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Generation of (*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene from C_{13}-Norisoprenoid Precursors

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Three C_{13}-norisoprenoid compounds, 3,6,9-trihydroxymegastigma-4,7-diene (**6**), 3,4,9-trihydroxymegastigma-5,7-diene (**4**), and the actinidols (**8**), have all been synthesized and subjected to acid hydrolysis. All three were shown to generate (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (**1**) under wine conservation conditions. At 45 °C, approximately 4000−5000 ng/L of **1** was formed from 1.0 mg/L of precursor, after 173 days, while at 25 °C more wine-like amounts (200−600 ng/L) were observed. A glucoside, 4,5-dihydrovomifoliol-C_9-β-D-glucopyranoside (**9b**), was isolated from grapevine leaves by multilayer coil countercurrent chromatography (MLCCC), and its stereochemistry was deduced as being (5*R*, 6*S*, 9*R*) by NMR and CD spectroscopy. Hydrolysis of this glucoside produced **1**, but in quantities insufficient to account for the levels observed in wine.

KEYWORDS: TPB; SIDA; polyphenols; grapes; precursors; C_{13}-norisoprenoids; MLCCC.

INTRODUCTION

(*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene (**1**, Figure 1) is a wine component which we recently identified in white wines (*1*). The aroma detection threshold of this compound was 40 ng/L in white wine, which places it among the most potent of wine odorants. It was generated by the heating of crude glycosidic extracts of both red and white varieties under acidic conditions (*1*). While **1** was found in all the crude glycoside hydrolysates, it was found only in white wine (at concentrations up to ∼250 ng/L), suggesting that there could be a chemical basis for its presence in the glycosidic fractions but not in red wine itself (*2*). We have subsequently shown that **1** reacts rapidly and immediately with wine polyphenols, which could well account for its absence in red wine. Studies on the occurrence and stability of **1** revealed that it is probably formed during wine aging, very like its C_{13}-norisoprenoid isomer 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN, **2**) (*2*).

C_{13}-Norisoprenoids are believed to arise from degradation of carotenoid precursors, and commonly accumulate in grapes as glycoconjugates (*3*). These glycoconjugates can then undergo acid-catalyzed transformations to produce other C_{13}-norisoprenoids. For example, Humpf et al. (*4*) have shown that TDN is generated from the glycoside **3** by heating at pH 2.5 under simultaneous distillation-extraction (SDE) conditions. Winterhalter and colleagues (*5*) have shown that both TDN and damascenone are generated from multiple precursor forms which appeared to be glycoconjugates involving different conjugating moieties.

It has previously been established that 3,4,9-trihydroxymegastigma-5,7-diene (**4**) as well as the dihydro analogue **5** are present in grapes, most probably as glycoconjugates (*6*). However, the rearranged compounds **6** and **7** have yet to be identified in grapes. Plucheoside B (triol **4** with β-D-glucose at C_3) was identified in the aerial part of *Pluchea indica* (*7*). Two monosaccharides of **4** [plucheoside B and alangionoside C (β-D-glucose at C_4)] as well as a disaccharide [ebracteatosie A (apiose and β-D-glucose at C_9)] were identified by Kanchanapoom et al. (*8*) in a Thai medicinal plant, *Acanthus ebracteatus*. Strauss et al. (*6*) investigated the hydrolytic behavior of both **4** and **6** to try and rationalize the formation of several C_{13}-norisoprenoids (Figure 2). Interestingly, one of the hydrolysis products they observed was an unknown (referred to by them as "compound 20") which had a similar mass spectrum to that of **1**. Mechanistically it would be quite straightforward for **1** to form from the triols **4** and **6**. The triols contain three allylic (or potentially allylic) alcohols that would be amenable to acid-catalyzed processes such as dehydration. Hydrolytically, triol **6** was shown to produce a complex mixture of products, including triol **4**, TDN (**2**), the actinidols (**8**), and several minor compounds, including the aforementioned "unknown 20". The actinidols (**8**) have been observed as volatiles in bottle-aged white wines, brandies, and heated muscat grape juices and in grapes (*9*), as well as in the acid hydrolysates of grape and wine glycoside extracts (*10*−*12*). They were originally isolated from

