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Isotopic Criteria in the Characterization of Aromatic Molecules. 1. Hydrogen Affiliation in Natural Benzenoid/Phenylpropanoid Molecules

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The site-specific isotope ratios of several families of aromatic molecules are analyzed in terms of hydrogen affiliation and discriminating potential. Among the aromatic molecules produced by plants, many are biosynthesized by the shikimate pathway, but the terpenic pathway also forms some compounds with a benzenic ring. In compounds of the phenylpropanoid family, specific hydrogen connections are determined with cinnamic acid, a key intermediate in the formation of a large number of aromatic molecules. Then affiliations through the phenylalanine precursor, back to the parent D-erythrose 4-phosphate and phosphoenolpyruvate molecules and finally to glucose, are considered. Typical isotopic profiles of the benzenic ring in natural, as compared to non-natural, molecules are defined. The dispersion observed in the $(D/H)_i$ ratios of the lateral chains is illustrative of diverse mechanistic responses and the role of exchange phenomena. The isotopic patterns of aromatic molecules pertaining to the terpenic family are drastically different from those of the shikimate descendants, and they exhibit much less variability. They enable the stereochemical affiliation of individual hydrogen atoms to be traced back first to the parent atoms in the common intermediate, geranyl diphosphate, then to the glyceraldehyde 3-phosphate and pyruvate couple involved in the DOXP pathway, and ultimately to the glucose precursor. The results illustrate the aptitude of the site-specific isotope ratios not only to authenticate natural with respect to chemical molecules but also to characterize different metabolic pathways and to reveal differences associated with the nature of the plant precursor.

KEYWORDS: Aromatic molecules; hydrogen affiliation; SNIF-NMR; origin inference; authentication

INTRODUCTION

The major aromatic constituents of essential oils pertain to the phenylpropanoid and terpenoid classes. The economical importance of these molecules in the field of aromas and flavors has encouraged the elaboration of various methods of chemical synthesis or hemisynthesis to complement natural plant production. The NMR investigation of site-specific natural isotope fractionation (SNIF-NMR) is known to provide powerful criteria, not only for authenticating the naturalness of molecules but also for inferring many features of their biosynthetic routes (*1*). Thus, we have shown, in the case of monoterpenes extracted from different kinds of plants, that a remarkably consistent scheme of hydrogen isotopic distribution can be established (*2*). This scheme defines mechanistic and stereochemical connections and is interpretable in terms of hydrogen affiliation to the ultimate carbohydrate precursors through a precise chain of intermediate molecules. Here, we extend the analysis to the case of aromatic molecules, either elaborated by different biochemical pathways or produced by hemisynthesis (**Table 1**). The isotopic parameters of several compounds resorting to the shikimate pathway have already been described, and some typical behaviors of the aromatic fragment have been emphasized (*3*−*15*). Thus, whereas the deuterium content of ring positions in natural monosubstituted compounds usually decreases in the order para > ortho > meta, the NIH shift (*16*, *17*) leads to the inverted order, meta > ortho, in para-substituted molecules. However it is risky to assign the samples on the sole basis of relative isotopic molar fractions. For instance, whereas the order para > ortho > meta is satisfied in benzaldehyde from *Cassia*, all investigated samples from *Prunus* are characterized by the order para > meta > ortho (*9*). Conversely, the sequence para > ortho > meta may also occur in certain products from chemical synthesis. It was therefore desirable to analyze and compare the isotopic parameters of aromatic molecules on a more general basis. To this aim, quantitative values of the isotope contents, $(D/H)_i$, were determined to further appraise mechanistic affiliation of natural molecules back to key intermediates and ultimately to carbohydrate precursors. Isotopic

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Table 1. Aromatic Compounds Investigated, Described by Their CAS Registry Number and Chemical Formula[a]

compound	CAS Registry No.	formula	1-substituent	OH	OCH_3	other
benzaldehyde	100-52-7	C_7H_6O	CHO			
p-hydroxybenzaldehyde	123-08-0	$C_7H_6O_2$	CHO	4		
vanillin	121-33-5	$C_8H_8O_3$	CHO	4	3	
methyl salicylate	119-36-8	$C_8H_8O_3$	$COOCH_3$	2		
piperonal	120-57-0	$C_8H_6O_3$	CHO			3,4 ($-OCH_2O-$)
estragole	140-67-0	$C_{10}H_{12}O$	$CH_2CH{=}CH_2$		4	
anethole	104-46-1	$C_{10}H_{12}O$	$CH{=}CHCH_3$		4	
methyl cinnamate	103-26-4	$C_{10}H_{10}O_2$	$CH{=}CHCO_2CH_3$			
safrol	94-59-7	$C_{10}H_{10}O_2$	$CH_2CH{=}CH_2$			3,4 ($-OCH_2O-$)
eugenol	97-53-0	$C_{10}H_{12}O_2$	$CH_2CH{=}CH_2$	4	3	
isoeugenol (*E*)	5932-68-3	$C_{10}H_{12}O_2$	$CH{=}CHCH_3$	4	3	
thymol	89-83-8	$C_{10}H_{14}O$	CH_3	3		4 $CH(CH_3)_2$
carvacrol	499-75-2	$C_{10}H_{14}O$	CH_3	2		4 $CH(CH_3)_2$

