JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Stable Isotope Labeling Pattern of Resveratrol and Related Natural Stilbenes

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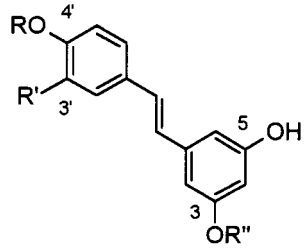
The stable isotope characterization of resveratrol **1** from *Polygonum cuspidatum* and of related natural stilbenes **11** and **12** obtained by hydrolysis of the corresponding glucosides **2** and **3** from *Rheum* is reported. The $C_6-C_2-C_6$ framework of suitably protected derivatives of **1**, **2**, and **3** has been degraded with ozone to the C_6-C_1 aldehydes **4**, **5**, **9**, and **10**, retaining all hydrogen atoms of the precursors. The natural and synthetic derivatives are characterized and distinguished by natural abundance deuterium nuclear magnetic resonance studies. In the case of anisaldehyde **4** the two series show, as expected, the characteristic difference of the aromatic labeling. The formyl deuterium contents of **4** and **5** from resveratrol are remarkably different, seemingly reflecting the different enrichments existing between positions 3 and 2, respectively, of the phenylpropanoid precursor. The positional $\delta^{18}O$ values of the extractive materials **1**−**3** were also determined. In this instance a selective deoxygenation procedure was adopted, leading from **1** to the products **6**, **7**, and **8**. The $\delta^{18}O$ values of the latter compounds reveal, respectively, those at position 4′ and positions 3 and 5 of **1**. Similarly, the phenolic products **11** and **12** were converted into **13** and **14**. From the $\delta^{18}O$ values of the single components it is possible to design a detailed map of the oxygen fractionations which characterizes the stilbenes **1**−**3**. In particular, the oxygen present at position 4′ of the phenylpropanoid moiety of **1**−**3** shows $\delta^{18}O$ values of +11.5, +1.8, and +6.7‰, respectively. Moreover, the phenolic oxygen atom at position 3′ of rhapontin **3** shows a value of +11.7‰. The data are compared with those previously obtained on structurally related compounds. These results show the utility of simple chemical degradations in the stable isotope characterization of structurally complex food components.

KEYWORDS: Resveratrol; rhapontin; stilbenes; natural; deuterium NMR; oxygen-18; isotopic characterization

INTRODUCTION

The distribution of the stable isotopes within the atomic species constituting an organic molecule is not random but depends instead upon the way in which it was constructed (*1*). Accordingly, through the determination of the site-specific isotopic enrichment of a compound it is possible to elucidate the nature of the operations that presided over the synthesis, thus revealing its origin. In this context we now report on the stable isotope characterization of resveratrol **1** from *Polygonum cuspidatum* (*2*) and of the related stilbenic glycosides **2** and **3** from *Rheum* (*3*, *4*). Resveratrol **1**, a long known plant secondary metabolite that received recent attention due to its biological activity (*5*), is commercially available as a food additive. There are two sources for this material: the costly plant extraction (*6*) and the chemical synthesis from two readily accessible C-6−C-1 precursors, that is, anisaldehyde **4** and 3,5-dimethoxybenzaldehyde **5** (*7*−*9*). Due to the preference of consumers for



1 R=R'=R"=H (resveratrol)
2 R=CH_3; R'=H; R"=β-glucopyranosyl
3 R=H; R'=OCH_3; R"=β-glucopyranosyl (rhapontin)

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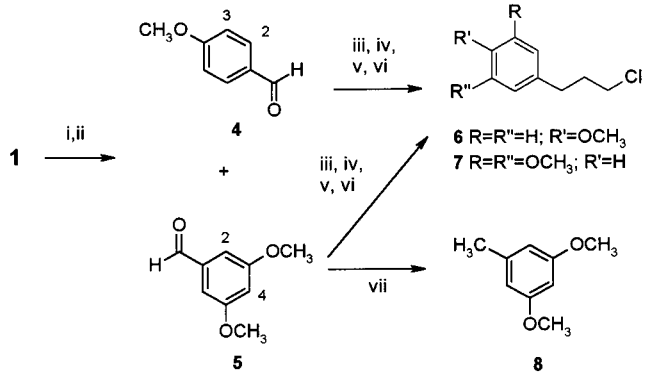
10.1021/jf011103j CCC: $22.00
Published on Web 04/06/2002

Scheme 1. Degradation of Resveratrol **1** into the Aldehydes **4** and **5** for the Deuterium Isotopic Measurements and to the Compounds **6**–**8** for the Positional ^{18}O Isotopic Determination[a]



[a] (i) diazomethane; (ii) O_3/CH_2Cl_2–MeOH 1:1, −78 °C; then Ph_3P; (iii) $Ph_3P{=}CHCO_2Et/CH_2Cl_2$; (iv) H_2/Pd–C/AcOEt; (v) $LiAlH_4$/THF; (vi) Ph_3P/CCl_4 reflux; (vii) H_2/Pd/C.

what is natural in food, it seemed desirable to verify if isotopic methods could be suitable also in the case of resveratrol **1** for the definition of its extractive rather than synthetic origin. Because resveratrol **1** is accessible in quantity only from *P. cuspidatum*, the structurally related stilbenes **2** and **3** were analyzed as comparison substances.

