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Procyanidins from the seeds of *Vitis amurensis*

Jiang-Nong Wang[a], Yoshio Hano[b], Taro Nomura[b,*], Ying-Jie Chen[c]

[a]*Department of Biological Science and Technology, Nanjing University, 22 Hankou Road, Nanjing 210093, People's Republic of China*
[b]*Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan*
[c]*Shenyang Pharmaceutical University, 103 Wenhua Road, Shenyang 110015, People's Republic of China*

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Abstract

Three procyanidins, procyanidin B-5 3′-*O*-gallate, vitisinol, and amurensisin, were isolated from the seeds of *Vitis amurensis*, whose structures were elucidated on the basis of spectral and chemical evidence. Vitisinol and amurensisin contain a spiro-type biflavanyl linkage and a biphenyl-lactone partial structure, respectively.

Keywords: *Vitis amurensis*; Vitaceae; Procyanidins; Procyanidin B-5 3′-*O*-gallate; Vitisinol; Amurensisin

1. Introduction

Vitis amurensis Rupr. (Vitaceae) is a wild vine distributed mainly from the north-east regions of China to northern Korea, its fruits are primarily used for wine brewing (Li, 1988). Additionaly, the vine, roots, and leaves have also been utilized in traditional Chinese medicine (Jiangsu New Medical College, 1977). A large number of tannins (including procyanidins) have been found in its fruits (Cathey & Singleton, 1969); however, there are no reports on the analysis of procyanidins in the seeds. Interestingly, some procyanidins from other species have been found to display anti-oxidant (Teissedre, Frankel, Waterhouse, Peleg & German, 1996) and anti-mutagenic effects (Liviero, Puglisi, Morazzoni & Bombardelli, 1994). In our survey of this Chinese medicinal resource, the chemical components of the seeds of *V. amurensis* were examined. This paper describes the isolation and the structure determination of three procyanidins, trivially named as procyanidin B-5 3′-*O*-gallate (**1**), vitisinol (**2**), and amurensisin (**3**), respectively.

2. Results and discussion

The ethanolic extract of the seeds of *V. amurensis* was partitioned between H_2O and chloroform and then between H_2O and ethyl acetate. Successive fractionation of the ethyl acetate soluble portion by Diaion HP-20 and gel filtration followed by HPLC purification afforded three procyanidins, procyanidin B-5 3′-*O*-gallate (**1**), vitisinol (**2**), and amurensisin (**3**), along with five known procyanidins, procyanidins B-1, B-2, B-3, B-4, and B-5 (Thompson, Jacques, Haslam & Tarnner, 1972). The known procyanidins were identified by comparison of their physical and spectroscopic data with those reported in the literature.

Procyanidin B-5 3′-*O*-gallate (**1**), an off-white powder, gave a positive reaction to methanolic ferric chloride and anisaldehyde–sulfuric acid tests, and its molecular formula was determined by HR-FABMS, $C_{37}H_{30}O_{16}$. The ^{1}H NMR spectrum exhibited signals due to two flavan-3-ol units and an aromatic ring, and the spectral pattern was similar to that of procyanidin B monogallate (Table 1) (Thompson et al., 1972). Appearance of the H-2 proton resonance as a broad singlet in both flavan-3-ol units suggested the presence of epicatechin moieties (Table 1) (Thompson et al., 1972; Fletcher, Porter, Haslam & Gupta, 1977). This

* Corresponding author. Tel.: +81-474-72-1207; fax: +81-474-76-6195.

E-mail address: nomura@phar.toho-u.ac.jp (T. Nomura).

