Two New Stilbene Dimer Glucosides from Grape (*Vitis vinifera*) Cell Cultures

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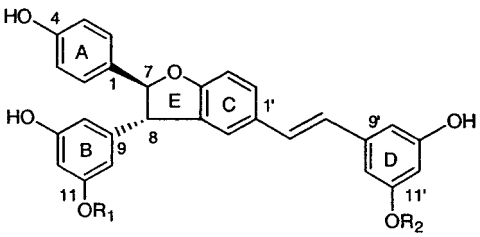
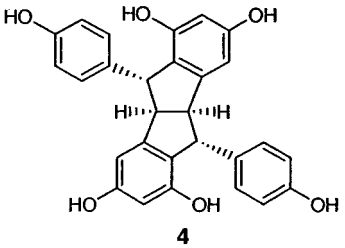
Two new stilbene dimer glucosides, resveratrol (*E*)-dehydrodimer 11-*O*-β-D-glucopyranoside (**1**) and resveratrol (*E*)-dehydrodimer 11′-*O*-β-D-glucopyranoside (**2**), were isolated together with the known resveratrol (*E*)-dehydrodimer (**3**) and pallidol (**4**) from *Vitis vinifera* cell cultures. The structures and stereochemistry of the new compounds were determined on the basis of spectroscopic data analysis. Compounds **1** and **2** are dimers that belong to a new type of oligostilbene formed from a resveratrol unit and a resveratrol glucoside unit. Compounds **1** and **3** exhibited nonspecific inhibitory activity against cyclooxygenase-1 and -2, with IC_{50} values in the range of 5 μM, whereas compound **4** was approximately 10-fold less active.

Stilbenes occur naturally in several plant families, such as the Dipterocarpaceae, Vitaceae, Cyperaceae, and Gnetaceae,[1,2] but grapes (*Vitis vinifera* L., Vitaceae) and products manufactured from grapes are considered the most important dietary sources of these substances.[3,4] A previous study has shown that (*E*)-resveratrol (3,5,4′-trihydroxy-*E*-stilbene), a grapevine phytoalexin, inhibits cyclooxygenase-1 (COX-1).[5] Earlier phytochemical studies on grape cell cultures have revealed that they biosynthesize resveratrol monomer derivatives.[6−8] Resveratrol can be biotransformed by *Botrytis cinerea*, a fungal grapevine pathogen, into resveratrol (*E*)-dehydrodimer (**3**), pallidol (**4**), leachinol F, and restrytisols A−C.[9,10]



1: R_1 = β-glc, R_2 = H
2: R_1 = H, R_2 = β-glc
3: R_1 = H, R_2 = H



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In our search for new cancer chemopreventive agents, a diverse group of natural products capable of mediating activities relevant to cancer chemoprevention has been isolated and characterized from plants.[11,12] In further work directed toward the search for novel natural product cancer chemopreventive agents, a fraction derived from an EtOAc-soluble extract of a grape cell culture was identified as a potent inhibitor of COX-1 activity (73% inhibition at 70 μg/mL). Besides resveratrol (*E*)-dehydrodimer (**3**), which was synthesized more than 20 years ago,[13] and pallidol (**4**),[14] two new glucosides (**1** and **2**) of **3** were isolated by activity-guided fractionation using the COX-1 inhibitory assay, and their structures were determined on the basis of the spectroscopic data analysis.

Compound **1** was purified by semipreparative reversed-phase HPLC, and its molecular formula of $C_{34}H_{32}O_{11}$ was established by positive HRFABMS (*m*/*z* [M + Na]$^+$, 639.1837). The ^{1}H and ^{13}C NMR data (Table 1) of **1** indicated that it is a dimer consisting of a resveratrol unit and a resveratrol glucoside unit. Assignments of all ^{1}H and ^{13}C NMR signals for **1** were made by the analysis of COSY, HMQC, and HMBC 2D NMR data. The ^{1}H NMR spectrum of **1** showed the presence of characteristic resonances in two distinct regions. The former, between δ_H 7.5 and δ_H 6.0, was constituted by a broad doublet at δ_H 7.43 (H-6′), a broad singlet at δ_H 7.26 (H-2′), and a doublet at δ_H 6.87 (H-5′) of an ABX-spin system of ring C, two doublets at δ_H 7.25 (H-2 and H-6) and δ_H 6.86 (H-3 and H-5) of an AA′XX′-spin system of a 1,4-disubstituted aromatic ring A, a triplet at δ_H 6.25 (H-12′) and a doublet at δ_H 6.53 (H-10′ and H-14′) of an AB_2-spin of ring D, three broad triplets at δ_H 6.52 (H-12), δ_H 6.44 (H-14), and δ_H 6.35 (H-10) of an ABC-spin system of ring B, and two coupled doublets at δ_H 7.06 (H-7′) and δ_H 6.90 (H-8′) with a large coupling constant (J = 16.3 Hz) for a trans olefinic proton system. The second region of the ^{1}H NMR spectrum of **1**, between δ_H 6.0 and δ_H 3.0, was characterized by two doublets at δ_H 5.52 (H-7) and δ_H 4.53 (H-8) for a dihydrobenzofuran moiety, one doublet at δ_H 4.94 for the anomeric proton of a glucose unit, and six protons of a glucosyl moiety between δ_H 4.0 and δ_H 3.0. Also, the ^{13}C NMR spectrum showed six signals at δ_C 101.43, 77.87, 77.56, 74.58, 71.16, and 62.49, which are characteristic for a glucose unit.[15] Moreover, enzymatic hydrolysis of **1** with β-glucosidase led to the generation of the aglycon, resveratrol (*E*)-dehydrodimer (**3**). The anomeric proton signal at δ_H 4.94 (1H, d, J = 7.6 Hz) is indicative of a β-configuration for the glucosyl bond.