In food chemistry, perhaps the most useful analytical method for the definition of the origin of an organic molecule is deuterium natural abundance ^2H NMR spectroscopy (SNIF-NMR) (*10*). However, a prerequisite for the applicability of this technique is that the deuterium signals in the NMR spectrum of the molecule under examination are dispersed enough to allow a proper integration of the individual signals. Unfortunately, this is not the case for resveratrol **1** and the related products **2** and **3**, which are characterized by the presence of only C−H signals on sp^2 carbon atoms, the ^1H NMR signals of which occur in a restricted region (for **1** between 6.20 and 7.40 ppm). It was therefore considered to be necessary to specifically degrade **1** and the structurally related natural compounds **2** and **3** to smaller molecules meeting the above-mentioned prerequisites, that is, (baseline) separation of the deuterium signals in the NMR spectrum. The procedure applied in the present instances simply consisted of the ozonolytic splitting of the double bonds of **1**, **2**, and **3**, respectively, performed on suitably protected derivatives. By these means, the molecules produced from the permethylated form of **1** are anisaldehyde **4** and 3,5-dimethoxybenzaldehyde **5**, respectively (**Scheme 1**). These two C-6−C-1 molecules are the same as those used in the chemical synthesis of **1** (*7*−*9*). Thus, comparison of the deuterium content of **4** and **5** from extractives **1**−**3** with chemically identical samples of synthetic origin would represent a comparison between extractive and synthetic specimens.

In addition to natural abundance deuterium NMR studies, $\delta^{18}O$ measurements were also carried out on **4** and **5** of natural and synthetic derivation for characterization purposes. The studies were done on specific degradation products of extractives **1**−**3**, allowing the assignment of the positional $\delta^{18}O$ values of the oxygen atoms attached to the sp^2 carbon atoms of these phenolic products. Despite the fact that isotopic oxygen depletion measurements are considered to be of paramount importance in defining the mechanism of natural phenomena in earth sciences (*11*), a limited number of applications of this diagnostic tool in the definition of the origin of oxygenated organic molecules have been reported to date. This might be due to the fact that in the case of polyoxygenated compounds only *positional* oxygen depletion values are significant for identification purposes (*12*). To specifically identify a single oxygen atom of the many present in a multiply oxygenated molecule, it is necessary to rely on the techniques of the chemical synthesis, which are sometimes difficult to apply. However, the diagnostic potential of the positional $\delta^{18}O$ measurements in the definition of the extractive rather than synthetic origin of molecules of importance in food chemistry has been recently demonstrated in the case of raspberry ketone and vanillin, phenolic compounds structurally related to **1**−**3** (*13*−*15*). Accordingly, in the present study **1**−**3** were specifically deoxygenated, thus allowing also the $\delta^{18}O$ values relative to the oxygen atoms present at different positions of the C-6−C-2−C-6 framework to be acquired. The results of the multiple isotope characterization of stilbenes **1**−**3** are presented below.

EXPERIMENTAL PROCEDURES

Origin of the Samples. Resveratrol from *P. cuspidatum* (from China) was a gift of Dr. P. Maggioni (Chemprogress, Sesto Ulteriano, Italy). Rhapontin, containing ∼20% of the β-glycoside of 4′-*O*-methylresveratrol, was a commercial sample. Natural anisaldehyde was from Robertet (Grasse, France), whereas the synthetic modifications were from Aldrich (Milwaukee, WI) and BBE (Milano, Italy), respectively. Synthetic 3,5-dimethoxybenzaldehyde was from Aldrich.

Degradation of Natural Resveratrol. *(a) Anisaldehyde **4** and 3,5-Dimethoxybenzaldehyde **5**.* Tri-*O*-methylresveratrol was obtained quantitatively by treating a dilute methanolic solution of resveratrol at 10 °C with a large excess of ethereal diazomethane over ∼24 h. The above material, 8.1 g (0.03 mol), in 80 mL of CH_2Cl_2/MeOH (1:1) was treated at −78 °C with ozonized oxygen until the appearance of a blue color. The ozone stream was replaced with nitrogen, and Ph_3P (7.8 g; 0.03 mol) in CH_2Cl_2 (30 mL) was added dropwise. After 30 min, the temperature was raised and the crude mixture was evaporated under vacuum at 30−40 °C. The residue was taken up with Et_2O/hexane, and the precipitated phosphine oxide was removed by filtration. The oily residue was chromatographed on SiO_2, eluting with increasing quantities of ethyl acetate in hexane to give anisaldehyde **4**, 3 g (73%), purified by bulb-to-bulb vacuum distillation, then mixed fractions, and, finally, 3,5-dimethoxybenzaldehyde **5**, 3 g (60%), mp 46−48 °C (from hexane). There was no formation of the acids or of the alcohols corresponding to **4** and **5**.

*(b) 3-(4-Methoxyphenyl)-1-chloropropane **6** and 3-(3,5-Dimethoxyphenyl)-1-chloropropane **7**.* Anisaldehyde **4** (2.5 g; 0.018 mol) in CH_2Cl_2 (30 mL) was treated at reflux for 5 h with carboxyethylmethylenetriphenylphosphorane (6.28 g; 0.018 mol). The reaction mixture was evaporated, and the residue was treated with Et_2O/hexane. The precipitated triphenylphosphine oxide was filtered, and the oily residue obtained upon evaporation of the solvent was chromatographed on a short SiO_2 column, yielding with hexane/AcOEt (6:4), ethyl *p*-methoxycinnamate, 2.8 g (76%): ^1H NMR (500 MHz, $CDCl_3$) δ 7.63 (1H, d, J = 16.2 Hz), 7.46 (2H, d, J = 8.2 Hz), 6.89 (2H, d, J = 8.2 Hz), 6.29 (1H, d, J = 16.2 Hz), 4.24 (2H, q, J = 6.7 Hz), 3.82 (3H, s, OCH_3), 1.32 (3H, t, J = 6.7 Hz). The latter material (2.4 g; 0.011 mol) in AcOEt (30 mL) was submitted to hydrogenation at room temperature in the presence of 10% Pd/C (0.2 g). At the end of the adsorption, the reaction mixture was filtered and evaporated, and the residue, 2.4 g, was treated at reflux in dry THF (50 mL) with $LiAlH_4$ (∼0.5 g; 0.012 mol) for 5 h. After usual workup, 3-(4-methoxyphenyl)propan-1-ol, 1.4 g (76%), was isolated after purification by SiO_2 column chromatography: ^1H NMR (500 MHz, $CDCl_3$) δ 7.09 (2H, d, J = 8.8 Hz), 6.81 (2H, d, J = 8.8 Hz), 3.76 (3H, s, OCH_3), 3.63 (2H, t, J = 6.2 Hz), 2.63 (2H, t, J = 7.5 Hz), 2.19 (1H, s, OH), 1.84 (2H, m).

