Application of Multilayer Coil Countercurrent Chromatography for the Study of *Vitis vinifera* Cv. Riesling Leaf Glycosides†

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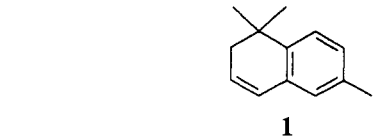
The glycosidic fraction isolated from *Vitis vinifera* cv. Riesling leaves by XAD-2 adsorption and methanol elution was separated by multilayer coil countercurrent chromatography (MLCCC) to identify genuine precursors of the off-flavor-causing compound 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN). A two-step procedure utilizing preparative MLCCC in combination with analytical MLCCC yielded two major vine leaf glycosides in pure form, i.e., the β-D-glucopyranosides of 3-oxo-7,8-dihydro-α-ionol (Blumenol C) and 3-oxo-4,5-dihydro-α-ionol, respectively. The determination of minor vine leaf constituents in separated MLCCC fractions was achieved after acetylation and subsequent HPLC purification of the per-O-acetylated derivatives. This procedure allowed the identification of 3,4-dihydroxy-7,8-dihydro-β-ionone 3-*O*-β-D-glucopyranoside as a major TDN precursor in Riesling leaves. In addition, the β-D-glucopyranosides of 3,4-dihydroxy-β-ionone and zingerone were identified.

INTRODUCTION

During the past years extensive research has been undertaken to further enhance the knowledge about "secondary" flavor formation in wine. As a result of the research efforts of different groups throughout the world, polyhydroxylated terpenoids (so-called "polyols"), which are often present in glycosidically bound form, have been elucidated as precursors to a range of potent wine volatiles (Gunata et al., 1993; Rapp et al., 1984; Sefton et al., 1989; Strauss et al., 1984, 1986a,b; Williams et al., 1980, 1989; Winterhalter, 1992). Although today a great number of glycoconjugated terpenoids have been detected in wine, it is only in a few cases that the entire structure of the intact glycoside has been fully characterized [e.g., Marinos et al. (1992); Sefton et al. (1992); Strauss et al. (1987a, 1988); Voirin et al. (1990); Williams et al. (1982)]. This discrepancy is caused by the complexity of the glycosidic isolates obtained from wine and due to the fact that the individual glycoconjugates of several key flavor compounds are present within this complex mixture in extremely minute amounts (parts per billion range).

The application of the preparative all-liquid chromatographic technique of countercurrent chromatography (CCC) has enabled a partial separation of the many glycoconjugates that exist in wine (Strauss et al., 1987a; Winterhalter et al., 1990a). In favorable cases even a complete separation has been achieved. Despite these efforts, the structures of important wine flavor precursors, e.g., those being responsible for the formation of the potent aroma compound β-damascenone, remain to be fully characterized (Sefton et al., 1989; Skouroumounis et al., 1992). Similarly, glycoconjugates, liberating the aromatic hydrocarbon 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) (1) during prolonged bottle storage of wine, still have to be elucidated. TDN with a low flavor threshold (20 ppb in wine) is regarded to be responsible for a "kerosene" off-flavor, which often occurs in aged Riesling wines (Simpson, 1978; Simpson and Miller, 1983; Strauss et al., 1987b).

In a preliminary study on the generation of TDN in Riesling wine, the presence of multiple precursors became obvious (Winterhalter et al., 1990b). Precursors of 1 are assumed to be various glycosides (i.e., mono- as well as disaccharides) of the C_{13} norisoprenoid structures 2,6,10,10-tetramethyl-1-oxaspiro[4.5]dec-6-ene-2,8-diol (2) and 3,4-dihydroxy-7,8-dihydro-β-ionone (3). After glycosidase treatment, both compounds have been most recently identified as new Riesling wine constituents (Winterhalter, 1991). In model hydrolytic reactions at wine/juice pH (i.e., pH 3.2) aglycons 2 and 3 have been shown to generate TDN as a major degradation product.



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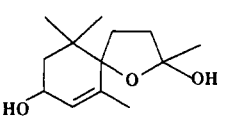

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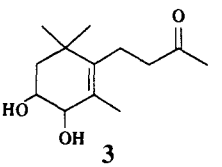

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Because wine contains only a low concentration of TDN, it was necessary to find a better suited substrate, which enables the isolation of sufficient quantities of TDN precursors for subsequent NMR studies. By using these glycosides as reference compounds, state-of-the-art MS techniques, e.g., FAB-MS and MS/MS, should then enable the unequivocal identification of the respective structures in wine (Marinos, 1992). As a convenient starting material for the isolation of TDN-generating glycoconjugates, Riesling leaves were chosen, since they have been found to generate 5–8 times more of TDN compared to Riesling wine itself. For the isolation of the precursors multilayer coil countercurrent chromatography (MLCCC) was applied. This all-liquid chromatographic technique enables efficient preparative separations of labile natural compounds under mild conditions (Fischer et al., 1991; Ito, 1986; Marston et al., 1990).