[a] The ring carbon atoms are numbered starting from the C-substituted site that is given the number 1. However, in **Table 3** the carbon atoms of the terpenic molecules, thymol and carvacrol, will be numbered as in ref *2*, according to their affiliation with the GPP precursor.

patterns of aromatic molecules biosynthesized by the shikimate and by the terpenic pathways were characterized and compared to those of non-natural samples. Special attention was given to the case of phenylpropanoid molecules existing under isomeric forms, such as the couples estragole/anethole and eugenol/isoeugenol.

MATERIALS AND METHODS

Preparation of Samples. Natural samples were extracted from plants or essential oils or were provided by aroma and perfume companies under their own guarantee of authenticity. Non-natural samples were purchased from chemical retailers or kindly provided by several companies. Some molecules produced by biotechnological processes may be categorized as natural.

The purity of commercial products was checked by gas chromatography. It was usually higher than 98%. The natural compounds were extracted by steam distillation or by solvents, and the organic layers were purified by preparative low-pressure liquid chromatography.

The isomerization of estragole into anethole was carried out by treatment with potassium hydroxide in diethylene glycol at 165 °C. After 12 h of reflux, estragole was transformed, nearly completely, into mostly (90%) *trans*-anethole. Eugenol was isomerized under similar conditions into isoeugenol, which was subsequently oxidized into vanillin. Whether or not vanillin obtained through heating of natural eugenol in basic medium followed by air oxidation may benefit from the "natural" status is a question relevant to official regulations. In Europe, control authorities admit that transformations carried out in conditions of "kitchen chemistry" preserve the natural character. The Industrial Organization of the Flavor Industry (IOFI) has produced several guidelines for naturalness, but, obviously, fixing limits to "kitchen chemistry" may be questionable!

SNIF-NMR Measurements. The resonance signals of the different isotopomers were first assigned by proton spectroscopy. The site-specific isotope ratios, $(D/H)_i$, were determined by ^2H NMR experiments at 76.77 or 61.4 MHz on AM500 or AM400 Bruker spectrometers respectively, at a temperature of 305−308 K. The spectra were recorded using a dedicated 10 mm probe equipped with a ^{19}F locking device. Fifty microliters of C_6F_6 was added for locking. When necessary, hydroxyl signals responsible for signal overlapping were moved by adding a small quantity of trifluoroacetic acid. When the internal referencing method was used, a precisely defined quantity of isotopically certified *N*,*N*-tetramethylurea (TMU), purchased from the European Institute for Reference Materials and Measurements, was added to the sample. Broadband ^1H decoupling was applied continuously. Relaxation delays of at least $5T_1$ (relaxation time) were applied after 90° pulses. Quantitative determinations were optimized by using a dedicated complex least-squares curve-fitting algorithm (*18*). The results are the average over three to five spectra.

Isotope Ratio Mass Spectrometry Determinations. The overall deuterium contents were measured by means of a VG SIRA9 isotope ratio mass spectrometer. The products were burnt in a Carlo Erba microanalyzer to give CO_2 and H_2O. Nitrogen oxides possibly occurring were reduced on copper catalyst to avoid subsequent poisoning. Water resulting from the combustion of the product at 550 °C was first separated from carbon dioxide by differential cooling and then Zn-reduced into hydrogen gas. The standard deviation of the deuterium determination, reported on the V.SMOW scale (*19*), was about 0.3 ppm.

Computation of Site-Specific Isotope Ratios. Two methods were used for determining the isotope ratios, $(D/H)_i$. In the first method, the area of the isotopomeric signals, *i*, is referred to that of the reference, TMU, according to eq 1

$$(D/H)_i = p^{WS}m^{WS}M^{A}S^{Ai}(D/H)^{WS}/p^{A}m^{A}M^{WS}S^{WS} \quad (1)$$

where WS and A stand, respectively, for the TMU reference and for the molecule investigated and *p*, *m*, *M*, and *S* are, respectively, the number of equivalent hydrogen atoms in site *i*, the weight of product used, the molecular mass, and the signal area of the isotopomer.

Another procedure, described by eq 2, was also applied in the case of overlap with the TMU signal and on condition that the molecule was devoid of exchangeable hydrogen atoms, such as those of hydroxyl groups.

$$(D/H)_i = f_i(D/H)_A/F_i \quad (2)$$

F_i, the statistical molar fraction corresponding to a random deuterium distribution, is given by eq 3

$$F_i = p_i/\Sigma p_i \quad (3)$$

where p_i is the number of equivalent hydrogen atoms at site *i*.