The latter material (1.4 g; 0.0084 mol), in CCl_4 (40 mL), was refluxed for 8 h with Ph_3P (2.2 g; 0.0084 mol). The reaction mixture was evaporated and the residue taken up with Et_2O/hexane. The precipitated triphenylphosphine oxide was removed by filtration, and the oil that remained upon evaporation of the solvent was chromatographed on SiO_2, eluting first with 5% AcOEt in hexane some unreacted triphenylphosphine and, by increasing the amount of AcOEt, 3-(4-methoxy-

phenyl)-1-chloropropane **6**, 1.2 g (78%), purified by bulb-to-bulb vacuum distillation, 99% by GC-MS: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.7 (2H, d, $J = 8.8$ Hz), 6.81 (2H, d, $J = 8.8$ Hz), 3.74 (3H, s, OCH_3), 3.47 (2H, t, $J = 6.7$ Hz), 2.68 (2H, t, $J = 7.3$ Hz), 2.03 (2H, m).

By repeating the same sequence, starting from 3,5-dimethoxybenzaldehyde **5** was obtained 3-(3,5-dimethoxyphenyl)-1-chloropropane **7**, 99% by GC-MS: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.36 (2H, d, $J = 2.1$ Hz), 6.32 (1H, t, $J = 2.1$ Hz), 3.78 (3H, s, OCH_3), 3.53 (2H, t, $J = 6.4$ Hz), 2.72 (2H, t, $J = 7.3$ Hz, 2.08 (2H, m).

*(c) 3,5-Dimethoxytoluene **9**.* 3,5-Dimethoxybenzaldehyde **5** (1.7 g; 0.01 mol), in 25 mL of absolute ethanol, was hydrogenated at room temperature in the presence of 10% Pd/C (0.5 g). At the end of the absorption, the filtered solution was evaporated and the residue distilled bulb-to-bulb under vacuum to provide 3,5-dimethoxytoluene **9**, 0.8 g (87%), 99% by GC-MS.

Degradation of Commercial Rhapontin. The commercial product (50 g) in 200 mL of pyridine was treated at 0 °C with 300 mL of acetic anhydride. After 24 h at room temperature, the reaction mixture was poured into ice water. The semisolid precipitate was decanted and taken up into AcOEt (500 mL). The organic phase was washed repeatedly with dilute HCl, $NaHCO_3$, and brine. The residue obtained upon evaporation of the dried solution was chromatographed on SiO_2 eluting with ~25% AcOEt in hexane the pentaacetate of the β-glucopyranoside of 4′-*O*-methylresveratrol (pentaacetate of **2**), 12.2 g (20%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43 (2H, d, $J = 8.6$ Hz), 7.02 (1H, d, $J = 16.3$ Hz), 6.96 (2H, m), 6.90 (2H, d, $J = 8.6$ Hz), 6.86 (1H, d, $J = 16.3$ Hz), 6.62 (1H, t, $J = 2.1$ Hz), 5.32−5.26 (2H, m), 5.18−5.11 (2H, m), 4.28 (1H, dd, $J = 5.8$ and 12.4 Hz), 4.18 (1H, dd, $J = 2.4$ and 12.4 Hz), 3.90 (1H, ddd, $J = 2.4$, 5.4, and 9.9 Hz), 3.83 (3H, s, OCH_3), 2.30 (3H, s, $COCH_3$), 2.07 (3H, s, $COCH_3$), 2.056 (3H, s,$COCH_3$), 2.055 (3H, s, $COCH_3$), 2.04 (3H, s, $COCH_3$). The amount of AcOEt in the eluting mixture was then increased, yielding the hexaacetate of rhapontin **3**, 30 g (62%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.29 (1H, dd, $J = 2.2$ and 8.5 Hz), 7.21 (1H, d, $J = 2.2$ Hz), 6.97 (1H, d, $J = 16.3$ Hz), 6.96−6.93 (3H, m), 6.84 (1H, d, $J = 16.3$ Hz), 6.63 (1H, t, $J = 2.1$ Hz), 5.32−5.26 (2H, m), 5.16 (1H, t, $J = 9.4$ Hz), 5.12 (1H, d, $J = 7.4$ Hz), 4.28 (1H, dd, $J = 5.7$ and 12.3 Hz), 4.18 (1H, dd, $J = 2.4$ and 12.3 Hz), 3.90 (1H, ddd, $J = 2.4$, 5.6, and 10.0 Hz), 3.85 (3H, s, OCH_3), 2.33 (3H, s, $COCH_3$), 2.30 (3H, s, $COCH_3$), 2.07 (3H, s, $COCH_3$), 2.06 (3, s, $COCH_3$), 2.05 (3H, s, $COCH_3$), 2.04 (3H, s, $COCH_3$).