EXPERIMENTAL PROCEDURES

General. NMR spectra were taken on Fourier transform AC 200 and WM 400 spectrometers. For DEPT experiments the Bruker standard impulse sequence was used. CD spectra were

† Part 2 in the series 1,1,6-Trimethyl-1,2-dihydronaphthalene (TDN) Formation in Wine. For part 1 see: *J. Agric. Food Chem.* **1991**, *39*, 1825–1829.

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recorded on an ISA Jobin Yvon CD 6 dichrometer. The reference glycosides 4–10 were commercially obtained (Serva).

Plant Material. Intact *Vitis vinifera* cv. Riesling leaves were plucked in September/October 1991 at a vineyard in Thüngersheim, Germany. After removal of the stems, the leaves were stored at −30 °C.

Isolation of Glycosidic Extracts from Riesling Vine Leaves. Deep-frozen leaves (10 kg) were crushed in a Braun blender. Portions of 1 kg were worked up as follows: After addition of MeOH (1 L/kg of leaves), stirring overnight at room temperature, and centrifugation (4000*g*, 30 min), the MeOH extract was concentrated *in vacuo*. The aqueous residue was extracted with pentane (200 mL), diluted with H_2O to a final volume of 1 L, and passed through a column (40 × 500 mm) of Amberlite XAD-2 resin (Gunata et al., 1985). After rinsing with distilled H_2O (2 L), the retained glycosides were eluted with MeOH (500 mL). The combined MeOH eluates from 10 kg of vine leaves were concentrated *in vacuo*. The remaining aqueous phase was extracted with Et_2O to remove any remaining volatiles and evaporated to dryness; 16 g of the so-obtained dark brown glycosidic extract (19 g in total) was separated by preparative MLCCC in portions of 2 g. Each sample was dissolved in the respective mobile phase and injected by means of a six-way valve using a 5-mL sample loop.

Multilayer Coil Countercurrent Chromatography (MLCCC) [Synonym: Centrifugal Partition Chromatography (CPC) or High-Speed Countercurrent Chromatography (HSCCC)]. An Ito multilayer coil separator–extractor (P.C. Inc., Potomac) with two different types of PTFE columns has been used.

Preparative separations with up to 2 g of glycosidic extracts have been carried out with a 75 m × 2.6 mm i.d. PTFE tubing (volume approximately 400 mL).

Analytical separations (up to 300 mg) have been achieved on a 160 m × 1.6 mm i.d. PTFE column (volume approximately 320 mL). Typical flow rates were 1.5–8 mL/min for preparative separations and 1–2 mL/min for analytical separations.

Selection of Biphasic Solvent Systems. First, the settling time of the equilibrated solvent phases was measured after gentle mixing (10 times) of the two phases in a test tube (settling times < 30 s are required for a satisfactory retention of the stationary phase in MLCCC). Second, partition coefficient (K) values were determined before and after partitioning of the solutes in the selected two-phase mixture using reversed-phase HPLC (250 mm × 4 mm i.d., Superspher 100 RP18 column, 4 μm, Knauer, Berlin, Germany). For this purpose 5 mg of the glycosidic mixture was dissolved in 1.00 mL of the thoroughly equilibrated aqueous layer, and an aliquot (20.0 μL) of this solution was injected into the HPLC system. The organic layer (1.00 mL) of the two-phase system was then added to the aqueous layer. After thorough partitioning of the solutes between the two layers, 20 μL of the aqueous layer was again analyzed by HPLC. K values were then estimated by comparison of the integrated peak areas before and after partitioning.

MLCCC Separations. Separations were carried out at the rotational speed of 800 rpm in either the "head-to-tail" or the "tail-to-head" elution mode, depending which layer of the biphasic system was selected as stationary phase (Conway, 1990). The separation was followed by UV detection using a variable-wavelength monitor (Knauer, Berlin). In the case of Riesling leaf glycosides separated fractions were further analyzed by thin-layer chromatography utilizing the lower phase from a mixture of $CHCl_3$–MeOH–H_2O (7:13:8) as developing solvent (vanillin/sulfuric acid detection).

Enzymatic and Acid Hydrolyses of Vine Leaf Glycosides. Aliquots ($^1/_{20}$ part) of the pooled MLCCC fractions were concentrated *in vacuo*, taken up in 20 mL of citric acid/phosphate buffer (pH 5.0), and incubated with either Rohapect D5L (0.1 mL, Röhm, Darmstadt) or β-glucosidase from sweet almond (30 mg, Serva) overnight at 37 °C. The liberated aglycons were extracted with Et_2O and, after drying (Na_2SO_4), analyzed by HRGC and HRGC–MS. For acid hydrolyses a further aliquot ($^1/_{20}$ part) was subjected to simultaneous distillation/extraction (SDE) at pH 3.2 using the distillation head described by Schultz et al. (1977).