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was supported by the ^{13}C-chemical shifts of the heterocylic ring carbons at C-2 and C-3 (Table 1) (Fletcher et al., 1977; Porter, Newman, Foo & Hemingway, 1982). Furthermore, the C-2 signal at δ 77.0 is also consistent with a 2,3-*cis*-3,4-*trans* stereochemistry (Fletcher et al., 1977). The HMBC spectrum supported the structure of compound **1** as an epicatechin dimer monogallate (Fig. 1). In the HMBC spectrum, the H-4 methine signal of the upper unit exhibited correlations with three carbons of the A ring in the lower flavan unit (Fig. 1). These correlations indicated that **1** has either a $C_{4\rightarrow6'}$ or a $C_{4\rightarrow8'}$ interflavanyl bond. Therefore, two possible dimeric procyanidins, procyanidin B-2 monogallate and procyanidin B-5 monogallate were initially considered for the structure of **1**. Hydrolysis of **1** by tannase afforded the dimeric procyanidin (**4**) and gallic acid. The dimeric procyanidin (**4**) was identified as procyanidin B-5 (Thompson et al., 1972; Nonaka, Kawahara & Nishioka, 1982) based on its ^{1}H NMR spectrum, in which the ^{1}H NMR chemical shift values of protons in the heterocyclic ring system of **4** were in good agreement with those of procyanidin B-5 rather than those of procyanidin B-2 (Thompson et al., 1972; Nonaka et al., 1982). A down field shift of H-3 in the lower epicatechin unit is indicative of an ester linkage of a galloyl group at C-3′ (Table 1). Thus, compound **1** was determined to be procyanidin B-5 3′-*O*-gallate.

Vitisinol (**2**), an off-white powder, gave a positive methanolic ferric chloride test. The molecular formula $C_{30}H_{22}O_{12}$ was determined by HR-FABMS, and the IR spectrum displayed absorption bands due to hydroxyl and aromatic groups as well as a significant band due to a γ-lactone carbonyl group (1785 cm^{-1}). The ^{1}H NMR spectral data of **2** were similar to those

Table 1
^{13}C NMR chemical shifts (ppm) for **1**

		CH connectivity[a]
Upper flavan unit		
C-2	77.0	4.94 *br s*
C-3	72.0	4.10 *br s*
C-4	37.3	4.64 *d* (J = 1.2)
C-5	158.8	
C-6	96.6	6.03 *d* (J = 2.4)
C-7	159.3	
C-8	96.0	6.06 *d* (J = 2.4)
C-9	157.7	
C-10	98.9	
C-1″	131.2	
C-2″	114.8	6.98 *d* (J = 1.7)
C-3″	145.3	
C-4″	145.5	
C-5″	115.5	6.76 *d* (J = 8.1)
C-6″	119.0	6.71 *dd* (J = 1.7, 8.1)
Lower flavan unit		
C-2′	77.9	5.08 *br s*
C-3′	69.3	5.50 *m*
C-4′	26.8	2.79 *dd* (J = 2.6, 17.5)
		3.00 *dd* (J = 4.3, 17.5)
C-5′	155.8	
C-6′	107.8	
C-7′	155.2	
C-8′	96.4	6.18 *s*
C-9′	154.9	
C-10′	99.7	
C-1‴	131.9	
C-2‴	115.1	7.06 *d* (J = 2.0)
C-3‴	145.2	
C-4‴	145.3	
C-5‴	115.2	6.74 *d* (J = 8.0)
C-6‴	119.1	6.87 *dd* (J = 2.0, 8.0)
Galloyl unit		
C-1	121.5	
C-2	109.8	7.05 *s*
C-3	145.8	
C-4	138.9	
C-5	145.8	
C-6	109.8	7.05 *s*
C-7	166.0	

[a] Measured in $(CD_3)_2CO$.