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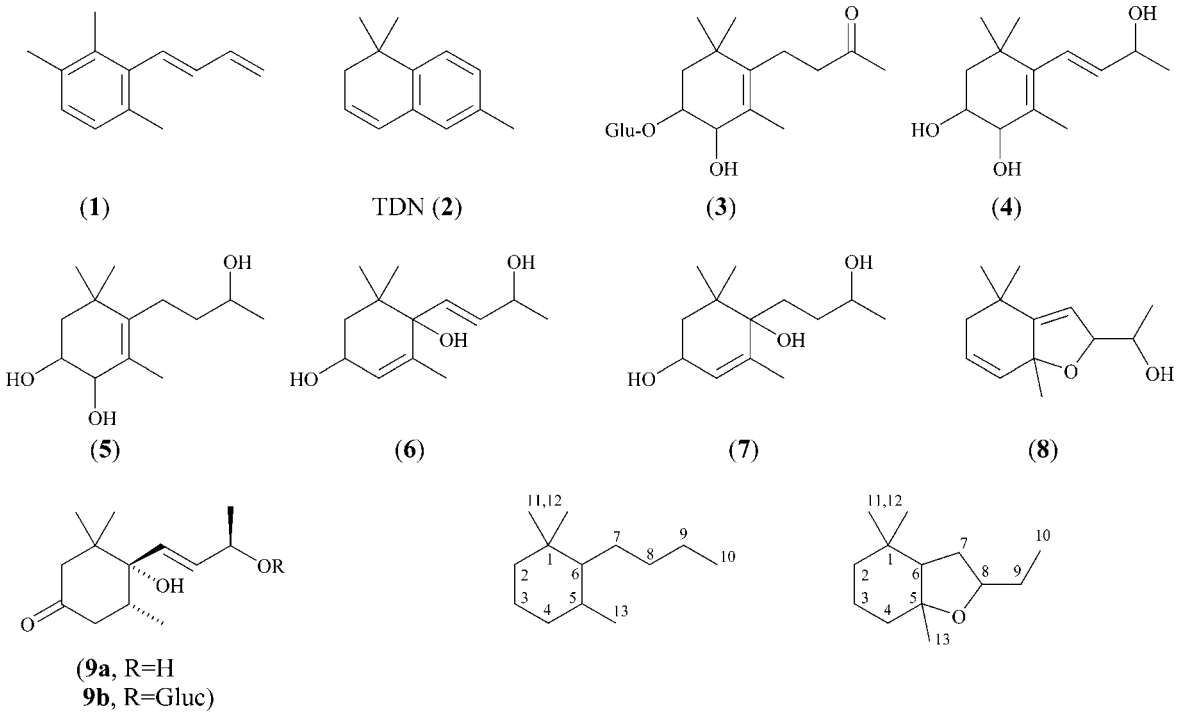


Figure 1. Structures (and numbering scheme) of compounds referred to in the text.

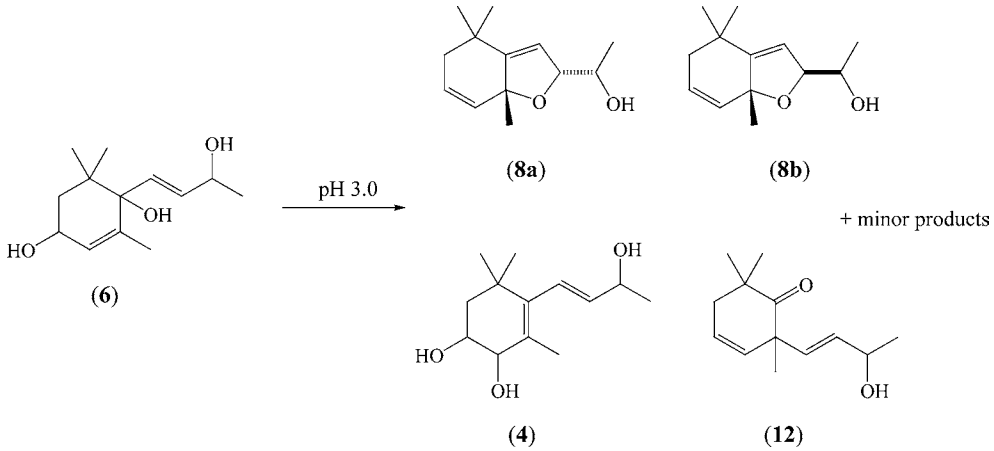


Figure 2. Hydrolysis products of **6**, as reported by Strauss et al. (*6*).

the leaves of *Actinidia polygama* by Sakan et al. (*13*) who also synthesized these compounds (*14*), although they did not determine the stereochemistry of either the natural or synthetic products. Subsequently, Dimitriadis et al. (*9*) used the same synthetic approach and reported that four isomeric actinidols were formed in a ratio of 40:53:3:4. They subsequently confirmed that the two major, naturally occurring, grape-derived isomers of the actinidols had a *trans* relationship between the C_5-methyl and the C_8-hydroxyethyl (*9*). Each of the three compounds **4**, **6**, and **8** could be considered potential precursors to **1**. This study was undertaken to determine the role of these three in generating **1** under wine conservation conditions.

MATERIALS AND METHODS

General. NMR spectra were recorded as solutions in chloroform-*d* and were obtained on a Varian Gemini spectrometer operating at either 300 MHz (^{1}H) or 75.5 MHz (^{13}C). Circular dichroism (CD) spectra were recorded on an Applied Photophysics π*-180 CD spectrometer, and specific rotations were recorded on a PolAAr 21 polarimeter. All reagents were purchased from Sigma-Aldrich. All solvents were of the highest commercial grade available. Diethyl ether and THF were distilled from sodium/benzophenone immediately prior to use. All organic solutions were dried over anhydrous sodium sulfate prior to filtration. Unlabeled (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (**1**) was prepared as described by Janusz et al. (*1*). Crude glycosidic extracts (from both fruit and leaves) were prepared as described earlier (*1*). GC−MS−O analysis of hydrolysates was conducted as described earlier. (*1*). GC−MS analysis of acid hydrolysates and quantification of **1** were conducted as described elsewhere (*2*). Buffered model wine solutions were prepared by saturating a 10% solution of ethanol in water with potassium hydrogen tartrate and adjusting the pH to the desired value with 10% aqueous tartaric acid solution.