Acetylation and Liquid Chromatographic Purification. Acetylation of the solvent-free MLCCC fractions was carried out at room temperature with Ac_2O/pyridine. After usual workup and flash chromatography (Still et al., 1978), the acetates were finally purified by HPLC on a Eurospher Si 100 (5 μm) column (250 mm × 4 mm i.d.; Knauer, Berlin) using pentane–Et_2O gradients.

Capillary Gas Chromatography (HRGC) and Capillary Gas Chromatography–Mass Spectroscopy (HRGC–MS). The same systems as described previously have been used (Winterhalter, 1991). The chromatographs were equipped with a 30-m J&W DB-5 fused silica capillary column (0.25 mm i.d. and 0.25-μm film thickness).

Thermospray–Mass Spectrometry (TS–MS). A Finnigan MAT 4500 mass spectrometer equipped with a thermospray bypass interface jet 220 was used (0.1 M NH_4Ac). Positive ions (70 eV) over a range m/z 100–900 were scanned.

Spectral Data for Isolated Compounds. NMR data given are for the per-O-acetylated derivatives, because of the better resolved ^1H NMR signals in the case of the sugar protons.

3-Oxo-7,8-dihydro-α-ionyl (Blumenol C) Tetra-O-acetyl-β-D-glucopyranoside (11): 132 mg; TS–MS (70 eV) pseudomolecular ion at m/z 558 $[M + NH_4]^+$; molecular mass 540 ($C_{27}O_{11}H_{40}$); ^1H NMR (200 MHz, $CDCl_3$) δ (coupling constant J in Hz) 0.98 and 1.02 (6H, 2s, 2 × CH_3-1), 1.08 (3H, d, J = 6.2, CH_3-9), 1.2–1.9 (5H, m, H_2-7, H_2-8, H-6), 1.95 (3H, d, J = 1.2, CH_3-5), 1.98–2.08 (13H, 4 acetates and partly obscured H_a-2), 2.34 (1H, d, J = 17.2, H_b-2), 3.59–3.73 (2H, m, H-9 and H-5′), 4.08 (1H, dxd, J = 12.3, 2.5, H_a-6′), 4.21 (1H, dxd, J = 12.3, 4.6, H_b-6′), 4.50 (1H, d, J = 7.9, H-1′), 4.91 (1H, dxd, J = 9.4, 7.9, H-2′), 5.05 (1H, dxd, J = 9.6, 9.4, H-4′), 5.18 (1H, t, J = 9.4, H-3′), 5.79 (1H, br s, H-4); ^{13}C NMR (50 MHz) δ 19.72 (CH_3-C5), 24.59 (CH_3-C9), 25.66 (27.13 and 28.79 (2 × CH_3 at C1), 36.18 (C8), 36.27 (C1), 47.19 (C2), 50.95 (C6), 62.02 (C6′), 68.55 (C4′), 71.48, 71.69, 72.86 (C2′/C3′/C5′), 75.58 (C9), 99.06 (C1′), 125.14 (C4), 165.90 (C5), 199.45 (C3), 20.55 and 169.1–170.60 (4 acetates). Assignments are based on DEPT experiments. CD data were identical with those previously published (Miyase et al., 1988).

3-Oxo-4,5-dihydro-α-ionyl Tetra-O-acetyl-β-D-glucopyranoside (12): 24 mg; TS–MS pseudomolecular ion at m/z 558 $[M + NH_4]^+$; molecular mass 540 ($C_{27}O_{11}H_{40}$); ^1H NMR (400 MHz, $CDCl_3$) δ 0.82 (3H, s, CH_3-1), 0.90 (3H, d, J = 5.8, CH_3-5), 0.97 (3H, s, CH_3-1), 1.25 (3H, d, J = 6.3, CH_3-9), 1.85 (1H, br d, J = 9.5, H-6), 1.99–2.06 (13H, 4 acetates and H_a-4), 2.12 (1H, dxd, J = 13.3, 2.4, H_a-2), 2.27 (1H, d, J = 13.3, H_b-2), 2.38 (1H, dxdxd, J = 13.5, 3.9, 2.3, H_b-4), 3.62 (1H, dxdxd, J = 9.7, 4.2, 2.5, H-5′), 4.06 (1H, dxd, J = 12.3, 2.5, H_a-6′), 4.20 (1H, m, H-9), 4.23 (1H, dxd, J = 12.3, 4.2, H_b-6′), 4.57 (1H, d, J = 8.0, H-1′), 4.99 (1H, dxd, J = 9.4, 8.0, H-2′), 5.11 (1H, dxd, J = 9.7, 9.5, H-4′), 5.19 (1H, dxd, J = 9.5, 9.4, H-3′), 5.35 (1H, br dxd, J = 15.4, 9.5, H-7), 5.60 (1H, dxd, J = 15.4, 6.8, H-8); ^{13}C NMR (100 MHz) δ 21.10, 21.27, 21.34, 30.65 (CH_3 at C1, C5, C9), 33.38 (C5), 38.24 (C1), 49.29, 55.84 (C2, C4), 56.87 (C6), 61.89 (C6′), 68.28 (C4′), 71.74, 71.86, 72.84 (C2′, C3′, C5′), 77.82 (C9), 99.61 (C1′), 130.70, 135.48 (C7, C8), 210.55 (C3), 20.58–20.72 and 169.17–170.60 (4 acetates) ^1H NMR data for the aglycon moiety are in good agreement with previously published data (Sefton et al., 1990).

