JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

(*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene: A Potent Grape-Derived Odorant in Wine

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(*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene (TPB) was identified as a potent odorant in acid hydrolysates of crude glycoconjugate fractions isolated from grapes and grape vine leaves. TPB was also identified in a Semillon wine, using gas chromatography/mass spectrometry, by co-injection with an authentic sample. TPB had an aroma detection threshold of 40 ng/L in a neutral white wine and the concentration of TPB in four out of five white wines analyzed ranged from 50 to 210 ng/L.

KEYWORDS: Wine; grape; odorant; glycoside; 2,3,6-trimethylphenylbutadiene

INTRODUCTION

Various sensory investigations (*1*−*5*) have indicated that volatile compounds, generated by wine acid-catalyzed hydrolysis of grape-derived involatile precursor forms (mostly glyco-conjugates), have a significant impact on the aroma of wines. Whereas enzyme preparations with glycosidase activity form aglycones from their glycoconjugate forms by breaking the glycosidic (i.e. acetal) linkage, the liberation of volatile compounds from glycoconjugates of alcohols by acid catalysis during wine maturation and conservation apparently only takes place with glycosides of activated (e.g. allylic) alcohols (*6*−*9*). This presumably entails cleavage of the ether bond rather than the glycosidic bond between glucose and the aglycone. Under acid conditions, this process can be accompanied by other transformations, including dehydration, cyclization, and re-arrangement reactions.

Several studies (*10*−*14*) have been conducted on the composition of grape-derived volatile compounds, liberated from crude glycoconjugate fractions by hydrolytic means. Although the composition of such hydrolysates is highly complex, the vast majority of the components of these hydrolysates are either odorless or have weak aromas only, and probably contribute little or nothing to wine aroma and flavor. The acid hydrolysis products 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), which can impart a kerosene-like character to aged Riesling wines (*15*, *16*), and β-damascenone, which has an intense fruity aroma (*8*, *16*), are likely to be contributors to the aroma and flavor of some wines. However, the majority of such hydrolysis products that contribute to wine aroma and flavor are probably yet to be identified.

We report here the identification of (*E*)-1-(2,3,6−trimethylphenyl)buta-1,3-diene (TPB, **1**) as a potent odorant in pH 3.2 hydrolysates of crude glycosidic extracts of grapes, and its subsequent identification and quantification in several white wines.

MATERIALS AND METHODS

Materials. The chemicals used were of the highest purity commercially available. All solvents were Mallinckrodt Nanopure grade, and verified for purity by GC/MS prior to use. Wine samples were obtained from retail outlets. Grape and grape vine leaf samples (Shiraz and Cabernet Sauvignon) were obtained from Nuriootpa in the Barossa Valley, South Australia.

Analysis by GC/O/MS. Gas chromatography/olfactometry/mass spectrometry (GC/O/MS) was carried out with a Hewlett-Packard (HP) 6890 gas chromatograph fitted with a Gerstel MPS2 autosampler, a Gerstal thermal desorption unit (TDU), and Gerstel programmed temperature vaporization (PTV) inlet (CIS-4). The GC was coupled to a HP 5973N mass spectrometer and Gerstel olfactory detector port (ODP2). The Gerstel MPS2 was fitted with a 70-μm carbowax/divinylbenzene stable flex (CW/DVB) fiber (Supelco, USA, Cat-No. 57337-U) and was operated in automated solid-phase microextraction (SPME) mode. The gas chromatograph was fitted with a ca. 30 m × 0.25 mm Phenomenex fused silica capillary column ZB-Wax with a 0.25 μm film thickness. A crosspiece was installed at the end of the analytical column, approximately 2.2 m of 220 μm i.d./320 μm o.d. deactivated fused silica tubing (SGE, Melbourne Australia) was connected from the crosspiece to the ODP, and approximately 2 m of 110 μm i.d./320μm o.d. deactivated fused silica tubing was connected from the crosspiece to the mass spectrometer, giving a split ratio of approximately 2:1 in favor of the ODP. The carrier gas was helium (BOC gases, Ultrahigh Purity) with a flow rate of 1.8 mL/min. For SPME, the headspace of the sample was extracted at 55 °C for 5 min and desorbed in the injector for 10 min. A direct SPME Injection Sleeve with an ID of 0.75 mm (Supelco, USA) was installed in the inlet and the splitter, at 28:1, was opened after 60 s. Injection was done in pulse splitless mode with an inlet pressure of 25.0 psi maintained until