Isolation of Glycoside 9b by Multilayer Coil Countercurrent Chromatography (MLCCC). *Separation 1: Gradient Chloroform/Methanol/Water (7/13/8 to 7/1/8).* The MLCCC apparatus was a Quattro (Analytical and Environmental Consultancy Services (AECS), Bridgend, UK) equipped with 4 coils on 2 holders (bobbins of 190 mm diameter). Each bobbin was composed of 2 coils (1.6 mm i.d. Teflon tubing) with volume capacities of 100 mL and 250 mL, respectively. The temperature of the cabinet containing the different coils was controlled by a thermostated cooling system (Ratek circulated cooling device), (Adelab, Australia). The whole system was equipped with a back-pressure regulator (250 psi) (Upchurch Scientific, New Zealand) set upstream of the bobbins. The individual separations were performed using one

100 mL coil only. The revolution speed was set at 800 rpm, the temperature was kept at 25 °C, and the flow rate was delivered at 1.5 mL/min by a Waters 600E pump. The elution was monitored by a GBC LC1210 UV−vis detector (320 nm) and UPC-900 detector (254 nm) (Amersham Pharmacia Biotech, Sweden). Chromatograms were recorded using Agilent Chemstation software. The Riesling glycosidic extract (approximately 300−400 mg) was dissolved in a (50/50 v/v) mixture of the upper and lower phases of the solvents, filtered through a 0.45 μm filter (Millipore) prior to injection through a (2 mL) Rheodyne injection loop. A methanol-gradient solvent system consisting of chloroform/methanol/water (7/*x*/8) was used. The proportion of methanol was reduced, at 30 min intervals, from 13 parts to, successively, 10, 7, 4, and one part (i.e., from $x = 13$ to $x = 1$, via $x = 10$, $x = 7$, and $x = 4$). The solvents were sonicated for 30 min before use. The elution mode of the mobile phase was tail-to-head, with the less dense layer (aqueous) being the mobile phase. Eighty fractions (3 mL) were collected with a fraction collector. Fractions were also screened by enzyme hydrolysis, followed by GC−MS analysis. In total, 9 runs of approximately 300−400 mg material per run were performed and combined based on their 254 nm detection. The average stationary phase retention was 88% before injection and 50% after injection.

Separation 2: Isocratic Chloroform/Methanol/Water (7/13/8). Fractions 11−25 (approximately 800−900 mg) from separation 1 were further separated on a 200 mL coil (2 × 100 mL coils joined in tandem) as per separation 1, but an isocratic solvent system was used with 13 parts methanol. The stationary phase retention was 50% before injection, and 24% after injection. A total of 120 fractions (3 mL) were collected.

Separation 3: Isocratic Butanol/Ethyl Acetate/Water (2/3/5). Fractions 51−60 from separation 2 (approximately 200−300 mg) were further purified on a 200 mL analytical column coil (2 × 100 mL coils joined in tandem) as per separation 1. However, an isocratic butanol/ethyl acetate/water (2/3/5) solvent system was used. The stationary phase retention before injection was 89% and 82% after injection. A total of 110 fractions (5 mL) were collected. The above 3 separations resulted in a clear oil for fractions 56−63 (31 mg, >95% purity) which was found to be 4,5-dihydrovomifoliol-β-D-glucopyranoside (**9b**). The spectroscopic data for **9b** was consistent with that reported by Inada et al. (*18*).

Enzyme Hydrolysis. The glycosidic extract (10−15 mg) was dissolved in pH 5 phosphate−citrate buffer (0.5 mL) and an equivalent mass of AR 2000 glycosidase enzyme preparation was added. The vial was capped under nitrogen, and the solutions were incubated for 16 h at 40 °C. The aglycones were then extracted with dichloromethane: pentane (1:2; 5 × 1 mL) and diethyl ether:dichloromethane (3:1; 2 × 1 mL) and filtered, and the organic portion was dried through a plug of cotton wool containing Na_2SO_4. Internal standard (25 μL of 600 mg/L 3,5-dimethylphenol) was added, and the extract was then analyzed directly by GC−MS.