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Table 1. ^{1}H and ^{13}C NMR Data of Compounds **1** and **2** in CD_3COCD_3[a]

	1		2	
position	δ_H	δ_C	δ_H	δ_C
1		132.51		132.19
2(6)	7.25 d (8.5)	128.55	7.25 d (8.6)	128.27
3(5)	6.86 d (8.5)	116.20	6.85 d (8.6)	115.81
4		158.44		158.99
7	5.52 d (7.8)	93.83	5.46 d (8)	93.75
8	4.53 d (7.8)	57.81	4.47 d (8)	57.49
9		145.27		144.90
10	6.35 t (2)	109.64	6.19 d (2.1)	107.06
11		160.15		159.88
12	6.52 t (2)	103.14	6.28 t (2.1)	102.00
13		159.60		159.88
14	6.44 t (2)	108.50	6.19 d (2.1)	107.06
1′		140.77		140.41
2′	7.26 brs	123.93	7.27 brs	123.60
3′		132.15		131.88
4′		160.57		160.61
5′	6.87 d (8.2)	110.21	6.88 d (8.2)	109.83
6′	7.43 brd (8.2)	128.65	7.44 brd (8.2)	128.76
7′	7.06 d (16.3)	129.10	7.14 d (16.3)	129.30
8′	6.90 d (16.3)	127.28	6.95 d (16.3)	128.20
9′		131.80		131.56
10′	6.53 d (2)	105.71	6.81 brs	107.83
11′		159.54		160.28
12′	6.25 t (2)	102.70	6.53 t (2)	103.50
13′		159.54		158.95
14′	6.53 d (2)	105.71	6.68 brs	106.03
Glc				
1	4.94 d (7.6)	101.43	4.94 d (7.4)	101.10
2		74.58		74.33
3		77.87		77.63
4		71.16		71.02
5		77.56		77.41
6		62.49		62.31

[a] TMS was used as the internal standard; chemical shifts are shown in the δ scale with J values (Hz) in parentheses.

Therefore, compound **1** could be proposed as a glycoside of resveratrol (*E*)-dehydrodimer (**3**).

The position of the glucose unit in **1** was determined by the HMBC NMR experiment, which showed a long-range correlation between the anomeric proton signal at δ_H 4.94 (H-1 of Glc) and C-11 at δ_C 160.15 of ring B. Also, the presence of an ABC-spin system of ring B in the ^{1}H NMR spectrum of **1** instead of an AB_2-spin system confirmed the position of the glucose moiety at C-11. The relative configuration of the chiral centers of ring E of **1** was deduced from the coupling constant (J = 7.8 Hz) between the H-7 and H-8 benzofuran protons, which was identical to the coupling constant found for **3**.[16] Therefore, the aromatic substituents of ring E were arranged in a trans configuration. Accordingly, the structure of **1** was assigned as resveratrol (*E*)-dehydrodimer 11-*O*-β-D-glucopyranoside.

Compound **2** was obtained as a minor constituent, and its molecular formula of $C_{34}H_{32}O_{11}$ was established by positive HRFABMS (*m*/*z* $[M + Na]^+$, 639.1890), again corresponding to a dimer of a resveratrol unit and a resveratrol glucoside unit. The ^{1}H and ^{13}C NMR data (Table 1) of **2** were closely comparable to those of **1** except for the signals of rings B and D. This suggested that compound **2** is a regioisomer of **1** with the position of the glucose unit transferred from C-11 to C-11′. On enzymatic hydrolysis of **2** with β-glucosidase, compound **3** was generated. The position of the glucose unit was confirmed by the HMBC NMR technique, which showed a three-bond correlation between the anomeric proton signal at δ_H 4.94 (1H, d, J = 7.4 Hz, H-1 of Glc) and C-11′ at δ_C 160.28 of ring D. Thus, the structure of **2** was concluded to be resveratrol (*E*)-dehydrodimer 11′-*O*-β-D-glucopyranoside. The relative trans configuration of the chiral centers of ring E was deduced in the same manner as **1**.