The actual molar fractions of the different isotopomers, f_i, are computed from the ^2H NMR spectrum obtained in the absence of internal reference, and the overall isotope ratio of the molecule, $(D/H)_A$, is measured by isotope ratio mass spectrometry.

RESULTS AND DISCUSSION

More than 80 products corresponding to 13 aromatic molecules, described in **Table 1**, with natural or semisynthetic status have been investigated by the SNIF-NMR method (*1*, *20*). The isotopic results are gathered in **Tables 2** and **3**, where they are compared with some data from the literature.

Influence of Exchange Phenomena. The investigation of aromatic molecules elaborated by chemical synthesis has pointed out the influence of exchange phenomena on the hydrogen isotope parameters (*21*). As a result of such exchanges, stimulated by the drastic conditions of industrial processes, the isotope ratios measured in chemical products may no longer be

Table 2. Site-Specific Natural Hydrogen Isotope Ratios of Aromatic Molecules[a] Biosynthesized by the Shikimate Pathway

molecule	*ortho*	*ortho′*	*meta*	*meta′*	*para*	O-alk	C-alk			
benzaldehyde	*2, 6*		*3, 5*		4	*CHO*				
bitter almond,[b] $n = 19$	126		130		166	136				
p-hydroxybenzaldehyde	*2, 6*		*3, 5*			*CHO*				
ex-vanilla beans,[d] $n = 10$	159		197			274				
vanillin	*2, 6*		5			OCH_3	*CHO*			
ex-beans,[l] $n = 49$	157		196			127	131			
ex-lignin,[l] $n = 64$	132		169			106	120			
isom ex-eug, $n = 3$	150		172			127	185			
isom ex-eug-C1[k]	151		190			131	180			
ex-isoeug,[m] $n = 5$	158		195			129	187			
methyl salicylate	*2*	*6*	*3*	*5*	*4*	OCH_3				
com,[c] $n = 3$		136	142	131	146	125				
piperonal	*2*	*6*	*5*			OCH_2	*CHO*			
com, $n = 8$	149	147	183			187	265			
estragole	*2, 6*		*3, 5*			OCH_3	*1′*	*2′*	*3′*	
fennel	157		186			139	210	158	63	
star anise, $n = 2$	147		173			127	183	145	73	
pine tree[e,f]	140		163			139	186	132	171	
tarragon	140		178			126	182	135	148	
com C1[g]	157		168			131	171	150	157	
com C2[g]	168		163			134	168	150	157	
com C3[g]	159		164			135	174	159	159	
com C4[g]	175		152			130	165	146	159	
com C5[g]	162		167			133	171	150	158	
anethole	*2, 6*		*3, 5*			OCH_3	*1′*	*2′*	*3′*	
fennel,[h] $n = 15$	142		196			132	142	145	119	
star anise,[h] $n = 20$	137		180			124	140	130	141	
green anise,[h] $n = 4$	142		195			121	151	127	146	
ex-est pine,[h] $n = 15$	136		175			135	191	137	150	
ex-est tarragon	137		186			127	187	137	149	
isom ex-est-C2	160		170			138	196	134	145	
isom ex-est-C4	173		157			134	193	139	146	
isom ex-est-C2 + HDO[i]	159		176			136	216	141	169	
chem synth, $n = 2$	145		135			136	88	127	147	
methyl cinnamate	*2, 6*		*3, 5*		*4*		*1′*	*2′*	CH_3	
eucalyptus	140		162		162		151	142	129	
basil	146		163		163		153	148	131	
biotech	159		157		157		158	153	141	
com	147		144		144		179	171	138	
safrol	*2*	*6*	*5*			OCH_2	*1′*	*2′*	*3′ t*	*3′ c*
sassafras oil	143	133	186			173	163	150	162	71
com, $n = 3$	110	130	246			176	183	146	118	151
com C1[n]	149.5	147.9	185.1			168.7	188.4	144.6	115.6	170.1
eugenol	*2, 6*		*5*			OCH_3	*1′*	*2′*	*3′*	
clove, $n = 3$	162		229			132	162	141	135	
clove oil, $n = 4$	158		198			128	156	136	126	
com,[j] $n = 9$	151		178			129	151	142	124	
com C1[k]	141		184			130	150	128	115	
isoeugenol		*2, 6, 5*				OCH_3	*1′*	*2′*	*3′*	
com, $n = 5$		170				130	*o*	166	128	
isom ex-eug-C1[k]		165				132	*o*	165	120	

