

## Stable Isotope Characterization of the *ortho*-Oxygenated Phenylpropanoids: Coumarin and Melilotol

ELISABETTA BRENNA,<sup>†</sup> GIOVANNI FRONZA,<sup>\*,‡</sup> CLAUDIO FUGANTI,<sup>†</sup>  
FRANCESCO G. GATTI,<sup>†</sup> VALENTINA GRANDE,<sup>†</sup> STEFANO SERRA,<sup>‡</sup>  
CLAUDE GUILLOU,<sup>§</sup> FABIANO RENIERO,<sup>§</sup> AND FRANCESCA SERRA<sup>§</sup>

Dipartimento di Chimica, Materiali e Ingegneria Chimica “Giulio Natta”, Politecnico di Milano, and  
Istituto di Chimica del Riconoscimento Molecolare, CNR, Via Mancinelli 7, 20131 Milano, and  
Physical and Chemical Exposure Unit, Institute for Health and Consumer Protection, Joint Research  
Centre, European Commission, BEVABS, TP281, Via Fermi 2, 21020 Ispra, Italy

The natural abundance  $^2\text{H}$  NMR spectra of extractive coumarin **10** and of its dihydroderivative melilotol **11** produced by baker's yeast reduction has been compared with synthetic materials. Diagnostic for the differentiation of **10** are the  $(\text{D}/\text{H})_{\beta}$  values, which are in the 128.1–133.6 ppm interval for the natural compounds but 258.5 and 189.8 ppm for the synthetic materials. Such a dramatic difference is also found for methyl cinnamate **12**, which shows  $(\text{D}/\text{H})_{\beta}$  values of 127.2 and 515.8 ppm, respectively. In extractive **10**, the ratio  $(\text{D}/\text{H})_{4(\text{para})}/(\text{D}/\text{H})_{6(\text{ortho})} = 1.24$  is similar to that observed in structurally related salicin and methyl salicylate. Coumarin **10** is transformed in salicyl alcohol **9**, providing diacetate **14**, showing in the natural series the trend  $(\text{D}/\text{H})_{3(\text{meta})} > (\text{D}/\text{H})_{4(\text{para})} > (\text{D}/\text{H})_{5(\text{meta})} \sim (\text{D}/\text{H})_{6(\text{ortho})}$ . A similar trend is shown also by the synthetic **10**. A clear distinction between extractive and synthetic **10** is obtained through  $\delta^{18}\text{O}$  determinations on **10** and on chroman **13**. The bulk  $\delta^{18}\text{O}$  values in the extractive series of **10** are 20.3, 23.6, and 22.6‰, while those of the aromatic oxygen are 2.3, 0.5, and  $-0.5\text{‰}$ . In the synthetic sample, the values are 12.6 and 5.6‰, respectively. As a final product, the reduction of **10** leads to the dihydroderivative **11**. Both the baker's yeast reduction and the catalytic hydrogenation lead to a marked decrease of the deuterium content of **11**, which is stronger for the  $\beta$ -position than for the  $\alpha$ -position.

**KEYWORDS:** Coumarin; extractive; synthetic; deuterium NMR; chroman; positional  $\delta^{18}\text{O}$  values; dihydrocoumarin; methyl cinnamate

### INTRODUCTION

The stable isotope distribution of a number of aroma compounds has been recently determined with the intent of differentiating materials of chemically identical molecules of natural or synthetic origin (1–3). In this context, the analysis of the natural abundance  $^2\text{H}$  NMR spectra together with the definition of the positional  $\delta^{18}\text{O}$  values achieved through selective chemical degradation appeared particularly useful. Relevant examples in the field of aromas are vanillin **1** (4, 5) and raspberry ketone **2** (6) and the antioxidant resveratrol **3** (7). These phenolic compounds share in nature a common derivation from C-6–C-3 L-phenylalanine via cinnamic acid **4** and the oxygenated derivative *p*-coumaric acid **5**. In the production of C-6–C-1 vanillin **1**, the latter compound undergoes a second hydroxylation to caffeic acid **6** and, in turn, is O-methylated at

position 3 to ferulic acid, the closest precursor of **1** (Figure 1) (8). The  $^2\text{H}$  NMR analyses and the determination of the positional  $\delta^{18}\text{O}$  values of extractive **1–3** have revealed the changes occurring in both the deuterium pattern of the aromatic ring during the introduction of the oxygen functions (9) and the extent of the isotopic fractionation of oxygen in the process (10).

