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Differentiation of Natural and Synthetic Phenylalanine and Tyrosine through Natural Abundance ^{2}H Nuclear Magnetic Resonance

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The natural abundance deuterium NMR characterization of samples of the amino acids tyrosine (**1**) and phenylalanine (**2**), examined as the acetylated methyl esters **4** and **6**, has been performed with the aim to identify by these means the contribution in animals of the hydroxylation of the diet L-phenylalanine (**2**) to the formation of L-tyrosine (**1**), a feature previously revealed on the same samples through the determination of the phenolic δ^{18}O values. The study, which includes also the NMR examination of benzoic acid (**5**) from **2** and of tyrosol (**7**) from **1**, substantially fails in providing the required information because the mode of deuterium labeling of tyrosine samples of different origins is quite similar but indicates a dramatic difference in the deuterium labeling pattern of the two amino acids **1** and **2**. The most relevant variation is with regard to the deuterium enrichments at the CH_2 and CH positions, which are inverted in the two amino acids of natural derivation. Moreover, whereas the diastereotopic benzylic hydrogen atoms of L-tyrosine (**1**) appear to be equally deuterium enriched, in L-phenylalanine (**2**) the $(D/H)_{3R} > (D/H)_{3S}$. Similarly, benzoic acid (**5**) shows separate signals for the aromatic deuterium nuclei, which are quite indicative of the natural or synthetic derivation. The mode of deuterium labeling of the side chain of **1** and **2** is tentatively correlated to the different origins of the two amino acids, natural from animal sources for L-tyrosine and biotechnological probably from genetically modified microorganisms for L-phenylalanine.

KEYWORDS: Tyrosine; phenylalanine; tyrosol; animal vegetal origin; aspartame; natural abundance deuterium NMR

INTRODUCTION

Adulteration of foodstuff is a matter of concern. Among the analytical methods developed up to now to address this issue, the determination of the stable isotope composition of relevant food components seems to be particularly effective (*1*, *2*). We have recently reported a study designed to define the animal rather than plant origin of the amino acid L-tyrosine (**1**) through the determination of the δ^{18}O values of the phenolic oxygen atom (*3*). The premise of this method is the existence in nature of two pathways regulating the formation of L-tyrosine (**1**), characterized by different sources for the phenolic oxygen, that is, leaf water versus molecular oxygen (*4*). The key advanced intermediate in the sequence starting from shikimic acid and phosphoenolpyruvate is arogenic acid (**3**), which provides both L-tyrosine (**1**) and L-phenylalanine (**2**) (**Figure 1**). L-Tyrosine (**1**) is obtained from **3** by loss of carbon dioxide and two hydrogen atoms. Conversely, L-phenylalanine (**2**) is formed when carbon dioxide is lost together with a molecule of water.

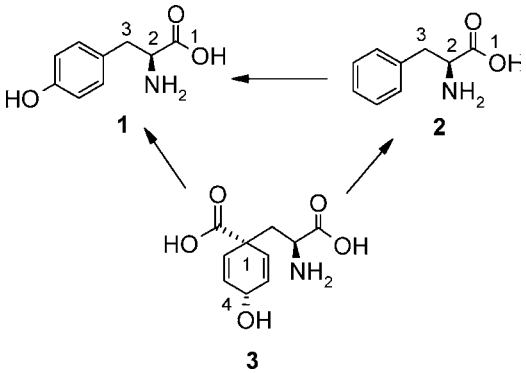

Figure 1. Biosynthetic pathways for the formation of tyrosine (**1**) and phenylalanine (**2**) from arogenic acid (**3**).

However, animals, for which L-tyrosine is not essential, are able to directly hydroxylate **2** to **1** (**Figure 1**), the extent of this activity depending upon the content of **1** in the diet (*5*). The δ^{18}O values of the phenolic oxygen of tyrosine and derivatives suggest that L-tyrosine from animals is a mixture of two

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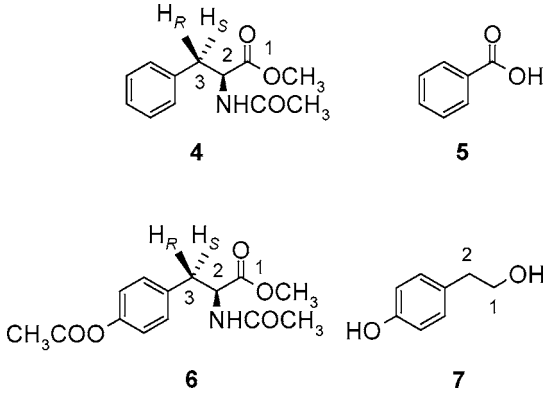

Figure 2. Products submitted to natural abundance ^{2}H NMR measurement.

materials of different origins, having, as a characteristic fingerprint, quite distinct δ^{18}O values. One of these is that of plant origin, acquired through the diet, directly formed from arogenic acid (**3**) and retaining as its phenolic oxygen atom that originally present in position 4 of shikimic acid; the other is that produced in the body from L-phenylalanine (**2**) of the diet by hydroxylation, thus incorporating atmospheric oxygen.