[a] The ring hydrogen atoms in *ortho*, *meta*, and *para* positions with respect to the lateral chain are numbered, respectively, 2/6, 3/5, and 4. The carbon chain itself is numbered, starting from the ring, 1′ , 2′, and 3′. Abbreviations are as follows: biotech, from biotechnology; com, commercial; ex-est, ex-estragole; ex-eug, ex-eugenol; chem synth, chemical synthesis; isom, from isomerization. [b] From ref *9*. [c] Average over three commercial samples (wintergreen). [d] From ref *8*. [e] Calculated from refs *3*, *4*, and *24*. [f] Estragole from turpentine (pine-tree). [g] Samples from commercial origin: C1 and C2 are from Aldrich, C3 and C4 are from Janssen, and C5 is from Sigma. [h] From refs *4* and *24*. [i] Slightly deuterium-enriched water (1000 ppm) was added to the medium. [j] Commercial samples provided by nine different firms; the isotopic results of site 5 are relatively dispersed. [k] Commercial sample of eugenol used in the isomerization experiments leading to isoeugenol and vanillin. [l] From ref *8*. [m] Obtained from five commercial samples of isoeugenol. [n] Among the four commercial samples, C1 exhibited significantly different values. [o] Poorly resolved spectra, mixture of cis and trans isomers.

representative of those of their precursors (*22*). In the mild conditions of natural biosyntheses, aromatic positions are less likely to be affected by exchange (except in cases of hydrogen transfers). However, subsequent extraction steps may be responsible for exchange in basic or acid media. Therefore, to preserve the reliability of the isotopic fingerprint, it is necessary to extract and purify the compound in experimental conditions that exclude the risk of exchange and avoid fractionation effects due to physical treatments. In the case of natural products from commercial sources, the possibility of isotopic perturbations due to the extraction process must be considered when apparently anomalous isotopic behavior is observed. In this respect, although most $(D/H)_i$ values measured in 16 samples of clove eugenol, either prepared in the laboratory or from commercial origin, are very consistent, the meta position exhibits an isotopic dispersion exceeding 20−30 ppm. As discussed later, variable participation of intermolecular exchange accompanying the NIH shift may be invoked to explain these differences. However, because this aromatic position is relatively exchangeable in the end products, variations observed in commercial samples may also be due to more or less drastic preparative conditions (*22*).

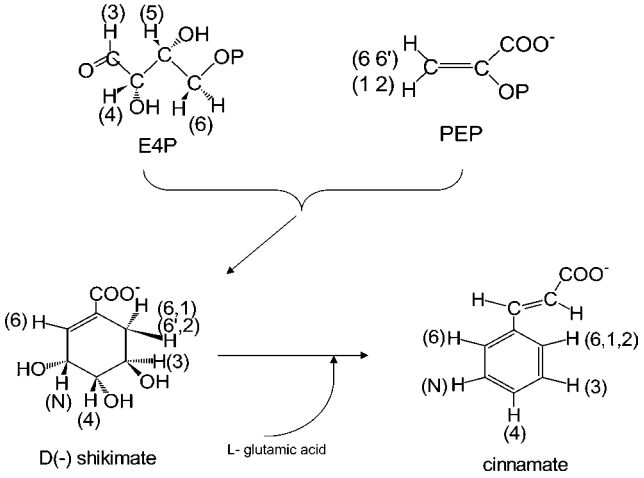


Figure 1. Isotopic affiliation of cinnamic acid to glucose through erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP) in the shikimate pathway. The numbers within parentheses denote the corresponding positions in glucose. 6 and 6′ correspond, respectively, to hydrogen 6-pro-S and hydrogen 6-pro-R of glucose. H-2 and, to a lesser extent, H-1 of glucose undergo partial intermolecular exchange in the glycolysis (*25*). (N) has been introduced by NADPH. The lateral chain of cinnamic acid is derived from that of phenylalanine, which results itself from L-glutamic acid.

Isotopic Profile of the Benzenic Fragment in Molecules Biosynthesized by the Shikimic Acid Pathway. The results collected in **Table 2** enable site-specific isotope contents to be compared in natural aromatic molecules from different origins. Although the absolute values of the isotope ratios, $(D/H)_i$, have been measured on different samples, in different experimental conditions, at different times, and with different spectrometers, they exhibit very consistent behaviors and are more informative than the relative molar fractions determined without referencing.

