Protocol for FAB MS/MS Characterization of Terpene Disaccharides of Wine†

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Application of fast atom bombardment tandem mass spectrometry (FAB MS/MS) in positive and negative ion modes to mixtures of glycosides obtained from Riesling wine after DCCC, HPLC, and flash chromatography provided the molecular mass of the glycosides, the sequence of sugar attachment, and information about the aglycons. Initial FAB MS/MS assignments were supported by ^{1}H NMR data on isolated and derivatized glycosides which allowed the characterization of (*E*)-2,6-dimethyl-1-*O*-[apiofuranosyl-(1→6)-β-D-glucopyranosyl]-octa-2,7-dien-6-ol and (*E,E*)-2,6-dimethyl-1-*O*-[apiofuranosyl-(1→6)-β-D-glucopyranosyl]-octa-2,6-dien-8-ol and the partial characterization of (*E*)-3,7-dimethyl-1-*O*-[arabinosyl-(1→6)-β-D-glucopyranosyl]-oct-2-en-7-ol, 7-[*O*-apiofuranosyl-(1→6)-β-D-glucopyranosyl]-*p*-menth-1-en-8-ol, and vomifoliol 9-*O*-arabinofuranosyl-D-glucopyranoside.

Keywords: *Glycoside; soft ionization mass spectrometry; wine*

INTRODUCTION

The current focus of flavor research on fruit and wine glycosidic flavor precursors has prompted a search for optimal methods to identify and analyze these constituents (Williams, 1993; Williams et al., 1993; Winterhalter, 1993). Analysis of the precursors is hampered in many cases by the heterogeneity of the glycosidic isolates, which are typically made up of structurally diverse aglycons in combination with several different glycosyl moieties. Soft ionization tandem mass spectrometry offers a new approach to analyze glycosidic mixtures, and we now describe the results of our studies with this technique applied to glycosidic precursors isolated from Riesling wine.

Our protocol combines partition liquid chromatographic techniques including droplet countercurrent chromatography (DCCC) and high-performance liquid chromatography (HPLC) with fast atom bombardment mass spectrometry (FAB MS) and tandem mass spectrometry (MS/MS). At each stage of the isolation procedure positive and negative FAB MS was used to target individual constituents in mixtures of glycosides present in the liquid chromatographic subfractions and to determine the molecular weights of the compounds. Parent and daughter ion experiments, in both positive and negative ion modes, gave further structural information on individual constituents in the mixtures.

Application of this strategy, in conjunction with proton nuclear magnetic resonance (^{1}H NMR) spectrometry, has permitted the characterization of two monoterpene apiosylglucosides, the partial characterization of a third monoterpene disaccharide glycoside, and the assignment of two other terpene disaccharide glycosides in Riesling wine.

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EXPERIMENTAL PROCEDURES

Preparation of concentrates from 60 L of an Australian Riesling wine and DCCC resolution of these concentrates has been previously described (Winterhalter et al., 1990). The subsequent chromatographic steps applied to the DCCC isolates are outlined in Figure 1 and detailed below.

Flash Chromatography. Flash chromatography of underivatized glycosides was carried out as described previously (Marinos et al., 1992a). Early eluting material off the DCCC (i.e., fractions 90–100, 107 mg) was separated into 25 fractions, from which fractions 6–15 were recombined and further purified by HPLC. For flash chromatography of acetylated glycosides, diethyl ether was the eluent, and using this solvent acetylated HPLC fraction 3 (2.4 mg) was separated into 30 × 1 mL fractions from which fractions 22–27 were found (by positive FAB MS examination) to contain glycosides of interest. Derivatized HPLC fraction 6 (7.3 mg) was similarly separated into 32 fractions from which fractions 12, 13, 16–20, and 22–25 were taken for investigation.

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 acetates, diethyl ether was employed as eluent.

HPLC of Glycosidic Fractions. The chromatographic conditions have been described previously (Marinos et al., 1992b). The combined flash chromatography fractions 6–15 (35.0 mg) were separated into eight new fractions with fraction 3, eluting at 13.5–15.0 min, containing glycosides **4** and **5**, and fraction 6, eluting at 18.0–19.0 min, containing glycosides **1**–**3**.

Gas Chromatography (GC) and GC–EIMS. Conditions were as described in Marinos et al. (1992a).

FAB MS. Analyses were made with a Finnigan TSQ-70 triple-stage quadrupole instrument under conditions previously described (Marinos et al., 1992b).