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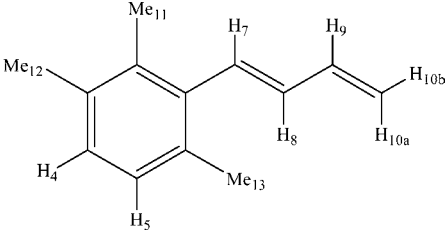† Flinders University of South Australia.
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splitting. The oven temperature was started at 80 °C, held at this temperature for 1 min, increased to 170 °C at 4 deg/min then to 250 °C at 50 deg/min, and held at this upper temperature for 10 min. The injector was held at 220 °C and the transfer line at 260 °C. Analysis by stir bar sorptive extraction (SBSE) was performed by placing a Gerstel Twister stir bar (10 mm long, coated with PDMS, 0.5 mm film thickness) into a 15 mL glass sample tube with an aluminum-lined cap (Supelco, USA) with 10 mL of wine. The samples were stirred at room temperature for 3 h, and the Twister was then removed, dried on a lint free tissue, then inserted into a glass desorption tube. The stir bar was thermally desorbed in the splitless mode with use of the following desorption temperature program: 20 °C, 60 deg/min increase to 180 °C, and then held there for 5 min. The desorbed solutes were cryofocused in the PTV inlet at −50 °C. The PTV inlet was then programmed to 250 °C at 12 deg/s and held for 5 min. Injection was done in solvent vent mode. The PTV was fitted with a glass liner with silanized glass wool (GERSTEL). The compounds were separated on the column described above, using helium (BOC gases, Ultrahigh Purity) carrier gas at 1.2 mL/min (constant flow). The oven temperature was started at 50 °C, held at this temperature for 1 min, increased to 150 °C at 10 deg/min, increased to 250 °C at 25 deg/min, and held at this temperature for 30 min. The transfer line was at 250 °C. Positive ion electron impact spectra at 70 eV were recorded in the range m/z 35−350 (cycle time 1.01 s). Quantitative analysis of TPB (**1**, **Figure 2**) in wines was carried out with instruments and operating conditions as described above (except that the GC column was connected to the MS only) with the following modifications: a HP 6890 series liquid injector was used and operated in fast liquid injection mode with a 10 μL syringe (SGE, Australia) fitted. The injector liner used was of deactivated borosilicate glass, 4 mm i.d., with a plug of silanized glass wool (2−4 mm), tapered at the column end. Wine samples were prepared for quantitative analysis by adding a solution of [^{2}H$_8$]-naphthalene (500 ng) in ethanol (50 μL) as internal standard to the wine (50 mL), which was then extracted with n-pentane (3 mL). A portion (2 μL) of the extract was then injected onto the GC. The splitter, at 42:1, was opened after 36 s. Fast injection was done in pulse splitless mode with an inlet pressure of 25.0 psi maintained until splitting. The carrier gas flow rate was 1.2 mL/min and the GC column was connected to a HP 5973 mass spectrometer. Positive ion electron impact spectra at 70 eV were recorded in the range m/z 35−350 for scan runs. For quantification of TPB, the mass spectrometer was operated in selected ion monitoring (SIM) mode. The ions monitored in SIM runs were: m/z 108 and 136 for [^{2}H$_8$]-naphthalene and 157, 142, 172 for TPB. Selected fragment ions were monitored for 30 ms each. The underlined ion for each compound was the ion typically used for quantitation, having the best signal to noise and the least interference from other wine components. The other ions were used as qualifiers. The analytical method was validated by a series of duplicate standard additions of unlabeled TPB (0.1 to 15.36 μg/L, $n = 9 \times 2$ for the analyte) to a dry red wine (12.5% ethanol, pH 3.6). The standard addition curves obtained were linear throughout the concentration range, with a coefficient of determination (r^2) of 0.997 and linear regression equation $y = 0.293x + 0.005$. The repeatability of the analysis was determined at two concentrations (0.5 and 10 μg/L) by spiking seven replicate aliquots of the same red wine with TPB. For seven replicate analyses of the wine spiked at the 0.5 μg/L level, the coefficient of variance (or relative standard deviation) was 8.5%. For seven replicate analyses of the wine spiked at the 10 μg/L level, the coefficient of variance (or relative standard deviation) was 8.4%. In the wine samples analyzed (**Table 2**), concentrations below 100 ng/L were determined by extrapolation and should be considered as estimates only. The concentration of TPB was estimated to be below 10 ng/L in those wines where it is indicated as not detected.