3,4-Dihydroxy-7,8-dihydro-β-ionone 3-O-β-D-Glucopyranoside (13): isolated as its pentaacetate (5.2 mg); TS–MS pseudomolecular ion at m/z 616 $[M + NH_4]^+$ indicating a molecular mass of 598 ($C_{29}O_{13}H_{42}$); ^1H NMR (400 MHz, $CDCl_3$) δ 1.05 and 1.06 (6H, 2s, 2 × CH_3-1), 1.58 (3H, s, CH_3-5), 1.67 (1H, br dxd, J = 12.7, 2.3, H_a-2), 1.87 (1H, t, J = 12.7, H_b-2), 1.99–2.08 (15H, 5s, 5 acetates), 2.15 (3H, s, CH_3-9), 2.17–2.55 (4H, m, H_2-7), H_2-8), 3.69 (1H, m, H-5′), 3.84 (1H, br dxt, J = 12.7, 3.8, H-3), 4.16 (2H, br d, J = 3.8, H_2-6′), 4.63 (1H, d, J = 7.8, H-1′), 4.95 (1H, dxd, J = 9.5, 7.8, H-2′), 5.01 (1H, dxd, J = 9.9, 9.4, H-4′), 5.18 (1H, dxd, J = 9.5, 9.4, H-3′), 5.36 (1H, br d, J = 3.8, H-4); ^{13}C NMR (100 MHz) δ 17.25 (CH_3-C5), 21.98 (C7), 26.82 and 28.95 (2 × CH_3-C1), 29.81 (CH_3-C9), 37.92 (C1), 40.56 (C2), 43.36 (C8), 62.37 (C6′), 68.73 (C4′), 70.22 (C4), 71.08 (C2′), 71.69 (C5′), 72.84 (C3′), 74.49 (C3), 100.79 (C1′), 124.09 (C5), 144.26 (C6), 207.80 (C9), 20.6–21.0 and 169.7–171.1 (5 acetates).

3,4-Dihydroxy-β-ionone 3-O-β-D-Glucopyranoside (14): isolated as its pentaacetate (3.2 mg); TS–MS pseudomolecular ion at m/z 614 $[M + NH_4]^+$ indicating a molecular mass of 596

($C_{29}O_{13}H_{40}$); ^1H NMR (400 MHz, $CDCl_3$) δ 1.10 and 1.11 (6H, 2s, 2 × CH_3-1), 1.57 (3H, s, CH_3-5), 1.75 (1H, br dxd, J = 12.7, 3.6, H_a-2), 1.93 (1H, t, J = 12.7, H_b-2), 1.99–2.07 (15H, 5s, 5 acetates), 2.30 (3H, s, CH_3-9), 3.70 (1H, dxt, J = 9.9, 4.1, H-5′), 3.91 (1H, br dxt, J = 12.7, 3.6, H-3), 4.17 (2H, br d, J = 4.1, H_2-6′), 4.65 (1H, d, J = 7.9, H-1′), 4.96 (1H, dxd, J = 9.5, 7.9, H-2′), 5.01 (1H, dxd, J = 9.9, 9.6, H-4′), 5.18 (1H, dxd, J = 9.6, 9.5, H-3′), 5.47 (1H, br d, J = 3.6, H-4), 6.10 (1H, d, J = 16.4, H-8), 7.12 (1H, d, J = 16.4, H-7); ^{13}C NMR (100 MHz) δ 18.94 (CH_3-C5), 27.19 and 27.51 (2 × CH_3-C1), 29.61 (CH_3-C9), 37.01 (C1), 40.48 (C2), 62.34 (C6′), 68.76 (C4′), 69.55 (C4), 71.08 (C2′), 71.78 (C5′), 72.86 (C3′), 74.13 (C3), 100.86 (C1′), 127.62 (C5), 133.86 (C8), 141.20 (C7), 142.19 (C6), 197.82 (C9), 20.6–21.0 and 169.5–171.1 (5 acetates).