^{1}H NMR. Spectra were recorded on a Bruker HR ACP 300 spectrometer operating at 300 MHz. Chemical shifts have been quoted in parts per million (ppm) downfield from tetramethylsilane. Multiplicities have been abbreviated as follows: s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet; br, broad; obsc, obscure.

Acetylation and Deacetylation Reactions. Acetylated derivatives were prepared with pyridine/acetic anhydride (1:1) under anhydrous conditions at room temperature overnight. Deacetylations were effected by treating the acetate deriva-

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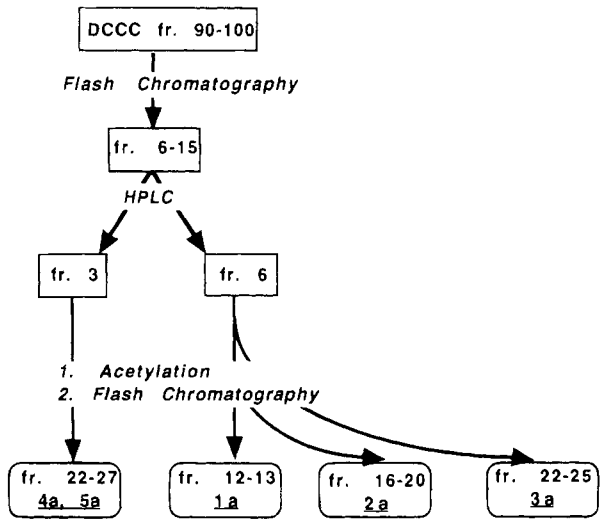


Figure 1. Partition liquid chromatography separation steps used to isolate glycosides and glycoside acetate derivatives from Riesling wine.

tives with methanolic sodium methoxide solution (1 mg of Na/mL) at room temperature overnight.

Trimethylsilyl Ethers (TMSi Derivatives). TMSi derivatives were prepared with Tri-Sil reagent (Pierce catalog no. 48999) under anhydrous conditions by heating the reaction mixture at 120 °C for 15 min.

Hydrolysis of Isolates. (*a*) *Enzymic hydrolyses* were carried out as described previously (Winterhalter et al., 1990) but using the fungal enzyme preparation Novoferm 12 (Novo Co.). The liberated aglycons were examined by GC–EIMS.

(*b*) *Acid hydrolyses* were performed by heating acidic solutions (pH 2.5, 0.035% perchloric acid aqueous solution) of the isolates at 100 °C for 15 min. The solutions were neutralized (equivalent molarity KOH), dried (stream of N_2), and desiccated overnight, after preparation of TMSi derivatives, the samples were analyzed by GC.

Acetolysis of Acetylated Glycosides. Portions of **1a** or **2a,** dried for 15 min at high vacuum, or Apiin (Carl Roth GmbH) were dissolved in 5 drops of a 0.1% perchloric acid solution in acetic anhydride. The reaction mixtures were left at room temperature for 30 h, and the progress of each was monitored by TLC (Tate and Bishop, 1962). When acetolyses were complete, the reaction mixtures were neutralized with CH_3COOK, excess acetic anhydride was destroyed by addition of methanol, and samples were dried (stream of N_2). The acetolysis products were redissolved in CH_2Cl_2, filtered, and examined by GC–EIMS. The four isomers of tetra-*O*-acetyl-apiofuranose showed almost identical fragmentation patterns as follows: *m/z* (relative intensity) 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).

RESULTS AND DISCUSSION

FAB MS/MS Experiments. A series of polar glycosidic fractions was obtained from Riesling wine by droplet countercurrent chromatography (DCCC, fractions 90–100) (Winterhalter et al., 1990) followed by flash chromatography on silica gel (Still et al., 1978). Monitoring flash fractions 6–15 (see Figure 1) by positive–negative FAB MS indicated the presence of a mixture of glycoconjugates with apparent M_rs of 464, 466, and 518. These M_rs were deduced from the observation of protonated $(M + H)^+$ and pseudomolecular 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 the following structural information. Positive ion mode MS/MS experiments on the protonated molecular ions of each glycoside indicated that all were pentosyl hexosides. The molecular ions yielded daughter ions at *m/z* 295 corresponding to a protonated anhydropentosylhexose and fragment ions for the individual sugars at *m/z* 163, 145 (hexose), 133, and 115 (pentose) (Paré et al., 1988). These positive mode daughter ion assignments were supported by the negative mode daughter fragment ions for both sugars at *m/z* 161, 119, 113, and 101 (hexose) and 149, 131 (pentose) (Domon and Costello, 1988a,b).