Additionally, two known compounds, resveratrol (*E*)-dehydrodimer (**3**) and pallidol (**4**), were isolated and identified by comparison of the observed ^{1}H and ^{13}C NMR data with literature values.[9,16,17] Using the ROESY NMR experiment and by J value comparison, the relative stereochemistry of resveratrol (*E*)-dehydrodimer (**3**) was confirmed. Thus, a ROE correlation between H-7 and H-10 (H-14) and the coupling constant (J = 8 Hz) between H-7 and H-8 clearly indicated a trans configuration of the chiral centers of ring E.[17] Resveratrol (*E*)-dehydrodimer (**3**) and pallidol (**4**) have not been demonstrated previously as constituents of *V. vinifera* or its cell cultures. Resveratrol (*E*)-dehydrodimer (**3**) was reported recently as a natural product from the lianas of *Gnetum hainanense* C. Y. Cheng.[16]

Compounds **1**, **3**, and **4** were evaluated for their cyclooxygenase-1 and -2 (COX-1 and -2) inhibitory activity. Compound **4** was marginally active, with IC_{50} values of 50 and 80 μM with COX-1 and -2, respectively. The isolated quantity of **2** was not sufficient to permit evaluation of biological activity. Compounds **1** and **3** demonstrated IC_{50} values of 5.2 and 4.3 μM, respectively, when evaluated with COX-1, and 7.5 and 3.7 μM, respectively, when evaluated with COX-2. Thus, inhibitory potential is reasonably strong with these test agents, but specificity is lacking in both cases.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Perkin-Elmer 241 polarimeter. UV spectra were recorded on a Beckman DU-7 spectrometer. IR spectra were taken on a JASCO 410 FT-IR spectrometer. NMR spectra were measured on a Bruker DRX-500 MHz spectrometer using a 2.5 mm or a 5 mm sample tube. EIMS and FABMS were obtained using a Finnigan MAT-90 mass spectrometer, and HRFABMS were obtained on a VG 7070-HF instrument. HPLC was performed using a Waters 515 pump and a Waters 2487 UV detector.

Cell Culture Material. Cell cultures of *V. vinifera* (L.) cv. Gamay Freaux var. Tenturier were established in 1978 from pulp fragments of young fruits and provided by C. Ambid (ENSA, Toulouse, France). Suspension cultures of *V. vinifera* were maintained as described previously.[18] Experiments were carried out by inoculating a 7-day-old cell suspension into an induction medium at a 1:8 (v/v) ratio, for each transfer.[18]

Extraction and Isolation. Frozen cells (2.5 kg, fresh weight) were extracted with acetone/water as reported previously.[6,7] The aqueous mixture was partitioned with ethyl acetate. The ethyl acetate extract was chromatographed over a Dowex-50 × 4-400 cation-exchange resin (Sigma, St. Louis, MO) and eluted by methanol/water. Crude polyphenols were eluted with 50% methanol. For further fractionation, the crude polyphenol-containing fraction was divided into three main subfractions by passage over Sephadex LH-20 and elution with MeOH/H_2O mixtures. Mixtures of (*Z*)-stilbenes and (*E*)-stilbenes were eluted by 20% MeOH and 30% MeOH, respectively, and were not investigated further. A mixture of stilbene dimers was eluted by 100% MeOH and further purified on Toyopearl HW-40S gel (Supelco, Bellefonte, PA), eluted with 100% MeOH, resulting in two main fractions. Final purification of the first fraction by HPLC resulted in the purification of compounds **1** (14 mg, 0.00056% w/w), **2** (0.6 mg, 0.00002% w/w), and **3** (13 mg, 0.00052% w/w) [column ODS-AQ Pack (YMC, Wilmington, NC), 20 × 250 mm i.d., C_{18}, 5 μm, 120 Å; guard column ODS-AQ Guard Pack (YMC, Wilmington, NC), 20 × 100 mm i.d.; linear gradient from 40% to 100% MeCN in H_2O (pH 2.4 with TFA), 30 min, 8 mL/min]. Pallidol (**4**,

12 mg, 0.00048% w/w) was purified from the second fraction using HPLC [50% MeCN in H_2O (pH 2.4 with TFA), 40 min, 8 mL/min].