*(a) Ozonolysis of the Pentaacetate of **2** and of the Hexaacetate of **3**: Anisaldehyde **4** and Acetylisovanillin **10**.* The pentaacetate of **2** (10 g; 0.015 mol) was submitted to the action of ozonized oxygen, as indicated above, eventually obtaining, after chromatographic separation, anisaldehyde **4**, 1.4 g (67%), and the 3,5-disubstituted benzaldehyde **9**, 4.5 g (60%): ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.92 (1H, s, CHO), 7.37 (1H, dd, $J = 1.2$ and 2.2 Hz), 7.34 (1H, dd, $J = 1.2$ and 2.1 Hz), 7.01 (1H, t, $J = 2.2$ Hz), 5.32−5.26 (2H, m), 5.18−5.12 (2H, m), 4.23 (1H, dd, $J = 5.5$ and 12.2 Hz), 4.19 (1H, dd, $J = 2.6$ and 12.2 Hz), 3.93 (1H, ddd, $J = 2.4$, 5.6, and 9.9 Hz), 2.32 (3H, s, $COCH_3$), 2.09 (3H, s, $COCH_3$), 2.06 (3, s, $COCH_3$), 2.05 (3H, s, $COCH_3$), 2.03 (3H, s, $COCH_3$). When this sequence was applied to the hexaacetate of rhapontin **3**, acetylisovanillin **10** and the aldehyde **9** were obtained in 62 and 59% yields, respectively.

*(b) Enzymic Hydrolysis of Commercial Rhapontin: 1-(4-Methoxyphenyl)-2-phenylethane **13**.* A mixture was made up composed of 18 g of commercial rhapontin, 1 g of emulsin (Fluka, Milano, Italy), distilled water (1 L), and acetone (0.1 L) at pH 6. After 24 h of stirring at room temperature, an additional quantity (0.5 g) of emulsin was added, and the incubation was continued for a further 48 h. The reaction mixture was extracted with AcOEt (3 × 400 mL). The residue obtained upon evaporation of the organic phase was chromatographed on SiO_2, giving 4′-*O*-methylresveratrol **11**, 1.3 g (60%) [^{1}H NMR (500 MHz, acetone-d_6) δ 8.11 (2H, s, 2 OH), 7.50 (2H, d, $J = 8.6$ Hz), 7.03 (H, d, $J = 16.3$ Hz), 6.91 (1H, d, $J = 16.3$ Hz), 6.91 (2H, d, $J = 8.6$ Hz), 6.54 (2H, d, $J = 2.1$ Hz), 6.27 (1H, t, $J = 2.1$ Hz), 3.80 (3, s, OCH_3)] and rhapontinin **12**, 5 g (56%) [^{1}H NMR (500 MHz, acetone-d_6) δ 8.38 (2H, s br, 2 OH), 7.66 (1H, s br, OH), 7.08 (1H, d, $J = 2.0$ Hz), 6.97 (1H, d, $J = 16.3$ Hz), 6.97 (1H, dd, $J = 2.1$ and 8.4 Hz), 6.91 (1H, d, $J = 8.4$ Hz), 6.88 (1H, d, $J = 16.3$ Hz), 6.53 (2H, d, $J = 2.1$ Hz), 6.25 (1H, t, $J = 2.1$ Hz), 3.84 (3H, s, OCH_3)]. Products **11** and **12** were converted, in separate experiments, into monooxygenated 1-(4-methoxyphenyl)-2-phenylethane **13** by first submitting the materials to catalytic hydrogenation, which saturated the double bond, followed by deoxygenation of the phenolic moieties by conversion into the corresponding phosphate ester, followed by cleavage with Li metal in liquid ammonia, operating exactly as described in the deoxygenation of vanillin (*13*), in ~35% overall yield: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.33 (2H, m), 7.25 (3H, m), 7.15 (2H, d, $J = 8.9$ Hz), 6.88 (2H, d, $J = 8.9$ Hz), 3.85 (3H, s, OCH_3), 2.94 (4H, m).

Conversion of Acetylisovanillin 10 into 3-(3-Hydroxy-4-methoxyphenyl)-1-chloropropane 14. Acetylisovanillin **10** was converted into 3-(3-hydroxy-4-methoxyphenyl)-1-chloropropane **14** by first reacting the aldehyde with carbethoxymethylenetriphenylphosphorane and converting the ethyl 3-acetoxy-4-methoxycinnamate obtained into **14** as described above for anisaldehyde and 3,5-dimethoxybenzaldehyde, respectively. The overall yield of **14** was ~30%, 99% by GC-MS: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.78 (1H, dd, $J = 1.8$ and 8.1 Hz), 6.78 (1H, d, $J = 8.1$ Hz), 6.67 (1H, dd, $J = 1.8$ and 8.1 Hz), 5.60 (1H, s, OH), 3.87 (3H, s, OCH_3), 3.51 (2H, t, $J = 6.4$ Hz), 2.68 (2H, t, $J = 7.3$ Hz), 2.04 (2H, m).

Natural Abundance Deuterium Nuclear Magnetic Resonance Measurements. Deuterium NMR data (46.076 MHz) were recorded at 308 K on a Bruker AC300 spectrometer equipped with a process controller, a 10 mm selective deuterium probehead, and a ^{19}F lock channel, under broad-band proton decoupling conditions.

Samples (0.3−0.9 g), hexafluorobenzene for ^{19}F lock (150 mg, Merck, Darmstadt, Germany) and tetramethylurea as internal (D/H) standard (50−150 mg), were carefully weighed directly into the NMR tube. Then 3 mL of acetonitrile was added as a solvent, and the tube was warmed and shaken until complete dissolution. For isovanillin samples, a 50:50 mixture of acetone/acetonitrile was used. Tetramethylurea (TMU) was a BCR reference product certified for a (D/H) value of 136.67 ppm.