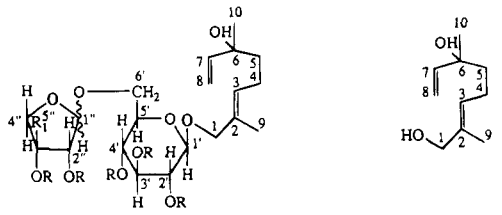
Fragmentation pathways of the glycosides in the positive and negative mode daughter ion spectra indicated that a pentose was the terminal unit in all cases. This was deduced from the observation of an anhydropentose either being readily lost as a fragment ion from the protonated or deprotonated molecular ions or being the ultimate daughter ion after subsequent elimination of the aglycon and the remaining sugar segment. The penultimate daughter ion in all cases in the positive ion mode experiments showed loss of hexose. These data suggested a linear pattern of substitution with the hexose sugar part being disubstituted.

Structural information for the aglycons was deduced from their apparent M_rs, 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. Thus, in the cases of glycoconjugates with apparent M_rs of 464 and 466 the appearance of ions for protonated aglycons at *m/z* 171 and 173 and/or fragment ions at *m/z* 153 $[(\text{aglycon} + H) - H_2O]^+$, 135 $[(\text{aglycon} + H) - 2H_2O]^+$, 155 $[(\text{aglycon} + H) - H_2O]^+$, and 137 $[(\text{aglycon} + H) - 2H_2O]^+$, suggest the presence of monoterpenediols of molecular weights 170 and 172, respectively. Several monoterpenediols with these M_rs have already been identified as aglycons in the glycosidic isolate of this wine (Winterhalter et al., 1990).

For the glycoside(s) with apparent M_r 518, the presence of a protonated ion for the aglycons at *m/z* 225 $[\text{aglycon} + H]^+$ suggested one or more C_{13} norisoprenoids of molecular weight 224. The observation of fragment ions at *m/z* 207 $[(\text{aglycon} + H) - H_2O]^+$, 189 $[(\text{aglycon} + H) - 2H_2O]^+$, and 161 $[(\text{aglycon} + H) - 2H_2O - CO]^+$ further suggested the presence of two hydroxyls and a carbonyl group in at least one of the putative C_{13} norisoprenoid aglycon skeletons. These data were consistent with the assignment of the glycoside(s) with apparent M_r of 518 as a conjugated form of vomifoliol (**5b**) (Figure 2). The latter compound was the only norisoprenoid of M_r 224 identified by GC–EIMS in the fungal glycosidase hydrolysate of the glycosidic mixture (Winterhalter et al., 1990).

After the preliminary FAB MS/MS examination, the flash chromatography fractions under investigation were further separated by chromatography on HPLC. The subfractions were monitored by positive–negative FAB MS, and those which contained glycoconjugates with the above-described mass spectrometric characteristics were acetylated and purified by flash chromatography. These chromatographic steps (see Figure 1) enabled isolation of disaccharide glycoside derivatives **1a–3a**, each in pure form as determined by TLC examination of the isolates and by the ^1H NMR data (discussed below). Glycoside acetates **4a** and **5a** were isolated as a mixture.

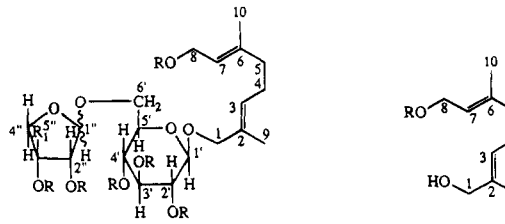
Characterization of Glycoside 1. The positive ion FAB MS/MS spectrum of hexaacetate **1a** showed (M +