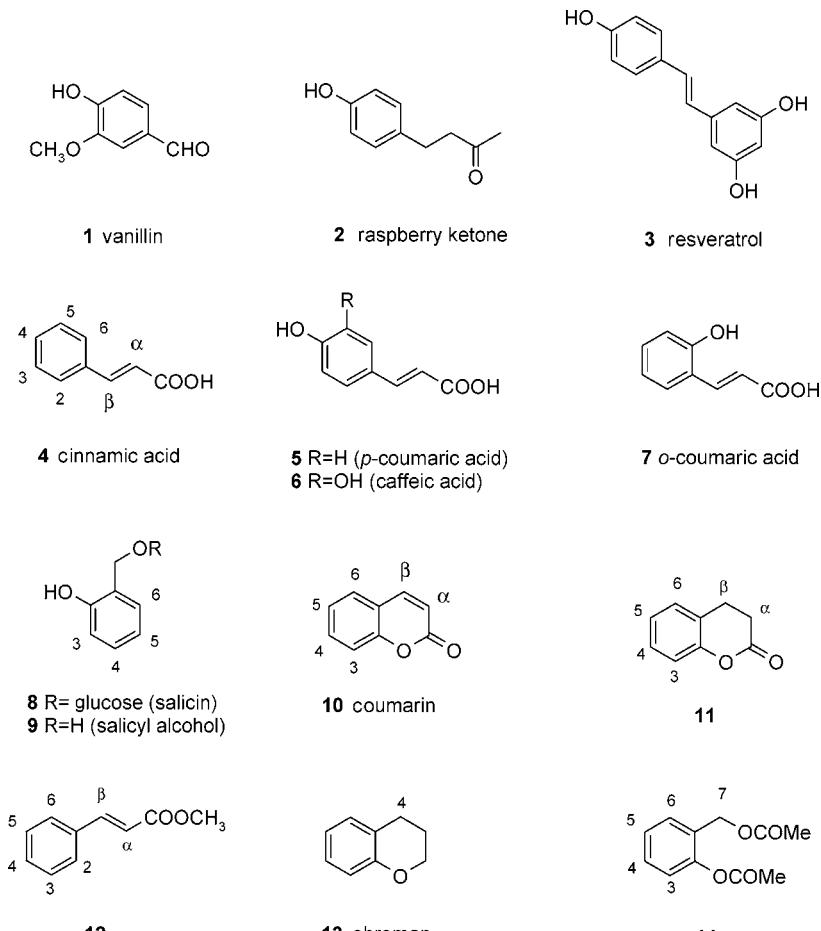
In this context, the pattern of natural abundance  $^2\text{H}$  of extractive and synthetic samples of the glycoside salicin **8** through NMR analyses has been recently determined (11). Indeed, salicyl alcohol **9**, the aglycone moiety of **8**, shares with **1–3** the derivation from cinnamic acid **4**. In this instance, cinnamic acid **4** is hydroxylated in position 2 to *o*-coumaric acid **7**, which subsequently undergoes C-2 chain shortening to the C-6–C-1 framework of **9** (Figure 1). The deuterium pattern of **9**, obtained by enzymic hydrolysis of extractive **8**, was determined through NMR analyses on the diacetyl derivative **14**. The main result was that  $(\text{D}/\text{H})_{4(\text{para})} > (\text{D}/\text{H})_{3(\text{meta})} \sim (\text{D}/\text{H})_{5(\text{meta})} > (\text{D}/\text{H})_{6(\text{ortho})}$ . Another result of this study (11) was that in these circumstances the H-3 and the H-5 of **9**, which are

\* To whom correspondence should be addressed. Tel: +39 02 23993027. Fax: +39 02 23993080. E-mail: giovanni.fronza@polimi.it.

<sup>†</sup> Politecnico di Milano.

<sup>‡</sup> CNR.

<sup>§</sup> European Commission.



**Figure 1.** Some biologically active phenolic phenylpropanoids and their relevant biosynthetic intermediates (1–10). Compounds 10–14 are the subject of this work.

ortho–para located with respect to the phenolic oxygen, undergo rapid equilibration with the hydrogen atoms of the solvent water under acid catalysis.

To achieve a better picture of the mode of formation of natural phenylpropanoids via the *o*-coumarate pathway, we submitted to stable isotope characterization coumarin 10, the lactone of the *Z*-isomer of *o*-coumaric acid, the impact flavor of Tonka bean absolute [*Dipteryx odorata* (Aubl.) Willd. (Fabaceae)], a widely used ingredient in the flavor industry (12). This study, which might be useful for the development of an analytical procedure allowing the differentiation of extractive molecules from the synthetic counterparts, is based on the isotopic characterization through natural abundance  $^2\text{H}$  NMR of the extractive and synthetic samples of coumarin 10 and further of a series of related compounds, including dihydrocoumarin (melilotol) 11 and methyl cinnamate 12 (Figure 1). In a subsequent experiment, coumarin 10 was degraded to chroman 13, which would retain the aromatic oxygen of 10. The  $\delta^{18}\text{O}$  value of chroman 13 of natural and synthetic derivation was determined in order to gain information on the oxygen fractionation occurring when 4 undergoes the transformation in *o*-coumaric acid 7.

## MATERIALS AND METHODS

**Origin of Samples and Preparation of Derivatives.** A total of 14 samples, including five samples of coumarin 10, three samples of dihydrocoumarin 11, four samples of diacetyl salicyl alcohol 14, and two samples of methyl cinnamate 12 were analyzed for the deuterium content (Table 1). The extractive coumarin was isolated from an aliquot

(6 g) of commercial Tonka bean absolute by liquid chromatography using a column with 200 g of  $\text{SiO}_2$  60 (Merck, Milano) with a hexane/ethyl acetate mixture 6/4. A homogeneous solid was recrystallized from the extract with a yield of 87%. Sample 10.1 was from material from V. Mane & Fils (Bar-sur-Loup), and samples 10.2 and 10.3 were from two different lots of absolute from Charabot (Grasse). Samples 10.4 and 10.5 were synthetic coumarins acquired from Fluka (Milano) and Carlo Erba (Milano).

The samples of dihydrocoumarin 11 were obtained by chemical and biochemical hydrogenation of extractive and synthetic coumarin. For the first process, 2 g of coumarin in 50 mL of ethyl acetate was hydrogenated at ambient conditions in the presence of 0.2 g of 10% Pd/C. After completeness of the reaction, the catalyst was removed by filtration, and the residue obtained by evaporation of the solvent was distilled bulb-to-bulb at 0.2 mm/Hg (oven temperature, 130 °C). The solid obtained on cooling was then used as such for the deuterium measurements. Alternatively, 5 g of coumarin dissolved in the minimum amount of ethanol was added dropwise to a stirred mixture of 2 kg of baker's yeast (DSM, Milano) and 0.2 kg of D-glucose mixed in 2.5 L of tap water at 35–38 °C. After 2 days under these conditions, 1 L of acetone was added, followed by 1 L of ethyl acetate. The mixture was filtered under vacuum through a large Celite pad. The organic phase was separated, and the lower layer was extracted twice by means of 1 L of ethyl acetate. The combined organic extract was washed with 5%  $\text{NaHCO}_3$  and 10%  $\text{NaCl}$  solution, dried over  $\text{Na}_2\text{SO}_4$ , and evaporated. The dihydrocoumarin 11 was obtained from the residue by liquid chromatography ( $\text{SiO}_2$ , ethyl acetate in hexane) and purified by bulb-to-bulb vacuum distillation, with a yield of 50%. The dihydrocoumarin produced from sample 10.1 of coumarin (extractive) by chemical hydrogenation was labeled sample 11.1, while the identical material produced by baker's yeast reduction was labeled sample 11.2. Sample