Zingerone Tetra-O-acetyl-β-D-glucopyranoside (**15**): 3.0 mg; ^1H NMR (400 MHz, $CDCl_3$) δ 2.03, 2.04, 2.07, 2.08, (4 × 3H, 4s, acetates), 2.14 (3H, s, H_3-1), 2.74 (2H, br t, J = 7, H_2-3), 2.84 (2H, br t, J = 7, H_2-4), 3.74 (1H, dxdxd, J = 9.8, 4.9, 2.5, H-5′), 3.80 (3H, s, CH_3O-7), 4.15 (1H, dxd, J = 12.2, 2.5, H_a-6′), 4.27 (1H, dxd, J = 12.2, 4.9, H_b-6′), 4.90 (1H, dxd, J = 7.9, H-1′), 5.16 (1H, dxd, J = 9.8, 9.5, H-C4′), 5.25–5.27 (2H, m, H-2′/H-3′), 6.67 (1H, dxd, J = 8.1, 2.0, H-10), 6.72 (1H, d, J = 2.0, H-6), 7.01 (1H, d, J = 8.1, H-9); ^{13}C NMR (100 MHz) δ 29.69 (C4), 45.16 (C3), 56.01 (CH_3O-C7), 61.94 (C6′), 68.44 (C4′), 71.21, 71.90, 72.63 (C2′/C3′/C5′), 100.98 (C1′), 112.99, 120.27, 120.38 (C6/C9/C10), 137.84, 144.38, 150.55 (C5/C7/C8), 207.77 (C2), 20.6 and 169.37–170.62 (4 acetates).

RESULTS AND DISCUSSION

The continuing studies on the formation of the off-flavor compound TDN (1) in wine (Winterhalter, 1991) required the application of a gentle preparative separation technique to fractionate glycosidic isolates obtained from Riesling vine leaves. The Riesling leaves were found to generate TDN in concentrations up to 1 mg/kg under SDE (pH 3.2) conditions. They were therefore considered a convenient substrate for the study of TDN-yielding progenitors. The all-liquid separation technique of MLCCC was selected due to the following reasons: (i) Compared with other CCC techniques, MLCCC is reported to possess a clearly enhanced resolving power (Ito, 1986). (ii) Using large-diameter coils, MLCCC enables preparative separations in a short time. (iii) There are no restrictions in the selection of biphasic solvent systems. (iv) most important, separations by MLCCC benefit from the absence of solid adsorbents; i.e., adsorption losses and formation of artifacts caused by active surfaces are eliminated. These advantages permit particularly gentle isolations, thus making MLCCC ideally suited to the analysis of labile natural compounds, such as, e.g., the sensitive TDN progenitors.

In the present study, MLCCC separations were carried out with a multilayer coil separator–extractor. This apparatus uses a Teflon tube, which is wrapped around a holder in several layers, as the separating column. This so-called "multilayer coil" contains a biphasic solvent mixture, which, due to the rotation of the coil in a planetary system, is exposed to quickly changing centrifugal field forces, thus providing a highly efficient partitioning of the solutes. The apparatus was originally designed by Ito et al. (1982). Detailed descriptions of the apparatus can be found in the literature (Conway, 1990; Ito, 1986; Mandava and Ito, 1988).

Separation of Model Glycosidic Mixtures. In preliminary experiments, the preparative capability as well as the separation efficiency of MLCCC was tested with model glycosides. The *preparative capability* of the apparatus was examined with a model mixture of phenolic glucosides, i.e., phenyl-β-D-glucose (**4**), salicin (**5**), and arbutin (**6**) (cf. Figure 1). For this separation a "preparative" coil, i.e., a 75 m × 2.6 mm i.d. PTFE tubing, was used. The mixture (300 mg of each compound) was baseline separated in less than 2 h using a standard solvent system for glycosides. In our subsequent analyses up to 2 g of glycosidic Riesling leaf isolates was successfully separated with the same type of column.

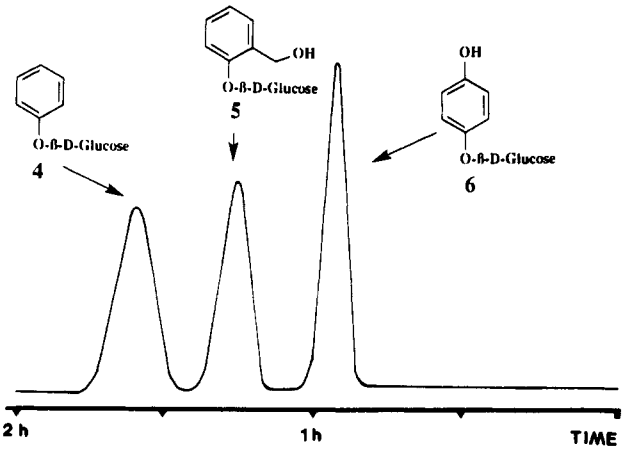
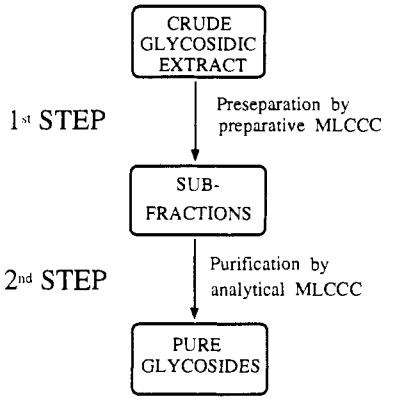