1: R=H, R_1=CH_2OH

1a: R=Ac, R_1=CH_2OAc

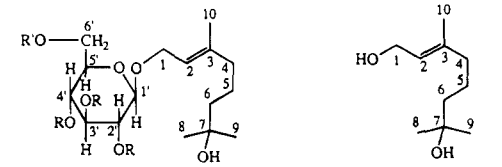
1b



2: R=H, R_1=CH_2OH

2a: R=Ac, R_1=CH_2OAc

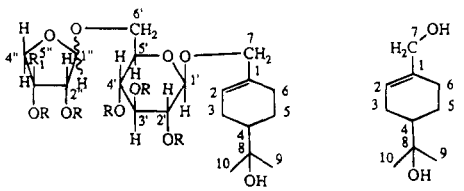
2b



3: R=H, R'=Arabinose

3a: R=Ac, R'=Tri O-Acetyl arabinose

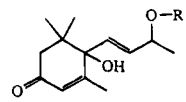
3b



4: R=H, R_1=CH_2OH

4a: R=Ac, R_1=CH_2OAc

4b



5: R=Arabinosyl-glucoside

5a: R=Hexa-O-acetyl arabinosyl-glucoside

5b: R=H

Figure 2. Structures of glycosides, glycoside acetate derivatives, and aglycons.

$H)^+$ = 717, $(M + Na)^+$ = 739, $(M + K)^+$ = 755, and a sequence of fragment ions at m/z 139, 259, 331, and 547 characteristic of a monosubstituted hexa-O-acetylated pentosyl hexoside (Williams et al., 1982). The adoption by the sugar moiety of all six acetates indicated the presence of a tertiary, or other unesterifiable, hydroxyl group in the presumed monoterpenediol aglycon part of the molecule.

The ^1H NMR spectrum of **1a** (see Table 1) showed aglycon signals assignable to 2,6-dimethylocta-2,7-diene-1,6-diol (Strauss et al., 1988). Importantly, an AB quartet at δ 3.93 and 4.12 was assigned as the signal for the geminally coupled methylene protons on C-1 bearing the glycosyl residue.

Two AB quartets for the protons on positions 4 and 5 of a tri-O-acetylated apiofuranoside (δ 4.12, 4.20 and 4.55, 4.74), along with signals corresponding to a tri-O-acetylated glucose that was not acylated on positions 1 and 6, suggested that the sugar moiety was an acetylated apiofuranosyl-(1→6)-glucopyranoside (Nishimura et al., 1990; Suzuki et al., 1988). A β-configuration for the glucose was evident from the doublet (J = 8.0 Hz) for the anomeric proton at δ 4.45. Proton NMR data do not provide unequivocal evidence for the stereochemistry of the glycosidic linkage of apiose (Suzuki et al., 1988). From a $^1H-^1H$ COSY spectrum of **1a** the coupling relationships of the individual protons were observed and found to accommodate the proposed structure (see Table 1).

Incomplete esterification of the tertiary hydroxyl group of apiose under mild conditions of acetylation (Iwagawa and Hase, 1983) or trifluoroacetylation (Voirin et al., 1990) of apiosylglucosides has been reported. In the present work, the flash chromatography step applied to the acetylated compounds removed partially derivatized compounds.

The observations of Bishop and Cooper (1963) on the rates of anomerization of pentofuranosides suggested a method to determine the stereochemistry of C-3 of the apiose moiety of disaccharide **1a.** On the basis of their data it was expected that a mild, kinetically controlled, acetolysis of a stereochemically pure apioside, e.g., apiin [for which the D-erythrofuranoside configuration has been established (Hulyalkar et al., 1965)], would liberate the acetate 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 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 **1a** in parallel with those from the acetolysis of a reference sample of acetylated 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 **1a** were identical to the two from apiin. Thus, the apiose in glycoside **1a**, like that in apiin, had 3-*C*-(hydroxymethyl)erythrofuranose stereochemistry.

Deacetylation of **1a** with NaOMe/MeOH produced a disaccharide **1,** with M_r of 464 and MS/MS characteristics similar to those described above for glycosides with apparent M_r of 464 in the original flash chromatography fractions. Acid hydrolysis of **1** and GC and GC–MS examination of the carbohydrates 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.

^1H NMR examination of glycoside **1** (Table 1) showed signals consistent with the apiofuranosyl-(1→6)-β-D-glucopyranoside structure. In particular, the erythrofuranose stereochemistry for apiose was indicated by the characteristic high field singlet at δ 3.60 for the equivalent protons on position 5 and an ABq at δ 3.94 and 4.07 for the protons on position 4 of the pentose moiety (Angyal et al., 1977; Bredenkamp et al., 1989; Snyder and Serianni, 1987; Suzuki et al., 1988).

Enzymic hydrolysis of **1** with a fungal enzyme preparation (Novoferm 12) liberated the aglycon which, on GC–EIMS, cochromatographed with and gave an identical spectrum to a reference sample of (*E*)-2,6-dimethylocta-2,7-diene-1,6-diol (**1b**) (Strauss et al., 1988).