4,5-Dihydrovomifoliol (9a). The glycoside (**9b**) (10 mg) was treated with AR 2000 as described above. δ_H ($CDCl_3$): 5.84 (1H, dd, J = 15.8, 5.6, H_8), 5.70 (1H, dd, J = 15.8, 1.1, H_7), 4.44 (1H, app quin, J = 6.1, H_9), 2.84 (1H, d, J = 13.5, H_{2a}), 2.15−2.50 (3H, m, $H_{4a,4b,5}$), 1.92 (1H, dd, J = 13.5, 2.2, H_{2b}), 1.33 (3H, d, J = 6.5, H_{10}), 0.97, 0.94 (6H, 2xs, $H_{11,12}$), 0.88 (1H, d, J = 6.5, H_{13}). MS *m*/*z* (%): 208 ((M−H_2O)$^+$, 9), 193 (2), 169 (7), 165 (13), 152 (9), 141 (31), 129 (27), 124 (33), 111 (31), 109 (51), 97 (33), 95 (42), 85 (100), 71 (71), 55 (35), 43 (71).

3,6,9-Trihydroxymegastigma-4,7-diene (6). Dehydroionone (**10**) (0.404 g, 2.13 mmol) was converted into 3,6-dihydroxymegastigma-4,7-dien-9-one (**11**) (299 mg, 63%) as described by Strauss et al. *(6).* A portion of this product (150 mg, 0.67 mmol) was dissolved in methanol (10 mL) and $CeCl_3 \cdot 7H_2O$ (93.5 mg, 0.25 mmol) and $NaBH_4$ (18 mg, 0.48 mmol) were added. After 10 min water (10 mL) was added slowly and the solution was saturated with NaCl. The reaction mixture was extracted with dichloromethane and diethyl ether, the extracts were combined, dried with Na_2SO_4 and concentrated in vacuo to yield a pale yellow oil (91 mg). The crude reaction mixture was purified by two silica chromatography columns (eluant, ethyl acetate) to yield the dienes (**6**) as a mixture of diastereomers (26 mg, 17%). δ_H ($CDCl_3$): (major isomer) 6.00−5.25 (3H, m, $H_{4,7,8}$), 4.50−4.00 (2H, m, $H_{3,9}$), 2.80−2.15 (3H, br s, 3O**H**), 1.88−1.44 (5H, m, $H_{2,13}$), 1.26 (3H, d, J = 6.5, H_{10}), 0.98, 0.89 (6H, 2s, $H_{11,12}$). δ_H (d_6−acetone): (major isomer) 5.84 (1H, ddd, J = 15.6, 5.9, 2.3, H_8), 5.61 (1H, dd, J = 15.5, 1.0, H_7), 5.48 (1H, m, H_4), 4.44−4.08 (2H, m, $H_{3,9}$), 3.88−3.28 (3H, br s, 3O**H**), 1.80−1.50 (5H, m, $H_{2,13}$), 1.22 (3H, d, J = 6.5, H_{10}), 0.99, 0.88 (6H, 2 s, $H_{11,12}$).

3,4,9-Trihydroxymegastigma-5,7-diene (4). The triols (**6**) (111 mg, 0.49 mmol) were dissolved in pH 3.0 aqueous buffer solution (14 mL) and heated on a boiling water bath in a stoppered round-bottom flask for 15 min. The reaction mixture was cooled and extracted with dichloromethane (2 × 10 mL) and chloroform (12 × 10 mL). The organic layers were combined, dried, and concentrated in vacuo to yield a yellow oil (93 mg). The crude reaction mixture was subjected to 2 silica chromatography columns (column 1 eluant, methanol/dichloromethane gradient; column 2 eluant, ethyl acetate) to give the triols (**4**) as a mixture of diastereomers (39 mg, 35%). δ_H ($CDCl_3$): (major isomer) 6.00 (1H, br d, J = 16.0, H_7), 5.53 (1H, dd, J = 16.0, 6.3, H_8), 4.37 (1H, app quin, J = 6.3, H_9), 4.00−3.70 (2H, m, $H_{3,4}$), 2.34 (3H, br, 3 O**H**), 1.82 (3H, br s, H_{13}), 1.76−1.40 (2H, m, $H_{2a,2b}$), 1.30 (3H, d, J = 6.3, H_{10}), 1.04, 1.01 (6H, 2s, $H_{11,12}$). δ_H (d_4-MeOH): (major isomer) 6.07 (1H, d, J = 16.1, H_7), 5.40 (1H, ddd, J = 16.1, 6.2, 1.7, H_8), 4.05 (1H, app quin, J = 6.3, H_9), 3.87 (1H, br d, J = 3.3, H_4), 3.79 (1H, m, H_3), 1.86 (3H, br s, H_{13}), 1.78 (1H, m, H_{2a}), 1.45 (1H, ddd, J = 12.3, 3.6, 1.3, H_{2b}), 1.29 (3H, d, J = 6.3, H_{10}), 1.07, 1.10 (6H, 2s, $H_{11,12}$).

A second fraction (31 mg) was also isolated with the hydroxyketone **12** as the major product, and the diastereomeric actinidols (**8a**/**b**) were isolated as a minor component. The spectroscopic data for **12** was in agreement with that of Strauss et al. (*6*).