**Table 1.** Origin and (D/H) Isotopic Ratios (Parts per Million)<sup>a</sup> of Coumarin **10**, Dihydrocoumarin **11**, and Diacetyl Salicyl Alcohol **14**

Coumarin <b>10</b>							
sample (origin)	(D/H) <sub>3,5</sub>	(D/H) <sub>4</sub>	(D/H) <sub>6</sub>	(D/H) <sub>Ar</sub> <sup>b</sup>	(D/H) <sub>β</sub>	(D/H) <sub>α</sub>	<i>R</i> <sub>4/6</sub> <sup>c</sup>
10.1 (natural from <i>F. tonka</i> ) <sup>d</sup>	157.7 (1.7)	166.3 (4.7)	128.1 (2.1)	152.4	131.9 (1.9)	111.2 (3.7)	1.30
10.2 (natural from Charabot) <sup>d</sup>	157.0 (2.6)	161.4 (4.3)	133.0 (3.2)	152.1	133.6 (2.9)	104.6 (4.6)	1.21
10.3 (natural from Charabot) <sup>d</sup>	155.2 (3.6)	159.7 (6.3)	131.0 (5.4)	150.3	128.1 (5.2)	116.0 (2.5)	1.22
10.4 (synthetic from Fluka)	167.5 (3.3)	158.6 (2.8)	146.9 (3.2)	160.1	258.5 (4.3)	146.9 (3.2)	1.08
10.5 (synthetic from Carlo Erba)	130.6 (1.0)	138.1 (4.6)	125.5 (3.5)	131.2	189.8 (4.3)	122.9 (2.7)	1.10
Dihydrocoumarin <b>11</b>							
sample (origin)	(D/H) <sub>3</sub>	(D/H) <sub>4,6</sub>	(D/H) <sub>5</sub>	(D/H) <sub>Ar</sub>	(D/H) <sub>β</sub>	(D/H) <sub>α</sub>	
11.1 (from 10.1 by H <sub>2</sub> /Pd reduction)	171.4 (3.4)	150.4 (2.8)	135.0 (3.8)	151.8	81.1 (3.3)	95.7 (3.0)	
11.2 (from 10.1 by b.y. reduction)	168.4 (4.4)	144.7 (3.7)	132.1 (3.7)	147.4	85.5 (0.2)	93.7 (1.1)	
11.3 (from 10.4 by H <sub>2</sub> /Pd reduction)	179.0 (3.5)	160.7 (3.3)	158.2 (2.5)	164.6	121.9 (2.1)	122.0 (1.9)	
Diacetyl salicyl alcohol <b>14</b>							
sample (origin)	(D/H) <sub>3</sub>	(D/H) <sub>4</sub>	(D/H) <sub>5</sub>	(D/H) <sub>6</sub>	(D/H) <sub>Ar</sub>	(D/H) <sub>7</sub>	<i>R</i> <sub>4/6</sub>
14.1 (natural from 10.1)	175.4 (5.7)	164.8 (3.2)	133.5 (3.2)	133.9 (4.5)	151.9	85.0 (2.0)	1.23
14.2 (natural from 10.2)	168.7 (3.7)	160.6 (5.0)	133.5 (4.9)	130.5 (3.2)	148.3	102.4 (3.3)	1.23
14.3 (natural from 10.3)	170.8 (5.1)	156.1 (2.3)	136.6 (2.8)	128.8 (3.2)	148.2	112.3 (4.0)	1.21
14.4 (synthetic from 10.4)	167.2 (2.5)	161.4 (1.3)	151.0 (1.3)	146.3 (6.6)	156.5	137.7 (2.5)	1.10

<sup>a</sup> The (D/H) standard deviations are reported within parentheses. <sup>b</sup> (D/H)<sub>Ar</sub> is the mean deuterium content of the aromatic nuclei calculated from the individual positional (D/H). <sup>c</sup> Ratio (D/H)<sub>4</sub>/(D/H)<sub>6</sub>. <sup>d</sup> Coumarin different lots.

11.3 was a dihydrocoumarin produced by chemical hydrogenation of a synthetic coumarin (sample 10.4).

The diacetate of salicyl alcohol **14** was obtained from coumarin by ozonolysis and NaBH<sub>4</sub> reduction, followed by a chemical acetylation. Three grams of coumarin dissolved in 100 mL of a 1:1 mixture of CH<sub>2</sub>Cl<sub>2</sub>–MeOH was submitted to the action of ozonized oxygen at –78 °C until the blue color persisted. Nitrogen was then flushed through, followed by the dropwise addition, at the same temperature, of an ethanolic solution of 0.9 g of NaBH<sub>4</sub>. The mixture was kept at room temperature overnight and then diluted with 100 mL of icy water. The organic phase was subsequently separated, and the reaction mixture was extracted twice with CH<sub>2</sub>Cl<sub>2</sub> (100 mL). The organic extract was washed with 10% NaCl solution and dried to give, upon evaporation of the solvent, salicyl alcohol in the solid phase. The crude material was treated with 15 mL of pyridine with 10 mL of acetic anhydride. After 24 h, the reaction mixture was evaporated under vacuum to give a residue, which was taken up with water and CH<sub>2</sub>Cl<sub>2</sub>. The organic extract was washed with cold diluted HCl and a saturated solution of NaHCO<sub>3</sub>. The oily residue obtained upon evaporation was separated by liquid chromatography on SiO<sub>2</sub> with 15% ethyl acetate in hexane providing the desired diacetate **14** in 60% yield. The latter was purified by bulb-to-bulb vacuum distillation, as above. Samples 14.1, 14.2, and 14.3 were derived in this way from coumarin samples 10.1, 10.2, and 10.3, respectively, while sample 14.4 was from the synthetic coumarin sample 10.4. The conversion of coumarin into chroman **13** (3,4-dihydro-2H-1-benzopyran) was achieved by the following procedure. Coumarin was chemically hydrogenated to dihydrocoumarin, as described above. The latter material (3 g) in diethyl ether (50 mL) was added dropwise under stirring to a boiling suspension of 2 g of LiAlH<sub>4</sub> in 50 mL of diethyl ether. After 5 h under these conditions, ethyl acetate was added to destroy the excess of hydride, followed by cold diluted HCl. The residue, obtained upon evaporation of the washed and dried extract, contained only oily 3-(2-hydroxyphenyl)-1-propanol. The crude material in 15 mL of pyridine was treated overnight at 0 °C with *p*-toluenesulfonyl chloride (4.7 g, 1.2 mol equiv). The mixture was poured onto crushed ice containing 10 mL of concentrated HCl, and the mixture was subsequently extracted with diethyl ether (2 × 150 mL). The washed and dried organic phase, after evaporation, gave an oily residue that was dissolved in 50 mL of 2-propanol and added dropwise under stirring conditions to 200 mL of 20% NaOH at 70–80 °C. After 5 h, the cooled reaction mixture was diluted with water and extracted with diethyl ether (2 × 150 mL). The organic residue was chromatographed on SiO<sub>2</sub> with 10% ethyl acetate in hexane to provide chroman **13** and further purified by bulb-to-bulb vacuum distillation (0.5 mm/Hg; oven

