**, 218, 254-260.
- Reinhold, V. N.; Carr, S. A. New mass spectral approaches to complex carbohydrate structure. *Mass Spectrom. Rev.* **1983**, 2, 153-221.
- Ribéreau-Gayon, P.; Peynaud, P. *Sciences et techniques du vin* Tome 1; Dumod, 1975.
- Ribéreau-Gayon, P.; Boidron, J. N.; Terrier, A. Aroma of muscat grape varieties. *J. Agric. Food Chem.* **1975**, 23, 1042-1047.
- Ribéreau-Gayon, P.; Glories, Y. Phenolics in grapes and wines. In *Proceedings, 6th Australian Wine Industry Technical Conference; 13-17 August 1986*; Lee, T. H., Ed.; Australian Industrial: Adelaide, SA, 1987: pp 247-256.
- Rommelspacher, H.; May, T.; Susilo, R. β -Carbolines and tetrahydroisoquinolines: detection and function in mammals. *Planta Med.* **1991**, 57, 85-92.
- Rose, M. E. Some modern mass spectrometric methods for steroids and terpenoids. *Biochem. Soc. Trans.* **1983**, 11, 561-564.
- Rose, M. E.; Veares, M. P.; Lewis, I. A. S.; Goad, J. Analysis of steroid conjugates by fast atom bombardment mass spectrometry. *Biochem. Soc. Trans.* **1983**, 11, 602-603.
- Sakata, I.; Iwamura, H. Synthesis and properties of menthyl glycosides. *Agric. Biol. Chem.* **1979**, 43, 307-312.

Salles, C.; Essaied, H.; Chalier, P.; Jallageas, J. C.; Crouzet, J. Evidence and characterization of glycosidically bound volatile components in fruits. In *Bioflavour '87*; Schreier, P. Ed.; Walter de Gruyter & Co: Berlin, New York, 1988: pp 145-160.

Salles, C.; Jallageas, J-C.; Fournier, F.; Tabet, J-C.; Crouzet, J. C. Apricot glycosidically bound volatile components. *J. Agric. Food Chem.* **1991**, 39, 1979-1983.

Santikarn, S.; Her, G. R.; Reinhold, V. N. Oligosaccharide structural studies by on-line HPLC-MS using fast atom bombardment ionization. *J. Carbohydrate Chem.* **1987**, 6, 141-154.

Sato, S.; Tadenuma, K.; Takahashi, K.; Nakamura, N. Configuration of sake taste. VI. Rough taste components. 3. Tetrahydroharman-3-carboxylic acid. *J. Soc. Brew. Japan* **1975**, 70, 821-824.

Schenck, G. O. Aufgaben und Möglichkeiten der präparativen strahlen-chemie. *Angew. Chem.* **1957**, 69, 579-599.

Schreier, P.; Drawert, F. Gaschromatographisch-massenspektrometrische untersuchung flüchtiger inhaltsstoffe des weines. I. Unpolare verbindungen des weinaromas. *Z. Lebensm. Unters.-Forsch.* **1974**, 154, 273-278.

Schreier, P.; Drawert, F.; Junker, A. Identification of 3,7-dimethyl-1,5,7-octatrien-3-ol as a volatile component of the grape- and wine aroma. *Z. Lebensm. Unters.-Forsch.* **1974**, 155, 98-99.

Schreier, P.; Drawert, F.; Junger, A. Identification of volatile constituents from grapes. *J. Agric. Food Chem.* **1976**, 24, 331-336.

Schreier, P. Flavor composition of wines: a review. *CRC Crit. Rev. Food Sci. Nutr.* **1979**, 12, 59-111.

Schreier, P.; Drawert, F.; Abraham, K.O. Identification and determination of volatile constituents in Burgundy *Pinot noir* wines. *Lebensm.-Wiss. u.-Technol.* **1980**, 13, 318-321.

Schreier, P. Volatile constituents in different grape species. In *Grape and wine centennial symposium proceedings; 18-21 June 1980; Webb, A. D., Ed.*; University of California: Davis, CA, 1982: pp 317-321.

Schreier, P. Formation of wine aroma. In *Proceedings, Alko Symposium on flavour research of alcoholic beverages; Helsinki 1984; Nykänen, L.; Lehtonen, P., Eds.*; Foundation for Biotechnical and Industrial Fermentation Research 3, 1984a: pp 9-37.

Schreier, P. *Chromatographic studies of biogenesis of plant volatiles*; A. Hüthig Verlag: Heidelberg, Basel, New York, 1984b: pp 75-76.