In phenylpropanoid molecules, synthesized according to the shikimate pathway, the aromatic ring is formed from D-erythrose-4-P (E4P) and phosphoenol pyruvate (PEP) (*23*), and its isotopic distribution can be affiliated with that of glucose as summarized in **Figure 1**. The hydrogen atom at position 4 (para), characterized by a relatively high D/H value (on the order of 155−165 ppm), is related to hydrogen-4 of glucose through hydrogen-2 of E4P. This result is in agreement with the relative enrichment (≈155−160 ppm) observed at this position in glucose from C3 plants (*24*). The hydrogen atoms at position 2 (ortho) are derived, on the one hand, from site 6 of glucose through site 4 of E4P and, on the other hand, from H-1 and H-6 pro-S or H-2 and H-6 pro-R of glucose through site 3 of phosphoenol pyruvate. It should be emphasized that, although differences of about 20 ppm are observed among the $(D/H)_{2,6}$ values of the various molecules, samples of a given molecular species, extracted from a given plant species, are characterized by relatively low dispersion. Thus, the mean $(D/H)_{2,6}$ values measured in 19 samples of benzaldehyde from *Prunus*, on 7 samples of anethole from star anise, on 4 samples of anethole from fennel, and on 5 samples of anethole from green anise are equal to 126, 137, 151, and 145 ppm, respectively, with corresponding standard deviations of 3.2, 4.7, 1.8, and 3.0 ppm. On this basis, when large differences in average values are observed, it may be concluded that the parent atoms are isotopically different. In this respect, it should be recalled that the isotopic distribution in plant glucose does not depend solely on the metabolic type, C3, C4, CAM.... For a given metabolic pathway, it may also vary significantly as a function of the physiology of the plant (*24*). For instance, differences in $(D/H)_6$ of glucose reaching 20 ppm are observed between grape sugar and beet sugar. The aromatic $(D/H)_{2,6}$ parameter may therefore provide information on the nature of the plant precursor. The isotope ratio $(D/H)_{3,5}$ associated with the position meta to the substituent in monosubstituted phenylpropanoid may also depend on the nature of the plant. Its value is about 115 ppm (SD = 4.4 for $n = 21$) in cinnamaldehyde from *Cassia*, whereas it reaches 130 ppm (SD = 2.1 for $n = 19$) in benzaldehyde from bitter almond. One of these atoms is affiliated with H_3 of glucose, which has been shown to vary from 124 to 151 ppm among beet, grape, and orange sugars. The other atom, which has been introduced by NADPH in the step of reduction of 3-dehydroshikimate to shikimate, should be relatively depleted (≈100 ppm) as shown for such an origin in refs *2*, *20*, *24*, and *25*.

From many studies of the mechanism of aromatic hydroxylation (*15*, *26*, *27*), the relative natural deuterium enrichment observed at the meta position of para-substituted phenylpropanoid molecules (*3*) can be attributed to the NIH shift, which involves a migration of the para hydrogen to the meta position in the in vivo para-hydroxylation of the aromatic ring. The mechanistic features of this phenomenon, which may occur with a variable degree of retention of the transferred atom, are still controversial (*5*, *16*, *26*−*28*). Because interpretations based on the sole relative values of the isotopic parameters (*10*) are ambiguous, we may consider absolute values of the isotope ratios to estimate whether the significant increase in the meta deuterium content of para-hydroxylated molecules results entirely from the transfer of a relatively ^{2}H-enriched para-hydrogen or whether significant KIE must be invoked. On the basis of the $(D/H)_{3,5}$ values measured in monosubstituted rings (**Table 2**) and taking into account an order of magnitude of 160−170 ppm for the isotope ratio of the para hydrogen, it is concluded that hydrogen migration occurs with a normal kinetic isotope effect, k_H/k_D, significantly higher than unity. This deuterium enrichment could fit with hydrogen abstraction from the meta position of an intermediate bearing the transferred para hydrogen. They are compatible with a mechanism involving elimination of one of the two meta hydrogen atoms in the enolization of a keto-tautomer intermediate resulting from a previous arene oxide or dihydrodiol intermediate (*16*, *26*, *27*). In this respect, it is noticeable that, in the case of hydroxylation of phenylalanine by tyrosine hydroxylase, partitioning of an arene oxide has been excluded on the basis of labeling experiments (*28*). The large natural variations (added to uncertainties associated with different experimental conditions) reflected by the isotopic results (**Table 2**) render rather speculative further quantitative estimations of both the KIE value and the percentage of intra- and intermolecular transfer, and, in the absence of such precise information, conclusions regarding a possible stereo selectivity of the hydrogen migration cannot be ascertained. Finally, it is remarkable that the values of $(D/H)_{3,5}$ associated with the two meta atoms of *p*-hydroxybenzaldehyde and with the single meta atom of vanillin biosynthesised by the same vanilla plant species are nearly equal, at about 198 ppm. This behavior suggests a lack of stereospecificity in the step of hydroxymethylation of *p*-coumaric acid leading to ferulic acid and vanillin. Similarly, it is noted that, in the case of safrol, where the two ortho sites are diastereotopic, close values of $(D/H)_2$ and $(D/H)_6$ are measured.

Another example that further illustrates the complexity of the mechanistic pathways is that of methyl salicylate. Several biosynthetic routes have been proposed (*23*, *29*−*31*). It is usually considered that phenylalanine and cinnamic acid act as precur-

sors, and benzaldehyde and benzoic acid have been mentioned as intermediates. However, a new route starting from isochorismate has been described recently (*31*). Whatever is the considered mechanism, hydrogen para to the carbonyl is affiliated with H_4 of glucose. The value of its isotope ratio (157 ppm for an authentic wintergreen sample) is consistent with this origin. In a mechanism involving monosubstituted intermediates such as benzoic acid or benzaldehyde and subsequent non-stereospecific hydroxylation, equal values of the isotope ratios of the two hydrogen atoms meta to the carbonyl would be expected. It is remarkable that the D/H ratios are unequal: about 136 ppm at position α to OH and 123 ppm at the other meta site for the considered wintergreen sample. The average value is consistent with that measured in the monosubstituted benzenoids. In the isochorismate pathway, which does not average the meta ratios, the corresponding hydrogen atoms are also affiliated, respectively, with NADPH and H_3 of glucose. In a hypothesis by which both the NIH hydroxylation and isochorismate pathways are active in a given plant, the internal meta D/H ratio should reflect their relative contributions.