temperature, 90 °C). Chroman samples 13.1–13.3 were produced from extractive coumarin samples 10.1–10.3, while sample 13.4 was obtained from the synthetic sample 10.4. The studied samples of methyl cinnamate **12** were a synthetic compound from Fluka at Milano (sample 12.1) and a commercially available “natural” product from H&R (sample 12.2).

The purity of the samples, checked by determination of melting point, thin-layer chromatography, and proton NMR spectroscopy, was 100%. To minimize eventual isotopic fractionation during the purification process, all compounds were treated in the same way. They were purified through column chromatography, pooling all pure fractions, and the crystallizations were carried out using the minimum amount of solvent to lower the loss of material in the mother liquors.

**NMR Experiments.** The <sup>2</sup>H NMR experiments were performed on a Bruker Advance 500 spectrometer equipped with a 10 mm probehead and a <sup>19</sup>F lock (C<sub>6</sub>F<sub>6</sub>) channel, under CPD (Waltz 16 sequence) proton decoupling conditions. The spectra were recorded at 300–310 K. The reference used for (D/H) calculations was *tert*-butyl disulfide calibrated against the official standard TMU (Community Bureau of References, BCR) with a certified (D/H) ratio (123.38 ppm). The spectra were recorded dissolving about 0.8 g of material in ca. 3.0 mL of solvent, adding 50  $\mu$ L of C<sub>6</sub>F<sub>6</sub> for the lock and ca. 120 mg of *tert*-butyl disulfide as internal standard [(D/H) 129.2 ppm]. The solvents used were acetone for **10**, **11**, and **14** and CH<sub>2</sub>Cl<sub>2</sub> for **12**.

At least three spectra were run for each sample collecting ca. 8000 scans using the following parameters: 6.8 s acquisition time, 1200 Hz width, 16 K time domain, 8 K FT data points, 3 s recycle delay. The longest relaxation time (*T*<sub>1</sub>) estimated previously (*JJ*) belongs to the methyl nuclei of the reference material (ca. 0.8 s). Thus, the repetition time of 9.8 s is more than enough to ensure a complete nuclear relaxation between two consecutive scans. Each free induction decay was Fourier transformed with a line broadening of 0.5–1.5 Hz, manually phased, and integrated after an accurate correction of the spectrum baseline. For partially overlapped signals, the peak areas were determined through the deconvolution routine of the Bruker NMR software using a Lorentzian line shape.

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

$$(D/H)_i = n_{WS} g_{WS} MW_L S_i (D/H)_{WS} / n_i g_L MW_{WS} S_{WS} \quad (1)$$

where WS stands for the working standard with a known isotope ratio (D/H)<sub>WS</sub> and *L* stands for the product under examination; *n<sub>WS</sub>* and *n<sub>i</sub>* are the number of equivalent deuterium atoms of the standard and of

the  $i$ -th peak;  $g_{ws}$  and  $g_L$  are the weights of the standard and the sample;  $MW_L$  and  $MW_{ws}$  are the corresponding molecular weights;  $S_i$  and  $S_{ws}$  are the areas of the  $i$ -th peak and of the standard, respectively.

The signal assignment for compounds **10–12** and **14** was obtained from the analysis of the proton spectrum taken in the same solvent and at the same concentration of the deuterium spectrum. The signals were assigned through chemical shift correlation experiments (COSY) and determination of the nuclear Overhauser enhancements (NOESY). The chemical shifts are expressed in ppm from internal TMS ( $\delta$ ), and the coupling constants are expressed in hertz. Coumarin **10** ( $\delta$ , acetone- $d_6$ , **Figure 1**): 7.83 ( $H_\beta$ ), 7.54 ( $H_6$ ), 7.48 ( $H_4$ ), 7.22 ( $H_3 + H_5$ ), 6.34 ( $H_\alpha$ );  $J_{3,4} = J_{4,5} = 7.9$ ,  $J_{4,6} = 1.8$ ,  $J_{5,6} = 7.7$ ,  $J_{\alpha,\beta} = 9.7$ . Dihydrocoumarin **11** ( $\delta$ , acetone- $d_6$ , **Figure 1**): 7.21 ( $H_2 + H_6$ ), 7.06 ( $H_5$ ), 6.94 ( $H_3$ ), 2.96 ( $CH_2\beta$ ), 2.70 ( $CH_2\alpha$ );  $J_{3,4} = 8.3$ ,  $J_{3,5} = 1.4$ ,  $J_{4,5} = J_{5,6} = 7.5$ ,  $J_{\alpha,\beta} \sim 7.6$ . Methyl cinnamate **12** ( $\delta$ ,  $CD_2Cl_2$ , **Figure 1**): 7.71 ( $H_\beta$ ), 7.54 ( $H_{2,6}$ ), 7.38 ( $H_{3,4,5}$ ), 6.48 ( $H_\alpha$ ), 3.79 ( $OCH_3$ );  $J_{\alpha,\beta} = 16.0$ . Diacetyl salicyl alcohol **14** ( $\delta$ , acetone- $d_6$ , **Figure 1**): 7.47 ( $H_6$ ), 7.39 ( $H_4$ ), 7.26 ( $H_5$ ), 7.14 ( $H_3$ ), 5.07 ( $CH_2\beta$ ), 2.29, and 2.02 (two  $COCH_3$  groups);  $J_{3,4} = 8.2$ ,  $J_{3,5} = 1.3$ ,  $J_{3,6} = 0.5$ ,  $J_{4,5} = 7.6$ ,  $J_{4,6} = 1.8$ ,  $J_{5,6} = 7.6$ .

