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The prediction of isotopic patterns in phenylpropanoids from their precursors and the mechanism of the NIH-shift: Basis of the isotopic characteristics of natural aromatic compounds

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Abstract

The theoretical ^{2}H-distribution in the aromatic ring of phenylpropanoids can be predicted from that of their precursors – erythrose-4-phosphate, phosphoenolpyruvate and NADPH – and by invoking the mechanism of the NIH-shift and implied deuterium isotope effects. For each position in the non-oxygenated ring, the predicted natural ^{2}H-abundance is in excellent agreement with experimental data obtained from quantitative ^{2}H NMR-measurements on natural compounds, especially concerning the relative ^{2}H-abundances $p > o \geqslant m$. For the *p*-hydroxylated derivatives, the experimentally determined ^{2}H-abundance sequence order $m > o$ can also be deduced, assuming an anisotropic migration (intramolecular isotope effect) of the *p*-hydrogen atom to the two differently ^{2}H-substituted *m*-positions during the NIH-shift (intramolecular hydrogen transfer) and an in vivo deuterium kinetic isotope effect of ~1.20 on the final hydrogen elimination from the proposed ketodiene intermediate.

The predicted ^{2}H-distribution pattern of methyl salicylate **10**, a representative of an *o*-hydroxylated natural compound, is in excellent agreement with that reported from ^{2}H NMR analyses. However, for salicyl alcohol, minor differences between the theoretical and experimentally determined pattern are found that cannot yet be satisfactorily explained. On the other hand, a very good agreement is found between the theoretical and experimental pattern of coumarin, provided a deuterium kinetic isotope effect of ~1.30 is assumed for the elimination of the H-atoms from the ketodiene intermediate. The secondary *m*-hydroxylation of *p*-coumaric acid in the biosynthesis of vanillin seems to proceed without large isotope effects. Parallel differences are also observed for the ^{18}O-kinetic isotope effects on the corresponding monooxygenase-catalysed reactions.

The results demonstrate convincingly that the mechanisms of these general reactions of the phenylpropanoid biosynthetic pathway are identical and follow general principles. Small observed differences between the ^{2}H-patterns of individual natural aromatic compounds originating from the same hydroxylation type can therefore be assigned to differences of the patterns of the precursors, the extent and the orientation of the hydrogen migration, and the kinetic isotope effect on the final hydrogen elimination.

The evidence for the existence of general systematic rules governing isotopic patterns in the shikimic acid pathway and its subsequent reactions is further supported by the recently reported ^{13}C-distribution pattern of vanillin, which is also in agreement with that predicted from the precursors.

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Hence, it is apparent that the systematics of the isotope patterns of phenylpropanoids are in line with the generally accepted biosynthetic reactions in the shikimic acid pathway and that this knowledge can strengthen their value as an essential support for the distinction of natural and synthetic aromatic compounds.

Keywords: Shikimic acid pathway; ^{2}H-Pattern; ^{13}C-Pattern; NIH-Shift; Natural aromatic compounds; Isotope effects

1. Introduction

Isotopic abundances and patterns are the most powerful means of assessing the authenticity and origin of a wide range of natural products (Schmidt et al., 1998). Whereas for most compounds the approach taken is purely empirical, using the comparison of unknown samples with authentic references, we have recently shown that, on the basis of the biosynthetic routes exploited and a knowledge of the reaction mechanisms involved, a set of systematic rules for the isotopic characteristics of a given group of compounds can be elaborated, providing a scientific basis for the interpretation of corresponding experimental data (Schmidt et al., 1995, 2001, 2003; Schmidt and Eisenreich, 2001). In this context we have above all shown that the dominant reasons for the origin of isotopic patterns of natural compounds (non-statistical intramolecular isotope distribution = the relative abundances of their isotopomers) are kinetic isotope effects and reaction mechanisms, especially in "unidirectional" (irreversible) processes (Schmidt, 2003).

The phenylpropanoids and their derivatives are among the most common active agents of spices, aromas and fragrances and provide a valuable target for authenticity testing (Schmidt et al., 1998). It has been demonstrated that the hydrogen patterns of the aromatic ring of these compounds originate from the precursors erythrose-4-phosphate, phosphoenolpyruvate and NADPH, and that they are modulated by the NIH-shift associated with the mechanism of the *p*-hydroxylation, wherein an oxygen atom is introduced from O_2. As a consequence of these processes, a relative deuterium abundance $p > o \geqslant m$ exists in the aromatic ring of non-hydroxylated natural compounds, and a deuterium abundance $m > o$ and a δ^{18}O-value of $\sim+6‰$ in the *p*-hydroxylated derivatives (Schmidt et al., 2001, 2003). In contrast, synthetic analogues show a statistical deuterium distribution (Schmidt et al., 2003) and more positive δ^{18}O-values (Schmidt et al., 2001).

Recently, data on *o*-hydroxylated products have become available. Brenna et al. (2004) report a relative deuterium abundance $p > m_3 \approx m_5 > o$ for salicyl alcohol (**9**, Fig. 1) from *Salix purpurea* L. The authors comment that their results "cannot be due to the ...NIH-shift because in this case the hydrogen migration should lead to a deuterium enrichment of position 3, leaving unchanged position 5" and suggest an equilibration with water as an explanation for their results. Le Grand et al. (2005) found slightly different relative abundance sequences for "methyl salicylate" **10**, respectively, $p > m_3 > m_5 > o$ from wintergreen oil and $p > m_3 > m_5 \approx o$ from birch bark oil, but they did not attempt a general interpretation of their results. However, data recently reported for the ^{2}H-pattern of coumarin **7** from tonka beans (*Fava tonka*) are confusing: some positions are in agreement with the above results but, at first glance, other positions are not (Brenna et al., 2005). So far, except for vanillin **5**, no data on *m*-hydroxylated natural compounds are available.