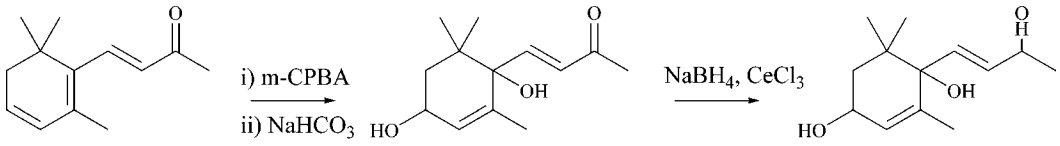
Actinidols (8). The triols (**6**) (70 mg, 0.31 mmol) were dissolved in pH 3.0 aqueous buffer solution (7 mL) and heated under reflux for 30 min. The reaction mixture was then cooled to room temperature and extracted with diethyl ether (2 × 5 mL). The hydrolysis and extraction step was then repeated 7 times. The organic layers were combined, dried, and concentrated in vacuo to yield the crude product (60 mg). This was purified by two silica chromatography columns (column 1 elutant, ethyl acetate; column 2 elutant, 30% ethyl acetate in pentane) to give the actinidols (**8a/b**) as a mixture of diastereomers (8 mg, 13%), in approximately a 1.3 to 1 ratio. The hydroxyketone (**12**) (18 mg, 31%) and diastereomeric 3,4,9-trihydroxymegastigma-5,7-dienes (**4**) (25 mg, 36%) were also isolated. δ_H ($CDCl_3$): (major isomer of **8**, consistent with isomer (Kovats index (KI) 1200) reported by Dimitriadis et al. (*9*)) 5.78 (1H, m, H_4), 5.62 (1H, dt, J = 10.3, 3.2, H_3), 5.30 (1H, br s, H_7), 4.60 (1H, dd, J = 4.9, 1.1, H_8), 3.60 (1H, qd, J = 6.3, 4.9, H_9), 2.01 (2H, dd, J = 3.2, 1.4, H_2), 1.44 (3H, s, H_{13}), 1.22 (3H, s, H_{11}), 1.17 (3H, d, J = 6.3, H_{10}), 1.14 (3H, s, H_{12}). δ_C ($CDCl_3$): 154.0, 132.3, 126.2, 117.5, 86.6, 86.2, 69.9, 43.3, 34.1, 28.7, 26.7, 26.4, 19.2. MS *m*/*z* (%): 193(2), 175(1), 164(14), 163(100), 157(1), 149 (12), 145-(13), 135(5), 131(5), 121(14), 119(7), 107(11), 105(12), 93(10), 91-(12), 79(10), 77(10), 55(6), 43(23).

δ_H ($CDCl_3$): (minor isomer of **8**, consistent with isomer (KI 1211) reported by Dimitriadis et al. *(9)*) 5.79 (1H, m, H_4), 5.59 (1H, dt, J = 10.0, 3.2, H_3), 5.37 (1H, br s, H_7), 4.75 (1H, dd, J = 3.0, 1.1, H_8), 3.81 (1H, qd, J = 6.6, 3.1, H_9), 2.01 (2H, dd, J = 3.2, 1.4, H_2), 1.45 (3H, s, H_{13}), 1.23 (3H, s, H_{11}), 1.15 (3H, d, J = 6.6, H_{10}), 1.14 (3H, s, H_{12}). δ_C ($CDCl_3$): 154.5, 132.0, 126.7, 115.5, 86.3, 86.0, 68.4, 43.3, 34.2, 28.6, 26.6, 26.2, 17.5. MS *m*/*z* (%): 193(3), 175(1), 164(15), 163-(100), 157(1), 149 (12), 145(13), 135(4), 131(4), 121(13), 119(7), 107-(9), 105(12), 93(9), 91(10), 79(6), 77(7), 55(3), 43(13).

Generation of 1 by Acid Hydrolysis. Solutions of each of the compounds, **4**, **6**, and **8**, as well as glycoside **9b**, were prepared by dissolving the particular compound (1.0 mg/L) in pH 3.2 model wine. The solutions were transferred into glass ampules and sealed under nitrogen. The ampules were heated at 25 °C, 45 °C or 100 °C. Duplicate ampules were removed at selected times, opened, and the concentration of **1** determined (*2*).

RESULTS AND DISCUSSION

Isolation of Pure 4,5-Dihydrovomifoliol-C_9-β-D-glucopyranoside. MLCCC was employed for the isolation of individual



(**10**) (**11**) (**6**)

Figure 3. Synthesis of triol **6**.

glycoside **9b** from a crude glycosidic mixture (*15*). GC−MS analysis (after enzyme hydrolysis) revealed that aglycone **9a** was a major component of all the crude glycosidic extracts studies, comprising both red and white varieties. As this particular component was most abundant in a 2001 South Australian Riesling leaf extract, this particular extract was chosen for MLCCC separation. The solvents and conditions used were adapted from those of Skouroumounis and Winterhalter (*16*).