Eight to 10 spectra were run for each sample, collecting 1600−2000 scans and using the following parameters: 6.8 s acquisition time, 0.05 s relaxation delay, 1200 Hz spectral width, 16K memory size, and 15 μs (90°) pulse length. Each FID was Fourier transformed with no zero filling (0.15 Hz/point digital resolution) and line broadening of 2 Hz.

Bruker WIN NMR software was used for FID processing and peak deconvolution.

Internal isotopic ratios are

$$R_{ij} = n_j S_i / S_j \quad (1)$$

where S_i is the area of the ith site, S_j is the area of a reference peak, and n_j is the number of isochronous hydrogens at the j site. A statistical distribution of deuterium among the n molecular sites would originate R_{ij} factors equal to the number of hydrogens of the corresponding ith sites.

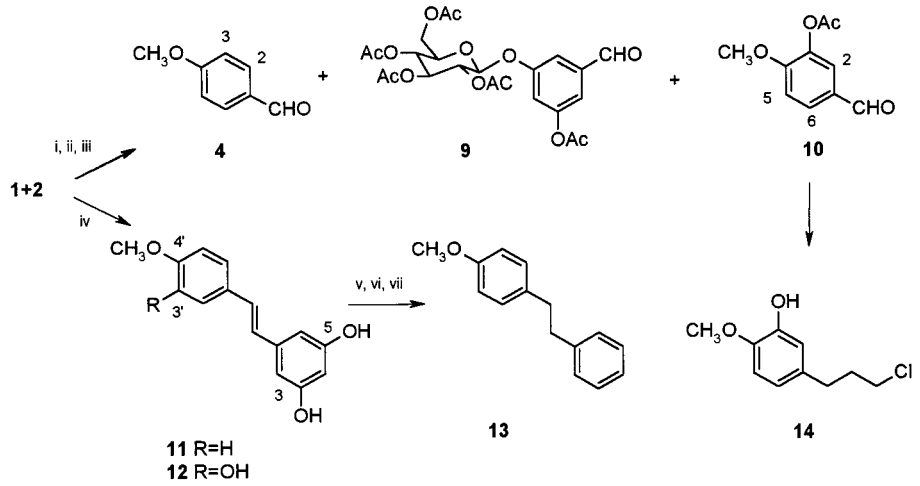
The absolute values of the site-specific (D/H) ratios were calculated according to the formula (*8*)

$$(\mathrm{D/H})_i = n_{\mathrm{WS}} g_{\mathrm{WS}} (\mathrm{MW})_{\mathrm{L}} S_i (\mathrm{D/H})_{\mathrm{WS}} / n_i g_{\mathrm{L}} (\mathrm{MW}_{\mathrm{WS}}) S_{\mathrm{WS}} P_{\mathrm{L}} \quad (2)$$

where WS stands for the working standard (TMU) with a known isotope ratio $(D/H)_{WS}$ and L for the product under examination; n_{WS} and n_i are the number of equivalent deuterium atoms of TMU and of the ith peak, respectively; g_{WS} and g_L are the weights of the standard and the sample, respectively; MW_L and MW_{WS} are the corresponding molecular weights; S_i and S_{WS} are the areas of the ith peak and of the standard, respectively; P_L is the purity of the sample, measured by capillary gas chromatography. $(D/H)_{WS}$ is the working standard isotope ratio as determined by isotope ratio mass spectrometry on the Vienna Standard Ocean Water (V-SMOW) scale (*14*).

Measurement of the ^{18}O/^{16}O Isotopic Ratios. The determination of the relative stable isotopic ratio was carried out using a Finnigan MAT Delta S mass spectrometer coupled with a pyrolysis unit including a commercially available pyrolysis system (Leco VTF900, St. Joseph, MI), fitted with a Carlo Erba S-200LS autosampler (ThermoQuest, San

Scheme 2. Degradation of the β-Glucopyranoside of 4′-*O*-Methylresveratrol **2** and of Rhapontin **3** to the Aldehydes **4** and **10** for the (D/H) Measurements and to Compounds **13** and **14** for the Positional ^{18}O Isotopic Determination[a]



[a] (i) Ac_2O/Pyr; (ii) O_3/CH_2Cl_2–MeOH 1:1, –78 °C; (iii) Ph_3P; (iv) emulsin, H_2O, pH 5.6; (v) H_2/Pd/C; (vi) $(EtO)_2POCl$/NaOH; (vii) Li/NH_3 (l).

Jose, CA). The analytical procedures for the determination of the $^{18}O/^{16}O$ ratio have been described in a previous study (*15*).

The values are expressed in the δ per mil (‰) scale versus the international standard V-SMOW (*14*), using the formula

$$\delta^{18}O\ (‰)_{V-SMOW} = [(R_{sample}/R_{V-SMOW}) - 1] \times 10^3 \qquad (3)$$

where $R = {}^{18}O/{}^{16}O$. The reproducibility of the measurements was estimated within ±0.7‰.

RESULTS AND DISCUSSION

Resveratrol **1** was first isolated over 60 years ago from *Veratrum grandiflorum* by Takaoka (*2*), who confirmed the structure through chemical synthesis from 4-hydroxybenzaldehyde and 3,5-dihydroxyphenylacetic acid. More recently, several additional more practical syntheses of **1** were reported (*7*−*9*) in which the $C_6-C_2-C_6$ skeleton is constructed by coupling C_6-C_1 synthons derived from anisaldehyde **4** and 3,5-dimethoxybenzaldehyde **5**, respectively. The present study concerns the isotopic characterization of extracted resveratrol from *P. cuspidatum* and of a commercial sample of rhapontin from *Rheum* (*3*). The latter material contains, in addition to rhapontin **3**, ~20% of product **2**, that is, the β-glucopyranoside of 4′-*O*-methylresveratrol. In addition to these natural stilbenes, a sample of extracted C_6-C_1 anisaldehyde **4** was also examined, together with synthetic samples of **4**, 3,5-dimethoxybenzaldehyde **5**, and acetyl isovanillin **10**, obtained in an unspecified manner.

