JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Differentiation of Extractive and Synthetic Salicin. The ^{2}H Aromatic Pattern of Natural 2-Hydroxybenzyl Alcohol

ELISABETTA BRENNA,[†] GIOVANNI FRONZA,*[,§] CLAUDIO FUGANTI,[†] FRANCESCO G. GATTI,[†] MATTEO PINCIROLI,[§] AND STEFANO SERRA[§]

Dipartimento di Chimica, Materiali e Ingegneria Chimica "Giulio Natta", Politecnico di Milano, Milano, Italy, and Istituto di Chimica del Riconoscimento Molecolare, CNR, Via Mancinelli 7, 20131 Milano, Italy

The natural abundance deuterium NMR characterization of four commercially available samples (Kahlbaum, Aldrich, Fluka, and Extrasynthèse) of salicin **1** in comparison with two extractive samples from *Salix humboldiana* and *Salix purpurea* L. and with a synthetic material, performed on the pentaacetate derivative **2** and on diacetyl salicyl alcohol **4**, is reported. Product **2** from *S. humboldiana* and the sample from salicin Kahlbaum show mean $(D/H)_{aromatic}$ values of 117 and 121 ppm, whereas, for the remaining, values of 146, 154, 153, and 150 ppm are observed, thus suggesting that salicin Kahlbaum is from extractive origin. The (D/H) values at positions 5′ and 6′ of the sugar moiety suggest a hypothesis on the origin of the glucose residue discriminating between those deriving from C3 or C4 plants. The analysis of **4**, obtained from **3**, formed in the β-glucosidase hydrolysis of salicin **1**, reveals in the natural samples from *S. purpurea* and from Kahlbaum the trend $(D/H)_4$(para) > $(DH)_3$-(meta) ∼ $(D/H)_5$(meta) > $(D/H)_6$(ortho), the first example of deuterium pattern of an ortho-oxygen-substituted phenylpropanoid. The three samples derived from commercial **1** (Aldrich, Fluka, and Extrasynthèse) and the synthetic sample show almost identical deuterium content at positions 4 and 6 (around 153 ppm), whereas for the two meta positions $(D/H)_3$ > $(D/H)_5$ (ca. 162 and 140 ppm, respectively). Product **4**, obtained from **3** submitted to acid-catalyzed deuteration, shows different deuterium incorporations in the two meta positions (which are ortho/para to the activating phenolic hydroxyl group), suggesting that possibly the deuterium abundance at the two meta positions may be affected by exchange phenomena with the medium.

KEYWORDS: Salicin; salicyl alcohol; phenylpropanoid; isotope ratio; deuterium pattern

INTRODUCTION

Salicin **1** is the bitter glucoside responsible for the long-known antipyretic and analgesic properties of the extracts of the bark and leaves of the plants of the genus *Salix*. This compound is considered to be the historic precursor of aspirin. Indeed, the pain relief activity of the infusion of the willow bark has been demonstrated since 1763 by Edward Stone. Chemical degradation of **1**, obtained in crystalline form in 1828 (*1*), indicated the presence in the molecule of a sugar and salicyl alcohol (*2*). Synthetic salicylic acid was sold in Germany by Bayer in 1859 as a component of several medications. By 1876, the physician Thomas MacLagan published a comparative study on the efficacy of salicin, salicylic acid, and sodium salicylate in treating rheumatic fever, showing the superiority of the former due to the lack of side effects. However, synthetic salicylic acid was by that time 10 times less expensive than extractive salicin **1**, with the consequence that it became the treatment of choice. The definitive success of synthetic salicylates as elective antipyretic drugs was reached in 1899 with the advent of aspirin, that is, *O*-acetylsalicylic acid (*1*). Some time after, it was demonstrated by chemical synthesis that salicin **1** is the β-D-glucopyranoside at the phenolic oxygen of salicyl alcohol (*3*, *4*).