Figure 1. MLCCC separation of phenolic β-D-glucopyranosides **4–6** (900 mg) using the preparative (2.6 mm i.d.) coil. Conditions: solvent system, $CHCl_3$–MeOH–H_2O (7:13:8); mobile phase, upper phase; flow rate, 1.5 mL/min; rotational speed, 800 rpm; UV detection, 220 nm.

Scheme I. Two-Step CCC Procedure for the Purification of Glycosides Consisting of an Initial Preparative MLCCC Separation of Crude Plant Extracts Followed by an Analytical-Scale Separation of Glycosidic Subfractions



We then investigated the possibility to further purify the preseparated glycosidic fractions according to Scheme I. For the purification step the MLCCC apparatus was equipped with an "analytical" coil, i.e., a 160 m × 1.6 mm i.d., PTFE tubing. Appropriate solvent systems were selected in accordance with the rules given by Oka et al. (1991): (i) For efficient separations, the partition coefficient (K) of the target compound should be close to 1. (ii) For satisfactory retention of the stationary phase, settling times of the solvent systems should be shorter than 30 s. (iii) To avoid excessive waste of one solvent, the mixture should provide equal volumes of both phases.

An example indicating the importance in choosing suitable solvent systems is outlined in Figure 2. An initial separation of nitrophenyl glycosides **7–10** using the preparative coil and the standard $CHCl_3$–MeOH–H_2O solvent system did not resolve the nitrophenyl glucoside **9** from the respective galactoside **10**. For these two structures an optimized system was selected according to the rules discussed above. Figure 2 shows the initial MLCCC separation with the preparative coil and the subsequent separation of the unresolved peak using analytical MLCCC and EtOAC–BuOH–H_2O (3:2:5) as

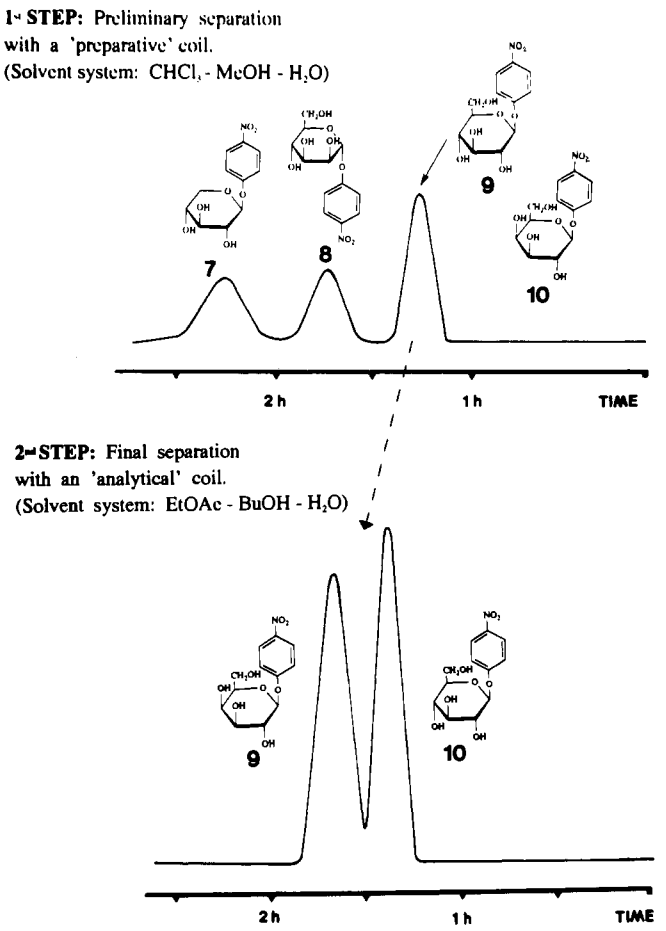


Figure 2. MLCCC separation of 4-nitrophenyl glycosides [4-nitrophenyl β-D-xylopyranoside (**7**), 4-nitrophenyl α-D-mannopyranoside (**8**), 4-nitrophenyl β-D-glucopyranoside (**9**), and 4-nitrophenyl β-D-galactopyranoside (**10**)]. First step: Presepparation with the preparative (2.6 mm i.d.) coil; solvent system, $CHCl_3$–MeOH–H_2O (7:13:8); mobile phase, upper phase; flow rate, 1.5 mL/min; rotational speed, 800 rpm. Second step: separation of the unresolved peak of glycosides **9**/**10** with the analytical (1.6 mm i.d.) coil; solvent system, EtOAc–BuOH–H_2O (3:2:5); mobile phase, upper phase; flow rate, 2.5 mL/min; rotational speed, 800 rpm; UV detection in both cases, 220 nm.

solvent system. In the latter case the glycosides, which differ only in the configuration of the hydroxyl group at C4, are nearly base-line separated in less than 2 h. This clearly demonstrates the *separation efficiency* of this technique.