Resveratrol **1** and the related products **2** and **3**, because they are highly unsaturated compounds, as indicated above, have NMR spectra not suitable for the determination of the deuterium content at natural abundance. Their degradation to smaller molecules using procedures not affecting the isotopic content was therefore necessary. The pathway followed involves cleavage with ozone of the double bond of suitably protected derivatives of **1**−**3**, which provides the C_6-C_1 aldehydes **4**, **5**, **9**, and **10**, retaining all of the hydrogen atoms originally present in the parent compounds. The cleavage with ozone of the ethylenic substrates was quantitative, and in the Ph_3P-mediated decomposition of the intermediate ozonides there is no formation of either the acids or the alcohols corresponding to the aldehydes **4**, **5**, **9**, and **10**. To avoid the danger of isotope fractionation during the purification processes, the materials were purified by chromatography, pooling all of the identical fractions.

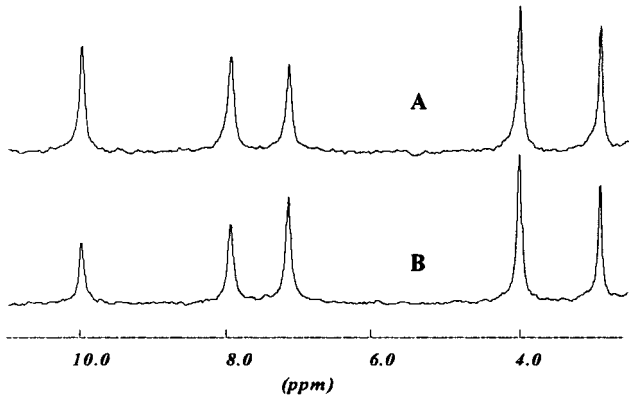


Figure 1. Natural abundance deuterium NMR spectra of anisaldehyde **4**: (A) synthetic from BBE; (B) natural from resveratrol **1**. The signal at 2.8 ppm is due to TMU (calibrated tetramethylurea used as internal reference).

Extracted resveratrol **1** was thus converted into the corresponding tri-*O*-methyl ether by treatment with ethereal diazomethane. Ozonolysis of the latter at low temperature, followed by triphenylphosphine treatment, yielded anisaldehyde **4** and 3,5-dimethoxybenzaldehyde **5**, respectively, separated by column chromatography (**Scheme 1**). Anisaldehyde **4** was similarly obtained by ozonolysis of the acetylated form of the β-glucoside **2**. In this instance, the crystalline aldehyde **9** was also isolated. Similarly, rhapontin **3**, once acetylated, on ozonolysis afforded acetylisovanillin **10** and, as for **2**, the aldehyde **9** (**Scheme 2**).

The natural abundance deuterium NMR spectra of anisaldehyde **4**, obtained from tri-*O*-methylresveratrol (**Figure 1B**) and from the peracetylated form of the glucoside **2**, respectively, were thus acquired and compared with those of a sample of natural anisaldehyde of extractive origin and of two synthetic samples (**Figure 1A**), respectively. Inspection of the results (**Table 1**) indicated in the synthetic series $(D/H)_2 > (D/H)_3$, whereas the reverse is true in the natural series (entries 3−5). However, for entries 1−4 the $(D/H)_2$ values are included between 140 and 147 ppm, whereas the value relative to **4** (entry 5) obtained from **2** is to some extent lower (129 ppm). The main numerical difference between the two series is in the $(D/H)_3$ values, which range from 135 to 130 ppm for entries 1 and 2 and up to 199, 195, and 167 ppm, respectively, for entries 3−5. Within the two sets, the difference in the (D/H) values of

Table 1. $(D/H)_i$ Isotopic Ratios[a] of Anisaldehyde **4** of Different Origin

entry	origin	$(D/H)_{formyl}$	$(D/H)_2$	$(D/H)_3$
1	synthetic (Aldrich)	288 (17)	147 (8)	135 (7)
2	synthetic (BBE)	268 (12)	140 (6)	130 (6)
3	natural (ex resveratrol **1** from *P. cuspidatum*)	184 (9)	145 (9)	199 (10)
4	natural (Robertet)	133 (5)	142 (6)	195 (5)
5	natural (ex resveratrol glucoside **2** from *Rheum*)	110 (7)	129 (5)	167 (5)

[a] $(D/H)_i$ values are expressed in ppm; the standard deviations are reported in parentheses.

Table 2. $(D/H)_i$ Isotopic Ratios[a] of 3,5-Dimethoxybenzaldehyde **5** of Different Origin

entry	origin	$(D/H)_{formyl}$	$(D/H)_2$	$(D/H)_4$
1	synthetic (Aldrich)	153 (6)	144 (6)	129 (18)
2	synthetic (Fluka)	130 (7)	129 (4)	134 (13)
3	natural (ex resveratrol **1** from *P. cuspidatum*)	96 (10)	140 (10)	134 (18)

[a] $(D/H)_i$ values are expressed in ppm; the standard deviations are reported in parentheses.

the aldehydic moiety is significant, going down from 288 and 268 ppm (synthetic products, entries 1 and 2) to 184, 133, and 110 ppm (natural derivation, entries 3−5), respectively.