Now, after almost 200 years, salicin **1** remains an appreciated natural remedy against fever and general indisposition, receiving renewed attention as a preventive against strokes and heart attacks. Over the years, the market requests for the activity associated with the glucoside have been traditionally met by concentrated extracts of the leaves and bark of plants of the genus *Salix* and by the crystalline material isolated therefrom. Recently, due to increasing demand, **1** is produced also by chemical synthesis, but consumers prefer to use natural products, as in foodstuffs. In this paper we propose an analytical methodology to characterize the natural or synthetic origin of **1**, based on the determination of the site-specific deuterium natural abundance by ^{2}H nuclear magnetic resonance, widely applied to the characterization of the origin of organic molecules

* Author to whom correspondence should be addressed (telephone +39 02 23993027; fax +39 02 23993080; e-mail giovanni.fronza@polimi.it).
[†] Politecnico di Milano.
[§] CNR.

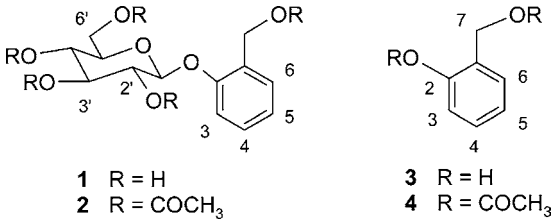
10.1021/jf0489320 CCC: $27.50
Published on Web 11/24/2004

(*5*, *6*). Furthermore, considering together the ^{2}H NMR data of **2** and **4** from the various samples of salicin **1**, it is possible not only to draw indications in favor of the extractive rather than synthetic origin of the glucoside but also to gain further insight into the aromatic deuterium pattern of the C-6−C-1, *o*-oxygen-substituted phenylpropanoid salicyl alcohol **3**.

MATERIALS AND METHODS

Origin of Samples and Preparation of Derivatives. A total of seven samples of salicin were examined. However, the amount of plant materials containing salicin with defined botanical origin was limited. Two extracts of the leaves of *Salix humboldiana* L. from Chile and *Salix purpurea* L. from Europe were obtained as gifts from Indena, Milano, Italy. Due to the low content of the desired glucoside, the whole material from the *S. humboldiana* extract was acetylated by direct treatment of the plant material with acetic anhydride and pyridine, followed by chromatography, and salicin was isolated as its crystalline pentaacetate **2**, which turned out to be the derivative of **1** actually suitable for the NMR studies. The salicin from *S. humboldiana* is designated sample 1.1. To this end, 80 g of extract in 200 mL of pyridine was treated dropwise under stirring at 0 °C with 220 mL of acetic anhydride. After 48 h at room temperature, the reaction mixture was concentrated under vacuum, and the dark oily residue was dissolved in 400 mL of CH_2Cl_2. The organic phase was washed twice with a cold saturated solution of $NaHCO_3$, cold 3% HCl, and brine. The residue obtained upon evaporation of the dried (Na_2SO_4) solution was column chromatographed on 450 g of SiO_2 with increasing amounts of ethyl acetate in hexane, eluting with a 8:2 mixture, a fraction (1.2 g) mainly composed of the pentaacetate **2**. Crystallization of this material from ethyl acetate (5 mL) at −20 °C provided 690 mg of **2**. The pentaacetate **2** thus obtained is designated sample 2.1. The *S. purpurea* extract was handled in a different way, to recover directly salicyl alcohol **3** by enzymic hydrolysis of salicin contained therein. The salicin from *S. purpurea* is designated sample 1.2. To this end 25 g of extract was taken up with 250 mL of 0.1 M acetate buffer, pH 5, and stirred for 24 h at 30−35 °C in the presence of 0.25 g of β-glucosidase from almonds (Fluka, Milano, Italy). The crude reaction mixture was extracted with CH_2Cl_2 (3 × 250 mL), and the dried organic phase was evaporated. Silica (200 g) column chromatography of the residue provided (hexane/ethyl acetate 2:8) salicyl alcohol **3**, 400 mg. This material was treated overnight with 5 mL of pyridine and 5 mL of acetic anhydride. The reaction mixture was evaporated under vacuum, and the residue was chromatographed through a short path of silica to provide (hexane/ethyl acetate 6:4) the diacetate **4**, 460 mg. Bulb-to-bulb vacuum distillation (oven temperature = 130 °C, 0.03 mm/Hg) provided the material analyzed as sample 4.2. Five additional samples of crystalline salicin were examined: sample 1.3, C.A.F. Kahlbaum, Adlershof by Berlin, Germany; sample 1.4, Aldrich, Milano, Italy; sample 1.5, Fluka, Milano, Italy; sample 1.6, Extrasynthèse, Genay, France; and sample 1.7, prepared as reported (*4*). The corresponding pentaacetate esters were analyzed as samples 2.3−2.7. Enzymic hydrolysis of salicin samples 3−6 provided salicyl alcohol **3**, indicated as samples 3.3−3.6. The diacetyl derivate of the latter materials constitute sample 4.3−4.6, respectively. Additionally, the diacetate of synthetic salicyl alcohol (Fluka) was analyzed as sample 4.8. Finally, synthetic salicyl alcohol, 2.4 g, was refluxed for 1 h in 1:1 methanol/2 N HCl (30 mL) to which had been added 10 mL of 99.8% deuterium oxide. At the end of the reaction, the mixture was poured under stirring into crushed ice−CH_2Cl_2. The organic phase was washed with a saturated solution of $NaHCO_3$, dried, and evaporated. The residue, upon acetylation, as above, provided sample 4.9.