However, we believe that a systematic isotopic relationship must exist in the biosyntheses of all these compounds and that, in analogy to the pattern of *p*-hydroxylated phenylpropanoids, the available data on the *o*- and the *m*-hydroxylations are directed by logical rules and can be fitted to a common picture. The aim of this paper is therefore to detect regularities and to find general explanations for the individual isotopic patterns of the aromatic ring of different relevant natural compounds. In this respect, it is necessary to take into account the detailed patterns of the non-hydroxylated aromatic ring and to understand the intrinsic eventualities of the mechanisms of the hydroxylation reactions. An important point in this context is that the aromatic ring of the non-oxygenated phenylpropanoids must have, on the basis of its biosynthesis, individual and different deuterium abundances in each position, but that the ^{2}H NMR-signals give only the mean abundances for the two *o*- and two *m*-positions. A further important influence is that the direction and extent of hydrogen retention in the course of the hydroxylation with NIH-shift will depend on the primary ^{2}H-pattern of the substrate, on the enzymes involved and external conditions, and on intramolecular and kinetic isotope effects associated with any hydrogen migration and elimination. We shall show that the reported experimental data can largely be interpreted on the basis of these influences. We shall also integrate into these considerations proof that the recently elucidated ^{13}C-pattern of vanillin **5** (Tenailleau et al., 2004) is in good agreement with that of its precursors (Roßmann et al., 1991), adding further support to the postulate that the isotope distribution pattern of phenylpropanoids is determined by logical correlations.

This paper combines the experimental data on isotope patterns of phenylpropanoids from several cited original papers of some of the authors and interprets them on the basis of mechanistic and kinetic parameters in the context of the involved biosynthetic reactions. All these data have been obtained by quantitative site specific ^{2}H and ^{13}C NMR-measurements, as cited in the corresponding references.

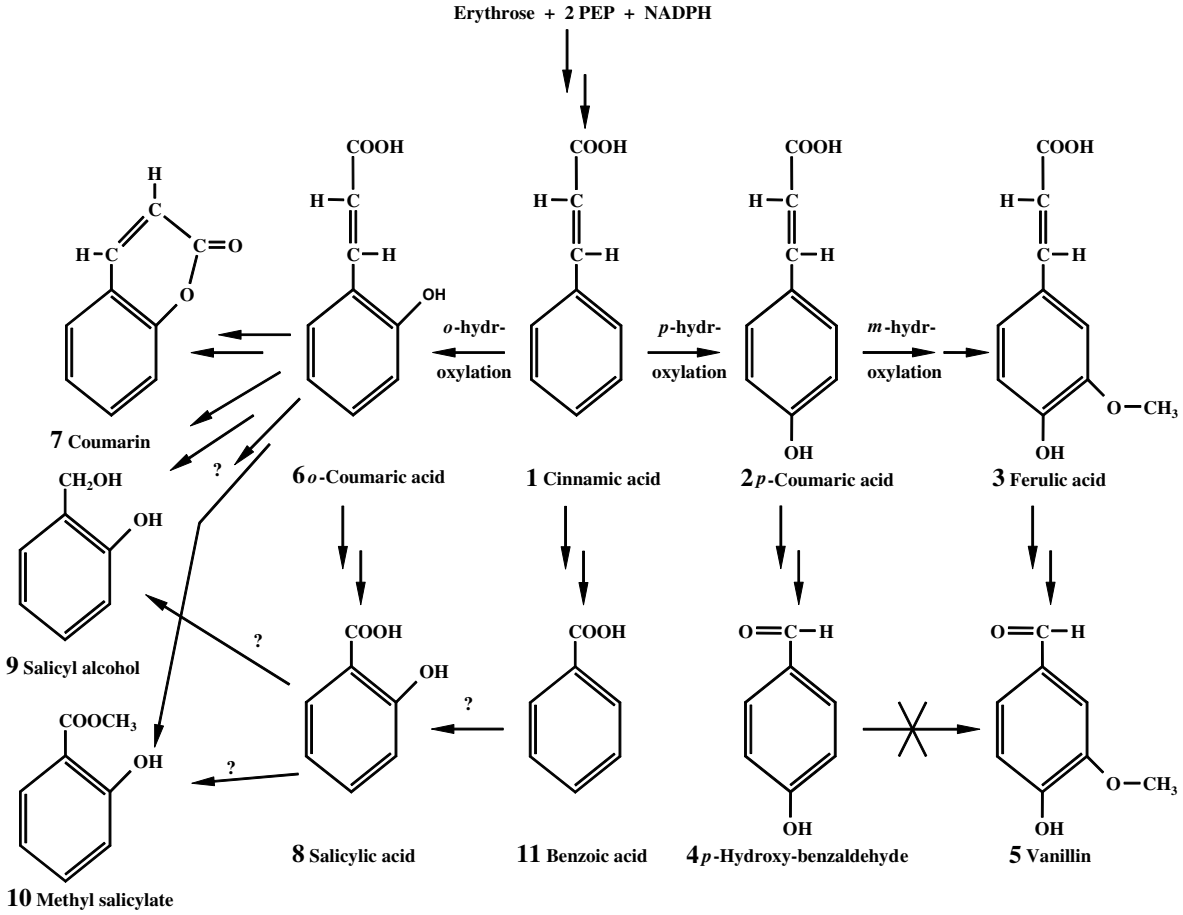

Fig. 1. Metabolic relationships of some phenylpropanoids and derivatives with *o*-, *p*- and *p*/*m*-oxygen functionalities as descendants of the common precursor cinnamic acid. For origin of the most important H-atoms in the shikimic acid pathway see Schmidt et al. (2003).

2. Results and discussion

2.1. Positional deuterium abundances in the aromatic ring of phenylpropanoids

As indicated, the isotope patterns of phenylpropanoids have exclusively been elucidated by quantitative NMR. In the present context, the ^{2}H-patterns of various compounds from different origins have to be compared. Therefore, relative isotope abundances or isotope patterns are used throughout but, where possible and informative, absolute values (ppm) will also be given. The general biosynthetic correlations between the compounds under consideration are outlined in Fig. 1. They are all derivatives of the common precursor, cinnamic acid **1**, and have all been obtained from C_3-plants.

While only one pathway is described for the biosynthesis of coumarin **7**, salicylic acid **8** and methyl salicylate **10** may be synthesised via two alternative pathways, probably depending on the plant (Richter, 1996). In all cases, however, the hydroxylations must be accompanied by the NIH-shift (from National Institutes of Health, where it was described for the first time, Daly et al., 1972), which means that the hydrogen atom to be substituted is not (completely) lost in the course of the hydroxylation but (partially) shifted to a neighbouring position, from which the original hydrogen atom is (partially) eliminated. Experiments with ^{2}H- or ^{3}H-labelled compounds prove that the degree of the label retention (preservation of the label of the substrate in the product) depends on the substrate, the enzyme and the external conditions, and can be anywhere between 0% and ~95% (Daly et al., 1972). The hydroxylation of cinnamic acid by plant enzymes is of especial importance in the present context: a retention of 84–90% for the *p*-hydroxylation of 4-^{3}H-cinnamic acid and a retention of 92% for the *o*-hydroxylation of 2,6-$^{3}H_2$-cinnamic acid were reported, whereas in the same plant (*Gaultheria*) for the *o*-hydroxylation of 2,6-$^{3}H_2$-benzoic acid a retention of only 15% has been observed (Ellis and Amrhein, 1971; Daly et al., 1972). The low retention found in salicylic acid after feeding 2,6-$^{3}H_2$-cinnamic acid has been taken as a proof that its biosynthesis passes via benzoic acid. The current availability of natural isotopic patterns from sufficient examples of *o*-hydroxylated natural compounds provides the opportunity to study the alternatives again under natural conditions and, vice versa, to explain and interpret the correlation of the natural isotopic distribution patterns with the biosyntheses.