Resveratrol (*E*)-dehydrodimer 11-*O*-*β*-D-glucopyranoside (1): powder; $[\alpha]^{20}_D$ −18.9° (*c* 0.38, MeOH); UV (MeOH) λ_{max} (log ϵ) 204 (4.73), 221 (4.58), 309 (4.43), 320 (4.39) nm; IR (neat) ν_{max} 3352, 2924, 1698, 1597, 1515, 1487, 1451, 1356, 1236, 1202, 1152 cm^{-1}; ^1H and ^{13}C NMR data of **1**, see Table 1; EIMS *m*/*z* 454 (15), 320 (40), 246 (28), 208 (45), 144 (100); FABMS *m*/*z* 639 $[M + Na]^+$, 616 $[M^\bullet]^+$, 455 (15), 406 (20), 329 (27), 307 (77), 289 (56), 176 (100); HRFABMS *m*/*z* calcd for $C_{34}H_{32}O_{11}Na$ 639.1833, found 639.1837.

Resveratrol (*E*)-dehydrodimer 11′-*O*-*β*-D-glucopyranoside (2): powder; $[\alpha]^{20}_D$ −12.0° (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 206 (4.88), 309 (4.23), 322 (4.21) nm; IR (neat) ν_{max} 3394, 2924, 1652, 1558, 1507, 1227, 1144, 1034 cm^{-1}; ^1H and ^{13}C NMR data of **2**, see Table 1; FABMS *m*/*z* 639 $[M + Na]^+$, 616 $[M^\bullet]^+$, 482 (72), 460 (58), 455 (28), 273 (95); HRFABMS *m*/*z* calcd for $C_{34}H_{32}O_{11}Na$ 639.1833, found 639.1890.

Resveratrol *E*-dehydrodimer (3): oil; $[\alpha]^{20}_D$ −1.7° (*c* 0.23, MeOH) [lit. $[\alpha]^{25}_D$ −1.15° (*c* 7.3, acetone)];[10] UV, IR, ^1H and ^{13}C NMR, and EIMS data, consistent with literature values.[10]

Pallidol (4): $[\alpha]^{20}_D$ 0° (*c* 0.45, MeOH) [lit. $[\alpha]^{20}_D$ 0° (MeOH);[14] $[\alpha]^{23}_D$ −36.3° (*c* 0.13, MeOH)[16]]; UV, IR, ^1H and ^{13}C NMR, and EIMS data, consistent with literature values.[14,16]

Enzymatic Hydrolysis of 1 and 2. Separate solutions of **1** (1 mg) and **2** (0.3 mg) in acetate buffer (pH 6) were treated with *β*-glucosidase (1 mg/mL) for 24 h at room temperature. Each reaction solution was evaporated to dryness with the resultant residue analyzed by HPLC [column Nova-Pak (Waters, Milford, MA), 3.9 × 300 mm i.d., C_{18}, 16 μm, 60 Å; 30% MeCN in H_2O, 0.5 mL/min, t_R 28.0 min] and TLC (Si gel, $CHCl_3$/MeOH, 5:1, R_f 0.35) to afford **3**.

Cyclooxygenase-1 and -2 Inhibition Assay. The effect of test compounds on cyclooxygenase-1 and -2 (COX-1 and -2) was determined by measuring PGE_2 production. Reaction mixtures were prepared in 100 mM Tris-HCl buffer, pH 8.0, containing 1 μM heme, 500 μM phenol, 300 μM epinephrine, sufficient amounts of COX-1 or COX-2 to generate 150 ng of PGE_2/mL, and various concentrations of test samples. The reaction was initiated by the addition of arachidonic acid (final concentration, 10 μM) and incubated for 10 min at room temperature (final volume, 200 μL). Then, the reaction was terminated by adding 20 μL of the reaction mixture to 180 μL of 27.8 μM indomethacin, and PGE_2 was quantitated by an ELISA method. Samples were diluted to the desired concentration with 100 mM potassium phosphate buffer (pH 7.4) containing 2.34% NaCl, 0.1% bovine serum albumin, 0.01% sodium azide, and 0.9 mM Na_4EDTA. Following transfer to a 96-well plate (Nunc-Immuno Plate Maxisorp, Fisher) coated with a goat anti-mouse IgG (Jackson Immuno Research Laboratories), the tracer (PGE_2-acetylcholinesterase; Cayman Chemical, Ann Arbor, MI) and primary antibody (mouse anti PGE_2; Monsanto, St. Louis, MO) were added. Plates were then incubated at room temperature overnight, reaction mixtures were removed, and wells were washed with a solution of 10 mM potassium phosphate buffer (pH 7.4) containing 0.01% sodium azide and 0.05% Tween 20. Ellman's reagent (200 μL) was added to each well, and the plate was incubated at 37 °C for 3−5 h, until the control wells yielded OD = 0.5−1.0 at 412 nm. A standard curve with PGE_2 (Cayman Chemical, Ann Arbor, MI) was generated on the same plate, which was used to quantify the PGE_2 levels produced in the presence of test samples. Results were expressed as a percentage, relative to control (solvent-treated) samples, and dose−response curves were constructed for the determination of IC_{50} values.

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