The purity of the samples was checked by the melting point determination, TLC, and proton NMR spectroscopy. To minimize eventual differential 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. The fact that the values of the total (D/H) ratios of the aromatic nuclei for the salicin pentaacetate **2** and for the corresponding derivative **4** reported in **Tables 1** and **2** are reasonably comparable shows clearly that no macroscopic isotope fractionation occurred during the processing of the samples.



1 R = H
2 R = $COCH_3$
3 R = H
4 R = $COCH_3$

Figure 1. Structures and numbering of salicin **1**, salicyl alcohol **3**, and their acetyl derivatives **2** and **4**.

NMR Experiments. The ^{2}H NMR experiments were performed on a Bruker Avance 500 spectrometer equipped with a 10 mm broad band probehead and a ^{19}F lock (C_6F_6) channel, under CPD (Waltz 16 sequence) proton-decoupling conditions. The spectra were recorded at 310 K for the salicin derivative **2** and at 298 K for **4** with the sample spinning at 18 Hz. The reference used for $(D/H)_i$ calculations was *tert*-butyl disulfide calibrated against the official standard TMU (Community Bureau of References, BCR) with a certified (D/H) ratio. The spectra were recorded by dissolving 0.4−1.0 g of material in ∼3.0 mL of solvent, adding 70 μL of C_6F_6 for the lock and 100−130 mg of *tert*-butyl disulfide as internal standard [(D/H) 129.2 ppm)]. The solvents used were $CHCl_3$ and CH_2Cl_2 for **2** and acetone for **4**.

At least five spectra were run for each sample, collecting 4000−8000 scans to reach a S/N > 100 (acetyl signals) and using the following parameters: 6.8 s acquisition time, 1200 Hz spectral width, and 16 K memory size. An estimation of the relaxation times (T_1) of the deuterium nuclei under examination showed that the longest T_1 belongs to the methyl nuclei of the reference material (∼0.8 s). Thus, the acquisition time of 6.8 s is long enough to ensure a complete relaxation of the spins between two successive pulses. Each FID was Fourier transformed with a line broadening of 1.5−2 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 XWIN NMR software (version 3.1) 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 $(D/H)_{WS}$ and L for the product under examination; n_{WS} and n_i 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 **2** and **4** was obtained from the analysis of the proton spectrum taken in the same solvent and at the same concentration of the deuterium spectrum using the values of the vicinal coupling constants and experiments of correlation of the chemical shift (COSY) and of determination of the nuclear Overhauser enhancements (NOESY). The chemical shifts are expressed in parts per million from internal TMS (δ) and the coupling constants in hertz. Pentaacetyl salicin **2** (δ, $CDCl_3$, numbering as reported in **Figure 1**): 7.35 (H_6), 7.28 (H_4), 7.09 (H_3 and H_5), 5.34−5.04 ($H_{1'}$, $H_{2'}$, $H_{3'}$, $H_{4'}$, and CH_2-7), 4.28 ($H_{6'b}$), 4.19 ($H_{6'a}$), 3,85 (H-$_{5'}$), 2.09, 2.07, 2.05, and 2.04 (5 $COCH_3$ groups); $J_{3,4}$ = 8.1, $J_{4,5}$ = 8.1, $J_{4,6}$ = 1.9, $J_{5,6}$ = 7.9, $J_{4',5'}$ = 9.8, $J_{5',6'a}$ = 2.9, $J_{5',6'b}$ = 5.4, $J_{6'a,6'b}$ = 12.3. Diacetyl salicyl alcohol **4** (δ, acetone-d_6, numbering as reported in **Figure 1**): 7.47 (H_6), 7.39 (H_4), 7.26 (H_5), 7.14 (H_3), 5.07 (CH_2-7), 2.29, and 2.02 (2 $COCH_3$ 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.