2.2. ^{2}H-pattern of non-oxygenated phenylpropanoids from the precursors

Three of the carbon-bound hydrogen atoms in the aromatic ring of phenylpropanoids, numbered 1, 2 and 4 in Fig. 2, originate from erythrose, one is from C-3 of phosphoenolpyruvate (PEP, numbered 3′), and one (3) from NADPH. Both carbon precursors are related to glucose, and their positional ^{2}H-abundances in the aromatic ring can thus be derived from this common origin. The corresponding positional values (ppm) have been calculated from quantitative positional NMR-data by Zhang et al. (2002) for glucose; the ppm-value of position C-3′ – the average of C-1 and C-6 of glucose – is diminished by 8 ppm (6%) relative to the glucose values, taking into account the well-known depletion of the methyl group of pyruvate, as demonstrated for example in the ^{2}H-abundance of the methylene group of the side chain of phenylpropanoids originating from the same source (Schmidt et al., 2003). As the original hydrogen atom at carbon

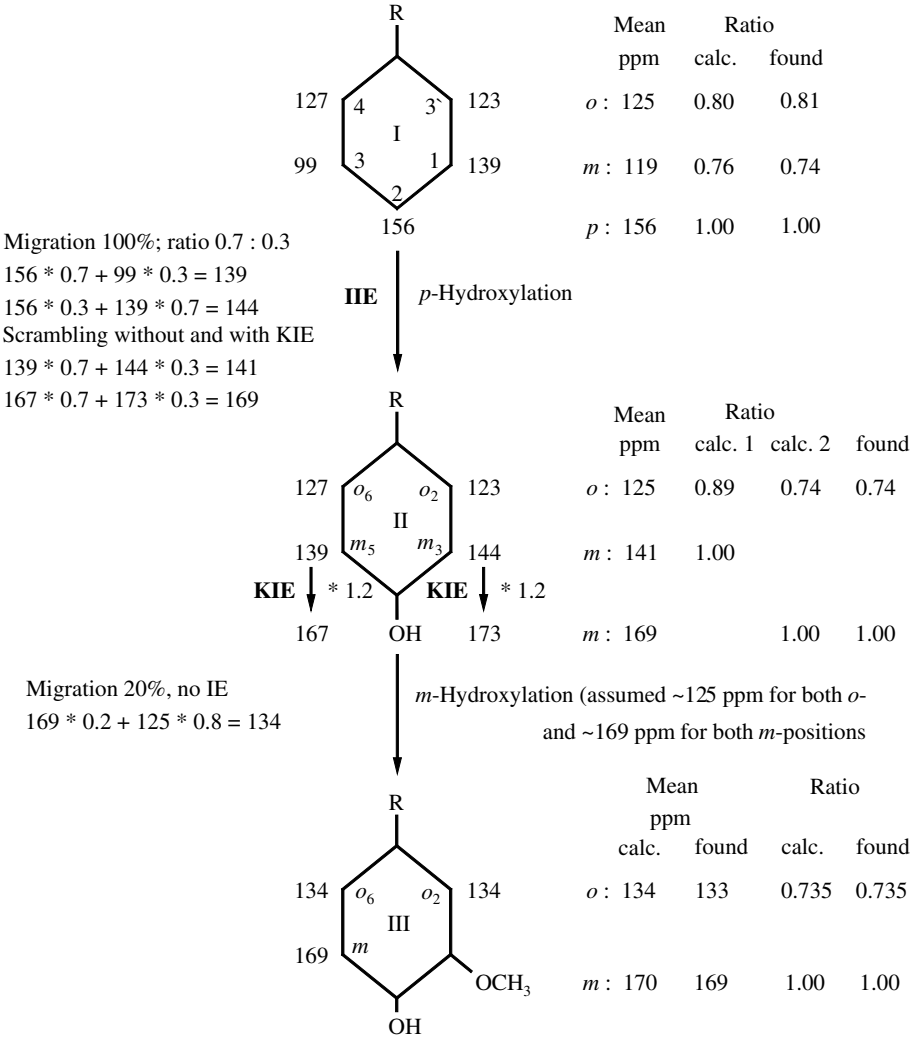

Fig. 2. Theoretical ^{2}H-patterns (overlap of relative deuterium isotopomer abundances) of the non-hydroxylated aromatic ring of phenylpropanoids, its *p*- or *p*/*m*-hydroxylated derivatives and their comparison with experimentally found patterns. Numbers in ring I (cf. Section 2.2) correlate the individual positions to those of the precursors erythrose-4-phosphate (positions 1, 2 and 4), phosphoenolpyruvate (position 3′) and NADPH (position 3). The numbers outside the ring are the average ^{2}H-abundances in ppm-values (relative standard deviations are within the range 1.3–1.7%) of these precursors as deduced from the glucose ^{2}H-pattern of C_3-plants (Zhang et al., 2002), assuming that positions 1, 2 and 4 correspond to positions C-3, C-4 and C-6 of glucose, position 3′ is C-3 of PEP, calculated from the average of glucose positions (C-1 + C-6) × 0.94, that of position 3 is from NADPH deduced from corresponding positions in fatty acids (Billault et al., 2001). The resulting mean values for the *o*:*m*:*p* positions and their ratios are compared to those of experimental average data from natural compounds (Schmidt et al., 2003). Ring II (cf. Section 2.3) demonstrates the theoretical positional ^{2}H-abundances after *p*-hydroxylation assuming a 100% migration of the *p*-H, from this a 70% to atom C-3 (m_5) and a 30% to atom C-1 (m_3). The comparison of the resulting average theoretical abundance ratio *m*:*o* = 1.00:0.89 with the experimental ratio of *p*-hydroxylated natural compounds *m*:*o* = 0.74 (Schmidt et al., 2003) suggests a deuterium isotope effect of 1.20 on the hydrogen abstraction. Ring III (cf. Section 2.5) shows the experimental and calculated isotope pattern of the aromatic ring of vanillin from a C_3-plant precursor (lignin) as an example of a compound of secondary *m*-hydroxylation. The numbers outside the ring are the average measured ^{2}H-abundances in ppm (relative standard deviations are: *o*-position, 2.0%; *m*-position, 3.5%). A migration ratio of 0.2:0.8 and a kinetic deuterium isotope effect of 1.00 are assumed on the *m*-hydroxylation. IIE, intramolecular; KIE, kinetic isotope effect.