The identity of the aglycone, 4,5-dihydrovomifoliol (**9a**), was confirmed by NMR and GC−MS analysis of the product after enzyme hydrolysis, and the latter was in broad agreement with that of Sefton et al. (*17*). The sugar unit in **9b** was assigned as β-D-glucopyranoside based on comparison of the ^{13}C and ^{1}H NMR data with the literature (*18,19*). Inada et al. (*18*) established, with the aid of NOESY NMR experiments, that the β-D-glucopyranose was attached at C_9. As our NMR data were identical with those reported, the sugar was also determined to be attached at the C_9 position.

The circular dichroism (CD) spectrum of the glycoside **9b** showed a positive Cotton effect at 290 nm (methanol) as was observed by both Inada et al. (*18*) and Otsuka et al. (*19*), as well as Tamaki et al. (*20*) for the analogous disaccharide. On the basis of application of the octant rule (*21, 22*), they determined the absolute stereochemistry at C_5 and C_6 to be *R* and *S*, respectively. Hence, the absolute stereochemistry of the glycoside **9b** isolated in the present study is also assigned as (5*R*,6*S*). Tamaki et al. (*20*) deduced the stereochemistry at C_9 as *R* based on the fact that the ^{13}C NMR data for the ring carbons were superimposable on those of alangionoside G and alangionoside I (*23*) which both had *R* stereochemistry at C_9. Meanwhile, Shen and Terazawa (*24*) deduced the stereochemistry as *R* by the Helmchen method.

Inada et al. (*18*) reported a relatively large specific rotation ($[\alpha]_D$ −35.1, *c* 0.11, methanol) for **9b**, whereas Otsuka et al. (*19*) reported a value ($[\alpha]^{24}_D$ ca. 0, *c* 0.71, methanol) closer to the one determined here ($[\alpha]_D$ −5.4, *c* 1.0, methanol). This supports Otsuka and colleagues' claim (*19*) that the optical rotation obtained by Inada et al. is not accurate, based on their calculations of the optical rotation using the Klyne rule from an analogous disaccharide, which they determined to be approximately −7°.

Sefton et al. (*17*) previously identified the glycoside **9b** (isolated as the tetraacetate) as a grape-derived product but were unable to determine C_5/C_6 relative stereochemistry, which differed between the aglycone obtained from the natural glycoside and a synthetic isomer. The C_5-secondary methyls of both the synthetic aglycone and the glucoside obtained in this study are equatorial**.** This suggests that the stereochemistry of the synthetic aglycone differs from that of the natural glucoside in that the C_6-side chain is equatorial in the latter but axial in the former. This assignment is supported by the ^{1}H NMR spectra of these compounds (the glucoside as its tetraacetate) in $CDCl_3$ and C_6D_6. For both solvents, the H_2 and H_4-axial hydrogens are significantly further downfield in the glucoside (as the tetraacetate) than they are in the synthetic aglycone, as expected for a 1,3-diaxial interaction with the C_6-hydroxyl in the former.

Hydrolysis of Glycoside 9b. Having isolated a pure fraction of the glycoside **9b**, we were keen to conduct some hydrolysis experiments to determine if it produced **1**, or indeed any other aromas worth pursuing. Preliminary hydrolysis of the isolated glycoside **9b** (at approximately 50 mg/L) gave **1** as one of the products identified by gas chromatograph−olfactometry (GC−O), with this compound being one of the strongest aromas in the hydrolysate. However, when this glycoside was hydrolyzed at a more wine-like concentration of 1 mg/L in model wine (pH 3.2) at 25 °C, 45 °C, or 100 °C, only low levels (∼20 ng/L) of **1** were observed. Therefore, although **9b** was initially considered a possible precursor to **1**, these results suggest that unrealistically high amounts of this glycoside would have to be present in wine to account for the levels observed in some white wines. (*11,12*). Thus, other more significant precursors to **1** must be present in glycosidic extracts and at least some wines.

Synthesis of C_{13}-Norisoprenoids 4, 6, and 8. The 3,6,9-triols (**6**) were synthesized (as a mixture of diastereomers) in two steps (Figure 3) from 3,4-dehydro-β-ionone (**10**) using the procedure of Strauss et al. (*6*). It was found not to be practical to attempt separation of the individual diastereomers, and the diastereomeric mixture was used in the hydrolytic study. In keeping with the observations of Strauss et al., when **6** was heated briefly to 100 °C at pH 3.0 the major products were as follows: unreacted **6**, rearranged triol **4** (as a mixture of diastereomers), the actinidols (**8**), and the rearranged ketone **12**. Pure fractions of both **4** and **8** were obtained as diasteromeric mixtures, after column chromatography. As with **6**, the subsequent hydrolysis experiments were performed on these mixtures.