Schulten, H.-R. Biochemical, medical, and environmental applications of field-ionization and field-desorption mass spectrometry. *Int. J. Mass Spectrom. Ion Phys.* **1979**, 32, 97-283.

Schwab, W.; Schreier, P. Aryl β -D-glucosides from *Carica papaya* fruit. *Phytochemistry* **1988**, 27, 1813-1816.

Schwab, W.; Schreier, P. Vomifoliol-1-O- β -D-xylopyranosyl-6-O- β -D-glucopyranoside: a disaccharide glycoside from apple fruit. *Phytochemistry* **1990a**, 29, 161-164.

Schwab, W.; Schreier, P. Studies on bound aroma compounds of sour cherry fruit (*Prunus cerasus* L.). *Z. Lebensm. Unters. Forsch.* **1990b**, 190, 228-231.

Sefton, M. A.; Skouroumounis, G. K.; Massy-Westropp, 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, 2071-2084.

Sefton, M. A.; Francis, I. L.; Williams, P. J. The volatile composition of Chardonnay juices: a study by flavour precursor analysis. *Am. J. Enol. Vitic.* **1992a** (submitted).

Sefton, M. A.; Winterhalter, P.; Williams, P. J. Free and bound 6,9-dihydroxymegastigm-7-en-3-one in *Vitis vinifera* grapes and wine. *Phytochemistry* **1992b**, 31, 1813-1815.

Self, R.; Eagles, J.; Mellon, F. A.; Fenwick, G. R. The potential of fast atom bombardment mass spectrometry in food and agricultural science. In *Mass spectrometry of large molecules*; Facchetti, S., Ed.; Elsevier Science: Amsterdam, 1985: pp 209-231.

Self, R.; Eagles, J.; Galletti, G. C.; Mueller-Harvey, I.; Hartley, R. D.; Lea, A. G. H.; Magnolato, D.; Richli, U.; Gujer, R.; Haslam, E. Fast atom bombardment mass spectrometry of polyphenols (syn. vegetable tannins). *Biomed. and Environ. Mass Spectrom.* **1986**, 13, 449-468.

Shimomura, H.; Sashida, Y.; Adachi, T. Phenolic glucosides from *Prunus grayana*. *Phytochemistry* **1987**, 26, 249-251.

Shimomura, H.; Sashida, Y.; Mimaki, Y.; Kudo, Y.; Maeda, K. New phenylpropanoid glycerol glucosides from the bulbs of *Lilium* species. *Chem. Pharm. Bull.* **1988**, 36, 4841-4848.

Shiraga, Y.; Okano, K.; Akira, T.; Fukaya, C.; Yokoyama, K.; Tanaka, S.; Fukui, H.; Tabata, M. Structures of potent antiulcerogenic compounds from *Cinnamomum cassia*. *Tetrahedron* **1988**, 44, 4703-4711.

Shosheyov, O.; Bravdo, B. A.; Siegel, D.; Goldman, A.; Cohen, S.; Ikan, R. Iso-geraniol (3,7-dimethyl-3,6-octadien-1-ol): a novel monoterpene in *Vitis vinifera* L. cv. Muscat Roy. *Vitis* **1990**, 29, 159-163.

Simpson, R. F.; Strauss, C. R.; Williams, P. J. Vitispirane: a C₁₃ spiro-ether in the aroma volatiles of grape juice, wines and distilled grape spirits. *Chem. Ind.* **1977**, 15, 663-664.

Simpson, R. F. 1,1,6-Trimethyl-1,2-dihydronaphthalene: an important contributor to the bottle aged bouquet of wine. *Chem. Ind.* **1978a**, 1, 37.

Simpson, R. F. Aroma and compositional changes in wine with oxidation, storage and ageing. *Vitis* **1978b**, 17, 274-287.

Simpson, R. F. Some important aroma components of white wine. *Food Technol. Aust.* **1979**, 31, 516-522.

Simpson, R. F.; Miller, G. C. Aroma composition of aged Riesling wine. *Vitis* **1983**, *22*, 51-63.

Simpson, R. F.; Miller, G. C. Aroma composition of Chardonnay wine. *Vitis* **1984**, *23*, 143-158.

Singleton, V. L.; Noble, A. C. Wine flavor and phenolic substances. In *Phenolic, sulfur, and nitrogen compounds in food flavors*; Charalambous, G.; Katz, I., Eds.; ACS Symposium series No 26; American Chemical Society: Washington, DC, 1976: pp 47-70.

Somers, T. C. In search of quality for red wines. *Food Technol. Aust.* **1975**, *27*, 49-56.