C-3 of erythrose is replaced in the course of the biosynthesis by a hydrogen atom from NADPH, the ^{2}H-abundance in this position has been taken from H-atoms in fatty acids (Billault et al., 2001) that are inserted from the same hydrogen source, the common NADPH-pool in photosynthesising plants, wherein large differences due to kinetic isotope effects are not to be expected (Schmidt et al., 2003).

The validity of the primary ^{2}H-pattern of non-hydroxylated phenylpropanoids (the relative abundance of ^{2}H-isotopomers) as deduced in the legend to Fig. 2 (ring I), is confirmed by comparing the calculated relative positional ratios for the positions p:o:m = 1.00:0.80:0.76 with that from experimental data, 1.00:0.81:0.74 (Schmidt et al., 2003). This deduced pattern will be the basis for the subsequent verification and interpretation of the patterns found for any oxygen-substituted phenylpropanoid.

2.3. Mechanism of the NIH-shift and deduction of the ^{2}H-pattern after p-hydroxylation

The mechanisms of the enzymatic hydroxylation of aromatic compounds have been the subject of many investigations. It turns out that the site of hydroxylation and the retention of an isotopic label are dependent on the origin (e.g. plant species) of the compound, the nature of the enzyme, and the level of substitution of the substrate. A causal phenomenon is the NIH-shift, the migration of the hydrogen atom to be substituted by oxygen into a neighbouring position and its partial retention.[1] An epoxide has originally been proposed as the most probable intermediate after the attack of the active oxygen species (Daly et al., 1972; Fitzpatrick, 1994; Manitto et al., 2000), although a primary cationic intermediate has also been put forward (Mitchell et al., 2003): in either case a ketodiene is formulated as a secondary intermediate (Fig. 3).

The substrates used in all these investigations were specifically and completely deuterated and/or tritiated in defined positions, and mostly direct non-competitive turnover rate measuring methods were used for the determination of isotope effects. The kinetic deuterium isotope effects of the hydrogen abstraction from the ketodiene intermediates of these substrates are estimated under in vitro conditions to attain values up to 4–5 (corresponding to a 75–80%

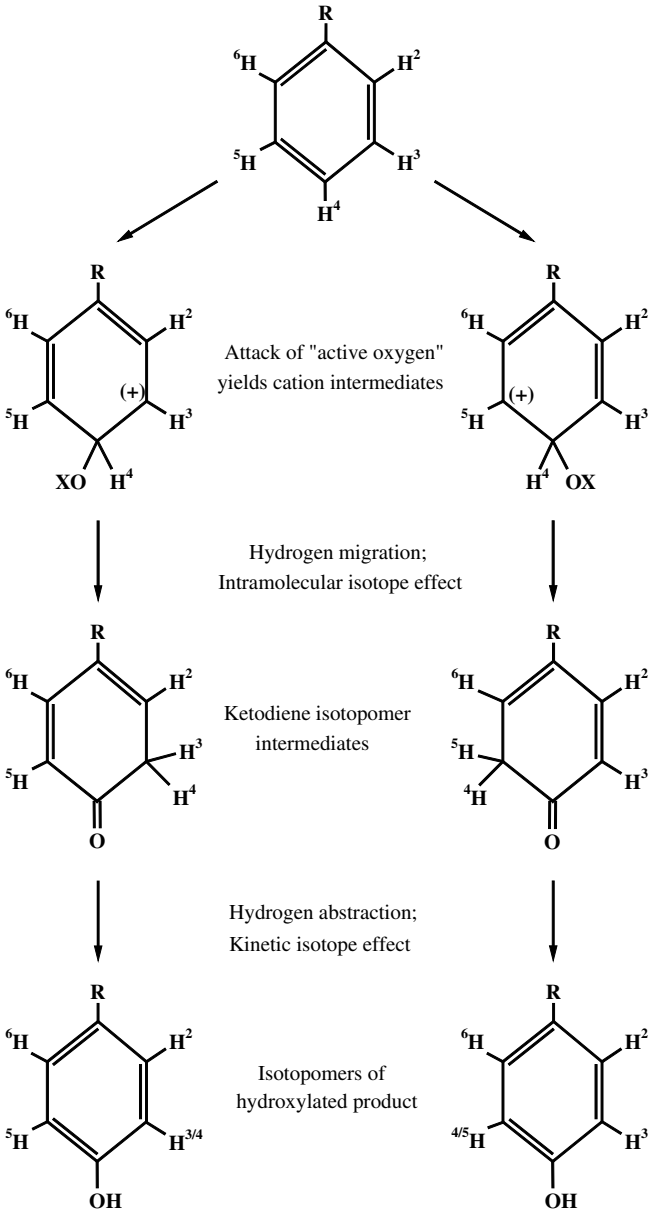

Fig. 3. Reaction mechanism of the enzyme-catalysed aromatic hydroxylation with NIH-shift. Adapted from Fitzpatrick (1994), Manitto et al. (2000) and Mitchell et al. (2003). The numbered hydrogen atoms in the individual positions represent isotopomers with defined natural deuterium abundances.

[1] The NIH-shift accompanying an aromatic hydroxylation has originally been exclusively studied with compounds, which had been completely ^{2}H-or ^{3}H-labelled, preferably in the position to be substituted by oxygen. The "retention" of the label of the position to be substituted by oxygen is its experimentally measured preservation in the product. It implies isotope effects on its "migration" to the neighbouring position (originally used synonymously for retention) and on the hydrogen abstraction from the intermediate ketodiene, the latter here called "kinetic isotope effect".

Intramolecular isotope effects are the ratios of rate constants for a reaction, in which isotopically different but normally chemically equivalent atoms or groups of the same molecule compete, e.g. the two *o*-positions of toluene or the three hydrogen atoms in the methyl group of this compound amongst each other (sometimes also these two differently positioned hydrogen atom kinds are compared). These isotope effects are often measured by the comparison of the turnover rates of a non-labelled compound and its completely (in the position in question) labelled isotopomer. This kind of measurement is called the non-competitive mode.