Hydrolysis of Aglycones 4, 6, and 8. Compounds **4**, **6**, and **8**, (each at 1 mg/L and pH 3.2) were separately subjected to acid hydrolysis in model wine at 25 °C and 45 °C, and the product mixtures were examined by GC−MS (*2*); all 3 norisoprenoids (hydrolyzed independently of each other) produced hydrolysates which had the same overall aroma, with descriptors including "cut-grass", "green", "metallic", "fly spray", and "fruity". Furthermore, all three hydrolysates were found to contain **1**. The two dienes **4** and **6** can be formed from each other under acidic conditions (*6, 9*), and it is possible that that they can also form from the actinidols (**8**), if formation of the latter is reversible (Figure 4). It is possible, therefore, to see similar product profiles irrespective of the chosen precursor.

Quantitative studies were carried out for each of the three suspected precursors, and all three generated **1** over time under acidic conditions at both temperatures employed. Trace levels (<100 ng **1** per mg starting material) of **1** were present in all zero time samples; these amounts are likely to have been generated during sample preparation as there was a small time delay (several hours) between preparation of the samples in model wine and their analysis. The amounts of **1** present in the

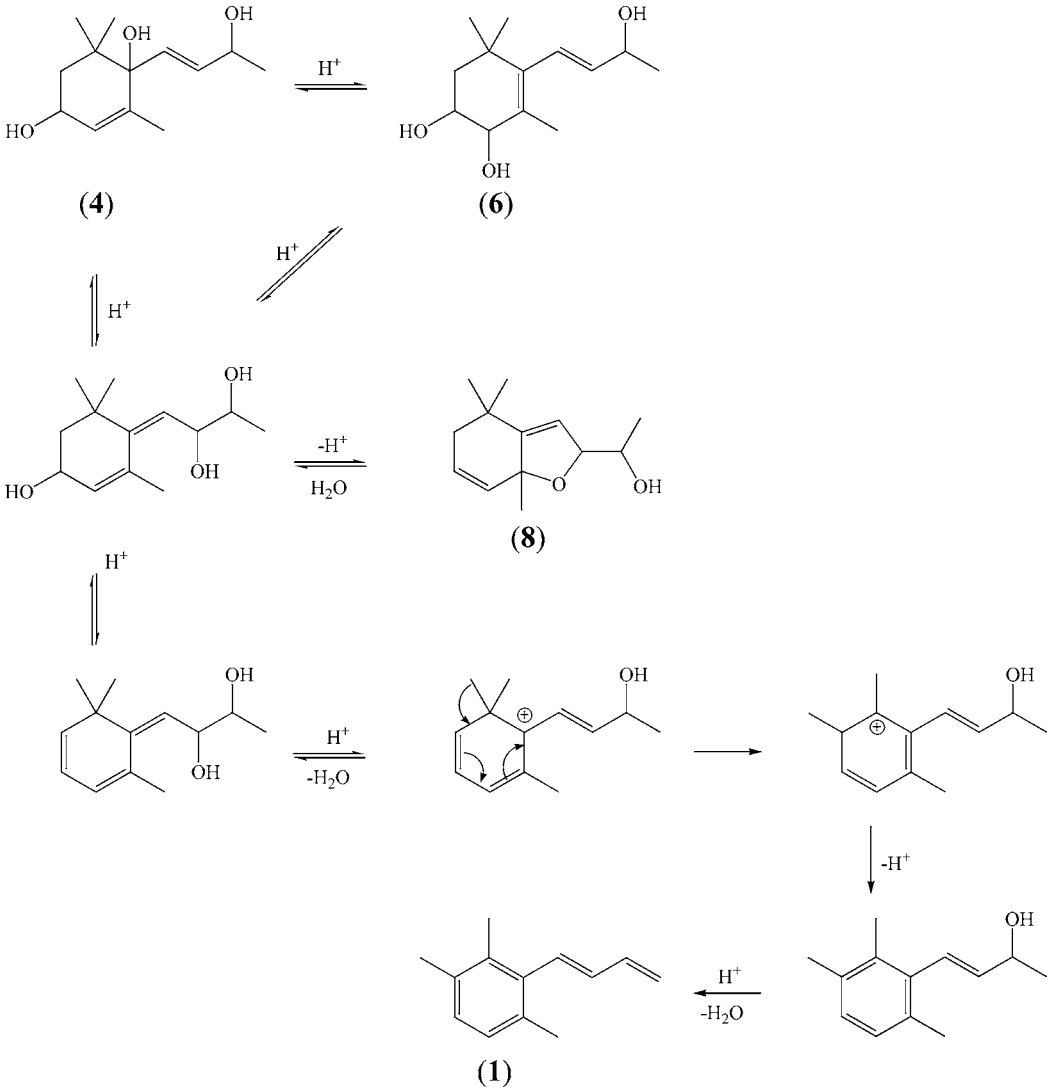


Figure 4. Proposed mechanism for the formation of **1** from **4**, **6**, and **8**.