RESULTS AND DISCUSSION

Pentaacetyl Salicin 2. The complex molecule of salicin **1** obtained was unsuitable for ^{2}H NMR studies, whereas more

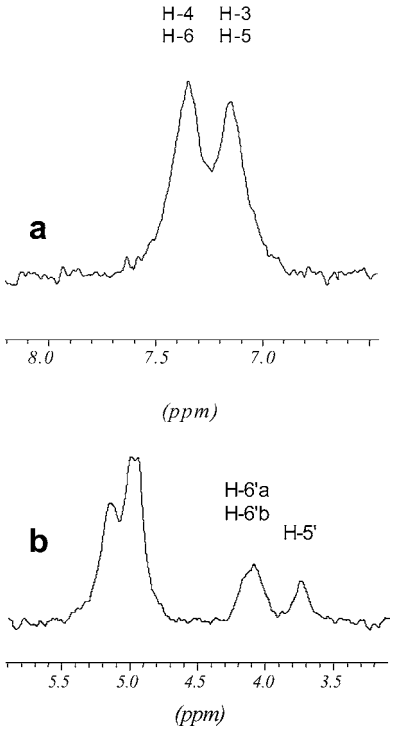


Figure 2. Natural abundance ^{2}H NMR spectra of the pentaacetyl salicin **2** from C.A.F. Kahlbaum (sample 2.3): (**a**) aromatic region (solvent CH_2Cl_2); (**b**) glucose and hydroxymethylene region (solvent $CHCl_3$).

resolved spectra were obtained with the pentaacetate derivative **2**. A typical ^{2}H NMR spectrum of **2** is reported in **Figure 2**. The spectrum shows three main separate regions that can be of significance. These are relative to the aromatic hydrogen atoms, to the CH groups at positions 1′-4′ of glucose plus the benzylic CH_2-7, and, finally, to the CH and CH_2 at positions 5′ and 6′, respectively, of the glucose moiety. The $(D/H)_i$ values of samples 2.1 and 2.3−2.7 are reported in **Table 1**.

A first clear distinction among the six examined samples appears from the total (D/H) values of the aromatic nuclei, which amount to ca. 120 ppm for extractive sample 2.1 and sample 2.3 and to 146−150 ppm for samples 2.4−2.7. The values relative to the four CH groups at positions 1′−4′ of glucose plus the benzylic CH_2 appear of limited significance, due to the too high number of atoms involved, whereas further interesting information can be drawn from that relative to positions 5′ and 6′ of the glucose residue. Recently Zhang et al. (*7*) reported a detailed investigation on the deuterium isotope pattern of hexoses of different origin using suitable derivatives in the attempt to characterize their metabolic pathway. Their data showed that the (D/H) ratios of the individual glucose positions are strongly influenced by the botanical nature of the plant from which the material was obtained. In particular, it appears that for glucose derived from C3 and CAM plants the deuterium content at position 5′ is greater than that at position 6′ (ratio $(D/H)_{6'}/(DH)_{5'}$ in the range of 0.8−0.9), whereas the opposite occurs for samples obtained from C4 plants (ratio in the range of 1.01−1.11). Among our samples a clear distinction can be found between entries 2.1 and 2.3 and entries 2.4−2.7. For samples 2.1 and 2.3 the ratio $(D/H)_{6'}/(DH)_{5'}$ is near 0.90, typical of glucose formed through the C3 metabolic pathway according to the nature of the plants of the genus *Salix*, whereas for samples 2.4−2.7 the ratio occurs in the range 1.10−1.30 (**Table 1**). This ratio, except that of entry 2.6, which appears to be abnormally high, fits reasonably well with that of ∼1.10 observed for glucose from C4 sugar cane (*7*). In conclusion, the data obtained for the samples in our hands of this rather complex molecule allow us to make a fair hypothesis about their origin: extractive from *Salix* plant for samples 2.1 (authentic sample from *S. humboldiana*) and 2.3 (C.A.F. Kahlbaum) and synthetic for samples 2.4−2.7 obtained possibly from glucose from sugar cane and synthetic salicyl alcohol.