Finally, it is recalled that the methyl group at the para-methoxy position of estragole results from a transfer, catalyzed by an *O*-methyltransferase, of a methyl group from *S*-adenosyl-L-methionine to the para-hydroxy position of chavicol (*32*). This para-methylation probably takes place after the formation of the allylic chain.

Isotopic Profile of the Lateral Chain of Molecules Biosynthesized by the Shikimic Acid Pathway. The $(D/H)_i$ values of the lateral chain of phenylpropanoid molecules usually exhibit larger dispersion than those of the benzenic fragment. Thus, the isotopic distribution measured in the allyl substituent is very different, not only among compounds such as estragole, eugenol, and safrol but also among molecules of the same molecular species elaborated by different plant species as is the case for estragole from fennel, star anise, tarragon, or pine tree (**Table 2**). However, it is noticeable that, in contrast to sites 1′ and 3′, the intermediate position 2′ is relatively stable in natural molecules.

Exchange phenomena, influenced by environmental or experimental conditions, may be responsible, at least partly, for the instability in these isotopic parameters. However, the observed variability also expresses complex architectures of the metabolic pathways. The mechanisms of formation of the lateral chain of benzenoid/phenylpropanoid molecules remain rather speculative. Many labeling experiments, conducted to determine to what extent the C6−C3 skeleton of phenylalanine is preserved in various metabolites, have illustrated different biosynthetic routes. For instance, phenylalanine transmits only a C6−C1 unit to the phenylpropanoid skeleton of the alkaloid ephedrine (*33*). More generally, both CoA-dependent β-oxidative and CoA-independent non-β-oxidative pathways are claimed to contribute to the formation of benzenoid compounds (*30*, *34*−*37*). These mechanisms may operate in parallel in the same plant and, in petunia flowers, for instance, the flux via the non-β-oxidative route, through the benzaldehyde intermediate, is privileged (*30*). Similarly, the biosynthesis of the C3 chain of allyl- and propenylphenylpropanoids is not fully elucidated. According to isotope tracer experiments, molecules such as estragole, eugenol, and anethole are derived from phenylalanine and cinnamic acid (*17*, *38*). However, although interconversion is possible in certain plants, allyl and propenyl derivatives may be biosynthesized by independent routes (*39*). Depending on the considered mechanism, the hydrogen atoms of the C3 fragment in the phenylalanine and cinnamic acid precursors are partly preserved or lost at different steps of the transformations. Several experiments performed on anethole concluded to full retention of the C6−C3 skeleton of phenylalanine. Moreover, although mechanistic details are still controversial, it has been concluded that hydrogen 2′ in anethole is fully affiliated with the corresponding hydrogen in the cinnamic acid intermediate (*23*, *39*, *40*). The isotope ratio of this hydrogen that varies between 130 and 145 ppm in fennel and star anise anethole is in reasonable agreement with the corresponding value in basil (148 ppm) and eucalyptus (142 ppm) cinnamate (**Table 2**). In the case of the allyl phenols, eugenol and estragole, the carbonyl carbon of cinnamic acid could have been lost and an extra carbon incorporated. Comparison of the isotopic parameters shows that tarragon estragole and its isomerized anethole exhibit rather consistent $(D/H)_i$ values. Thus, the $(D/H)_{2'}$ value, which was equal to 135 ppm in tarragon estragole, is preserved at 137 ppm in the isomerized anethole. In contrast, very different profiles are observed in natural anethole−estragole couples directly extracted from a given plant species. Moreover, it should be emphasized that the isotopic distribution in the allyl fragment considerably depends not only on the type of compound, estragole or eugenol, but, for a given molecule, on the nature of the plant.

To further investigate the allyl/propenyl transformation, we have carried out isomerization experiments on several samples of estragole extracted from tarragon oil or obtained from commercial sources. The results given in **Table 2** show that the isomerization process introduces moderate enrichment at the leaving site 1′ accompanied by deuterium impoverishment at site 3′. When the experiment is conducted in a medium very slightly enriched in deuterium, a significant increase in $(D/H)_{1'}$ and to a lesser extent $(D/H)_{3'}$ is observed (**Table 2**). Allylic isomerization into anethole being only partly intramolecular, the isotope ratios of the lateral chain, especially that of the 1′ position, are expected to be influenced by the experimental conditions of the semisynthesis. Moreover, these conditions also determine the percentages of cis and trans isomers formed and their possible interconversion.