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Table 2
^{13}C NMR chemical shifts (ppm) for **2** and **3**[a]

	2	CH connectivity	**3**[b]	CH connectivity
C-2	78.6	4.81 *br s*	80.8	4.97 *br s*
C-3	66.4	4.17 *m*	68.4	4.30 *m*
C-4	28.6	2.66 *d* (2H, J = 1.2)	30.2	2.78 *dd* (J = 2.2, 16.7)
				2.82 *dd* (J = 4.4, 16.7)
C-5	157.2		158.1	
C-6	90.9	6.12 *s*	97.4	5.95 *d* (J = 2.2)
C-7	152.1		158.4	
C-8	105.9		96.8	5.96 *d* (J = 2.2)
C-9	152.8		158.5	
C-10	104.1		100.9	
C-1′	131.2		137.4	
C-2′	114.2	6.78 *d* (J = 2.0)	115.5	7.20 *d* (J = 1.4)
C-3′	145.3		146.2	
C-4′	145.1		139.9	
C-5′	115.6	6.74 *d* (J = 8.2)	113.7	
C-6′	119.4	6.46 *dd* (J = 2.0, 8.2)	118.2	8.68 *d* (J = 1.4)
C-1″	179.1		118.1	
C-2″	61.2		145.8	
C-3″	94.2	5.82 *s*	142.0	
C-4″	106.5		148.3	
C-5″	154.8		109.1	7.43 *s*
C-6″	96.8	5.99 *d* (J = 2.0)	121.3	
C-7″	160.9		164.4	
C-8″	91.2	6.05 *d* (J = 2.0)		
C-9″	163.5			
C-10″	128.8			
C-11″	113.9	6.77 *d* (J = 1.8)		
C-12″	145.5			
C-13″	145.2			
C-14″	115.3	6.59 *d* (J = 8.3)		
C-15″	118.1	6.55 *dd* (J = 1.8, 8.3)		

[a] Measured in $(CD_3)_2CO$.
[b] CD_3OD.

of procyanidin dimers, in which two sets of ABC type aromatic signals, *meta*-coupled aromatic proton signals, an aromatic singlet, and characteristic signals due to H-2, 3, and 4 of an epicatechin unit were observed (Table 2). Signals due to H-2, H-3, and H-4 of the other flavan unit were not observed; instead, only a singlet at δ 5.82 was noted. The ^{13}C NMR spectrum of **2** exhibited 30 carbon signals, whose correlations to proton signals were determined by 2D NMR spectroscopic techniques (^{13}C–^{1}H COSY and HMBC) (Table 2 and Fig. 2(a)). In the HMBC spectrum, the proton signal at δ 5.82 was correlated with the quaternary carbon signals at δ 179.1, assignable to the γ-lactone carbonyl group, and at δ 61.2 of a spirocenter carbon. In addition, the singlet signal at δ 5.82 was correlated with ABC-type aromatic ring carbons and with C-8 of the epicatechin unit (Fig. 2(a)). These data suggested compound **2** to be an analogue of larixinol (**5**), a spiro-type biflavanyl compound isolated from *Larix gmelini* (Shen, Haslam, Falshaw & Begley, 1986). The ^{1}H and ^{13}C NMR chemical shifts of **2** were in agreement with those of **5** reported in the literature (Shen et al., 1986), except for the B and B′ rings both being 3,4-dihydroxyphenyl groups in **2** (Table 2). From the above data, two possible formulae (**2** and **2′**) were considered for the structure of vitisinol. In the 2D NOESY spectrum, observation of a significant NOE between H-2 of the epicatech in unit at δ 4.81 and the H-15″ of the B′ ring at δ 6.55 suggested an angular structure of over a linear one (Fig. 2(b)). The structure of vitisinol is thus represented by **2**. Although its absolute stereochemistry is unknown, the relative stereochemistry is presumably the same as that of larixinol (**5**) based on optical rotation data ($[\alpha]_D - 90^\circ$ in **2** and -150° in **5**).