Diacetyl Salicyl Alcohol 4. In the ^{2}H NMR spectrum of **2** the aromatic signals appear in two poorly resolved groups corresponding to the positions 3,5 and 4,6 of the ring, whereas it was desirable to obtain a deeper insight on the origin of the samples to examine a derivative of salicin in which the four individual signals are separated. The diacetate **4** turned out to be the compound of choice. The natural abundance ^{2}H NMR spectra of a selected set of specimens of **4** are reported in **Figure 3**, whereas in **Table 2** are reported the $(D/H)_i$ of samples 4.2−4.6 and 4.8. Samples 4.2−4.6 were obtained by acetylation of salicyl alcohol from the enzymic hydrolysis of the corresponding salicin, whereas sample 4.8 was obtained by acetylation of commercial salicyl alcohol.

From the figures of **Table 2** the samples can be clearly divided in two groups as already observed for salicin pentaacetate **2**. The extractive sample 4.2 and the product of Kahlbaum 4.3 are rather similar, showing both a $(D/H)_4$ value higher than that of the other positions (141 and 137 ppm, respectively) and a $(D/H)_3$ value similar to $(D/H)_5$ (127 and 135 ppm, respectively, for sample 4.2 and 119 and 115 ppm, respectively, for sample 4.3). The second group is formed by the synthetic samples 4.4−4.6 and 4.8, which are characterized by a mean $(D/H)_{total}$ for the aromatic protons included in the range of 148−157 ppm, deuterium enrichments for the positions 3, 4, and 6 within the ranges of 156−168, 150−163, and 147−156 ppm, respectively, and rather low deuterium contents for the position 5 (137−143 ppm).

The deuterium labeling pattern observed for the natural salicyl derivatives 4.2 and 4.3 here examined depends on the biochemical pathway leading to the formation of salicin **1** in nature (*8*). The C-6−C-1 aromatic moiety **3** arises from the C-6−C-3 shikimate-derived cinnamic acid **5**, via *o*-coumaric acid **6** and cleavage to salicyl aldehyde **7**, its actual precursor (**Figure 4**).

It has been proved in several instances (*6*, *9*−*12*) that the deuterium distribution along the aromatic ring in natural phenylpropanoids is not statistical, but, as a consequence of the

Table 1. Origin and $(D/H)_i$ Isotopic Ratios (Parts per Million) of Pentaacetyl Salicin **2**

sample (origin)	$(D/H)_{3,4,5,6}$[a]	$(D/H)_{7,1',2',3',4'}$[a]	$(DH)_{5'}$[a]	$(D/H)_{6'a,6'b}$[a]	$(D/H)_{6'}/(DH)_{5'}$[a]
2.1 (*S. humboldiana*)	117 (3.3)	133 (4.5)	123 (4.5)	111 (2.7)	0.90
2.3 (C.A.F. Kahlbaum)	121 (2.2)	149 (5.1)	140 (4.3)	128 (4.4)	0.91
2.4 (Aldrich, Milano)	146 (3,4)	158 (2.2)	115 (2.9)	132 (2.5)	1.15
2.5 (Fluka, Milano)	154 (1.9)	149 (2.7)	126 (5.4)	142 (4.9)	1.13
2.6 (Extrasynthèse,Genay)	153 (3.9)	142 (5.6)	113 (5.2)	147 (5.8)	1.30
2.7 (synthetic)	150 (1.8)	133 (2.6)	125 (4.5)	137 (3.2)	1.10

[a] Within parentheses are reported the $(D/H)_i$ standard deviations.