Intermolecular isotope effects are the ratio of turnover rates of a mixture of non-labelled and labelled molecules. They are measured by the determination of the label of substrate or product after 0% or 100%, respectively, and partial turnover; this is called a competitive mode of measurement. In the present context, the "intramolecular isotope effect" is used for the ratio of the migration to two equivalent neighbouring positions. It is here in reality pseudo-intramolecular because the molecular population of a natural abundance system is very complex and the reaction in question is simultaneously an intramolecular and an intermolecular competition. The experimental conditions are always competitive.

deuterium retention), whereas intramolecular isotope effects, measured as the relative turnover rates of differently labelled substrates, were close to unity and normal or inverse (Mitchell et al., 2003).

In contrast, the data to be interpreted in the present context are from a substrate representing a mixture of isotopomers, where those containing deuterium are at very low concentrations (mixture of isotopomers at natural abundance) and which are converted under unknown but, in any case, competitive conditions. The isotope effects deduced from the patterns of substrate and product can thus only be empirical overall practical ones, summarising the intramolecular isotope effect of an anisotropic hydrogen migration to sterically equivalent but isotopically different positions (additionally belonging to different isotopomers) and of the kinetic isotope effect of the final hydrogen abstraction.

Starting from these prerequisites and the above given general average pattern of the non-oxygenated aromatic ring, the theoretical pattern of a product after *p*-hydroxylation can be deduced. For practical reasons, a total 100% migration will be assumed during the NIH-shift. It will additionally be assumed that the two *o*-positions are not involved in the reaction, and their ^{2}H-abundances will be scrambled (mean value 125 ppm, Fig. 2) in the course of the reaction but that both *m*-positions are integrated in the NIH-shift. Furthermore, the non-statistical ^{2}H-distribution on these two *m*-positions of "naturally-labelled" phenylpropanoids will very probably cause an intramolecular isotope effect on the hydrogen shift from the ^{2}H-enriched *p*-position, with a preferred deuterium migration to the *m*-position with the lower deuterium content (faster reaction of the corresponding isotopomer), hence the highest protium content (Daly et al., 1972). Reed et al. (1973) observed a decrease of the ^{3}H-retention during the *p*-hydroxylation of 4-^{3}H-cinnamic acid by chick pea microsomes from 90–92% to 67–68%, when the *m*-positions of the substrate were substituted by deuterium. This could be due, at least partially, to an isotope effect on the migration. In the present calculations a ratio of 70%:30% will empirically be assumed for the migration of the *p*-deuterium to the two differently deuterated *m*-positions (keeping in mind that in reality the intramolecular isotope effect is a complex overlap of the inter- and intramolecular competitive reaction of several isotopomers).

On the basis of these assumptions, the ^{2}H-abundances after the migration accompanying the hydroxylation (ring II in Fig. 2) are calculated for position m_5 $(156 \times 0.7 + 99 \times 0.3) = 139$ ppm and for position m_3 $(156 \times 0.3 + 139 \times 0.7) = 144$ ppm: this leads to a mean value for the *m*-position of $(139 \times 0.7 + 144 \times 0.3) = 141$ ppm and a ratio $m{:}o = 1.00{:}0.89 = 1.12$ or $o{:}m = 0.89{:}1.00$, respectively.

However, the experimentally found *m*:*o* ratio for *p*-hydroxylated phenylpropanoids (average of the values from Table 1 of Schmidt et al. (2003) except those for *p*-hydroxybenzaldehyde and vanillin), is 1.00:0.74 (for estragole even 1.00:0.63 (Manitto et al., 2000)), suggesting an in vivo kinetic isotope effect of ~1.20 on the hydrogen abstraction. Hence, a final average ^{2}H-abundance in the *m*-position of 169 ppm results (average experimental value after lit. cited in (Schmidt et al., 2003) is 164 ppm). This is supported by the experimental value $k_1/k_2 = 1.22$ measured for the hydroxylation of ^{2}H-labelled phenylalanine by tyrosine hydroxylase (Fitzpatrick, 1994). It is noteworthy that a kinetic isotope effect on the elimination of a hydrogen atom from the proposed intermediate ketodiene has even been postulated as the sole reason for the ^{2}H-enrichment of natural estragole in the *m*-position (Manitto et al., 2000). Therefore, an estimated value $k_H/k_D = 1.20$ associated with the H-elimination in the course of the NIH-shift during the *p*-hydroxylation of natural compounds is reasonable and will be taken into account in the context of the analysis of the *o*-hydroxylation.

2.4. The NIH-shift and o-hydroxylation

The ^{2}H-pattern of the aromatic ring as the starting point for the deduction of the ^{2}H-patterns of products of *o*-hydroxylation must be identical to that used previously (Fig. 2). The assumptions are again that the total migration is 100% and that the reaction will not affect either the *p*- or the second *o*-position (which are not directly involved). As the two *o*-positions have nearly the same ^{2}H-abundance, a statistical orientation of the hydroxylation is to be expected, as the primary attack of the activated oxygen species will probably also be statistically determined. In contrast, for estimating the effect of the NIH-shift, the differences in the deuterium concentration at the two *m*-positions must be taken into consideration. Hence, as before, we have to expect a preference for the more depleted site for the migration, and again assume a ratio 70%:30% for the hydroxylation of the alternative *o*-positions of the molecule.

The expected values in ppm are calculated as before (Fig. 4). Again, we have to average the equivalent positions of the two resulting isotopomer molecules and we have to multiply the obtained abundance values in the *m*-position by the estimated isotope effect of 1.20 for the hydrogen abstraction. The resulting theoretical *o*-hydroxylated molecule predicts a sequence of the ^{2}H-abundance of the product $p > m_3 > m_5 \geqslant o$, meeting quite satisfactorily the data reported for the samples of natural methyl salicylate **10** from wintergreen ($p > m_3 > m_5 > o$) and from birch bark oil samples ($p > m_3 > m_5 \approx o$) (Le Grand et al., 2005).