Table 1. 300 MHz ^{1}H NMR Data for Compounds 1, 1a, 2a, 3a, and 4a (Chemical Shift δ Values; Couplings in Hertz)

H	1 (D_2O)	1a ($CDCl_3$)	1a ^{1}H–^{1}H COSY	2a ($CDCl_3$)	3a ($CDCl_3$)	4a ($CDCl_3$)
aglycon						
1	4.11, 4.20, ABq (11.1)	3.93, 4.12, ABq (11.9)	H-1b/H-1a/H-9	3.90, 4.25, ABq (11.9)	4.15, dd (10.8, 2.1) 4.30, dd (10.8, 13.2)	
2					5.6, m	5.67, m ($W_{1/2}$ = 9.5)
3	obsc	5.40, br t (7.0)		5.40, m		1.43, m
4	2.05–2.15, m	obsc		obsc	obsc	obsc
5	1.58–1.63, m	obsc		obsc	obsc	obsc
6					obsc	1.43, m
7	5.96, dd (17.4, 11.1)	5.90, dd (17.6, 10.7)	H-8a/H-8b	5.68, m		3.95, 4.15, ABq (11.4)
8	obsc	5.05, dd (10.7, 1.3); 5.21, dd (17.6, 1.3)	H-8a/H-8b/H-7	4.60, br d (8.0)	1.23, s	
9	1.66, br s	1.57, br s		1.70, br s	1.23, s	1.23, s
10	1.29, br s	1.27, br s		1.40, br s	1.63, s	1.23, s
glucose						
1′	4.39, d (8.5)	4.45, d (8.0)	H-2′	4.45, d (8.0)	4.50, d (7.9)	4.47, d (8.1)
2′	3.2–4.0, m	4.95, dd (9.4, 8.0)	H-1′/H-3′	4.95, dd (8.0, 9.7)	4.93, dd (7.9, 9.3)	4.87–4.99, m
3′	3.2–4.0, m	5.16, t (9.4)	H-2′/H-4′	5.17, br t (9.7)	5.25, t (9.3)	5.30, t (9.5)
4′	3.2–4.0, m	4.90, t (9.4)	H-3′/H-5′	4.91, br t (9.4)	4.89, br t (9.3)	4.87–4.99, m
5′	3.2–4.0, m	3.54–3.65, m	H-4′/H-6′a/H-6′b	3.55–3.70, m	3.59, m	3.5–3.8, m
6′a	3.2–4.0, m	3.54–3.65, m	H-5′/H-6′b	3.55–3.70, m	3.80, dd, (11.5, 4.6)	3.5–3.8, m
6′b	3.2–4.0, m	3.54–3.65, m	H-5′/H-6′a	3.55–3.70, m	3.70, br d (11.5)	3.5–3.8, m
pentose						
1″	obsc	5.32, br s (<1)		5.32, br s (<1)	5.01, s	5.43, s
2″	3.2–4.0, m	5.05, br s (<1)		5.01, br s (<1)	5.13, br s	5.01, s
3″					3.50–4.20, m	
4″	3.95, 4.07, ABq (10.3)	4.12, 4.20, ABq (10.6)	H-4″a/H-4″b	4.20, 4.30, ABq (10.5)	3.50–4.20, m	4.12, 4.20, ABq (10.5)
5″	3.60, s	4.55, 4.74, ABq (12.5)	H-5″a/H-5″b	4.55, 4.75, ABq (11.9)	3.50–4.20, m	4.54, 4.74, ABq (12.5)
acetates		1.99–2.08, 6 × s		1.97–2.08, 6 × s	1.90–2.10, 6 × s	1.97–2.09, 6 × s

These data established the structure of **1** as (*E*)-2,6-dimethyl-1–*O*-[apiofuranosyl-(1→6)-β-D-glucopyranosyl]-octa-2,7-dien-6-ol.

An alternative disaccharide glycoside of aglycon **1b**, conjugated through the tertiary hydroxyl group at position 6, was recently characterized in *Cynanchum hancockianum* (Lou et al., 1993)

Characterization of Glycoside 2. Positive mode FAB MS examination of the derivative **2a** indicated a heptaacetate [$(M + H)^+$ = 759]; the spectrum also showed the characteristic disaccharide sequence of fragment ions at *m/z* 259, 331, and 547 analogous to those seen in the spectrum of **1a**. It followed that the seventh acetate group of **2a** was associated with the aglycon.