Separation of Riesling Leaf Glycosides. The two-step CCC procedure (cf. Scheme I) was then also applied to the separation of glycosidic isolates obtained from *V. vinfera* cv. Riesling leaves. In an effort to identify genuine precursors of the off-flavor compound TDN (**1**), a preliminary separation of the glycosidic mixture was carried out with the preparative coil. The large-scale prefractionation was achieved within 4 h, using $CHCl_3$–MeOH–H_2O (7:13:8) as solvent system and the less dense layer as mobile phase. The separation was monitored by UV detection and also checked by TLC. MLCCC fractions with similar R_f values were then pooled in eight combined fractions, i.e., MLCCC fractions I–VIII. These combined fractions were first screened (SDE pH 3.2) for their potential to generate TDN and then further purified with the analytical coil using appropriate solvent systems. A representative example for the purification of major vine leaf glycosides is shown in Figure 3. For this analytical separation EtOAc–BuOH–H_2O (3:2:5) was used as solvent system with the more dense layer acting as mobile phase.

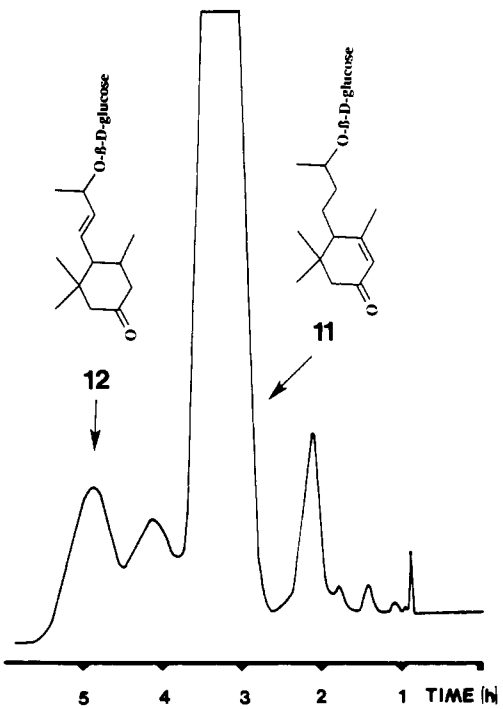


Figure 3. Separation of MLCCC fraction V with the analytical (160 m × 1.6 mm) coil. Conditions: solvent system, EtOAc–BuOH–H_2O (3:2:5); mobile phase, lower phase; flow rate, 2.5 mL/min; rotational speed, 800 rpm; UV detection, 190 nm.

The main peak yielded an almost pure compound (132 mg, purity >95%) which by means of its ^1H and ^{13}C NMR data was identified as 3-oxo-7,8-dihydro-α-ionyl (Blumenol C) β-D-glucopyranoside (**11**). Concerning the stereochemistry at C6, CD data revealed *R* configuration. Enzymatic hydrolysis (sweet almond emulsin) liberated the C_{13} norterpenoid ketoalcohol **11a** together with small amounts (3%) of 3-oxo-α-ionol (**16a**). Minor signals in the NMR spectrum of compound **11**, i.e., at 5.57 (dxd) and 5.87 ppm (br s), were therefore assigned to arise from the respective 3-oxo-α-ionyl β-D-glucopyranoside (**16**).

In addition to the major glucoside **11**, MLCCC fraction V also yielded the structurally related compound **12**. 3-Oxo-4,5-dihydro-α-ionyl β-D-glucopyranoside (**12**) was obtained in high purity when it crystallized. Glucoside **12** is a potential precursor of isomeric megastigma-6,8-dien-3-ones, aroma volatiles most recently identified in quince brandy (Näf et al., 1990). The known flavor properties of the latter ketones add significance to the identification of glucoside **12**, which is reported for the first time in nature. β-Glucosidase treatment of the mother liquor of **12** liberated two isomers of 3-oxo-*retro*-α-ionol (**17a**), thus indicating the occurrence of the respective β-D-glucoside **17** in Riesling leaves. The purification of these minor glycoconjugates, which are apparently also involved in flavor formation (Schumacher, 1968), and experiments for clarifying the absolute stereochemistry of aglycon **12a** are the subjects of continuing research.