Amurensisin (**3**), a white amorphous powder, gave positive methanolic ferric chloride and anisaldehyde–sulfuric acid tests. The molecular formula $C_{22}H_{16}O_{10}$ was elucidated by FABMS [m/z 445 $(M + Na)^+$]; the ^{1}H NMR spectrum of **3** showed five aromatic proton signals, comprising two sets of *meta*-coupled signals at δ 5.95, 5.96 (each 1H, *d*, $J = 2.2$ Hz), 7.20, 8.68 (each 1H, *d*, $J = 1.4$ Hz) and a singlet at δ 7.43, and aliphatic protons due to a –CH_2–CH–CH– moiety at δ 2.78 (1H, *dd*, $J = 2.2$ and 16.7 Hz), 2.82 (1H, *dd*, $J =$ 4.4 and 16.7 Hz), 4.30 (1H, *m*), and 4.97 (1H, *br s*) (Table 2). The ^{1}H NMR spectral data suggested that compound **3** is a flavan-3-ol derivative with an additional aromatic ring. The ^{13}C NMR spectrum of **3** exhibited 22 carbon signals, which were correlated with various protons by analysis of 2D HMQC and HMBC spectra (Table 2 and Fig. 3). In the HMBC spectrum, the H-2 proton at δ 4.97 correlated with the B-ring carbons at δ 115.5 and 118.2 (Fig. 3). Furthermore, the proton signal of the additional aromatic ring at δ 7.43 showed a significant correlation with the carbon at δ 164.4, assignable to an α, β-unsaturated ester carbonyl group which exhibited an absorption band at 1698 cm^{-1} in the IR spectrum. These HMBC correlations and the coupling constants (< 1 Hz) between the H-2 and H-3 of the flavan-3-ol unit revealed that compound **3** consists of an epicatechin unit and a gallic acid unit. Balas, Vercauteren and Laguerre (1995) reported that the coupling constant between H-2 and H-3 depends on the conformation of the heterocyclic ring, in which the H-2 proton of the terminal catechin unit in a catechin trimer peracetate appeared as a

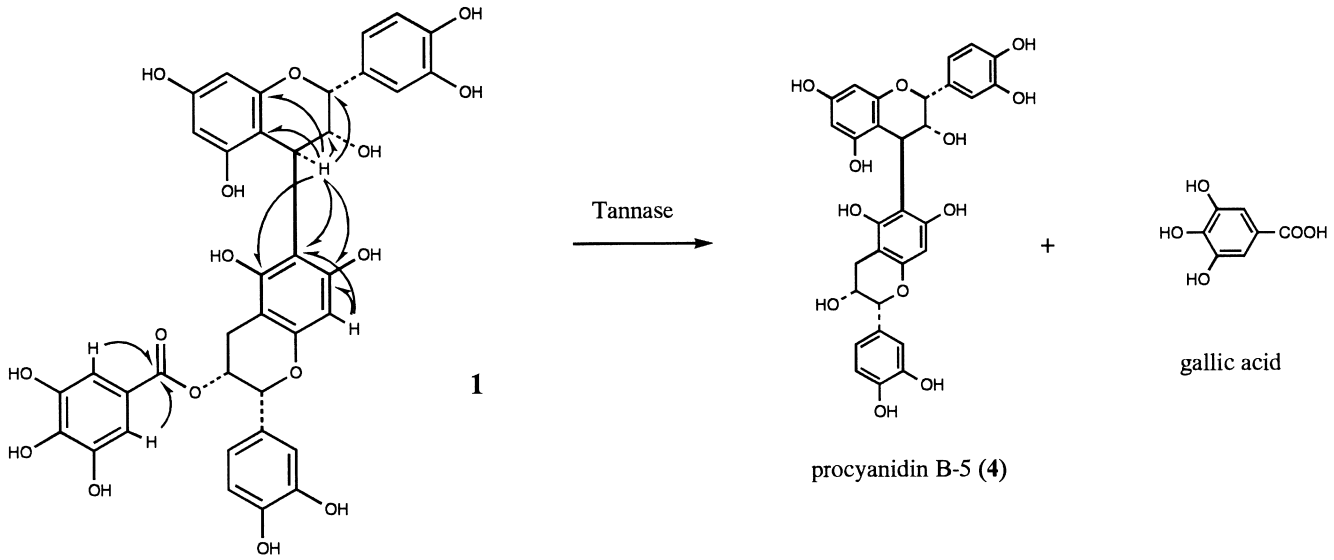
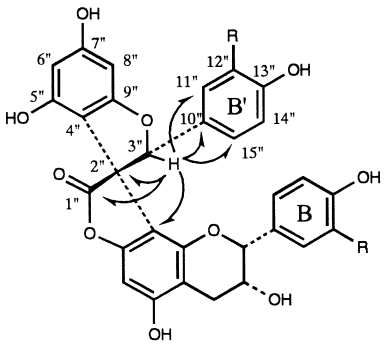
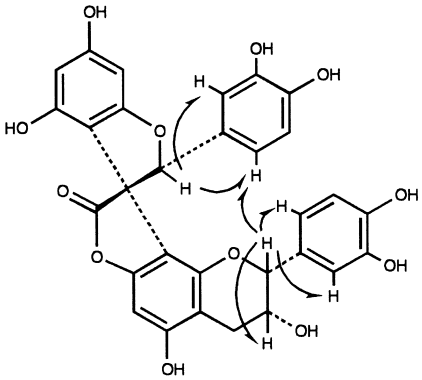