The ^{1}H NMR signals of **2a** given in Table 1 suggested 2,6-dimethylocta-2,6-diene-1,8-diol as a likely candidate for the aglycon (Strauss et al., 1988). The signals observed for the sugar moiety were in good agreement with data previously published for acetylated apiofuranosyl-(1→6)-glucopyranosides (Nishimura et al., 1990; Suzuki et al. 1988) as well as those seen in the spectrum of glycoside **1a**. The coupling constant (J = 8.0 Hz) observed for the anomeric proton of glucose suggested that it had the β-configuration. The similarity in long-range coupling of the AB quartet system at δ 3.90 and 4.25 to its counterpart in the spectrum of **1a,** and also its upfield chemical shift in relation to the more complex signal of the two allylic protons of the C=CHCH$_2$OAc system at δ 4.60, suggested that the aglycon was glycosylated via the C-1 hydroxyl rather than that on C-8. From the above data **2a** was assigned as hepta-O-acetylated 2,6-dimethyl-*1-O*-[apiofuranosyl-(1→6)-β-D-glucopyranosyl]octa-2,6-dien-8-ol.

Acetolysis of **2a** liberated only two anomers of acetylated apiose which, as described above, were identified by GC and GC–MS comparison with the reference sample as those of the erythrofuranose isomer.

Deacetylation of **2a** produced a disaccharide glycoside **2**, with M_r of 464 and MS/MS characteristics similar to those described for glycosides with apparent M_r of 464 in the original flash chromatography fractions.

Enzymic hydrolysis of **2** with Novoferm 12 liberated an aglycon which, under GC–EIMS conditions, gave a spectrum identical to, and cochromatographed with, a reference sample of (*E,E*)-2,6-dimethylocta-2,6-diene-1,8-diol (**2b**) (Strauss et al., 1988). These data allowed **2** to be assigned as the (*E,E*)-2,6-dimethyl-1-*O*-[apiofuranosyl-(1→6)-β-D-glucopyranosyl]-octa-2,6-dien-8-ol.

Partial Characterization of Glycoside 3. For derivatized glycoside **3a** the FAB mass spectrum suggested a hexaacetate [$(M + H)^+$ = 719]. The presence of fragment ions at *m/z* 259, 331, and 547, similar to those seen in the FAB spectra of acetates **1a** and **2a**, implied that **3a** was also a monosubstituted hexa-O-acetylated pentosyl hexoside (Williams et al., 1982). Also, like derivative **1a**, the production of a hexaacetate from glycoside **3** indicated the presence of an unesterified, presumably, tertiary hydroxyl group in the aglycon.

The ^{1}H NMR signals of **3a** given in Table 1 were assignable to 3,7-dimethyloct-2-ene-1,7-diol as the aglycon (Ohloff et al., 1964). For the sugar moiety, signals for a tri-O-acetylated glucopyranose substituted at positions 1 and 6 were observed. The greater multiplicity of the pentose protons in comparison with the analogous signals observed for **1a** and **2a**, and the absence of two AB quartets for the protons on positions 4 and 5 of a tri-O-acetylated apiofuranoside, excluded the possibility that apiose was the terminal unit. The signals were instead consistent with those of arabinose. The coupling constant of the anomeric proton of glucose indicated a β-configuration for that sugar. The high field chemical shift of the allylic protons on position 1 of the aglycon, as well as the observation of only six acetates, implied that the monoterpene was glycosylated via the primary hydroxyl.

Deacetylation of **3a** produced a disaccharide glycoside of M_r of 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 of 466.

Enzymic hydrolysis of **3** with Novoferm 12 liberated an aglycon which, on GC–EIMS, cochromatographed with and gave a spectrum identical to a reference sample of (*E*)-3,7-dimethyloct-2-ene-1,7-diol (**3b**) (Strauss et al., 1987a). Finally, acid hydrolysis of **3** liberated only two sugars which, as TMSi derivatives, were identified by GC and GC–MS comparison with reference sugars as arabinose and glucose. Thus, glycoside **3** was assigned as (*E*)-3,7-dimethyl-1-*O*-[arabinosyl-(1→6)-β-D-glucopyranosyl]-oct-2-en-7-ol. On the basis of previous research which established an α-furanosyl structure for the arabinose moieties in grape monoterpene disaccharide glycosides (Williams et al., 1982), a similar disaccharide is assumed for glycoside **3**.