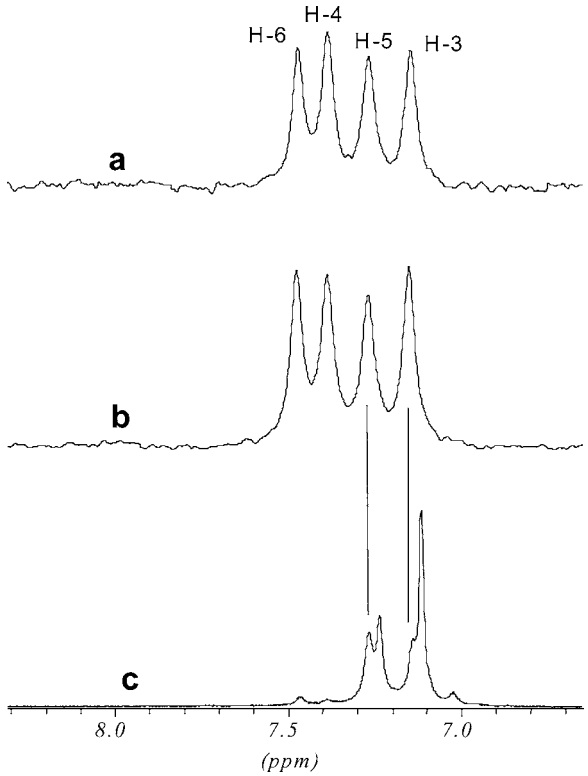


Figure 3. Natural abundance ^{2}H NMR spectra (acetone) of salicyl alcohol derivative **4**: (**a**) sample 4.3 from salicin Kahlbaum; (**b**) synthetic sample 4.8; (**c**) synthetic sample 4.9 from the acid-catalyzed exchange of salicyl alcohol with deuterated water. The signal multiplicity is due to the presence of mono- and dideuterated species (see text).

mode of labeling of the sugar fragments providing shikimic acid (*13*), the deuterium enrichment follows the trend para > ortho > meta (the positions are referred to the alkyl chain). In contrast, identical materials of synthetic origin display very similar deuterium contents in all positions. For para-hydroxylated natural phenylpropanoids it was shown that the trend of deuterium enrichments is different, that is, meta > ortho. This reversed effect is due to the well-known shift (*6*) of a proton from the para to the meta position accompanying the enzymic para-hydroxylation (NIH shift). In our case the hydroxylation process occurs in a position ortho to the alkyl chain of **5** to produce the intermediate **6**. In the absence of hydrogen migration the labeling pattern of **6** should reflect the above rule, as far as the deuterium distribution is concerned, that is, para > ortho > meta. Instead, samples 4.2, from salicin **1** from *S. purpurea*, and 4.3 from salicin Kahlbaum show a different trend with $(D/H)_4$(para) > $(DH)_3$(meta) ~ $(D/H)_5$(meta) > $(D/H)_6$(ortho). Thus, for these samples the deuterium enrichments seem to follow the course meta > ortho as for para-hydroxylated compounds. However, such an effect cannot be due to the above-

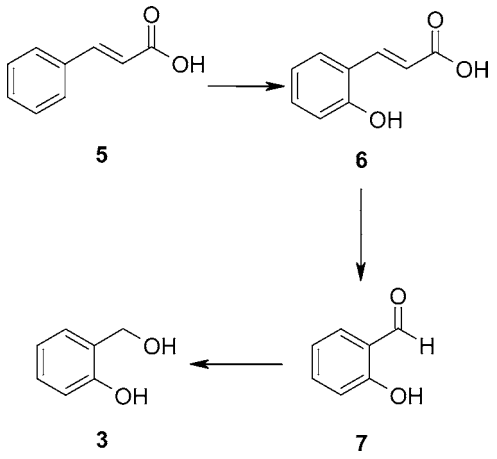


Figure 4. Biosynthetic pathway for the formation of salicyl alcohol **3** from cinnamic acid **5**.

mentioned NIH shift because in this case the hydrogen migration should lead to a deuterium enrichment of position 3, leaving unchanged position 5.