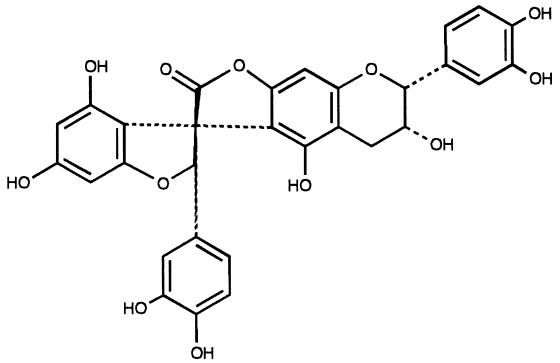
Fig. 1. HMBC correlations of H-4 in **1** and treatment of **1** with tannase.



2 : R = OH

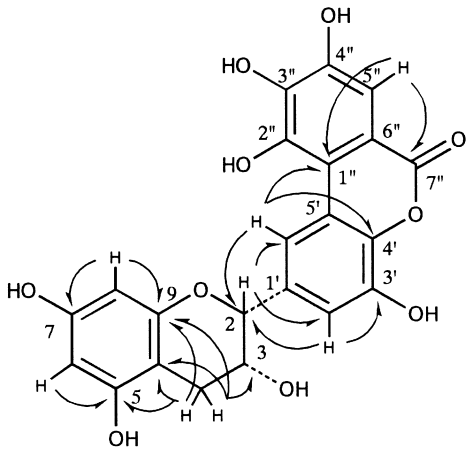
5 : R = H

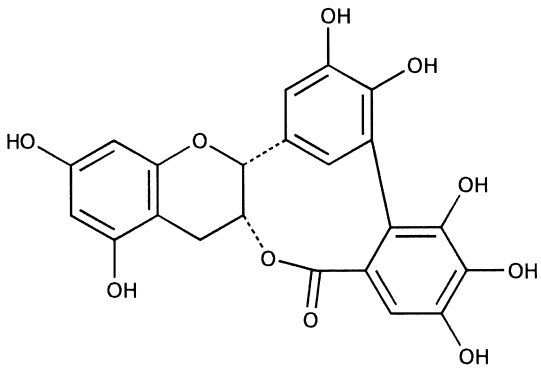




2 **2'**

Fig. 2. (a) HMBC correlations of H-3″ in **2**. (b) NOESY correlations for **2**.