The deuterium labeling pattern of the aromatic part of **1** and **2**, as measured on anisaldehyde **4**, is similar to that of raspberry ketone [4-(*p*-hydroxyphenyl)butan-2-one] from *Taxus baccata* and *Aeonium lindleyi* (*13*, *14*) and is in perfect agreement with measurements made on 4-hydroxybenzaldehyde accompanying vanillin in *Vanilla* pods (*18*). More problematic is the interpretation of the formyl deuterium enrichment values of anisaldehyde **4** from **1** and **2** in comparison with those of vanillin and 4-hydroxybenzaldehyde from *Vanilla* (*18*) and of benzaldehyde from different natural sources (*19*, *20*). The deuterium content of **4** obtained from **1** (entry 3) is very different from that measured on **4** obtained from **2** (entry 5). The same difference was measured between vanillin and 4-hydroxybenzaldehyde from *Vanilla* (*18*). Aldehyde **4** retains, in the formyl moiety, the hydrogen atom of the stilbene framework of **1** and **2.** This hydrogen originates from position 3 of the biosynthetic precursor *p*-coumaric acid, and thus the formyl deuterium values of entries 3 and 5 seemingly should reflect the differences in the deuterium enrichment at the corresponding position of *p*-coumaric acid. It follows from these observations that both the aromatic and the methine moieties of resveratrol **1** from *P. cuspidatum* and of its glucosidated 4′-*O*-methyl ether **2** from *Rheum* possess a different mean deuterium enrichment, the major element of distinction being the position α to the *p*-substituted ring.

The (D/H) values of 3,5-dimethoxybenzaldehyde **5**, obtained from permethylated **1**, and of two synthetic samples (**Table 2**), are nearly indentical as far as the aromatic positions are concerned, whereas the $(D/H)_{formyl}$ of the material from resveratrol **1** (entry 3) is 96 ppm, significantly lower with respect to those (153 and 130 ppm, respectively) of the synthetic counterparts (entries 1 and 2). Following the above reasoning, the formyl hydrogen of **5** obtained from resveratrol **1** reflects the deuterium enrichment at position 2 of the side chain of *p*-coumaric acid. Unfortunately, aldehyde **9** from acetylated **2** gave an NMR spectrum unsuitable for a quantification of the deuterium at the position of interest, that is, the formyl moiety.

Rhapontin **3** was then submitted to the above degradative sequence. Its hexaacetate, with ozone, provided almost quantitatively aldehyde **9** and acetylisovanillin **10** (**Scheme 2**). NMR analysis was made in comparison with a synthetic sample. The S/N ratio in the NMR spectra of **10** from rhapontin was rather low, owing to the low sample quantity, so standard deviations were quite high. However, **Table 3** shows clear differences between the two origins. Comparison of the data again indicates as the major element of distinction the formyl site enrichment, being 319 ppm for the synthetic material and only 130 ppm for the product isolated from **3**.

Table 3. $(D/H)_i$ Isotopic Ratios[a] of Acetylisovanillin **10** of Different Origin

entry	origin	$(D/H)_{formyl}$	$(D/H)_6$	$(D/H)_2$	$(D/H)_5$
1	synthetic (Fluka)	319 (15)	142 (14)	160 (8)	131 (5)
2	natural (ex rhapontin **3** from *Rheum*)	130 (27)	155 (15)	145 (17)	173 (32)

[a] $(D/H)_i$ values are expressed in ppm; the standard deviations are reported in parentheses.

Table 4. $(D/H)_i$ and R Isotopic Ratios[a] of Isovanillin of Different Origin

entry	origin	$(D/H)_{formyl}$	$(D/H)_2$	$(D/H)_5$	$R_{formyl,5}$	$R_{2,5}$
1	synthetic (Fluka)	272 (16)	119 (40)	138 (16)	1.96	1.73
2	natural (ex neohesperidin from *Citrus*)	105 (8)	143 (6)	157 (16)	0.67	1.83

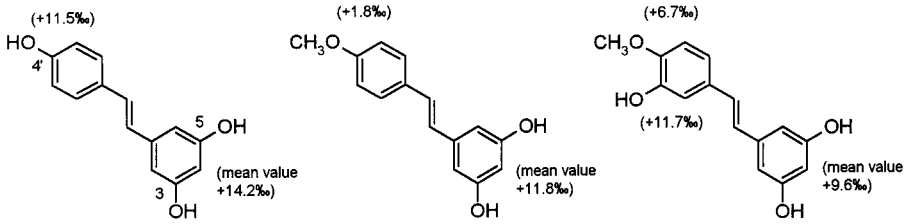
[a] $(D/H)_i$ values are expressed in ppm; the standard deviations are reported in parentheses.

Finally, for comparison purposes, isovanillin from the basic cleavage of neohesperidin from unripe bitter orange was also submitted to deuterium NMR studies, in comparison with a synthetic sample. Unfortunately, owing to the low solubility, part of the sample crystallized at the bottom of the NMR tube during measurements, so in this case we prefer to rely on internal ratios R and not on (D/H). The (D/H) and the R values of synthetic and "extractive" isovanillin are reported in **Table 4**. $R_{formyl,5}$ is the ratio between the formyl group and meta hydrogen enrichment. $R_{2,5}$ is the ratio between ortho and meta hydrogen enrichment. $R_{formyl,5}$ shows a strong depletion of deuterium in the formyl site of the natural sample. The same trend is also observed on $(D/H)_{formyl}$ of the "natural" material, which is much lower with respect to that of the correponding synthetic counterparts, as occurs in the case of anisaldehyde **4**.

Isovanillin is obtained from neohesperidin through basic treatment, the latest stage of the sequence being a retro-aldol C−C cleavage similar to that occurring in the generation of benzaldehyde from cinnamaldehyde (*19*). From a chemical point of view, it is expected that the basic treatment may cause equilibration with the water hydrogen atoms of the aromatic protons in positions ortho and para to the phenolic oxygen, whereas the hydrogen of the formyl group of isovanillin should be the one present in position 3 of the chain of *p*-coumaric acid as in the case of **1**−**3**.