Preparation of Acid Hydrolysates of Crude Glycoconjugate Fractions from Grapes and Grape Vine Leaves. Cabernet Sauvignon or Shiraz grapes (1 kg) were crushed by hand, sulfur dioxide (200 mg/L) was added, and the juice (600 mL) was clarified by centrifugation (8000*g*, 20 min). The juice was then stirred overnight with poly-vinylpolypyrrolidine (PVPP, 30 g), further clarified by centrifugation, then passed down a column packed with XAD2 resin (added as 100 mL of aqueous slurry). The column was washed with water (400 mL),

Table 1. NMR Parameters for TPB (**1**) (300 MHz (^{1}H), 75 MHz (^{13}C), Acetone-d_6)

signal[a]	δ[b] (multiplicity[c])	signal[a]	δ
		C$_1$	138.3
		C$_2$	135.6
		C$_3$	135.6
H$_4$	6.98 (d) A part of AB quartet	C$_4$	129.9
H$_5$	6.96 (d) B part of AB quartet	C$_5$	128.8
		C$_6$	134.8
H$_7$	6.71 (br d)	C$_7$	133.4
H$_8$	6.28 (dddd)	C$_8$	136.8
H$_9$	6.64 (dddd)	C$_9$	139.2
H$_{10a}$	5.31 (dddd)	C$_{10}$	117.9
H$_{10b}$	5.18 (dddd)		
H$_{11}$	2.19, s	C$_{11}$	17.8
H$_{12}$	2.22, s	C$_{12}$	21.2
H$_{13}$	2.22, s	C$_{13}$	21.8

[a] Assignments were made with the aid of COSY HETCOR and HMBC [600 MHz (^{1}H), 150 MHz (^{13}C)] experiments. [b] δ values in ppm. [c] $J_{9,10a} = 16.9$ Hz; $J_{7,8} = 16.1$ Hz; $J_{9,10b} = J_{8,9} = 10.4$ Hz; $J_{4,5} = 7.8$ Hz; $J_{10a,10b} = 1.9$ Hz; $J_{7,9} = J_{7,10a} = J_{7,10b} = J_{8,10a} = J_{8,10b} = 0.8$ Hz.

then dichloromethane (400 mL), then eluted with methanol (1 L). The methanol eluate was concentrated to dryness in vacuo to yield the crude glycosidic fraction. Cabernet Sauvignon and Shiraz leaves (1.5 kg) were frozen with liquid nitrogen, powdered in a mortar and pestle, and extracted with methanol (5 L) over 3 days. The filtrate was then concentrated in vacuo, redissolved in distilled water (1.5 L), and stirred with PVPP (150 g) overnight. The PVPP was removed by centrifugation and the juice passed twice through a column packed with XAD-2 (ca. 600 mL aqueous slurry) and then through a second XAD-2 (200 mL slurry) column. The crude glycosidic fraction was then recovered from the columns as described above. For preparation of acid hydrolysates, crude XAD-2 extracts (10−30 mg) of grape juice were dissolved in buffered model wine (10 mL, 10% aqueous ethanol, pH adjusted to 3.2 with potassium hydrogen tartrate and tartaric acid) and the solution was heated to 100 °C overnight. Leaf glycosides (10−30 mg) were heated to this temperature in the model wine (10 mL) for 3 days.

Synthesis of (*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene (TPB, 1). Megastigm-4-en-7-yne-6,9-diol (**2**, **Figure 2**) was prepared as described previously (*17*). *p*-Toluenesulfonic acid (approx 2−3eq) was added to a solution of the diol (**2**, 0.2 g) in CDCl$_3$ (14 mL). The reaction mixture was heated to 50 °C overnight (NMR monitoring), then quenched with aqueous sodium hydroxide (10%, a few drops), washed with brine, dried with sodium sulfate, and concentrated in vacuo to yield the crude product as a clear orange oil. This oil was subjected to column chromatography on silica (eluant X4) and the main product distilled at 90−100 °C at 2.8 mm mercury, yielding (*E*)-1-(2,3,6−trimethylphenyl)buta-1,3-diene (**1**, 10%). m/z (%), 172 (M$^+$, 35), 158 (13), 157 (100), 156 (10), 143 (15), 142 (85), 141 (30), 129 (13), 128 (15), 127 (100), 115 (20), 91 (5), 77 (6). Kovats GC retention indices, 1486 (DB-1701) and 1830 (ZB-Wax). For NMR data, see **Table 1**.