3 **3'**

Fig. 3. HMBC correlations for **3**.

broad singlet having a small coupling constant with the H-3 proton, as well as a long-range W-coupling with the H-4 β proton. However, in such a conformation of the catechin unit, the H-4 protons (δ 2.27, 2.67) caused a higher field shift (Δ ca. 0.4–0.5 ppm) as compared with those in the normal conformation, because of a diamagnetic effect arising from the B ring in an axial orientation. In the ^{1}H NMR spectrum of **3**, the H-4 protons resonated at δ 2.78 and 2.82, respectively. This fact, coupled with the appearance of H-2 as a broad singlet, indicated that **3** undoubtedly contains an epicatechin unit. The gallic acid unit at the C-5′ position, as well as the lactonization at the C-4′ position, were determined from simultaneous correlations of the H-6′ and the H-5″ protons to the carbon at δ 118.1 (C-1″). The other possible structure (**3′**) was eleminated, since no acylation shift was observed for H-3, in contrast to that noted for acylation of epicatechin (δ 4.22) to form epicatechin-3-*O*-gallate (δ 5.55) (Nonaka, Kawahara & Nishioka, 1983). Amurensisin with a biphenyl lactone structure has thus been represented in relative configuration by the formula **3**.

In the present study on the components of the seeds of *Vitis amurensis*, we report three new procyanidins, procyanidin B-5 3′-*O*-gallate (**1**), vitisinol (**2**), and amurensisin (**3**). Epigallocatechin gallate has been found to inhibit tumor promotion on mouse skin (Yoshizawa, Horiuchi, Fujiki, Yoshida, Okuda & Suginuma, 1987). The structural similarity of compound **3** to epigallocatechin gallate suggests that it has the potential to be a cancer chemopreventive agent, this will be the subject of further study.

3. Experimental

3.1. General procedures

Optical rotations were obtained on a JASCO DIP-370 digital polarimeter, whereas UV and IR spectra were recorded on Shimadzu UV-265 and Bruker IFS-55 spectrophotometers, respectively. NMR and MS spectra were measured on JEOL JNM EX-400 FTNMR and JEOL JMS DX-303 spectrometers, respectively. Wakogel C-200 and B-5FM (silica gel, Wako Pure Chemicals, Osaka, Japan) were used for column chromatography and TLC, respectively. HPLC was carried out on an SSC Flow System E-3100 (Senshu Science, Tokyo, Japan) equipped with an SSC-3000B UV detector. Capcell PAK C_{18} (Shiseido Co., Tokyo, Japan) and C/N Nucleosil $5C_{18}$ (Waters Co., Tokyo, Japan) were used as immobilized phase for HPLC.

3.2. Plant material

The seeds of *V. amurensis* were collected in the suburbs of Tonghua, Jilin province, China, in August 1994, and identified by Prof. Ze-rong Jiang, Shenyang Pharmaceutical University. An authentic specimen (TH 940816-1) was deposited in Shenyang Pharmaceutical University.