Assignment of Glycosides 4 and 5. In the FAB MS spectrum of the mixture of compounds **4a** and **5a** two ions at *m/z* 717 $(M + H)^+$ and 771 $(M + H)^+$ were observable corresponding to protonated molecular ions of hexa-O-acetylated derivatives of the glycosides with apparent M_rs of 464 (**4**) and 518 (**5**), respectively, in the original DCCC flash chromatography isolates. The observation of fragment ions (i.e., FAB MS/MS) at *m/z* 259, 331, and 547, like those seen in the spectra of **1a**–**3a** and characteristic of hexa-O-acetylated pentosyl hexosides, indicated that in both cases all six acetates were on the sugar moiety. These data implied that the aglycon moiety of **4a**, presumably a monoterpenediol from the FAB MS/MS data, had an unesterifiable, probably tertiary, hydroxyl group. The data for **5a** could similarly be interpreted as being those of a glycoside of a C_{13} norisoprenoid of molecular weight 224 with two oxygens resistant to acetylation in addition to the oxygen atom involved in glycosylation. This latter was consistent with the proposal made above on the basis of the FAB MS/MS analysis of the original DCCC flash chromatography isolates—that glycoside **5** was a conjugate of vomifoliol.

The ^{1}H NMR spectrum of the approximately 4:1 mixture of **4a** and **5a** allowed signals for the first compound to be assigned, and these are given in Table 1. Two tertiary methyl groups on a carbon bearing oxygen were evident at δ 1.23, along with an AB quartet system at δ 3.95 and 4.15 for the geminally coupled allylic protons on a carbon bearing oxygen and an unresolved signal for an olefinic proton of a trisubstituted double bond at δ 5.67. The observation in the ^{1}H NMR spectrum of only one olefinic proton suggested that the aglycon, which, from the FAB MS/MS data, was presumed to be a monoterpenediol at the oxidation state of the linalool oxides, was monocyclic. Accordingly, a *p*-menthenediol structure was assigned to the aglycon of **4a**.

Observable signals of the minor constituent in the mixture were assigned to a methyl on a carbon bearing oxygen (δ 1.40) and to an olefinic methyl group (δ 1.60) and were attributed to glycoside derivative **5a**.

Signals for the sugar moiety in the ^{1}H NMR spectrum of the mixture of **4a** (major component) and **5a** (minor component) were similar to those discussed above for the acetylated apiofuranosyl glucopyranosides **1a** and **2a**. The appearance of only two signals for anomeric protons, i.e., one for glucose (δ 4.47) and another for apiose (δ 5.43), in a ratio of 1:1, as well as the approximately 1:1 ratio of signals for the sugar moiety and the aglycon with the *p*-menthenediol structure, suggested that only signals for the sugar moiety of the dominant component **4a** were observable in the spectrum. Thus, **4a** was tentatively assigned as a hexa-*O*-acetylapiofuranosyl β-D-glucopyranoside of a *p*-menthenediol. The relatively high field chemical shift of the AB quartet system at δ 3.95 and 4.15, together with the substituted 2-propanol system at δ 1.23 and the presence of only six acetates, suggested that the *p*-menthenediol was glycosylated via the primary hydroxyl at C-7.

Deacetylation of the mixture produced two disaccharide glycosides with apparent M_rs of 464 and 518 [$(M + H)^+ = 465$, $(M + H)^+ = 519$] and MS/MS characteristics similar to those observed for the glycosides with apparent M_rs of 464 and 518 in the original DCCC flash chromatography isolates.

Enzymic hydrolysis of the mixture with Novoferm 12 liberated two aglycons which were assigned as *p*-menth-1-ene-7,8-diol (**4b**) and vomifoliol (**5b**) from their EI mass spectra by comparison with published data (Strauss et al., 1987b; Versini et al., 1991). Acid hydrolysis of the mixture and examination of the liberated sugars as the TMSi derivatives by GC–MS showed anomers of two major sugars, apiose and glucose, and a minor one, arabinose. Thus, glycoside **4** was assigned as the 7-*O*-[apiofuranosyl-(1→6)-β-D-glucopyranosyl]-*p*-menth-1-en-8-ol.

The nature of the minor constituent (acetylated glycoside **5a**) remains uncertain because of the incomplete ^{1}H NMR data. The available data are consistent with it being the acetate of vomifoliol 9-*O*-arabinofuranosyl-*O*-glucopyranoside. By analogy with the prior FAB MS discussion, the position of glycosylation in **5a** was interpreted as being through the secondary alcohol, leaving a free tertiary –OH group. The documented occurrence of 6-*O*-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranosides in grapes makes it reasonable to suggest that **5** is a member of the same series of (1→6) linked disaccharides (Williams et al., 1982).