Identification of TPB in a Semillon Wine. A 1996 commercially produced Semillon wine (50 mL) was extracted with 3 mL of pentane. Isooctane (3 drops) was added to approximately 1 mL of this extract, which was then concentrated with a gentle stream of nitrogen to approximately 0.5 mL. This concentrate was loaded onto a preparative TLC plate (Merck 20 × 20 cm^2 silica gel 60, F 254 nm) that had been prewashed with ethanol and air-dried. The TLC plate was developed in pentane. The fraction from the plate corresponding to TPB was isolated with pentane (5 × 3 mL; the R_f of TPB was determined on a

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separate plate, developed in another tank to avoid cross-contamination). Isooctane (3 drops) was added to this pentane extract, which was then concentrated under nitrogen to ca. 50 μL and analyzed by GC/MS in scan mode as described above.

Aroma Threshold of TPB. The aroma threshold of (*E*)-1-(2,3,6−trimethylphenyl)buta-1,3-diene (TPB) in a young (<12 months old) neutral dry white wine (Chenin Blanc, pH 3.5, 11.5% ethanol) and in unbuffered 10% aqueous ethanol was determined according to the American Society for Testing and Materials (ASTM) method E 679-79, using 24 judges. The judges were of European origin, aged between 20 and 50, with similar numbers of males and females. The white wine had a free and total sulfur dioxide content of ca. 20 and 180 mg/L, respectively. Wines were presented in ascending order of TPB concentration, at 10, 30, 90, 270, 810, and 2300 ng/L for both the white wine and the unbuffered model wine. Panellists smelt, but did not taste the samples. Those who could detect the spiked wines at all of these concentrations were then tested at lower concentrations of TPB. The same panellists were used for threshold determinations in both media. The best estimate threshold for each panellist was the geometric mean of the highest concentration missed and the next higher concentration tested. The group threshold was calculated as the geometric mean of the individual best estimate thresholds.

RESULTS AND DISCUSSION

As part of our continuing investigations into the contribution of grape glycoconjugates to wine flavor, we recently conducted a GC/Olfactometry/mass spectrometry (GC/O/MS) assessment of pH 3.2 hydrolysates of fractions isolated by XAD2 resin from crude extracts of grapes and grape vine leaves. In every hydrolysate examined by GC/O/MS, we observed a strongly odorous zone with an aroma varyingly described by several assessors as "green", "cut-grass", "tobacco", "insecticide", "chemical", and "pungent". This strong aroma was detected just before elution of β-damascenone on a DB 1701 column and just after this compound, which was easily recognized by its familiar stewed apple/quince-like odor, on a Carbowax column. In some GC/O runs, there was an overlap between the two aromas, giving rise to many complex odor descriptors.

In GC/O/MS of diluted samples, this "green", "cut-grass" aroma coincided with a peak with a mass spectrum (**Figure 1**) that was very similar to that of 1,1,6-trimethyl-1,2-dihydronaph-thalene (TDN, *15*), but was clearly not this compound as an authentic sample of the latter eluted at an earlier retention time on both columns. TDN was also seen as a much larger but much more weakly smelling peak in all chromatograms. The mass spectrum of the highly odiferous peak did not appear to match that of any other known wine component.