**Isotopic Oxygen Determinations.** The determination of  $\delta^{18}O$  of aromatic compounds by IRMS is fast and does not generally require any manipulation of the sample. The only constraint is posed by unintentional absorption of water that must be avoided; this problem can be easily prevented by keeping the samples in a dry chamber. For the analytical determination of  $^{18}O$ , the samples were pyrolyzed in a TC/EA high-temperature conversion elemental analyzer (ThermoFinnigan) and measured with a DeltaPlus XP. The method of measurement of  $\delta^{18}O$ , due to the absence of nitrogen, which is the only element that could cause an isobaric interference during the measurement of CO, followed the standard settings suggested by ThermoQuest, pyrolyzing the samples in a ceramic tube filled with glassy carbon chips at 1450 °C.

The results of oxygen isotope ratio analyses are reported in per mil (‰) relative to V-SMOW, defined as 0‰ point in the  $\delta^{18}O$  scale. The precision (standard deviation) for analysis of the laboratory standards (beet and cane sugar previously intercalibrated in collaboration with 15 different laboratories taking part in the European Project Sugar 18O-SMT4-CT98-2219) is  $\pm 0.18\text{‰}$  ( $n = 10$ ). To evaluate the precision of the analyses of the unknown samples, several replicates ( $n = 5$ ) were performed giving as a result a standard deviation of  $\pm 0.20\text{‰}$ , similar to the values obtained for the internal standards. The rest of the samples were analyzed in duplicate or triplicate and calculated against reference gases calibrated with an international reference material (benzoic acid IAEA-601,  $\delta^{18}O = +23.3\text{‰}$ ).

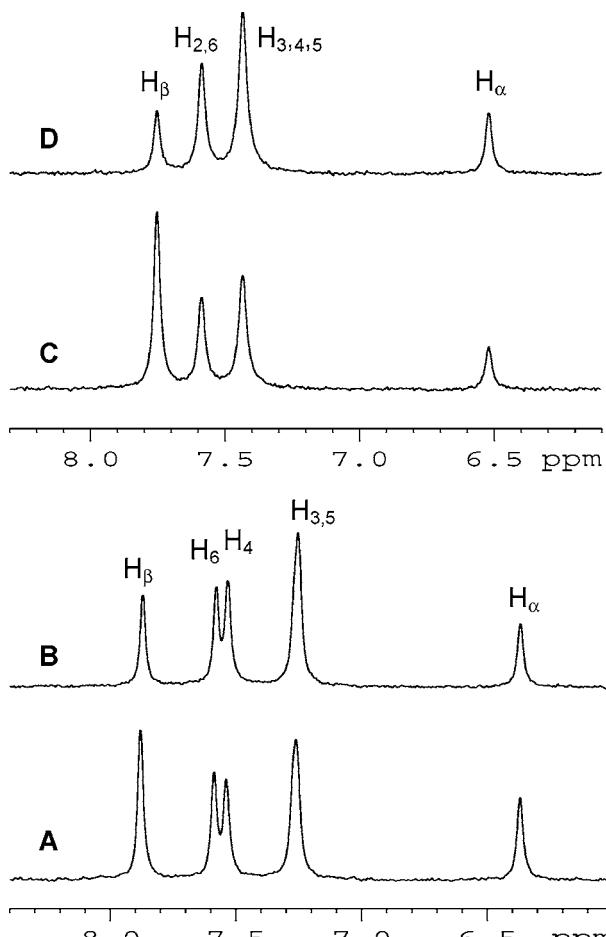
All of the results were calculated according to the following equation:

$$\delta \text{‰} = [( \text{sample ratio} - \text{reference ratio} ) / \text{reference ratio}] \times 1000 \quad (2)$$

## RESULTS AND DISCUSSION

**Coumarin.** Records concerning the natural occurrence of coumarin **10** and of its dihydroderivative melilotol **11** date back to the beginning of the chemical literature (13, 14). Both products are present in a large number of plant extracts and *Fava tonka* absolute has entered food flavors as a source of coumarin. This compound was one of the first synthetic chemicals available, being produced by condensation of salicylic aldehyde with acetic anhydride in the presence of sodium acetate (15). The coumarin samples of the present study were natural materials isolated by chromatography from commercially available *F. tonka* absolute and commercial synthetic samples. Examples of the natural abundance  $^2\text{H}$  NMR spectra of extractive and synthetic coumarin (samples 10.1 and 10.4) are reported in **Figure 2**, while in **Table 1** are included the  $(D/H)$  values relative to the five examined samples.