This agreement of the ^{2}H-concentration sequence is further underlined by the following details. As expected, in any of the natural samples analysed, the *p*-position has the highest and the *o*-position has the lowest deuterium content. In the case of the methyl salicylate **10** samples from *Betula lenta* L. (Le Grand et al., 2005), even the absolute values (152 and 122.6 ppm, ratio 0.81) are practically identical to the predicted ones (156 and 124 ppm, ratio 0.80). The corresponding ratio for methyl salicylate **10**

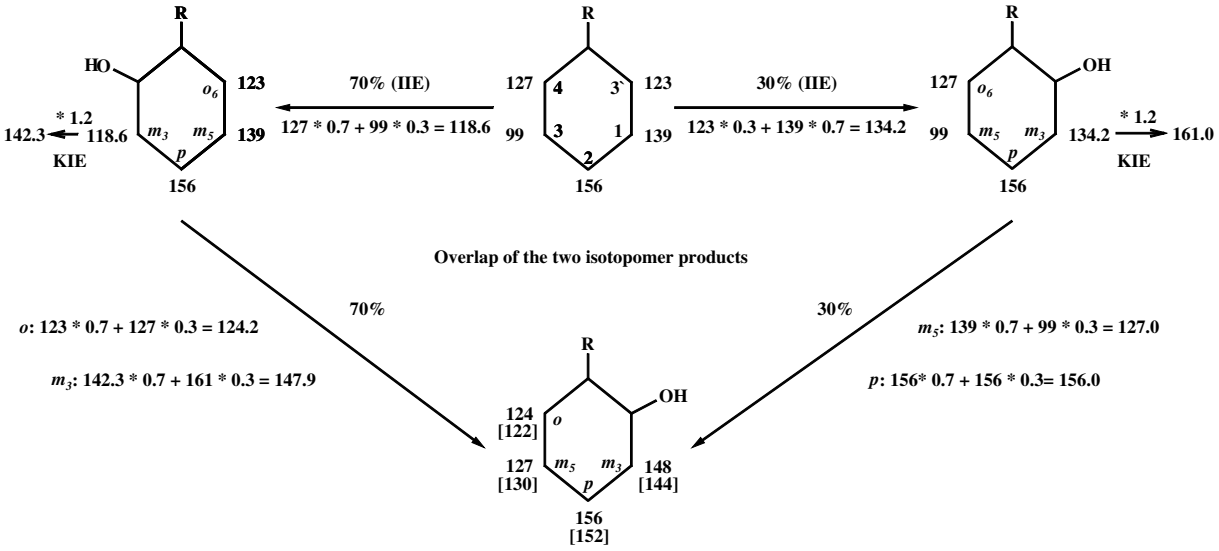

Fig. 4. Deduction of the theoretical ^{2}H-pattern of the aromatic ring of *o*-hydroxylated phenylpropanoids. Origin of positions and relative positional ^{2}H-abundances are as previously derived (Fig. 2). It is again assumed that the substitution by the hydroxyl group and the H-migration are preferably orientated towards the molecule site with the lower ^{2}H-abundance (position 3, intramolecular isotope effect corresponding to a faster reaction of the corresponding isotopomer). A 70%:30% migration and a kinetic isotope effect on the hydrogen elimination of 1.20 are taken as the basis of the calculations. The overlap of the resulting isotopomers yields a pattern which is within 4 ppm identical to that of the experimental average values of 9 samples of natural methyl salicylate from wintergreen oil (numbers in [ ] after (Le Grand et al., 2005)), and the theoretical sequence of the abundances meets satisfactorily that of the other available natural compounds.

from wintergreen oil (Le Grand et al., 2005) is 0.76 and for two natural salicyl alcohol **9** samples (Brenna et al., 2004) are 0.75 and 0.79, respectively. All calculated positional values for the four positions of methyl salicylate **10** agree within 4 ppm with the average values of 9 samples from wintergreen oil (Fig. 4). Hence it is shown that the above prerequisites and proposed effects on ^{2}H-distribution lead to theoretical values that are close to those experimentally determined. The small differences between predicted and experimental values are not greater than those among the natural samples from different origins, a variability probably due to small differences in the patterns of the precursors, the ratio of orientation of the hydrogen migration (intramolecular isotope effect or competition between isotopomers) and the isotope effect on the hydrogen elimination.

The latter could especially be true for the data for salicyl alcohol **9** ($p > m_3 \approx m_5 > o$) (Brenna et al., 2004), since, as discussed above, alternative routes for biosynthesis via benzoic and salicylic acid (Daly et al., 1972), imply only about 15% retention (Fig. 1). However, a corresponding calculation taking this retention rate into consideration and assuming other migration ratios, does not lead to a satisfactory result. Therefore, in this case "the effect (of the NIH-shift) on the aromatic deuterium distribution of the *o*-hydroxylation in phenylpropanoids is [still] a blind spot" (Brenna et al., 2004) and further explanations must be sought.

At first glance the pattern of the aromatic ring of coumarin **7** (Brenna et al., 2005) does not fit into the proposed scheme either. The sequence of the deuterium abundance in the aromatic ring as indicated by the authors ($m_3 > p > m_5 \approx o$) looks completely different from that found for the compounds discussed above. An initial approach to overcome this problem concerned the ^{2}H-abundances of the precursor, cinnamic acid. In the *p*- and the not-substituted *o*-positions it should be identical to those of the product coumarin. As a matter of fact, the ratio of the experimental ^{2}H-abundances in these positions ($o{:}p = 131{:}162 = 0.809$) is identical to that of methyl salicylate ($o{:}p = 122{:}152 = 0.803$). However, the absolute values of these positions are, in coumarin, 7% higher than in methyl salicylate ($131{:}122 = 1.074$ and $162{:}152 = 1.066$, respectively; average value 1.07). Hence, one can presume that the precursor of the coumarin sample was generally enriched by 7% in ^{2}H relative to that of methyl salicylate (most probably due to climatic reasons). In order to take, at least partially, this enrichment into account, the ppm-values in any position of the "general precursor" were multiplied by an "enrichment factor" 1.035 (half of 7%) in order to obtain a suitable coumarin precursor (Fig. 5).

