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BIPHENYL-LINKED BIFLAVANOIDS FROM GRAPE POMACE

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Key Word Index—*Vitis vinifera*; Vitaceae; grape pomace; oxidative coupling; biphenyl linkage; biflavanoids.

Abstract—Two novel biphenyl linked biflavanoids commonly associated with polyphenol oxidase-coupled products were isolated from Chardonnay grape pomace. Their chemical structures were established by long-range H,C couplings as epicatechin [6′ → 8]-epicatechin and epicatechin [6′ → 8] catechin. The disproportionate representation of the epicatechin unit as compared with catechin among the isolated biflavanoids could not be accounted for by their relative concentration in the pomace and suggested that the 2,3-*trans* configuration provided more steric constraints than the 2,3-*cis* one during grape polyphenol enzymatic oxidation of the B-ring leading to the quinone intermediate. © 1998 Elsevier Science Ltd. All rights reserved

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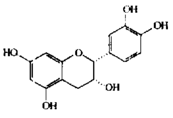
INTRODUCTION

Grape polyphenolics have received considerable attention recently due to their reported health benefits and much media publicity has been focused on what is commonly referred to as the French Paradox [1]. The latter came about as a result of a global epidemiological study called the MONICA project, a world-wide monitoring system for cardiovascular diseases under the World Health Organization [2], which showed that the mortality rate from ischaemic heart disease in France was dramatically lower than that in other Western countries, such as the USA or UK, despite a comparable high intake of saturated fat and serum cholesterol levels to those of these countries. This anomaly has been postulated to be a result of the high consumption of red wine in France, and further boosted by subsequent research which showed that red wine could indeed inhibit the oxidation of LDL or low density lipoprotein [3], a critical step in the development of atherosclerosis [4, 5]. Wine, and in particular red wine, contains large amounts of polyphenolics [6] and the more active fractions in inhibiting LDL oxidation *in vitro* have been associated with flavanol compounds, such as catechin and proanthocyanidin oligomers [7]. This class of compounds has also been shown to be effective as free radical scavengers, as well as inhibitors of oxidative enzymes, such as xanthine oxidase [8, 9].

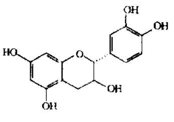
The present study is part of a research programme investigating the phenolic constituents in various grape residues generated during wine production and assessing their potential as an additional source of polyphenolics. In this work, the isolation and identification of two unusual dimeric flavanols is reported.

RESULTS AND DISCUSSION

The biflavanoids which were detected by their relatively high mobility on cellulose TLC developed with dilute aqueous acetic acid as solvent, co-occurred in a narrow chromatographic fraction with several low M_r polyphenols, including catechin, epicatechin and flavanoid glycosides. Further chromatographic treatment of this fraction on Sephadex LH20 employing 50% aqueous ethanol and on MCI gel CHP 20P failed to resolve the mixture. However, rechromatography of the mixture on Sephadex LH20 with 15% aqueous ethanol yielded epicatechin (**1**) and catechin (**2**) followed by the biflavanoid (**3**) and other chromatographic fractions, one of which contained a



(1)



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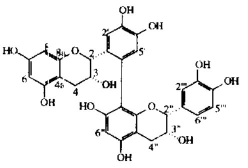

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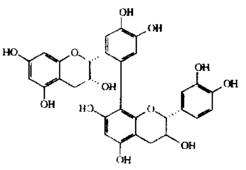

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highly mobile spot characteristic of a biflavanoid. This latter fraction was purified on an MCI Gel CHP 20 column using 30% aqueous methanol to give compound (**4**), which was the first to be eluted.