The mass spectrum of this unknown component of the hydrolysates was essentially the same as that of a byproduct we had previously encountered during our synthesis of the damascenone precursor (**3**, **Figure 2**, *17*). Initial attempts to produce **3** by acid-catalyzed dehydration of **2** with *p*-toluene-sulfonic acid had resulted in over-reaction and the production of predominantly butadiene (**1**). The structure of **1** was unambiguously established as (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB) by its proton and carbon NMR spectra (**Table 1**). The geometry of the C7−C8 olefinic linkage is assigned the *E*-configuration on the basis of the magnitude of the H7−H8 coupling constant (16.1 Hz). The arrangement of the three methyl groups on the aromatic ring was suggested on mecha-nistic grounds and confirmed by a HMBC NMR experiment; long-range correlations between H13 and C6/C5 as well as correlations between H12 and C3/C4 are only possible if the methyl substitution pattern is as indicated. Were the three methyl substituents actually contiguous, then we would expect only one long-range ring-carbon/methyl-hydrogen correlation. That the
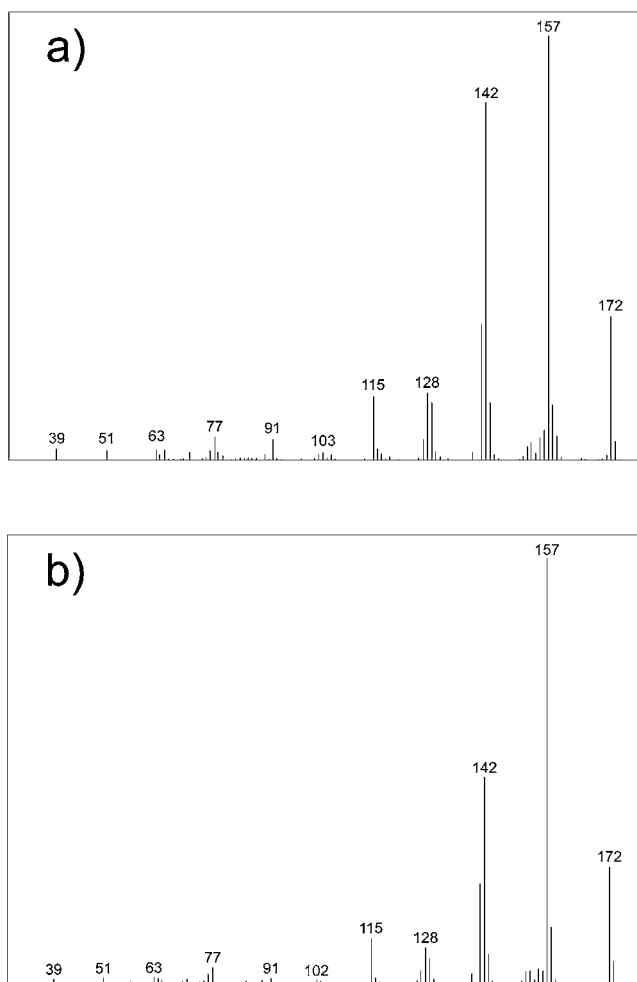
Figure 1. Mass spectrum of (a) (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB, **1**) and (b) 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN).
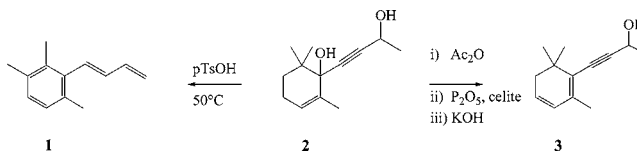
Figure 2. Synthesis of (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB, **1**) and megastigma-3,5-dien-7-yn-9-ol (**3**).

four substituents are themselves contiguous (as in **1**) is evident from the size of the coupling between H4 and H5. This authentic sample of **1** had an identical mass spectrum, retention time, and GC/O profile to the unknown component of the hydrolysates on both the DB1701 and Carbowax columns.

TPB was also detected in a commercial Semillon with use of stir bar sorptive extraction (*18*), although this compound coeluted with other wine components desorbed from the stir bar. When a pentane extract of the same wine was concentrated and subjected to fractionation by preparative thin-layer chro-matography, TPB could be clearly identified by GC/MS in the isolated fraction. The TPB peak in this fraction was sym-metrically enhanced by co-injection with an authentic sample, and both the authentic sample and the wine component had identical mass spectra. To our knowledge, this is the first time TPB has been observed as a wine component.

TPB was subsequently quantified in several wines by GC/MS, using selected ion monitoring and d_8-naphthalene as an internal standard. It was found at a concentration ranging from 50 to 210 ng/L in four of the five white wines examined. No

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Table 2. Concentration of (*E*)-1-(2,3,6-Trimethylphenyl)buta-1,3-diene (TPB, **1**) in Commercial Wines

wine	concn[a] (ng/L)
1997 Riesling	60
1996 Chardonnay	100
1997 Chardonnay	nd
1996 Semillon	210
1994 Chardonnay	50
1997 Cabernet Sauvignon	nd
1996 Cabernet Sauvignon	nd
1988 Shiraz	nd
1996 Merlot	nd
1995 Cabernet Sauvignon	nd
1995 Shiraz	nd

[a] nd = not detected.
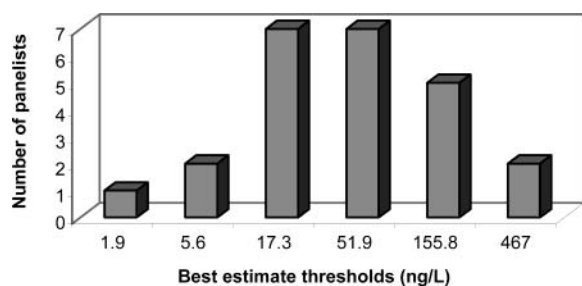
Figure 3. Best-estimate aroma thresholds of (*E*)-1-(2,3,6-trimethylphenyl)-buta-1,3-diene (TPB, **1**) in a neutral dry white wine.

trace (<ca. 10 ng/L) was detected in the six red wines analyzed (**Table 2**), nor in the young white wine used for aroma threshold determination (below).