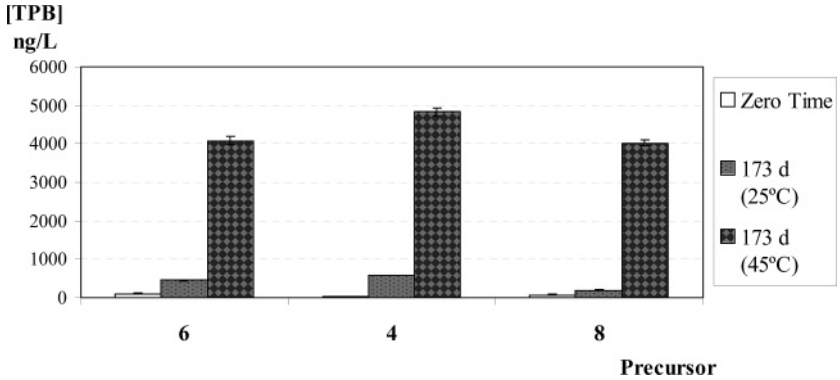


Figure 5. Production of **1** from **4**, **6**, and **8** (at 1 mg/L) at 45 °C and 25 °C after 6 months. All data are an average of 2 replicates.

hydrolysates after 6 months are shown in Figure 5. After 6 months at 45 °C, 4000−5000 ng/L of **1** was present, irrespective of the precursor. All three precursors also generated **1** at the lower temperature of 25 °C. Under these more typical maturation conditions, the precursors (1 mg/L) formed much more wine-like amounts (200−600 ng/L) of **1** (*1*,*2*).

TPB Generation from Crude Glycosidic Extracts. Compound **1** was first identified in the acid hydrolysates of both red and white crude glycosidic extracts (*1*). These hydrolysates were examined qualitatively by GC−MS−O, which indicated the presence of a powerful odorant. We have now analyzed hydrolysates of such extracts using the stable isotope dilution assay (SIDA) method developed specifically for **1** (*2*), to assess their potential as a precursor pool to this compound. Both grape and leaf glycosidic extracts were studied, with the results being shown in Table 1.

Similar amounts of **1** were produced from the grape extracts of all varieties, approximately 5000−10000 ng of 1 per gram of crude glycosidic material after heating for 3 months at 45 °C. However, the true amount of **1** generated is likely to be

Table 1. Amounts of **1** Generated by Acid Hydrolysis of Crude Fruit and Leaf Glycosidic Extracts

	fruit			leaf	
sample[a]	ng/g[b,c]	ng/kg[d]	ng/L[e]	ng/g[b,c]	ng/kg[f]
2000 S	7733	1036	2378	958	9415
2001 S	5267	1448	3092	2217	13905
2000 CS	7308	782	1554	1127	14234
2001 CS	8983	1249	2429	1285	20537
2001 R	7100	nd[g]	nd[g]	1169	19067
2001 M	9715	nd[g]	nd[g]	9450	29077
2001 T	7592	nd[g]	nd[g]	5827	30301

[a] S (Shiraz), CS (Cabernet Sauvignon), R (Riesling), M (Muscat Rose), T (Traminer). [b] Hydrolyzed approximately 0.1 mg extract per milliliter of model wine (pH 3.2) at 45 °C for 3 months; average of 2 replicates. [c] Mass of **1** produced per gram of crude glycosidic extract. [d] Mass of **1** produced per kg of fruit. [e] Mass of **1** produced per liter of juice. [f] Mass of **1** produced per kilogram of leaves. [g] Not determined.

substantially higher than the amounts actually measured, as **1** is both generated and degraded simultaneously (*2*). In keeping with earlier reports which suggest that grapevine leaves are a particularly rich source of glycosides (*16*), the yield of crude glycosidic extract from the leaf samples was much higher (approximately 2 orders of magnitude) than from the corresponding fruit samples. In terms of the actual amount of **1** produced hydrolytically, when "standardised" against the original masses employed, the leaf samples demonstrate a greater potential (approximately 10-fold) for generating **1** than their corresponding fruit counterparts.

Although the purified glucoside **9b** generated **1** on acid hydrolysis, and although **1** undoubtedly contributed to the overall aroma of the hydrolysates of **9b**, the amounts formed were small, and this compound cannot account for the amounts of **1** seen in the acid hydrolysates of crude glycosidic fractions obtained from grapes and vine leaves (Table 1).

Three C_{13}-norisoprenoid compounds, 3,6,9-trihydroxymegastigma-4,7-dienes (**6**), 3,4,9-trihydroxymegastigma-5,7-dienes (**4**), and the actinidols (**8**), all generated **1** at wine pH, in significant quantities. All three formed **1** over time at both ambient and elevated temperatures. Glycoconjugates of these compounds could account for the amounts of **1** found in the hydrolysates of the crude grape glycosidic extracts (Table 1). Such glycoconjugates may be the only important precursors, or there may be others, as yet unidentified. Even if all potential precursors were to be identified, it would be difficult, if not impossible, to assess the amount of **1** which can potentially form in wine by examining grape extracts. The processes of depletion of **1** are concomitant with its generation and cannot as yet be accurately mimicked experimentally. Diene **1** was only one of several interesting compounds that have been investigated in the acid hydrolysates of crude glycosidic extracts from Shiraz and Cabernet Sauvignon, and there are other aromas in these hydrolysates requiring further investigation.

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