The ^{13}C chemical shifts of compound (**3**) appeared as duplicated epicatechin carbon signals (Table 1), particularly in the high field region (28 to 80 ppm) associated with the heterocyclic C-ring, where the C-2, C-3 and C-4 carbon resonances showed up as twin peaks of comparable intensity. The remaining carbon signals were more or less consistent with the chemical shift values for the phloroglucinol A-ring and the catechol B-ring of flavan-3-ols, except for the presence of two quaternary carbons (established by DEPT) observed at 108.1 and 125.2 ppm at the expense of an unsubstituted aromatic carbon from each of the A- and B-ring, suggesting a biphenyl type linkage between the two epicatechin moieties. Examination of the B-ring methine carbons showed that there were only five high-field carbon signals (115.0 to 120.0 ppm) associated with the C-2′ and C-5′ and C-6′ carbons of the two catechol rings, instead of the expected six, thus indicating the point of biphenyl linkage to be at one of these positions. It appeared that the exact point of linkage could be readily deduced by examination of their proton-coupling patterns, as *ortho*-coupling for linkage at C-2′, a *meta*-coupling for linkage at C-5′ and no proton-coupling for C-6′. The two protons in question identified at δ 6.42 and 6.93 were observed as singlets, which are consistent with C-6′ as the attachment point. This B-ring bonding position was corroborated by inverse long-range proton-coupling experiments (HMBC), which also established the carbon chemical shift at 119.9 ppm was that of the C-5′ methine carbon where substitution at the adjacent C-6′ by a flavan group had induced a downfield shift, so that the observed peak now occurred in the same general region commonly observed for the unsubstituted C-6′ of the catechol B-ring (Table 1). Evidence for the presence of a flavan substituent on the B-ring can be obtained from the long-range interactions between the H-5′ (δ 6.62) and the quaternary A-ring carbon (108.1 ppm), which was the point of interflavanoid bonding. The remaining outstanding issue was whether this linkage point in the phloroglucinol A-ring was at C-6 or C-8. To resolve this question the oxygenated carbons in the A-ring needed to be unambiguously assigned. This was forthcoming from the HMBC data which linked the most upfield oxygenated A-ring peak at 153.6 ppm with the pyran H-2″ proton (δ 4.68), thus establishing the signal as that of C-8″. This 153.6 ppm carbon was not coupled to the only A-ring proton and so the linkage at C-6 was the likely possibility. In a similar manner, and by virtue of its long-range couplings with the H-4″ protons (δ 2.6–2.8), the chemical shift at 156.9 ppm was assigned to the A-ring C-5″, which in turn was further coupled to the only A-ring proton singlet (δ 6.10), thus defining the latter-mentioned proton signal as belonging to H-6″ and, therefore, confirming the position of the interflavanoid bond as being at C-8. Compound (**3**) was therefore the novel biflavanoid, epicatechin [6′ → 8]-epicatechin. This biflavanoid constitution was also confirmed by electrospray mass spectrometry, which gave the quasimolecular ion $[M-H]^-$ at *m/z* 577.

Table 1. ^{13}C NMR chemical shifts of compounds **1**–**4** in MeOH-d_4

C	**1**	**2**	**3**	**4**
C-2	79.9	82.6	78.0	78.1
C-3	67.5	68.7	65.8	66.0
C-4	29.3	28.3	29.3	29.2
C-4a	100.1	101.0	99.8	100.2
C-5	157.4*	157.4*	157.7	157.9
C-6	96.5	96.5	96.0	96.3
C-7	158.0*	157.6*	157.7	158.0
C-8	96.0	95.7	96.2	96.3
C-8a	157.7	156.8*	157.3	157.6
C-1′	132.3	132.1	131.7	132.3
C-2′	115.4	115.3	115.8	116.2
C-3′	145.8	146.1	145.5*	145.4*
C-4′	145.9	146.1	145.0*	145.2*
C-5′	116.0	117.3	119.9	120.0
C-6′	119.5	120.2	125.2	125.2
C-2″			79.4	82.3
C-3″			66.7	68.4
C-4″			28.9	27.4
C-4a″			100.5	101.2
C-5″			156.9	156.9
C-6″			96.3	96.3
C-7″			154.9	155.2
C-8″			108.1	108.1
C-8a″			153.6	153.3
C-1‴			131.9	132.5
C-2‴			115.0	114.9
C-3‴			145.0*	146.1
C-4‴			145.5*	146.1
C-5‴			116.0	116.0
C-6‴			119.6	119.9

* Assignment not established.