Stern, D. J.; Lee, A.; McFadden, W. H.; Stevens, K. L. Volatile from grapes. Identification of volatiles from Concord essence. *J. Agric. Food Chem.* **1967**, *15*, 1100-1103.

Stevens, K. L.; Bomben, J.; Lee, A.; McFadden, W. H. Volatiles from grapes. Muscat of Alexandria. *J. Agric. Food Chem.* **1966**, *14*, 249-252.

Still, W. C.; Kahn, M.; Mitra, A. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* **1978**, *43*, 2923-2925.

Strack, D.; Heilemann, J.; Wray, V.; Dirks, H. Structures and accumulation patterns of soluble and insoluble phenolics from Norway spruce needles. *Phytochemistry* **1989**, *28*, 2071-2078.

Strauss, C. R. Structure and chemistry of flavour precursors in grapes. Thesis, University of Adelaide, 1983.

Strauss, C. R.; Williams, P. J.; Wilson, B.; Dimitriadis, E. Formation and identification of aroma compounds from non-volatile precursors in grapes and wine. In *Proceedings, Alko Symposium on flavour research of alcoholic beverages; Helsinki 1984*; L.Nykanen and P. Lehtonen, Eds.; Foundation for Biotechnical and Industrial Fermentation Research, 3, 1984: pp 51-60.

Strauss, C. R.; Wilson, B.; Gooley, P. R.; Williams, P. J. Role of monoterpenes in grape and wine flavor. In *Biogenesis of Aromas*; Parliment T. H.; Croteau, R., Eds.; ACS

Symposium Series No. 317, American Chemical Society: Washington, DC, 1986a: pp 222-242.

Strauss, C. R.; Dimitriadis, E.; Wilson, B.; Williams, P. J. Studies on the hydrolysis of two megastigma-3,6,9,-triols rationalizing the origins of some volatile C₁₃ norisoprenoids of *Vitis vinifera* grapes. *J. Agric. Food Chem.* **1986b**, 34, 145-149.

Strauss, C. R.; Gooley, R. P.; Wilson, B.; Williams, P. J. Application of droplet counter current chromatography to the analysis of conjugated forms of terpenoids, phenols, and other constituents of grape juice. *J. Agric. Food Chem.* **1987a**, 35, 519-524.

Strauss, C. R.; Wilson, B.; Williams, P. J. Flavour of non-muscat varieties. In *Proceedings, 6th Australian Wine Industry Technical Conference; 13-17 August 1986*; Lee, T. H., Ed.; Australian Industrial: Adelaide, SA, 1987b: pp 117-120.

Strauss, C. R.; Wilson, B.; Williams, P. J. 3-Oxo- α -ionol, vomifoliol and roseoside in *Vitis vinifera* fruit. *Phytochemistry* **1987c**, 26, 1995-1997.

Strauss, C. R.; Wilson, B.; Anderson, R.; Williams, P. J. Development of precursors of C₁₃ nor-isoprenoid flavorants in Riesling grapes. *Am. J. Enol. Vitic.* **1987d**, 38, 23-27.

Strauss, C. R.; Wilson, B.; Williams, P. J. Novel monoterpene diols and diol glycosides in *Vitis vinifera* grapes. *J. Agric. Food Chem.* **1988**, 36, 569-573.

Suga, T.; Aoki, T.; Hirata, T.; Lee, Y. S.; Nishimura, O.; Utsumi, M. Biotransformation of foreign substrates with callus tissues. Transformation of terpineols with tobacco suspension cells. *Chem. Lett.* **1980**, 15, 229-230.

Surman, D. J.; Vickerman, J. C. Fast atom bombardment quadrupole mass spectrometry. *J. Chem. Soc., Chem. Commun.* **1981**, 7, 324-325.

Takahashi, N. Introduction. In *Chemistry of plant hormones*; Takahashi, N., Ed.; CRC press: Boca Raton, Florida, 1986; p 9.

Takayama, M.; Fukai, T.; Nomura, T. Fast atom bombardment mass spectrometry of prenyl flavonoids using thioglycerol as liquid matrix. *Shitsuryo Bunseki* **1987**, 35, 210-214.

Tamura, J.; Sakamoto, S.; Kubota, E. A newly developed direct analysis system using thin layer chromatography/fast atom bombardment mass spectrometry and its some applications. *Analisis* **1988**, 16, LXIV-LXIX.

Terrier, A.; Boidron, J. N.; Ribéreau-Gayon, P. Teneurs en composés terpéniques des raisins de *V. vinifera*. *C. R. Acad. Sc. Paris* **1972**, t 275, Ser. D, 941-944.