CONCLUSION

This work demonstrates that FAB MS and MS/MS can rapidly provide reliable structural information on the molecular mass of glycosides, the number of sugars involved in conjugation, the sequence in which the sugars are attached, and information about the aglycon moieties. Although the data provided by FAB MS and MS/MS experiments alone allow only tentative structural assignments, it can be obtained under experimental circumstances that (a) require a minimal amount of sample; (b) require minimal sample preparation time by avoiding the necessity for chemical derivatizations, and (c) provide time efficiency because the analyses are performed on mixtures. For the purposes of this study, the reliability of the initial assignments was supported by the isolation of individual derivatized glycoconjugates and their further structural characterization by ^{1}H NMR spectroscopy.

There are, however, limitations to the protocol that must be recognized. Thus, the number of molecular ions observed in the FAB spectrum of a mixture may not necessarily reflect the qualitative composition of that mixture. For example, the presence of several isomeric compounds in a mixture results in the observation of only a common molecular ion as was the case with glycosides **1**, **2**, and **4**. Additionally, the intensity of the individual pseudomolecular ions observed in a FAB mass spectrum of a mixture is dependent on the solubility of each compound in the liquid matrix em-

ployed and on the presence of other competing solutes in the matrix. Nevertheless, by following the MS of the targeted constituents through successive chromatographic stages, the spectra, particularly those from the MS/MS experiments, become progressively more diagnostic.

The protocol is a significant advance in the methods available for the analysis of glycosidic flavor precursors of grapes and wines. Earlier, a two-dimensional analysis, in which the DCCC partition functions of intact glycosides were plotted against the GC partition functions of hydrolytically liberated aglycons, revealed the range of glycoconjugates for each volatile aglycon that was present in a sample (Winterhalter et al., 1990). The use of FAB MS/MS on the intact glycosides as described here provides an off-line, third dimension in such an analytical regimen for the study of fruit and wine flavor precursors.

The study confirms the presence in Riesling wine of several glycosidic constituents that had been inferred from our previous research (Winterhalter et al., 1990). Glycoconjugates more polar than those of monosaccharides (i.e., eluting in DCCC fractions 91–100) and which, on enzymic hydrolysis, gave the monoterpenediols **1b**, **2b**, and **3b** as well as vomifoliol (**5b**) were observed in this particular Riesling wine in the two-dimensional analysis of the glycosides discussed above. The *p*-menthenediol (**4b**), which was recorded in that earlier study as unknown *p*-menthenediol isomer 3, was also found in abundance as a glycoconjugate eluting in the polarity range expected for a disaccharide derivative. However, in the 2D study (Winterhalter et al., 1990), **4b** was not detected in the specific fractions 91–100, possibly because of its low concentration in those fractions.

A disaccharide glycoside of diol **1b** has already been found in Riesling juice and assigned as the 6-*O*-α-L-arabinofuranosyl-(1→6)-β-D-glucopyranoside on the basis of low field ^{1}H and ^{13}C NMR studies (Strauss et al., 1988). The finding, in a Riesling wine, of an apiosylglucoside of the same diol, i.e. **1b**, demonstrates the common occurrence of disaccharides of this particular monoterpene.

The identification of apiosylglucosides **1**, **2**, and **4**, along with the original discovery of a glycoconjugate involving this disaccharide moiety in grapes (Voirin et al., 1990) and later findings (Baumes et al. 1994), confirms apiosylglucosides as ubiquitous in *Vitis vinifera*. The presence of these glycosides, together with arabinosyglucosides **3** and **5**, in a wine establishes that the labile furanosyl linkage (Mendicino and Picken, 1965) is stable enough to permit these disaccharides to survive the processes of winemaking.

It is important to note that although apiosidase activity has been reported as rare in commercial enzyme preparations (Gunata et al., 1993), the fungal enzyme Novoferm 12 used here was capable of hydrolyzing the apiosylglucosides **1**, **2**, and **4**.

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Supplementary Material Available: TLC R_f values and positive and negative ion FAB mass spectra of **1, 1a, 2, 2a, 3, 3a, 4, 4a, 5, and 5a** (4 pages). Ordering information is given on any current masthead page.

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