The positional $\delta^{18}O$ values of **1** and that of the aglyconic part of **2** and **3** were finally determined. This goal was achieved by measuring the global values of a set of products derived from the above materials by selective deoxygenation. To avoid the handling of compounds unsuitable for the measurements due to high volatility, anisaldehyde **4** and 3,5-dimethoxybenzaldehyde **5**, obtained from resveratrol **1**, were converted to the compounds **6** and **7**, respectively, which retain only the original oxygen atom in the aromatic ring and possess suitable molecular characteristics. Thus, extractive resveratrol **1**, possessing $\delta^{18}O = +11.8‰$, was converted, via **4** and **5** (**Scheme 1**), into 3-(4-methoxyphenyl)-1-chloropropane **6**, $\delta^{18}O = +11.5‰$, and 4-(3,5-dimethoxyphenyl)-1-chloropropane

Chart 1. Positional $\delta^{18}O$ Values of Resveratrol **1** Isolated from *P. cuspidatum* and of 4′-*O*-Methylresveratrol **11** and Rhapontinin **12** Obtained by Enzyme Hydrolysis of the Corresponding Glucosides from *Rheum*



7, $\delta^{18}O = +15.2‰$. Moreover, 3,5-dimethoxybenzaldehyde **5** derived from **1** was converted by catalytic hydrogenation into 3,5-dimethoxytoluene **8** with $\delta^{18}O = +13.2‰$ (**Scheme 1**). The latter material and the chloro derivative **7** retain the two oxygen atoms at positions 3 and 5 of resveratrol **1** (mean $\delta^{18}O = +14.2‰$), whereas **6** conserves the phenolic oxygen at position 4′ of the stilbene moiety (**Scheme 1**).

The determination of the positional $\delta^{18}O$ values of **2** and **3** required a different degradation sequence. To start, commercial rhapontin was submitted to hydrolysis in the presence of emulsin. The reaction is very slow, but eventually the aglycons **11** and **12**, later separated by column chromatography, were obtained. 4-*O*-Methylresveratrol **11** showed $\delta^{18}O = +8.5‰$, whereas the value for rhapontinin **12** was +9.4‰. Subsequently, diphenol **11** and triphenol **12**, in separate experiments, were submitted to selective deoxygenation by means of cleavage with Li metal in liquid ammonia of the corresponding phosphate esters (**Scheme 2**), both yielding monooxygenated 1-(4-methoxyphenyl)-2-phenylethane **13**. The product from **2** showed $\delta^{18}O = +1.8‰$, whereas the identical material from **3** possessed a value of +6.7‰. Moreover, acetylisovanillin **10**, obtained from rhapontin **3**, was converted by chain elongation, reduction, and transformation of the primary alcohol into the chloride **14** (**Scheme 2**), showing a $\delta^{18}O$ value of +9.2‰.

From consideration of the values relative to **13** and **14** it follows, by calculation, that the oxygen at position 3′ of **3** possesses a $\delta^{18}O$ value of +11.7‰. Moreover, when the global values of **12**−**14** are considered, the mean $\delta^{18}O$ value for the oxygen atoms at positions 3 and 5 of **12** results in a value ca. +9.6‰. In summary, it follows that the $\delta^{18}O$ values of the aromatic oxygen atom at position 4′ of **1**, **2**, and **3** are +11.5, +1.8, and +6.7‰, respectively. The oxygen atoms in the malonate-derived part of these molecules, that is, those at positions 3 and 5, show, for the same compounds, mean values of +14.2, +11.8, and +9.6‰, respectively. Finally, the oxygen atom at position 3′ of rhapontin **3** shows a value of +11.7‰ (**Chart 1**).

The selected oxygen depletion figures found for the oxygen atoms located at different positions of the examined stilbenes have been, in part, calculated on the basis of the biochemical mechanisms for natural formation of oxygenated organic molecules (*12*). The oxygen depletion values for position 4′ of **1**−**3** can be compared with those obtained for structurally related plant phenolics of relevance in food chemistry. The $\delta^{18}O$ value (6.7‰) of rhapontin **3** is in line with those found for the corresponding position of vanillin from *Vanilla* pods and from lignin (∼6.5‰) (*15*). The one from resveratrol **1** (+11.5‰) fits with those observed in the *Citrus* glycosides naringin and neohesperidin (14 and 11‰, respectively) (*14*). Finally, the ^{18}O content of the phenol of **2** (+1.8‰) is in line with those observed in the two specimens of raspberry ketone from *T. baccata* and *A. lindleyi* examined so far (−0.8 and +0.6‰) (*13*, *14*).

In conclusion, the approach to the stable isotope characterization of the practically important food components **1**−**3** based on selective degradation, followed by isotopic measurements of the derived fragments, seems to be satisfactory. Indeed, by ozonolytic splitting of the unsaturated products a set of simple aldehydes are obtained, on which the deuterium analysis can be performed quite easily. Additionally, the acquisition of the positional $\delta^{18}O$ values of selected degradation products might be of substantial utility for identification purposes. Additionally this kind of analysis, at variance with the deuterium NMR, requires only a minute amount (few milligrams) of material. The progressive deoxygenation procedure here applied allows the *positional* rather than *global* $\delta^{18}O$ values to be obtained, thus dramatically enlarging the diagnostic capacity of the investigation.

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Received for review August 14, 2001. Revised manuscript received February 13, 2002. Accepted February 14, 2002. We thank CNR Progetto Finalizzato Biotecnologie for financial support.

JF011103J