Because one of the sources of vanillin is natural eugenol from clove, we have also investigated the isomerization of eugenol into isoeugenol followed by oxidation into vanillin (**Table 2**). In spite of the poor signal resolution of the isoeugenol ^2H NMR spectrum, it is checked that the average isotope ratio of the aromatic sites is preserved in the reactions. As in the case of the isomerization of estragole, the situation is complicated by exchange phenomena and by cis−trans isomerism. Finally, the isotopic profile of hemisynthetic vanillin from natural eugenol is relatively close to that of natural vanillin from vanilla beans, but the isotope ratio of the carbonyl hydrogen is significantly higher in eugenol vanillin. To the extent that the experimental conditions of the isomerization and oxidation steps leading from eugenol to vanillin involve chemical assistance, the product should not benefit from the natural label. However, taking into account the official exception of "kitchen chemistry" discussed under Materials and Methods, the criteria required to confer a "natural" status to a given in vitro molecular transformation are still open to dispute.

Comparison of Shikimic and Terpenic Aromatic Molecules. Although most aromatic molecules arise from the common biosynthetic intermediate, phenylalanine, or its close precursor, shikimic acid, some of them pertain to the class of terpenes. We have shown that nonaromatic members of the monoterpene family are related by remarkably consistent sets of isotopic parameters and that the isotopic patterns enable

Table 3. Comparison of the $(D/H)_i$ (Parts per Million) Isotopic Parameters of Thymol and Carvacrol to Those of Two Nonaromatic Monoterpenes, Carvone and α-Pinene[a] (*2*)

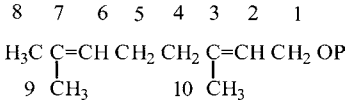
carvone[b]	C1, C5(ax)	C1, C5(eq)	C2				C6	C8	C9	C10
NMR site	*5*	*4*	*1*				*3*	*2*	*6*	*7*
4*S* (+)	152.7	165.8	28.6				103.0	160.0	102.1	122.0
n = 3	9.6	4.7	8.2				18.3	7.0	1.1	1.8
4*R* (–)	150.0	155.5	29.3				127.1	148.0	93.7	109.3
n = 2	0.1	6.8	2.2				2.2	4.2	7.6	3.7

α-pinene[b]	C1(en)	C1(ex)	C2	C4	C5(en)	C5(ex)	C6	C8	C9	C10
NMR site	*9*	*2*	*6*	*1*	*4*	*3*	*5*	*8*	*10*	*7*
1*R*,5*R* (+)	149.1	153.3	40.8	124.2	148.1	165.5	140.1	91.6	110.6	115.8
n = 4	4.6	2.3	4.1	10.4	4.9	6.5	6.5	4.1	2.9	1.7
1*S*,5*S* (–)	159.1	151.0	37.7	93.3	162.9	157.3	152.9	90.5	111.9	119.5
n = 3	3.3	4.0	7.7	1.2	7.9	7.0	5.1	4.8	3.8	5.9

thymol	C2	C4	C5	OH	C7	C8, C9	C10
NMR site	*3*	*2*	*1*	*4*	*5*	*7*	*6*
nat A	38	177	170	175	90	100	109
nat B	42	172	151	181	93	98	108
nat C	57	176	149	181	94	100	110
synth, *n* = 3	139	152	154	186	151	135	143

carvacrol	C1	C2	C5	OH	C7	C8, C9	C10
NMR site	*2*	*1*	*3*	*4*	*5*	*7*	*6*
nat A	216	22	212	139	93	107	112
nat B	190	32	220	162	95	106	110
synth C	182	71	181	191	112	113	125
synth D	183	136	153	142	138	132	133

[a] The natural dispersion is illustrated by results measured on three samples of thymol, A, B, and C, and two samples of carvacrol, A and B. As in ref *2*, the atoms are numbered according to their affiliation to the GPP precursor.



Among the samples from non-natural origin, relatively close values are measured on three samples of thymol, but the two carvacrol samples C and D are discriminated. The NMR signals are numbered in the order of decreasing chemical shifts. [b] From ref *2*.