At first, we consider the deuterium values of positions 4 and 6 of **10**, i.e., those not activated toward acid-catalyzed proton exchange. In natural samples 10.1–10.3, the  $(D/H)_{4(\text{para})}$  and



**Figure 2.** Natural abundance  $^2\text{H}$  NMR spectra of coumarin **10** (acetone): (A) synthetic sample 10.4 and (B) extractive sample 10.1. Natural abundance  $^2\text{H}$  NMR spectra of methyl cinnamate **12** ( $CD_2Cl_2$ ): (C) synthetic sample 12.1 and (D) natural sample 12.2.

the  $(D/H)_{6(\text{ortho})}$  (positions referred to the alkyl side chain) range from 166.3 to 159.7 and from 133 to 128.1 ppm, respectively. These values are higher than those previously determined for the corresponding positions of salicin **8**. Indeed, in the latter instance, values around 139 and 107 ppm, respectively, were measured (11). However, if we consider the ratio  $R_{4/6}$  between  $(D/H)_{4(\text{para})}$  and  $(D/H)_{6(\text{ortho})}$  for the two biosynthetically related products **8** and **10**, mean values of 1.28 and 1.24 are obtained (**Table 1**). For the synthetic samples, the same ratio is 1.03 and 1.09, respectively. Very recently, Le Grand et al. (16) in a study on the origin of methyl salicylate, a natural product sharing with salicin and coumarin many steps of the biosynthetic pathway, proposed the ratio  $R_{4/6}$  as a simple and significant parameter to distinguish between samples of natural and synthetic derivation. This parameter in the case of methyl salicylate showed values around 1.0 for the synthetic samples and around 1.25 for the natural ones, in good agreement with those determined by us for salicin **8** ( $\Delta R_{4/6} = 0.25$ ). For coumarin, the difference between natural and synthetic samples is less marked ( $\Delta R_{4/6} = 0.15$ ) but still significant.

The signals of H-3 and H-5 are unresolved in coumarin **10**. However, the  $(D/H)_{H-5+H-3(\text{meta})}$  remains in the narrow range of 157.7–155.2 ppm for all three extractive samples. Strangely, the two synthetic samples, 10.4 and 10.5, showed different aromatic deuterium patterns:  $(D/H)_{6(\text{ortho})} = 146.9$  and 125.5 ppm and  $(D/H)_{4(\text{para})} = 158.6$  and 138.1 and  $(D/H)_{H-5+H-3(\text{meta})} = 167.5$  and 130.6 ppm, respectively. The deuterium signals

**Table 2.** Origin and (D/H)<sub>i</sub> Isotopic Ratios (Parts per Million)<sup>a</sup> of Methyl Cinnamate **12**

sample (origin)	(D/H) <sub>2,6</sub>	(D/H) <sub>3,4,5</sub>	(D/H) <sub>A<sub>f</sub></sub> <sup>b</sup>	(D/H) <sub>β</sub>	(D/H) <sub>α</sub>	(D/H) <sub>OMe</sub>
12.1 (synthetic from Fluka)	150.5 (1.7)	144.0 (2.4)	146.6	515.8 (1.9)	121.6 (3.7)	143.8 (2.6)
12.2 (natural commercial from H&R)	126.3 (0.7)	150.4 (1.7)	140.7	127.2 (1.2)	131.2 (5.1)	121.1 (2.3)

<sup>a</sup> The (D/H)<sub>i</sub> standard deviations are reported within parentheses. <sup>b</sup> (D/H)<sub>A<sub>f</sub></sub> is the mean deuterium content of the aromatic nuclei calculated from the individual positional (D/H)<sub>i</sub>.

of the double bond of the extractive samples 10.1–10.3 differed substantially from 10.4 and 10.5, obtained by synthesis. Specifically, samples 10.1–10.3 showed a (D/H)<sub>CH-β</sub> around 131 ppm. The corepective values in samples 10.4 and 10.5 were 258.5 and 189.8 ppm, respectively. When the α-position is considered, the two sets appear less differentiated.

More detailed information on the aromatic deuterium pattern of **10** comes from the examination of the spectra of **14**, the diacetyl derivative of salicyl alcohol **9**, the material used in the characterization of salicin **8**, showing separated signals for the four aromatic hydrogen atoms (11). The (D/H)<sub>i</sub> values of samples 14.1–14.4, prepared from the coumarin samples 10.1–10.4, are reported in **Table 1**. The total (D/H)<sub>aromatic</sub> is in satisfactory agreement with the data of the precursors. In detail, the (D/H)<sub>6(ortho)</sub> and the (D/H)<sub>4(para)</sub> in 14.1–14.4 were quite similar to those observed in 10.1–10.4, while the (D/H)<sub>5(meta)</sub> and the (D/H)<sub>3(meta)</sub>, for the extractive samples, fell around 134 and 172 ppm, respectively. Similarly, in the synthetic sample 14.4, the corresponding values were 151.0 and 167.2 ppm, respectively. Thus, in all of these samples, position 3 of the salicyl moiety, which is ortho to the phenolic oxygen, is more deuterium enriched than position 5, para to the activating group. These positions for the two samples of **14** derived from extractive salicin **8** showed a much more similar degree of deuterium labeling, since the (D/H)<sub>3(meta)</sub> and the (D/H)<sub>5(meta)</sub> were 127 and 119 ppm and 134 and 115 ppm, respectively. The (D/H)<sub>7</sub> values relative to the benzylic position are without significance in the considered situation because one of the two hydrogen atoms comes from the NaBH<sub>4</sub> used in the reduction of the intermediate ozonide.

So far, it seems quite difficult to attribute a biosynthetic relevance to the fact that in natural coumarin position 3, ortho to the oxygen atom introduced in the framework of cinnamic acid **4** when it is converted into *o*-coumaric acid **7**, is more deuterium-enriched than position 5, symmetrical to position 3 in precursor **4**, because the same is not true in salicin **8**. It is worth noting that in our exchange experiments, in which salicyl alcohol was incubated with deuterated water in the presence of mineral acid, position 3 was more rapidly labeled than position 5 (11).

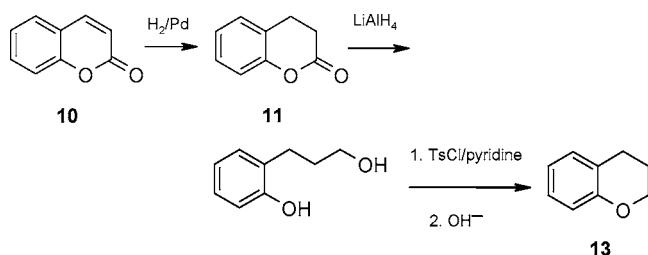
In these experiments, the observation made on salicin and on methyl salicylate (16) was, however, confirmed as follows: H-4 (para) is more deuterium-enriched than H-6 (ortho). As a general trend, it can be deduced that the ratio *R*<sub>4/6</sub> of the natural *o*-hydroxylated phenylpropanoids (range 1.19–1.30) is greater than that of the synthetic samples (range 1.0–1.11) (**Table 1**). This behavior is partially similar to that of the natural phenylpropanoids, for which as a consequence of the modality of labeling of the sugar fragments, providing shikimic acid (17), the deuterium enrichment follows the trend para > ortho > meta.