Alternatively, a possible reason for effect on the deuterium content at positions 3 and 5 of salicyl alcohol **3** may be the occurrence of proton exchange phenomena with the medium. In fact, the salicyl alcohol submitted to acid-catalyzed exchange with deuterated water shows a huge incorporation of deuterium atoms in positions 3 and 5, which are ortho−para with respect to the activating phenolic hydroxyl group, as shown in the spectrum of sample 4.9 reported in **Figure 3c**. There is, however, a kinetic preference for position 3. It is worth noting in the spectrum of **Figure 3c** that the deuterium signals of both positions 3 and 5 are split (peaks at 7.266 and 7.236 ppm for D-3 and 7.140 and 7.116 ppm for D-5). Reasonably, the doubling of the signals should be caused by the presence in solution of mono- and dideuterated species. The signals of the dideuterated species should resonate at higher fields with respect to the monodeuterated one due to the mutual ^{2}H/^{2}H isotope effect on the chemical shift. The deuteration experiments indicate that the nonequivalent meta positions are activated by the ortho−para phenolic hydroxyl group toward acid-catalyzed proton exchange. This implies that the deuterium content at positions 3 and 5 may be influenced by the medium as a consequence of possible equilibrium with water at some time of the history of the molecule.

The remaining examined products (samples 4.4−4.6 and 4.8) show very similar total deuterium contents much higher than that of the natural products, from which they can be easily distinguished. The isotope abundances at positions 4 and 6 are almost identical (around 153 ppm), whereas the two meta positions display $(D/H)_3$ > $(D/H)_5$ (ca. 162 and 140 ppm, respectively). Possibly also for these samples the differentiation may be due to exchange phenomena that occur, as shown by

Table 2. Origin and $(D/H)_i$ Isotopic Ratios (Parts per Million) of Diacetyl Salicyl Alcohol **4**

sample (origin)	$(D/H)_3$ (meta)[a,b]	$(D/H)_4$ (para)[a,b]	$(D/H)_5$ (meta)[a,b]	$(D/H)_6$ (ortho)[a,b]	$(D/H)_{total}$ (aromatic)[a,c]	$(DH)_7$ (CH_2OAc)
4.2 (from **1** *S. purpurea* L.)[d]	127 (3.8)	141 (4.3)	134 (4.0)	106 (3.2)	127 (3.8)	128 (2.4)
4.3 (from **1** C.A.F. Kahlbaum)[d]	119 (3.2)	137 (3.9)	115 (2.6)	108 (3.8)	120 (2.9)	133 (1.9)
4.4 (from **1** Aldrich)[d]	164 (4.3)	156 (2.9)	143 (3.8)	147 (1.8)	153 (2.8)	144 (2.2)
4.5 (from **1** Fluka)[d]	158 (3.0)	155 (3.3)	140 (2.6)	152 (3.0)	151 (2.5)	138 (2.4)
4.6 (from **1** Extrasynthèse)[d]	156 (1.3)	150 (2.0)	137 (2.2)	150 (3.9)	148 (2.8)	144 (1.8)
4.8 (from **3** Fluka)[e]	168 (4.0)	163 (3.3)	141 (3.9)	156 (4.2)	157 (3.9)	145 (2.8)

[a] The $(D/H)_i$ standard deviations are reported within parentheses. [b] Ortho, meta and para positions are referred to the hydroxymethylene substituent. [c] $(D/H)_{total}$ is calculated from the individual positional deuterium contents. [d] Obtained by acetylation of salicyl alcohol from the enzymic hydrolysis of the corresponding salicin **1**. [e] Obtained by acetylation of commercial salicyl alcohol.

the partially exchanged sample 4.9, with a kinetic preference for position 3 (**Figure 3c**).