individual hydrogen sites to be connected to the sugar precursor atoms through a complex mechanistic route (*2*). With a view to illustrate the potential of the isotopic profile to distinguish terpenic and phenylpropanoid aromatic molecules, we have extended our study of monoterpenes to the case of the aromatic compounds, thymol and carvacrol. To facilitate the mechanistic comparison with the nonaromatic molecules (*2*), the numbering of the hydrogen atoms was referred to that of the geranyl diphosphate (GPP) intermediate. It is immediately obvious from a comparison with carvone and pinene, for instance (**Table 3**), that the isotopic data of thymol and carvacrol remarkably fit the typical monoterpenic profile and are largely different from those of phenylpropanoid molecules. As are other cyclic molecules, such as limonene, thymol is derived from the α-terpinyl cation intermediate. The results show that no scrambling occurs in the course of aromatization through γ-terpinene and *p*-cymene (*41*). All aromatic sites of thymol and carvacrol can be affiliated, according to the strategy described in ref *2*, to their different parent sites in GPP (**Table 3**), and the results corroborate a formation through the deoxyxylulose phosphate (DOXP) pathway (*42*−*45*). In particular, the very low $(D/H)_2$ ratio (30−50 ppm) is necessarily assigned to the hydrogen introduced with a strong isotope effect at a late step of the DOXP sequence. The hydroxylation sites are immediately identified through the isotopic pattern. Once position C_2 has been located, it becomes obvious that stereospecific substitution of the hydroxyl group occurred at carbon ex-1 of GPP in thymol and at carbon ex-4 of GPP in carvacrol (and not at position 2). As discussed in the case of other cyclic monoterpenes, such as cineole, the $(D/H)_i$ parameters also reflect enantiomeric selectivity toward chiral linalyl and terpinyl precursors. The relatively high value of $(D/H)_4$ (175 ppm) suggests that the preserved hydrogen is the ex-*pro-S* atom of GPP, which can be affiliated formally to positions 3 and 4 of glucose with participation of exchange, in particular at the aldolization step. However, kinetic isotope effects associated with hydrogen elimination at sites 1, 4, and 5 must also be considered because the D/H values at these positions are significantly higher than those measured in nonaromatic monoterpenes. Unlike other investigated monoterpenes, both thymol and carvacrol have a hydrogen atom at position 7. The $(D/H)_7$ value, close to 92 ppm [to be compared to one of the $(D/H)_{meta}$ values of monosubstituted phenylpropanoid], indicates that this hydrogen, which was absent in the GPP and terpinyl precursors, has been introduced subsequently with a significant direct isotope effect.

Thanks to highly stereospecific chains of transformations, aromatic terpenic molecules are characterized by very reproducible and informative isotopic patterns. It is also immediately obvious (**Table 3**) that the isotopic parameters provide unambiguous criteria for distinguishing natural from non-natural samples.

Discriminating Potential of Isotopic Patterns. Simple inspection of the site-specific isotope ratios of aromatic molecules enables phenylpropanoid and terpene biosynthetic pathways to be unambiguously identified. However, the isotopic

framework in molecules derived from the shikimic pathway lacks the remarkable overall consistency characterizing the terpenic family. Isotopic affiliation of the alkyl chain of benzenoid/phenylpropanoid compounds is complicated by possible contributions of different biosynthetic pathways and by sensitivity to exchange phenomena. In spite of these difficulties, the isotopic profile provides efficient criteria, not only for discriminating natural, hemisynthetic, and chemical origins but also, in the case of natural and biotechnological compounds, for identifying the plant precursor of molecules such as estragole or anethole and possibly their geographical origin. Usually, this distinction cannot be achieved securely on the sole basis of the isotopic molar fractions of the benzenic fragment. In para-substituted molecules, for instance, the usual behavior $(D/H)_{meta} > (D/H)_{ortho}$ exhibited by natural compounds is also frequently observed in chemical samples. However, when the whole set of isotopic parameters is considered, unambiguous authentication criteria can be defined and discriminant statistical analyses of the data usually define well-separated groups as a function of the biosynthetic or chemical pathways. In a number of cases some specific isotopic parameters are highly characteristic of a defined origin. For instance, very high D/H values (>300 ppm) usually exclude the possibility of a natural origin.

On the basis of the present results, a further step in the exploitation of isotopic data of natural aromatic molecules could be reached by carrying out quantitative studies of isotopic responses in well-defined systems. Thus, comparative investigations involving several metabolites extracted from a given plant species, grown in controlled environments, should provide better appraisal of the fluxes through different mechanistic routes and of the role of plant physiology and environmental factors.

NOTE ADDED AFTER REVIEW

One of the reviewers drew our attention to a recent publication concerning phenylpropanoid. This reference (*46*), which was not available to us when we submitted this paper, presents quantitative interpretations of isotope ratios based on previously published experimental data from various sources. The authors insist, as we do, on the role of the NIH shift. However, we disagree with the overall analysis that is founded on a very confusing and basically erroneous discussion of isotope effects at natural abundance. In particular, it does not make sense to consider that, at natural abundance, reactions are oriented by differences in isotope contents at competitive sites—in the hypothesis of 100% migration (arbitrarily retained) a possible intramolecular isotope effect accompanying hydrogen migration is in no way accessible from the D/H ratios of products—we cannot adhere to the definition "the intramolecular isotope effect is in reality, a complex overlap of the inter- and intramolecular competitive reactions of several isotopomers".... Moreover, we think that, in the present case, an experimental data set built from literature results obtained in various conditions is not consistent enough to derive mechanistically reliable predictive factors. The SNIF-NMR method is an extremely powerful tool for the inference of atom affiliations and for the elucidation of many mechanistic features of product elaboration (*2*, *22*, *24*, *25*). However, as further demonstrated in this work, many parameters are likely to influence final fractionation values. Therefore, great care must be taken not to overestimate the quantitatively predictive potential of isotopic data obtained in unknown or heterogeneous conditions.

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