Support of this view derives from the <sup>2</sup>H NMR analyses carried out on methyl cinnamate **12**. The spectra of the synthetic and natural samples 12.1 and 12.2 are reported in **Figure 2**, and the (D/H)<sub>i</sub> values are collected in **Table 2**. The synthetic sample 12.1 shows a (D/H)<sub>2,6(ortho)</sub> = 150.5 and (D/H)<sub>3,4,5(meta+para)</sub> = 144.0 ppm, while for natural 12.2 the corresponding values

were 126.3 and 150.4 ppm, respectively. This result confirms the hypothesis that the aromatic moiety of the synthetic product is labeled in a uniform way, whereas the sugar-derived aromatic framework of 12.2 is characterized by a deuterium enrichment for the positions 2 and 6 much smaller than the one of the three remaining hydrogen atoms in positions 3, 4, and 5. Interestingly, the (D/H)<sub>β</sub> of 12.1 was 515.8 ppm, dramatically higher than the 127.2 ppm value of the natural material 12.2. On the other hand, the (D/H)<sub>α</sub> values of the two samples were 121.6 and 131.2 ppm, values consistent with the ones observed in the corresponding position in samples 10.1–10.5 (**Table 1**). The significant deuterium enrichment for positions β in synthetic **10** and **12**, as compared with the natural counterparts, might be the consequence of a kinetic isotopic effect in the condensation, which drives the conversion of salicyl aldehyde and benzaldehyde into **10** and **12**, respectively. It is worth noting that the (D/H)<sub>α</sub> values of extractive coumarin **10** and of natural methyl cinnamate **12** are all in the range of 131.2–104.6 ppm. Samples of L-phenylalanine of natural origin display in the corresponding position (D/H) values of the same magnitude (9).

**Melilotol.** Melilotol **11** is present in small amounts in many essential oils, and it is the component responsible for the therapeutic effect of the extract of *Melilotus officinalis* (L.) Lam. (Leguminosae). The preparation of aliquots of **11** by enzymic reduction of easily accessible extractive coumarin **10** might be economically interesting, because the material produced in this way is considered “natural”. In this case, the determination of the profile of the deuterium natural abundance of **11** should be a sound method to demonstrate the origin of the product obtained by different methods. Accordingly, **11** was prepared by chemical hydrogenation and baker’s yeast reduction of the coumarin sample 10.1 (samples 11.1 and 11.2, respectively). Chemical hydrogenation of the synthetic coumarin 10.4 provided the third material included in the study (sample 11.3).

The (D/H)<sub>i</sub> values of samples 11.1–11.3 are reported in **Table 1**. As expected, the pattern of the aromatic moiety of **11** corresponded to that of the parent unsaturated precursors. The part of the spectrum that might be of some interest in the differentiation of the various samples is the one concerning the CH<sub>2</sub>-α and CH<sub>2</sub>-β. The data indicate that samples 11.1 and 11.2 obtained by chemical and biochemical hydrogenation, respectively, have almost identical values, and therefore, they are indistinguishable one from the other. It should be noted that the (D/H)<sub>β</sub> and (D/H)<sub>α</sub> values of 11.1–11.3 are smaller than those of their precursors 10.1 and 10.4. The lower deuterium content exhibited by the side chain methylene groups of samples 11.1 and 11.3, obtained by catalytic reduction, is due to the combined effect of the isotopic composition of the hydrogen employed, a gas produced from methane of petrochemical origin, depleted in deuterium and of a deuterium kinetic isotope effect. In the biological reduction of the carbonyl-activated double bond (sample 11.2), it is expected that the α-hydrogen derives from water, while the one in the β-position is delivered by the intermediacy of the reduced nicotine cofactor(s) (18). A deuterium kinetic isotope effect is expected to be present for both processes (19).



**Figure 3.** Conversion of **10** into **13** by hydrogenation, reduction, and cyclization.

**Table 3.** Positional and Global  $\delta^{18}\text{O}$  Values (‰) for Coumarin **10**

sample (origin)	C=O	ArO	total
10.1 (natural from <i>F. tonka</i> ) <sup>a</sup>	38.3	2.3	20.3
10.2 (natural from Charabot) <sup>a</sup>	46.7	0.5	23.6
10.3 (natural from Charabot) <sup>a</sup>	45.7	-0.5	22.6
10.4 (synthetic from Fluka)	19.6	5.6	12.6

<sup>a</sup> Different lots.

**Determination of the Positional  $\delta^{18}\text{O}$  Values of Coumarin.** To complete the isotopic characterization of coumarin **10**, the positional  $\delta^{18}\text{O}$  values of extractive and synthetic samples were determined. For this purpose, **10** was converted to chroman **13**, which retains the aromatic oxygen atom of **10**. The transformation was performed chemically, hydrogenating **10** to dihydrocoumarin **11**. The latter compound, on LiAlH<sub>4</sub> reduction, provided 3-(2-hydroxyphenyl)propan-1-ol. This material, treated with 4-toluenesulfonyl chloride in pyridine, gave the primary tosylate, whose ring closed to chroman **13** under basic conditions (Figure 3). Samples 10.1–10.4 and samples 13.1–13.4 were subsequently submitted to the isotopic oxygen determinations. The bulk and positional  $\delta^{18}\text{O}$  values of coumarin samples 10.1–10.4 are reported in Table 3. The numerical data representing the  $\delta^{18}\text{O}$  values of the carbonyl carbon were calculated from the  $\delta^{18}\text{O}$  values of **10** and **13**.