3.3. Extraction and isolation

The dried seeds of *V. amurensis* (10 kg) were coarsely ground and extracted three times with 80% EtOH for 1 day. The 80% EtOH solution was concentrated under reduced pressure to give a suspension. Successive extraction of the suspension yielded a $CHCl_3$-soluble fraction (10 g), an EtOAc-soluble fraction (123 g), and an *n*-BuOH-soluble fraction (200 g). The EtOAc-soluble fraction (123 g) was subjected to CC over Diaion HP-20 (500 ml) with H_2O–MeOH as eluent to afford five fractions [100% H_2O (5 l), 20% MeOH (5 l), 40% MeOH (5 l), 60% MeOH (5 l), 100% MeOH (5 l)]. The fraction (45 g) eluted with H_2O was subjected to Sephadex LH-20 (200 ml) chromatography eluted with EtOH followed by further fractionation on MCI gel CHP-20P (100 ml) to afford to known procyanidins B-1 (20 mg), B-2 (40 mg), B-3 (10 mg), B-4 (10 mg), and B-5 (200 mg); each was identified by comparison of their physical and spectral data with those reported in the literature. Fractions (40 g) eluted with 40% and 60% MeOH–H_2O were combined and passed through a Sephadex LH-20 (200 ml) column with H_2O and H_2O–MeOH as eluent. The fraction eluted with 100% H_2O (frs. 1′–10′, 1.3 g) was further subjected to CC on MCI gel CHP-20P (100 ml) with 30% MeOH–H_2O to afford 32 fractions (frs. 1″–32″). HPLC purification [solvent system; 2.5% AcOH–80% aq. CH_3CN (4:1), column, CAPCELL PAK C_{18}, $10\phi \times 250$ mm), flow rate, 2 ml/min] of fraction 18″ (30 mg) yielded vitisinol (**2**, 10 mg). Combined fractions eluted with 30% MeOH–H_2O during Sephadex LH-20 chromotography (frs. 28′–30′, 5 g) were next treated with TOYOPEARL HW-40 (200 ml) using 60% MeOH–H_2O and then MeOH–H_2O–acetone (8:1:1, 3:1:1, and 4:3:3) as eluents to afford fractions 1″–9″. Further gel filtration of fraction 9″ with TOYOPEARL HW-40 afforded amurensisin (**3**, 3 mg). The combined fractions 6″–8″ (20 mg) were subject to an additional chromatographic step using MCI gel CHP-20P to yield procyanidin B-5 3′-*O*-gallate (**1**, 15 mg).

3.4. Procyanidin B-5 3′-O-gallate (*1*)

Off-white powder; $FeCl_3$and anisaldehyde–sulfuric acid tests: positive; $[\alpha]_D^{22} + 61^\circ$ (*c* 0.12, MeOH), UV λ_{max} (MeOH) nm (log ε): 211 (4.95), 279 (4.20), IR

ν_{max} (KBr) cm^{-1}: 3340 (*br*), 2853, 1691, 1605, 1519, 1446, 1373, 1340, 1318, 1294, 1234, 1200, 1147, 1112, 1030, FABMS: *m/z* 731 $[M + H]^+$, HR-FABMS: *m/z* 731.1586 $[M + H]^+$ ($C_{37}H_{31}O_{16}$, requires 731.1621).

3.5. Hydrolysis of **1** with tannase

A solution of **1** (2 mg) and tannase (5 mg) in H_2O (5 ml) was left standing for 3 h at 35°C, after which water was removed in vacuo. The EtOH soluble part of the reaction mixture was then subjected to CC over Sephadex LH-20 (5 g) (eluent; EtOH) to give two substances, which were identical with gallic acid and procyanidin B-5, respectively.

3.6. Vitisinol (**2**)

White powder; $FeCl_3$ test: positive; $[\alpha]_D^{22} - 90°$ (*c* 0.1, MeOH), UV λ_{max} (MeOH) nm (log ϵ): 210 (4.63), 280 (4.12); IR ν_{max} (KBr) cm^{-1}: 3378 (*br*), 1785, 1624, 1521, 1469, 1364, 1284, 1197, 1140, 1114, 1089; CD ($c = 6.9 \times 10^{-6}$ mol, MeOH): $[\theta]_{214} + 26057$, $[\theta]_{232} + 70887$, $[\theta]_{246} - 312455$, $[\theta]_{288} - 153857$. FABMS: *m/z* 575 $[M + H]^+$. HR-FABMS: *m/z* 575.1185 $[M + H]^+$ ($C_{30}H_{23}O_{12}$, requires 575.1193).

3.7. Amurenisin (**3**)

White powder; $FeCl_3$ and anisaldehyde–sulfuric acid tests: positive; $[\alpha]_D^{22} - 47°$ (*c* 0.03, MeOH) UV λ_{max} (MeOH) nm (log ϵ): 207 (4.95), 262 (4.20), 32 (4.05); IR ν_{max} (KBr) cm^{-1}: 3381 (*br*), 2853, 1698, 1604, 1531, 1436, 1351, 1294, 1206, 1153, 1109; FABMS: *m/z* 445 $[M + Na]^+$.

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