Combining together these observations with those above relative to salicin pentaacetate, it is possible to conclude that among the salicin samples of unknown origin that of Kahlbaum is of extractive source, whereas the commercial products from Aldrich, Fluka, and Extrasynthèse are obtained by chemical synthesis using, seemingly, D-glucose from sugar cane (*7*). Moreover, the site-specific deuterium abundance of the aromatic ring for the natural samples shows a distribution which differ from that of the other known phenylpropanoids (*6*). From a mechanistic point of view the present study might hold some relevance, because it reveals for the first time the natural abundance aromatic deuterium pattern of an ortho-oxygen-substituted phenylpropanoid. The effect on the aromatic deuterium distribution of the ortho-hydroxylation in phenylpropanoids is a blind spot in biosynthesis (*6*), and the present results on salicyl alcohols can be helpful to this end.

ACKNOWLEDGMENT

Prof. Gianluca Nasini, CNR, Milano, and Indena spa, Milano, are acknowledged for the gift of the plant materials.

LITERATURE CITED

(1) Jourdier, S. A miracol drug. *Chem. Br.* **1999**, issue 2, chembytes e-zine, issues 1999.

(2) Piria, A. Untersuchungen über Salicin. *Annalen* **1845**, *56*, 35−77.

(3) Irvine, J. C.; Rose, R. E. The constitution of salicin. Synthesis of pentamethyl salicin. *J. Chem. Soc.* **1906**, *89*, 814−822.

(4) Kunz, A. Studies on salicin I. Exceptional rotations of the halogeno-tetra-acetyl derivatives of salicin, a new synthesis of salicin. *J. Am. Chem. Soc.* **1926**, *48*, 262−268.

(5) Martin, G. J.; Martin, M. L. Stable isotope analysis of food and beverages by NMR spectroscopy. *Annu. Rep. NMR Spectrosc.* **1995**, *31*, 91−104.

(6) Schmidt, H.-L.; Werner, R. A.; Eisenreich, W. Systematics of ^{2}H patterns in natural compounds and its importance for the elucidation of biosynthetic pathways. *Phytochem. Rev.* **2003**, *2*, 61−85.

(7) Zhang, B.-L.; Billault, I.; Li, X.; Mabon, F.; Remaud, G.; Martin, M. L. Hydrogen isotopic profile in the characterization of sugars. Influence of the metabolic pathway. *J. Agric. Food Chem.* **2002**, *50*, 1574−1580.

(8) Haslam, E. *Shikimic Acid, Metabolism and Metabolites*; Wiley: Chichester, U.K., 1993.

(9) Aleu, J.; Fronza, G.; Fuganti, C.; Serra, S.; Fauhl, C.; Guillou, C.; Reniero, F. Differentiation of natural and synthetic phenylacetic acids by ^{2}H NMR of the derived benzoic acid. *Eur. Food Res. Technol.* **2002**, *214*, 63−66.

(10) Brenna, E.; Fronza, G.; Fuganti, C.; Pinciroli, M. Differentiation of natural and synthetic phenylalanine through natural abundance ^{2}H nuclear magnetic resonance. *J. Agric. Food Chem.* **2003**, *51*, 4866−4872.

(11) Hagedorn, M. L. Differentiation of natural and synthetic benzaldehydes by ^{2}H nuclear magnetic resonance. *J. Agric. Food Chem.* **1992**, *40*, 634−637.

(12) Remaud, G.; Debon, A. A.; Martin, Y.; Martin, G. G.; Martin, G. J. Authentification of bitter almond oil and cinnamon oil: Application of the SNIF-NMR method to benzaldehyde. *J. Agric. Food Chem.* **1997**, *45*, 4042−4048.

(13) Walsh, C. *Enzymatic Reactions Mechanism*; Freeman: San Francisco, CA, 1979; pp 923−929.

Received for review June 30, 2004. Revised manuscript received September 22, 2004. Accepted September 24, 2004. MURST-COFIN is acknowledged for financial support.

JF0489320