The ^{13}C NMR spectrum of compound (**4**) was very similar to that of the biflavanoid (**3**). The most apparent differences in the spectra of the two compounds were at the upfield region of the spectra associated with the carbons of the pyran C-ring, and the lowfield position of the C-2 carbon (82.3 ppm). The large proton-couplings of the attached hydrogen (δ 4.51, $J = 6.1$ Hz) indicated that the configuration of the pyran ring was 2,3-*trans* or the flavanoid moiety was a catechin unit. The other set of C-ring carbon signals (29.2, 66.0 and 78.1 ppm) was assigned to the epicatechin moiety by comparison with the corresponding values of epicatechin itself and that of compound (**3**) (Table 1). In addition, the presence of the two quaternary carbon peaks at 108.1 and 125.2 ppm, which were characteristic of the biphenyl positions in the phloroglucinol A-ring and the catechol B-ring, respectively, indicated that the catechin and epicatechin units were similarly linked together by a biphenyl bond, as in (**3**). This biflavanoid constitution was also confirmed by electrospray mass spectrometry with the observation of a $[M—H]^-$ at *m/z* 577. In order to establish the position of the interflavanoid bonding, it was necessary to define the proton chemical shifts of the two C-ring protons. In this instance, where the signals were crowded together, the ^{1}H-^{1}H-COSY data were somewhat complex but however, this was clarified with TOCSY, which enabled the resolution of the two sets of C-ring protons. The assignment of the phloroglucinol A-ring and the catechol B-ring carbons were made based on long-range H,C couplings (HMBC) with these C-ring protons. These data indicated that the H-2 proton (δ 4.51) of the catechin moiety was coupled to the high field signal at 153.3 ppm, establishing the latter signal to be the C-8a. However, this carbon was not associated with the only singlet aromatic proton (δ 5.99) in the same A-ring, hence this proton had to be the H-6, this assignment being confirmed by the observation of H,C couplings between the proton and the quaternary C-4a (101.2 ppm). This relationship established C-8 as the point of linkage in the catechin moiety. The similarity of the chemical shift values of the B-ring carbons of the epicatechin moiety in (**4**) with corresponding unit in (**3**), suggested the other point of attachment was likely to be at C-6′. This was established unambiguously by long-range correlation experiments which showed the H-2 (δ 4.70) of the epicatechin moiety was coupled to the quaternary C-1′ (132.3 ppm) and C-2′, as well as the bridging carbon (125.2 ppm). The latter bridging carbon was, as expected, also associated with H-2′ (δ 7.09), thus confirming (**4**) to be the novel epicatechin-(6′ → 8)-catechin.

The chemical structures of (**3**) and (**4**) are analogous to the biphenyl-linked dicatechin first reported by Weinges and Huthwelker [10] among the products resulting from the action of peroxidase and, more recently, by Guyot and co-workers [11] using grape polyphenoloxidase, on catechin. This dimer has also been prepared by chemical oxidation of catechin with potassium ferricyanide [12]. It has been proposed that the mechanism for the formation of the dicatechin involved the oxidation of the B-ring catechol to the corresponding *O*-quinone, which was then followed by nucleophilic attack by the reactive C-8 carbon of another catechin unit in a Michael-type addition reaction to the quinone B-ring [11]. From these reports, it would appear that the new biflavanoids are probably a result of enzymic oxidation of catechin and epicatechin by grape polyphenol oxidase after disruption of the intact fruit.

It is interesting to observe that while catechin and epicatechin were isolated in comparable quantities in Chardonnay pomace, epicatechin was the more frequent participant in the oxidative biphenyl linkages, while catechin was restricted to the reaction involving the phloroglucinol A-ring only in the present study. Catechin apparently can participate in the bipyhenyl linkages at both aromatic rings with reasonable facility as reported earlier [10–12] but it would appear that its epimer was a better substrate for the quinone intermediate under these conditions, probably due to some steric constraints as a result of its 2,3-*trans*-configuration.

EXPERIMENTAL

General. ^{13}C NMR spectra were recorded in MeOH-d_4. MS were obtained by ES-MS. TLC were performed on Schleicher and Schnell cellulose plates developed with HOAc–H_2O (3:47) and visualized with vanillin–HCl spray.

Extraction and isolation. Chardonnay pomace (5 kg) from the Dry River Vineyard, Martinborough, NZ, was transferred into a bucket immediately after pressing, covered with 80% EtOH (10 l) and left to stand at room temp. for 7 days. A portion (2 l) of the extract was concd and the residue diluted with H_2O to 200 ml and defatted with hexane (3 × 200 ml). The resulting soln was then added to a Sephadex LH 20 column (60 × 45 cm) and the column developed with MeOH–H_2O (1:1). Frs of 1 l were collected. Frs 6–10 which contained flavan-3-ols and flavanoid glycosides, as indicated by TLC monitoring, were combined, concd and the residue rechromatographed using aq. EtOH (1:1). This resulted in an early fr. rich in flavan-3-ols, which was again added to another Sephadex LH 20 column prepd with aq. EtOH (3:1) to give homogenous TLC samples.