Contrary to major vine leaf constituents such as glucosides **11** and **12**, which could be obtained in high purity after the two-step MLCCC procedure, less abundant glycosides—including the targeted TDN progenitors—were more difficult to analyze. A considerable generation of **1** was observed from MLCCC fraction IV. Hence, a purification of this complex fraction by analytical MLCCC with EtOAc–BuOH–H_2O-based solvent systems was attempted. In this case, however, the resolving power of MLCCC did not allow the final purification of the precursor glycoside. NMR data still indicated the presence of two

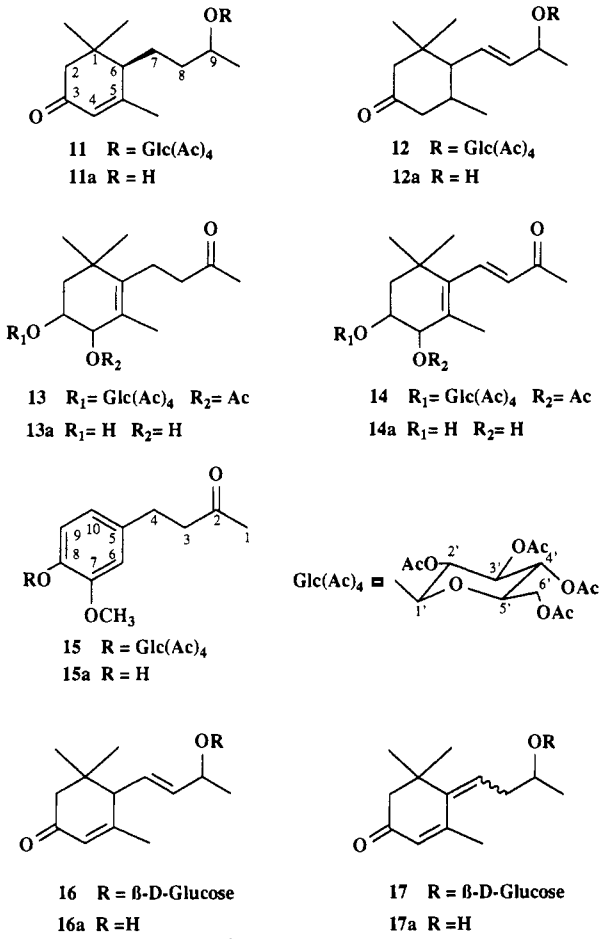


Figure 4. Structures of Riesling leaf glucosides 11–15, as well as the tentatively identified conjugates 16 and 17.

additional glycosides as impurities. It was therefore necessary to peracetylate the mixture and to use analytical HPLC for further analysis. Normal-phase HPLC successfully separated three peaks, among which the major one was the known TDN precursor 3,4-dihydroxy-7,8-dihydro-β-ionone 3-*O*-β-D-glucopyranoside (**13**). Glucoside **13** was first identified in *Epimedium diphyllum* (Miyase et al., 1989) and more recently isolated from red currant leaves (Humpf et al., 1991). Model hydrolytic studies with aglycon **13a** confirmed its role in the formation of **1**. In addition to **13** the structurally related compound 3,4-dihydroxy-β-ionone 3-*O*-β-D-glucopyranoside (**14**) as well as zingerone β-D-glucopyranoside (**15**) were identified (cf. Figure 4). These new vine leaf glucosides which have previously been isolated from *Sonchus asper* (Shimizu et al., 1989) and the needles of *Pinus contorta* (Higuchi and Donnelly, 1977), respectively, are again potential flavor precursors. The rearranged form of aglycon **14a**, i.e., 3,6-dihydroxy-α-ionone, yielded the odoriferous ketone 4-(2′,3′,6′-trimethyl)-but-3-en-2-one as a major breakdown product (Winterhalter, 1992), and zingerone (**15a**) is the well-known pungent principle of ginger root (Connell and Sutherland, 1969).

Conclusion. Due to the gentle operation conditions, the application of CCC techniques is steadily increasing. It has been demonstrated in this study that the combination of preparative MLCCC with analytical MLCCC facilitates the analysis of aroma-relevant glycoconjugates in vine leaves. Major vine leaf glycoconjugates could be obtained by this approach in high purity, thus enabling immediate structure elucidation by NMR methods. For less abundant glycosides, including the TDN-generating glucoconjugate **13**, acetylation and subsequent HPLC purification had to be carried out. Contrary to Riesling wine in which disaccharidic conjugates predominate, the most abundant conjugates identified so far in Riesling leaves are monosaccharides. Nevertheless, mass spectral data indicate that—to a lesser extent—disaccharidic conjugates are also present in the leaves. Thus, future application of MLCCC will be directed to the investigation of the more polar glycosidic fractions of Riesling leaves.

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