Even so, a calculation as before with a 70%:30% migration ratio in both directions and the overlap of the two resulting isotopomers yields a coumarin **7** with a theoretical pattern that is far from that experimentally determined: varying the migration ratios did not alter this situation. However, the rather large experimental ^{2}H-abundance at the m_3-position ($(D/H)_3 = 171$ ppm) suggests another explanation: a larger kinetic isotope effect. This possibility is also suggested by the fact that – as in other cases – the average ^{2}H-abundance of the aromatic ring of the (natural) hydroxylated product exceeds that of the (theoretical) non-hydroxylated precursor. Further support is provided by the difference of the kinetic ^{18}O-isotope effects on the hydroxylation reactions: whereas the kinetic isotope effect on the

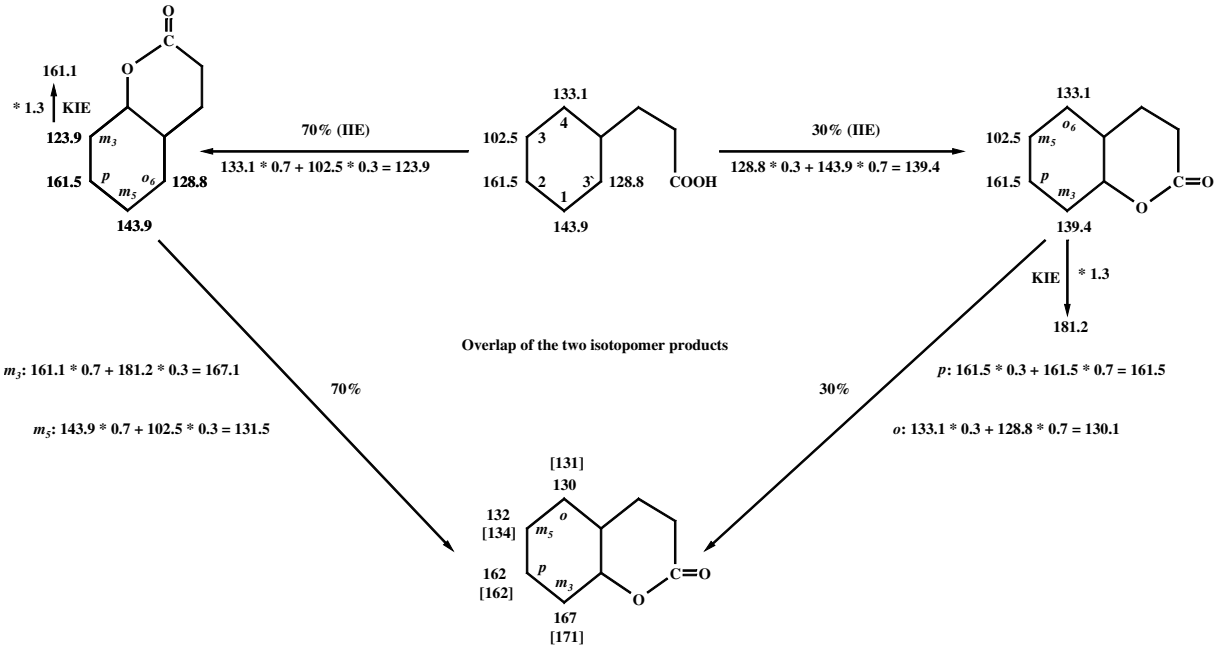

Fig. 5. Deduction of the theoretical ^{2}H-pattern of the aromatic ring of natural coumarin. As the absolute ^{2}H-abundances of the natural compound in the *p*- and *o*-position are slightly higher than those of the theoretical model, all values of the non-hydroxylated precursor have been adapted by multiplication with an "enrichment factor" of 1.035. The relative orientation of the hydroxylation and the calculations are treated as before, with the exception that a kinetic isotope effect of 1.30 is assumed for the hydrogen abstraction. The resulting pattern is very similar to the experimental data from (Brenna et al., 2005, values in [ ]) and the positional sequence identical to that deduced from these values.

p-hydroxylation of vanillin – as calculated from the reported δ^{18}O-value (Fronza et al., 2001) – is 1.018, it is 1.023 for the *o*-hydroxylation of coumarin **7** (Brenna et al., 2005). If a deuterium kinetic isotope effect of 1.30 is used to make the calculation in Fig. 5, then the resulting theoretical pattern is in quite good agreement with that obtained experimentally. The calculations, as demonstrated in Fig. 5, have also been made on the basis of a 7% and 0% ^{2}H-enrichment, respectively, and on other isotope effects, but the results were less satisfactory. In any event, the results may indicate *o*-hydroxylation of some substrates to be more sensitive to deuterium substitution than is the *p*-hydroxylation.

2.5. Expectations and results on the m-hydroxylation

So far, no data on the deuterium pattern of *m*-hydroxylated natural plant products are available. It appears that in plants *m*-hydroxylation is preferably a secondary *p*- or *o*-hydroxylation relative to an already-introduced primary hydroxyl group. However, *m*-hydroxylations of deuterated mono- and disubstituted aromatic substances by hepatic monooxygenases are mostly accompanied by small deuterium retentions. So, for example, the retention rate for the *m*-hydroxylation of 5-^{2}H-salicylic acid is 0% (Daly et al., 1972). From a calculation according to the above scheme assuming a total migration of 30% (equal to both sides) and a kinetic isotope effect of 1.05, an abundance sequence $p > o_2 > o_6 > m$ for an *o*-hydroxylated product is predicted, which has to be confirmed or rejected by experimental data.

Vanillin **5** can be considered as a product of the secondary *m*-hydroxylation of a *p*-hydroxylated precursor, although the exact details of the sequence of methoxylation are still disputed (Funk and Brodelius, 1992). In *Vanilla* beans, vanillin is accompanied by *p*-hydroxybenzaldehyde **4**. Whereas the ^{2}H-patterns of the aromatic rings of both these compounds are absolutely identical, the ^{2}H-abundance at their carbonyl groups is completely different (Remaud et al., 1997; Schmidt et al., 2003; John and Jamin, 2004). Therefore *p*-hydroxybenzaldehyde **4** cannot be the precursor of vanillin **5**, but vanillin **5** must rather be synthesised from *p*-coumaric acid **2** via ferulic acid **3**, respectively, chain shortening being subsequent to *m*-hydroxylation. (The proposal that 3,4-dimethoxycinnamic acid is an intermediate (Funk and Brodelius, 1992) is now considered improbable).

Vanillin from C_3-plant lignin is certainly derived from ferulic acid, and for reasons of comparability only the deuterium pattern of this compound (Remaud et al., 1997) will be considered in the present context. The reported absolute ^{2}H-abundance in the *m*-position of the aromatic ring (169 ppm) is identical to that as expected for *p*-hydroxylated compounds from C_3-plants (169 ppm, see above). Furthermore, the corresponding *m*:*o* ratio (1.00:0.79) is quite similar to that of these plants (1.00:0.74). As the experimental ^{2}H-abundance of the *o*-position (133 ppm) is only slightly higher than the corresponding average value of *p*-hydroxylated precursor compounds (125 ppm, see Fig. 2), it can be concluded that the hydroxylation of *p*-coumaric acid at the *m*-position proceeds with only a small ^{2}H-migration and/or a small isotope effect on the H-abstraction. As

indicated above, this conclusion is both in line with general experience on *m*-hydroxylation, especially of disubstituted benzene derivatives (small retention rate, Daly et al., 1972), and with the fact that vanillin and *p*-hydroxybenzaldehyde from *Vanilla* have absolutely identical ^{2}H-abundances in the *m*- and *o*-positions. Reasons for the lack of an NIH-shift in this hydroxylation cannot be given but it may be possible that the involved enzyme introduces oxygen by another mechanism, e.g. a direct mode (Hanzlik et al., 1984; Mitchell et al., 2003).