Epicatechin (**1**). Freeze-dried powder (550 mg). R_f 0.30. ^{13}C NMR: δ 29.3 (C-4), 67.5 (C-3), 79.9 (C-2), 96.0 (C-8), 96.5 (C-6), 100.1 (C-4a), 115.4 (C-2′), 116.0 (C-5′), 119.5 (C-6′), 132.3 (C-1′), 145.8, 145.9 (C-3′, C-4′), 157.4, 157.7, 158.0 (C-5, C-7, C-8a). ^{1}H NMR: δ 2.7–2.9 (*m*, H-4), 4.17 (*bs*, H-3), 4.81 (obscured by H_2O peak, H-2), 5.92 (*bs*, H-6), 5.94 (*bs*, H-8), 6.77 (*m*, H-2′, H-5′), 6.98 (*s*, H-6′).

Catechin (**2**). Freeze-dried powder (490 mg). R_f 0.48. ^{13}C NMR: δ 28.3 (C-4), 68.7 (C-3), 82.6 (C-2), 95.7 (C-8), 96.5 (C-6), 101.0 (C-4a), 115.3 (C-2′), 117.3 (C-5′), 120.2 (C-6′), 132.1 (C-1′), 146.1 (C-3′, C-4′), 156.8, 157.4, 157.6 (C-5, C-7, C-8a). ^{1}H NMR: δ 2.5–2.96 (*m*, H-4), 4.04 (*m*, H-3), 4.64 (*d*, $J = 7.1$ Hz, H-2), 5.94 (*bs*, H-6), 6.00 (*bs*, H-8), 6.7–6.8 (*m*, H-2′, H-5′), 6.89 (*s*, H-6′).

Epicatechin (6′ → 8)*epicatechin* (**3**). Freeze-dried powder (19 mg). R_f 0.78. ^{13}C NMR: δ 28.9, 29.3, 65.8, 66.7, 79.4, 78.0, 96.0, 96.2, 96.3, 99.8, 100.5, 108.1, 115.0, 115.8, 116.0, 119.6, 119.9, 125.2, 131.7, 131.9, 145.0 × 2, 145.5 × 2, 153.6, 154.9, 156.9, 157.3, 157.7 × 2. ^{1}H NMR: δ 2.3–2.6 (*m*, 2 × H-4), 2.6–2.8 (*m*, 2 × H-4″), 3.93 (*bs*, H-3), 4.10 (*bs*, H-3″), 4.60 (*bs*, H-2), 4.68 (*bs*, H-2″) 5.71 (*d*, $J = 2.5$ Hz, H-8), 5.79 (*d*, $J = 2.5$ Hz, H-6), 6.10 (*s*, H-6″), 6.50 (*dd*, $J = 2.0$ and 8.5 Hz, H-6‴), 6.50 (*d*, $J = 8.5$ Hz, H-5‴), 6.62 (*s*, H-5′), 6.67 (*d*, $J = 2.0$ Hz, H-2‴) and 7.12 (*s*, H-2′). ES-MS: $[M—H]^-$ *m/z* 577.

Epicatechin (6′ → 8)*catechin* (**4**). Freeze-dried powder (21 mg). R_f 0.75, ^{13}C NMR: δ 27.4, 29.2, 66.0, 68.4, 78.1, 82.3, 96.3 × 3, 100.2, 101.2, 108.1, 114.9, 116.0, 116.2, 119.9, 120.0, 125.2, 132.3, 132.5, 145.2, 145.4, 146.1 × 2, 153.3, 155.2, 156.9, 157.6, 157.9, 158.0. ^{1}H NMR: δ 2.38–2.55 (*m*, 2 × H-4), 2.47–2.62 (*m*, 2 × H-4″), 3.88 (*bs*, H-3), 3.90 (*m*, H-3″), 4.51 (*d*, $J = 6.1$ Hz, H-2″), 4.70 (obscured by H_2O, H-2), 5.79 (*d*, $J = 2.5$ Hz, H-8), 5.82 (*d*, $J = 2.5$ Hz, H-6), 5.99 (*s*, H-6″), 6.43 (*dd*, $J = 2.3$, 8.5 Hz, H-6‴), 6.57–6.58 (*m*, H-5′, H-2‴, H-5‴), 7.09 (*s*, H-2′). ES-MS: $[M—H]^-$ *m/z* 577.

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