The aroma detection threshold of the authentic TPB was determined for both a neutral white wine and unbuffered 10% aqueous ethanol (model wine). In determining the sensory properties of reference compounds, it is important to ensure that they do not contain impurities which could modulate their sensory impact. The reference sample of TPB, which was >95% pure by NMR and GC, gave only a single aroma-active region, coinciding with the dominant peak, during GC/O on both the DB1701 and Carbowax columns. Importantly, the sample of TPB contained no trace of damascenone detectable by GC/O or selected ion chromatograms.

The aroma detection threshold of TPB in a neutral white wine was 40 ng/L. The distribution of best-estimate thresholds is shown in **Figure 3**. This makes it among the most potent of volatile wine components, comparable to β-damascenone, β-ionone, and wine lactone (*19*, *20*). Informal descriptors used by the sensory panel to distinguish spiked from unspiked white wines ranged from "floral", "geranium", and "tobacco" at the lower concentrations to "pungent", "very green", "unpleasant", "plastic", and "insecticide" at concentrations of 270 ng/L and above. Surprisingly, despite using the same panellists and stock solutions, the aroma detection threshold of TPB in the model wine was an order of magnitude higher: 430 ng/L. Informal comments from the sensory panel indicated that the harsh aroma of the ethanol in the model wine (but not the white wine) had a masking effect. It is also possible that other wine components have a synergistic effect on the sensory impact of TPB. Such observations show the need for caution in extrapolating sensory data from model wines to real wines or, indeed, from one wine to another (other examples of sensory detection thresholds varying in different media are given in *21*).

TPB has been previously reported as a potential staling compound in beer, having a leather/geranium odor (*22*), although

no structural or formal sensory information was provided to support this statement. Kleipool et al. (*23*) demonstrated the presence of TPB in rum and cognac by comparison with an authentic sample synthesized by a different route to the one we report here. They described TPB as having a "harsh and metallic character—remarkably strong for a hydrocarbon". De Rijke and Ter Heide (*24*) also included TPB in a list of compounds isolated from rum and cognac but did not discuss this compound.

Not withstanding the small number of samples analyzed, the presence of TPB in four out of five white wines and in all of the hydrolysates (which were from berry and leaf samples obtained from Cabernet Sauvignon and Shiraz vines) but in none of the red wines examined is intriguing. While it is plausible that the samples analyzed may not be typical of these varieties, it is also possible that the absence of TPB in the red wines is because this highly conjugated molecule can easily be protonated and the resulting cation is able to react with compounds (e.g. nucleophilic polyphenols) that are present in red wines at higher concentration than in white wines or model wine hydrolysate solutions. Whether or not this is the case, such considerations illustrate the inadequacy of relying solely on compositional or sensory analysis of hydrolysates formed in model wines to predict the composition or sensory properties of real wines, in which biological and chemical transformations of hydrolysis products can compete with their generation. Nevertheless, such model studies can still provide pointers toward possible important wine flavor compounds, as has clearly been the case in this study.

The structure of TPB (**1**) and its facile formation from the diol (**2**) indicate that it may belong to the class of compounds known as C_{13} norisoprenoids. These compounds are presumed to be formed by oxidative biodegradation of plant carotenoids followed by further enzyme-mediated transformations and, in some cases, by mild acid-catalyzed dehydration and rearrangement reactions (*25*). While grapes and grape vine leaves are known to yield a rich variety of such compounds, only three, β-damascenone, TDN, and β-ionone, have hitherto been implicated in wine flavor (*16*, *19*, *20*). Studies on potential precursors to **1**, currently underway in our laboratory, should indicate whether this compound also belongs to this group.

Abbreviations Used. TPB, (*E*)-1-(2,3,6-trimethylphenyl)buta-1,3-diene; GC/O/MS, gas chromatography/olfactometry/mass spectrometry; TDU, thermal desorption unit; PTV, programmed temperature vaporization; SPME, solid-phase microextraction; SBSE, stir bar sorptive extraction.

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