A closer look at the whole set of values of **10** allows the identification of two distinct groups, represented by samples 10.1–10.3 of extractive origin (mean value 22.1‰) and by the sample 10.4 produced by chemical synthesis (12.6‰). Taking into consideration also the positional values, the first set is characterized by an isotope ratio mean value of 0.6‰ of the aromatic oxygen atom, while a value of 5.6‰ is observed for the synthetic material 10.4. Moreover, the carbonyl oxygen in samples 10.1–10.4 displays a mean value of 43.5‰, considerably higher than the value of 19.6‰ of sample 10.4. Therefore, the positional  $\delta^{18}\text{O}$  values are relevant for the determination of the origin of coumarin. Additionally, the present data provide information on the possible mechanism of introduction of the oxygen function in the aromatic ring of cinnamic acid **4** during the conversion into *o*-coumaric acid **7**. The mean  $\delta^{18}\text{O}$  value of 0.6‰ here observed for the aromatic oxygen atom of extractive **10** is consistent with the values of phenolic oxygen of *p*-coumarate-derived products such as raspberry ketone **2**, -0.8 and +0.6‰ (6), and vanillin **1**, 5.3 and 6.3‰ (5), respectively, thus suggesting a similar mechanism for the oxygen activation, i.e., the direct introduction of the O-atom into the aromatic ring from O<sub>2</sub> through the action of a specific enzyme. Seen together, the results of the multiple isotope characterization of coumarin **10** and related materials so far reported are not only useful for differentiating extractive natural products from the synthetic ones but also provide insights into the means of formation of natural products.

## LITERATURE CITED

- Schmidt, H.-L.; Werner, R. A.; Eisenreich, W. Systematics of <sup>2</sup>H patterns in natural compounds and its importance for the elucidation of biosynthetic pathways. *Phytochem. Rev.* **2003**, *2*, 61–85.
- Schmidt, H.-L.; Werner, R. A.; Rossman, A. <sup>18</sup>O Pattern and biosynthesis of natural plant products. *Phytochemistry* **2001**, *58*, 9–32.
- Martin, G. J.; Martin, M. L. Stable isotope analysis of food and beverages by NMR spectroscopy. *Annu. Rep. NMR Spectrosc.* **1995**, *31*, 91–104.
- Remaud, G. S.; Martin, Y. L.; Martin, G. G.; Martin, G. J. Detection of sophisticated adulterations of natural vanilla flavors and extracts: Application of the SNIF-NMR method to vanillin and *p*-hydroxybenzaldehyde. *J. Agric. Food Chem.* **1997**, *45*, 859–866.
- Fronza, G.; Fuganti, C.; Serra, S.; Burke, A.; Guillou, C.; Reniero, F. The positional  $\delta^{18}\text{O}$  values of extracted and synthetic vanillin. *Helv. Chim. Acta* **2001**, *84*, 351–358.
- Fronza, G.; Fuganti, C.; Pedrocchi-Fantoni, G.; Serra, S.; Zucchi, G.; Fauhl, C.; Guillou, C.; Reniero, F. Stable isotope characterization of raspberry ketone extracted from *Taxus baccata* and obtained by oxidation of the accompanying alcohol (betuligenol). *J. Agric. Food Chem.* **1999**, *47*, 1150–1155.
- Fronza, G.; Fuganti, C.; Serra, S.; Cisero, M.; Koziet, J. Stable isotope labeling of resveratrol and related natural stilbenes. *J. Agric. Food Chem.* **2002**, *50*, 2748–2754.
- Haslam, E. *Shikimic Acid. Metabolism and Metabolites*; Wiley: Chichester, United Kingdom, 1993.
- Brenna, E.; Fronza, G.; Fuganti, C.; Pincioli, M. Differentiation of natural and synthetic phenylalanine and tyrosine through natural abundance <sup>2</sup>H nuclear magnetic resonance. *J. Agric. Food Chem.* **2003**, *51*, 4866–4872.
- Fronza, G.; Fuganti, C.; Schmidt, H.-L.; Werner, R. A. The  $\delta^{18}\text{O}$ -value of the *p*-OH group of *L*-tyrosine permits the assignment of its origin to plant or animal sources. *Eur. Food Res. Technol.* **2002**, *215*, 55–58.
- Brenna, E.; Fronza, G.; Fuganti, C.; Gatti, F. G.; Pincioli, M.; Serra, S. Differentiation of extractive and synthetic salicin: The <sup>2</sup>H aromatic pattern of natural 2-hydroxybenzyl alcohol. *J. Agric. Food Chem.* **2004**, *52*, 7747–7751.
- Bauer, K.; Garbe, D.; Surburg, H. *Common Fragrance and Flavor Materials*; Wiley-VCH: Weinheim, Germany, 2001; p 221.
- Bleitreu, H. Untersuchungen über Cumarin. *Liebig's Ann.* **1846**, *59*, 177–198.
- Phipson, T. L. Über das melilotol. *Chemisches Central-Blatt.* **1875**, 646.
- Perkin, W. H. Bildung von Cumarin und zimmtsäure, und anderen analogen säure aus aromatischen aldehyden. *Ber.* **1877**, *10*, 299–300.
- Le Grand, F.; George, G.; Akoka, S. Natural abundance <sup>2</sup>H-ERETIC NMR authentication of the origin of methyl salicylate. *J. Agric. Food. Chem.* **2005**, *53*, 5125–5129.
- Walsh, C. *Enzymic Reactions Mechanism*; Freeman: San Francisco, CA, 1979; pp 923–929.
- Sedgwick, B.; Morris, C. Stereochemical course of hydrogen transfer catalysed by enoyl reductase enzymes of the yeast fatty acid synthetase. *J. Chem. Soc., Chem. Commun.* **1980**, 96–97.
- Fronza, G.; Fuganti, C.; Guillou, C.; Reniero, F.; Joulain, D. Natural abundance <sup>2</sup>H nuclear magnetic resonance study of the origin of raspberry ketone. *J. Agric. Food Chem.* **1998**, *46*, 248–256.

Received for review July 28, 2005. Revised manuscript received October 5, 2005. Accepted October 5, 2005. COFIN "Aromi e Fragranze" is acknowledged for partial financial support.