2.6. Carbon isotope pattern of the aromatic ring of vanillin deduced from precursors

Vanillin **5** is also the first compound for which the complete ^{13}C-pattern has been elucidated by quantitative ^{13}C NMR (Tenailleau et al., 2004). As in the case of the deuterium patterns, a comparison with the δ-values of precursors only makes sense for the carbon atoms within the aromatic ring because the side chain has been subjected to secondary changes, which might have been accompanied by isotope effects. The origin of the carbon atoms is as indicated above and their δ^{13}C-values have been deduced from the pattern of glucose (Roßmann et al., 1991), as previously described.

Fig. 6 shows a comparison of data for vanillin **5** from different origins with that as deduced from the ^{13}C-pattern of C_3-glucose. Except for position 1 (C-2′ of PEP) of the CAM-product, for which a sugar reference is not available, the coincidence of the phenylpropanoid ring from C_3- and C_4-plant origin with that as expected from glucose is evident, whereas the synthetic product shows a more or less statistical ^{13}C-distribution. The question remains as to why the average δ^{13}C-values for both natural sources are so negative in comparison with that of C_3-glucose, even if the well-known ^{13}C-depletion of the methoxy group is taken into account; from a biochemical point of view they are to be expected nearer 28‰ and 15‰, respectively.

3. Conclusions

The ^{2}H- and the ^{13}C-patterns of the aromatic ring of phenylpropanoids can be primarily deduced from that of its precursors in the shikimic acid pathway. Any secondary changes in the course of hydroxylations can be explained on the basis of the stereoselectivity of the enzymes involved and the mechanism of the catalysed reactions, notably the NIH-shift and the implied isotope effects. This means that the first approach of the active oxygen species probably invokes an oxygen but not a hydrogen isotope effect, that the migration of the substituted hydrogen atom is accompanied by an intramolecular hydrogen isotope effect with a preference for the hydrogen to shift to the more ^{2}H-depleted neighbouring position, and that the final hydrogen abstraction prefers protium, leading to a kinetic isotope effect. As the mechanism of the monooxygenase reaction must be identical for the *p*- and the *o*-hydroxylation, but possibly not for the *m*-hydroxylation, small differences in the ^{2}H-patterns of individual aromatic compounds can be assigned to a combination of factors: variation of the primary pattern of the aromatic ring; the

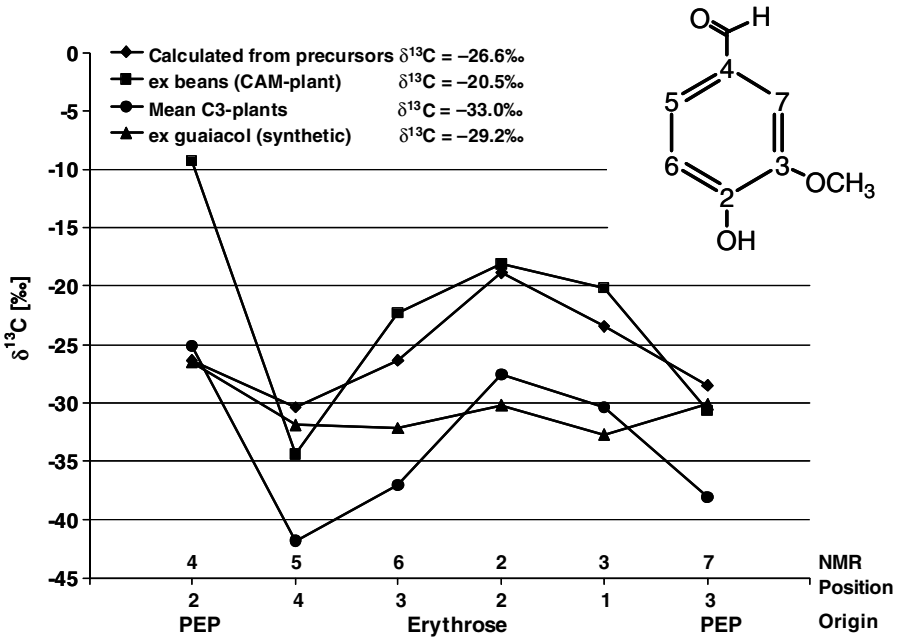

Fig. 6. Comparison of the ^{13}C-pattern of vanillin from C_3- and CAM-plant origin and from synthesis ex-guaiacol as measured by quantitative ^{13}C NMR (Tenailleau et al., 2004) and as calculated from the precursors and their correlation to glucose (after Roßmann et al., 1991). Experimental data are expressed as specific isotopic deviations (δ‰) and relative standard deviations vary between 0.05% and 0.5%. In the abscissa, the positions of the origin of the C-atoms and of their positions in the NMR-spectra are given. With the exception of position 4 of the product from *Vanilla* beans, all experimental data coincide with biosynthetic predictions, whereas the data for the synthetic product shows a more or less statistical distribution.

non-isotropic orientation and the extent of the hydrogen migration (intramolecular isotope effect); the kinetic ^{2}H-isotope effect on the hydrogen elimination from the proposed ketodiene intermediate of the reaction (estimated in vivo isotope effect = 1.2–1.3). This proposal is supported by the existence of corresponding small differences in the kinetic ^{18}O-isotope effects on the monooxygenase reaction. For example, the δ^{18}O-value of the *p*-hydroxyl group of vanillin **5** is +5.8‰ (Fronza et al., 2001), corresponding to $k_{16}/k_{18} \approx 1.018$, whereas that of the *o*-hydroxyl group of coumarin is +0.8‰ (Brenna et al., 2005), corresponding to $k_{16}/k_{18} \approx 1.023$.

Therefore, it is apparent that the characteristics of the NIH-shift is the dominant influence on the formation of the ^{2}H-patterns associated with *p*-hydroxylation (Schmidt et al., 2001), *o*-hydroxylation and probably *m*-hydroxylation by monooxygenases acting on natural aromatic compounds from the shikimic acid pathway. Understanding the fundamental principles of these correlations should make it possible to distinguish between natural and synthetic products on a sound theoretical scientific basis.

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