Tate, M. E.; Bishop, C. T. Thin layer chromatography of carbohydrate acetates. *Canadian J. Chem.* **1962**, 40, 1043-1048.

Tate, M. E. Separation of myoinositol pentaphosphates by moving paper electrophoresis (MPE). *Anal. Biochemistry* **1968**, 23, 141-149.

Unger, S. E. Isomer differentiation in fast atom bombardment mass spectrometry/mass spectrometry. *Intern. J. Mass Spectrom. Ion Processes.* **1985**, 66, 195-208.

Vérette, E.; Noble, A. C.; Somers, C. T. Hydroxycinnamates of *Vitis vinifera*: sensory assessment in relation to bitterness in white wines. *J. Sci. Food Agric.* **1988**, 45, 267-272.

Versini, G. Sull' aroma del vino "Traminer aromatico" or "Gewürztraminer". *Vignevini* **1985**, 5, 57-65.

Versini, G.; Rapp, A.; Reniero, F.; Mandery, H. Structural identification and presence of some *p*-menth-1-enediols in grape products. *Vitis* **1991**, 30, 143-149.

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

Voirin, S., Baumes, R., Bayonove, C.; M'Bairaroua, O.; Tapiero, C. Synthesis and n.m.r. spectral properties of grape monoterpenyl glycosides. *Carbohydrate Res.* **1990b**, 207, 39-56.

Wagner, R.; Dirniger, N.; Fuchs, V.; Bronner, A. Study of the intervarietal differences in the concentration of volatile constituents (linalool and geraniol) in the aroma of the grape. Interest of such analyses for the appreciation of the quality of the harvest. *International O.I.V. symposium*, Cape Town, South Africa, 1977: pp 137-142.

Waldmann, D.; Winterhalter, P. Identification of a novel vitispirane precursor in Riesling wine. *Vitis*, 1992, in press.

Wang, W. T.; LeDonne, N. C.; Ackerman, B.; Sweeley, C. C. Structural characterization of oligosaccharides by high-performance liquid chromatography, fast-atom bombardment-mass spectrometry, and exoglycosidase digestion. *Anal. Biochem.* **1984**, 141, 366-381.

Weeks, W. W. Carotenoids. A source of flavor and aroma. In *Biogenaration of Aromas*; Parliment, T. H.; Croteau, R., Eds.; ACS Symposium Series No. 317; American Chemical Society: Washington, DC, 1986: pp 157-166.

Welch, R. C.; Johnston, J. C.; Hunter, G. L. K. Volatile constituents of the Muscadine grape (*Vitis rotundifolia*). *J. Agric. Food Chem.* **1982**, 30, 681-684.

Wildenradt, H. L.; Christensen, E. N.; Stackler, B.; Caputi, A. Jr.; Slinkard, K.; Scutt, K. Volatile constituents of grape leaves. I. *Vitis vinifera* variety "Chenin Blanc". *Am. J. Enol. Vitic.* **1975**, 26, 148-153.

Williams, A. A. Recent developments in the field of wine flavour research. *J. Inst. Brew.* **1982**, 88, 43-53.

Williams, D. H.; Bradley, C.; Bojesen, G.; Santikarn, S.; Taylor, L. C. E. Fast atom bombardment mass spectrometry: a powerful technique for the study of polar molecules. *J. Am. Chem. Soc.* **1981**, 103, 5700-5704.

Williams, D. H.; Fleming, I. *Spectroscopic methods in organic chemistry*; Mc Graw-Hill Ltd, Maidenhead, England, 1987; pp 1-28.

Williams, D. H.; Stone, M. J.; Hauck, P. R.; Rahman, S. K. Why are secondary metabolites (natural products) biosynthesized? *J. Nat. Prod.* **1989**, 52, 1189-1208.

Williams, P. J.; Strauss, C. R.; Wilson, B. New linalool derivatives in Muscat of Alexandria grapes and wines. *Phytochemistry* **1980a**, 19, 1137-1139.

Williams, P. J.; Strauss, C. R.; Wilson, B. Hydroxylated linalool derivatives as precursors of volatile monoterpenes of muscat grapes. *J. Agric. Food Chem.* **1980b**, 28, 766-771.

Williams, P. J.; Strauss, C. R.; Wilson, B. Classification of the monoterpenoid composition of muscat grapes. *Am. J. Enol. Vitic.* **1981**, 32, 230-235.

Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. Use of C₁₈ reversed-phase liquid chromatography for the isolation of monoterpene glycosides and nor-isoprenoid precursors from grape juice and wines. *J. Chromatogr.* **1982a**, 235, 471-480.

Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. Novel monoterpene disaccharide glycosides of *Vitis vinifera* grapes and wines. *Phytochemistry* **1982b**, 21, 2013-2020.

Williams, P. J.; Strauss, C. R.; Wilson, B.; Massy-Westropp, R. A. Glycosides of 2-phenylethanol and benzyl alcohol in *Vitis vinifera* grapes. *Phytochemistry* **1983**, 22, 2039-2041.

Williams, P. J.; Strauss, C. R.; Wilson, B.; Dimitriadis, E. Origins of some volatile monoterpenes and nor-isoprenoids in grapes and wines—biosynthetic and biogenetic considerations. In *Topics in Flavour Research*; Berger, R. G.; Nitz, S.; Schreier, P., Eds.; Eichhorn, H, 1985; pp 336-352.

Williams, P. J.; Strauss, C. R.; Aryan, A. P.; Wilson, B. Grape flavour - a review of some pre- and postharvest influences. In *Proceedings, 6th Australian Wine Industry Technical Conference; 13-17 August 1986*; Lee, T. H., Ed.; Australian Industrial: Adelaide, SA, 1987; pp 111-116.

Williams, P. J.; Strauss, C. R.; Wilson, B. Developments in flavour research on premium varieties. In *Proceedings, 2nd international symposium on cool climate viticulture and oenology; 11-15 January 1988*, Smart, R.; Thornton, R.; Rodriguez, S.; Young, J., Eds.; New Zealand Society for Viticulture and Oenology: New Zealand, 1988; pp 331-334.

Williams, P. J.; Sefton, M. A.; Wilson, B. Nonvolatile conjugates of secondary metabolites as precursors of varietal grape flavor components. In *Flavor chemistry: Trends and developments*; Teranishi, R.; Buttery, R. G.; Shahidi, F., Eds.; ACS Symposium Series No. 388; American Chemical Society: Washington, DC, 1989: pp 35-48.

Williams, P. J. Hydrolytic flavor release in fruit and wines through hydrolysis of non-volatile precursors. In *Flavor research workshop*; Acree, T. E., Ed.; ACS professional reference book series; American Chemical Society: Washington DC, 1992: in press.

Williams, P. J.; Sefton, M. A.; Francis, I. L. Glycosidic precursors of varietal grape and wine flavor. In *Flavor precursors. Thermal and Enzymatic Conversions*. Teranishi, R.; Takeoka, G.; Güntert, M., Eds.; ACS symposium series No. 490; American Chemical Society: Washington, DC, 1992a: pp 74-86.

Williams, P. J.; Sefton, M. A.; Marinos, V. A. Hydrolytic flavour release from non-volatile precursors in fruits, wines and some other plant-derived foods. In *Recent developments in flavor and fragrance chemistry*; 3rd Haarmann and Reimer international symposium; 12-15 April, 1992, Kyoto, Japan, 1992b: in press.

Wilson, B.; Strauss, C. R.; Williams, P. J. Changes in free and glycosidically bound monoterpenes in developing muscat grapes. *J. Agric. Food Chem.* **1984**, 32, 919-924.

Winterhalter, P.; Schreier, P. Free and bound C₁₃ norisoprenoids in Quince (*Cydonia oblonga*, Mill.) fruit. *J. Agric. Food Chem.* **1988**, 36, 1251-1256.

Winterhalter, P.; Sefton, M. A.; Williams, P. J. Volatile C₁₃-norisoprenoid compounds in Riesling wine are generated from multiple precursors. *Am. J. Enol. Vitic.* **1990a**, 41, 277-283.

Winterhalter, P.; Sefton, M. A.; Williams, P. J. Two-dimensional GC-DCCC analysis of the glycoconjugates of monoterpenes, norisoprenoids, and shikimate-derived metabolites from Riesling wine. *J. Agric. Food Chem.* **1990b**, 38, 1041-1048.

Winterhalter, P.; Sefton, M. A.; Williams, P. J. A new ^{13}C -norisoprenoid intramolecular acetal in Riesling wine. *Chem. Ind.* **1990c**, (July), 463-464.

Winterhalter, P. 1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN) formation in wine. 1. Studies on the hydrolysis of 2,6,10,10-tetramethyl-1-oxaspiro[4.5]dec-6-ene-2,8-diol rationalizing the origin of TDN and related C_{13} norisoprenoids in Riesling wine. *J. Agric. Food Chem.* **1991**, 39, 1825-1829.

Winterhalter, P.; Harmsen, S.; Trani, F. A C_{13} -norisoprenoid gentiobioside from Quince fruit. *Phytochemistry* **1991**, 30, 3021-3025.