The diagnostic potential of site-specific deuterium distribution data obtained by natural abundance ^{2}H NMR measurements in the definition of the origin of food components is well-known (*2*). This technique was applied in previous studies to the characterization of several amino acids, showing that wide variations of deuterium content occur in samples of different origins (*6*, *7*). We now present a ^{2}H NMR study of L-tyrosine (**1**) and L-phenylalanine (**2**) performed with the aim to identify the relative proportions of the material directly derived from arogenic acid and that produced via phenylalanine by hydroxylation in tyrosine samples extracted from animal sources.

MATERIALS AND METHODS

Samples. A total of 31 samples were examined. These included eight phenylalanine samples [sample 1, commercial L-phenylalanine certified as natural; sample 2, D,L-phenylalanine from Fluka (Milano, Italy); sample 3, L-phenylalanine of undefined origin; sample 4, D,L-phenylalanine from Aldrich (Milano, Italy); sample 5, L-phenylalanine from aspartame from HSC; sample 6, l-phenylalanine from aspartame from Miwon; sample 7, L-phenylalanine from aspartame from Ajinomoto; sample 8, L-phenylalanine from aspartame from Caremoli (all aspartame samples were gifts from Perfetti, Lainate, Italy)], eight benzoic acid samples (samples 1−8) produced by oxidation of phenylalanine samples 1−8, nine tyrosine samples [sample 1, D,L-tyrosine from Merck (VWR International, Milano, Italy); sample 2, L-tyrosine from hen feathers; sample 3, D,L-tyrosine from Fluka (Milano, Italy); sample 4, L-tyrosine from Fluka (Milano, Italy); sample 5, L-tyrosine from Fluka (different production batch, Milano, Italy); samples 6−8, L-tyrosine from human hair (three different batches); sample 9, L-tyrosine of unspecified animal origin], and six tyrosol samples [sample 1, tyrosol from *Ligustrum ovalifolium*; samples 2−4 and 6, tyrosol obtained from tyrosine samples 1, 5, 2, and 6, respectively; sample 5, tyrosol from L-tyrosine from Aldrich (Milano, Italy)].

Solubility problems prevented the direct acquisition of the NMR spectra of the two amino acids as such. Accordingly, **1** and **2** were converted through reported procedures into the corresponding highly soluble acetylated methyl esters **6** and **4**, respectively (**Figure 2**).

***N*-Acetylphenylalanine Methyl Ester 4.** In a typical experiment (*8*), phenylalanine (3 g; 0.018 mol) was added at 0 °C in portions under stirring to methanol (100 mL) to which had previously been added $SOCl_2$ (8 mL). The mixture was kept at room temperature for 24 h and then evaporated to dryness under vacuum. The residue was taken up in methanol and evaporated, repeating the operation three times. The solid residue was suspended in CH_2Cl_2 (100 mL) and treated under stirring at 0 °C with acetic anhydride (10 g; 0.098 mol), followed by pyridine (15 g; 0.189 mol). After 24 h at room temperature, the reaction mixture was poured onto crushed ice containing $NaHCO_3$ (5 g; 0.059 mol). The organic phase was separated, and the aqueous phase was extracted with CH_2Cl_2 (100 mL × 2). The combined organic extract, washed twice with cold 5% HCl (100 mL), saturated aqueous $NaHCO_3$, and water (100 mL each), was dried (Na_2SO_4) and evaporated. The residue was crystallized from hexane−ethyl acetate to give the desired *N*-acetylphenylalanine methyl ester **4** (3 g; 75%): ^{1}H NMR (CH_2Cl_2) δ 7.30−7.14 (5H, m, phenyl), 6.63 (1H, s br, NH), 4.79 (1H, m, CH-2), 3.68 (3H, s, OCH_3), 3.13 (1H, dd, J = 5.9 and 13.6 Hz, H_{3R}), 3.02 (1H, dd, J = 7.1 and 13.6 Hz, H_{3S}), 1.92 (3H, s, $COCH_3$). L-Phenylalanine samples from aspartame (α-L-aspartyl-L-phenylalanine methyl ester) were obtained as follows: aspartame (9.8 g; 0.033 mol) was refluxed for 6 h with 150 mL of 20% aqueous NaOH. The cooled solution was brought to pH 5−6 with 20% HCl. The precipitated L-phenylalanine was collected by suction and repeatedly washed on the filter with water (4.5 g; 83%).

Benzoic Acid (5) from Phenylalanine (2). The oxidation of phenylalanine samples was performed in alkaline aqueous solution with 3% potassium permanganate as previously reported for phenylacetic acid (*9*) (yeld ∼90%): ^{1}H NMR of **5** (DMSO) δ 7.92 (2H, m, H_{ortho}), 7.53 (1H, m, H_{para}), 7.42 (2H, m, H_{meta}).

***N*,*O*-Diacetyltyrosine Methyl Ester (6).** The transformation of tyrosine **1** into **6** was performed as reported for the preparation of **4** from **2** (yield = 84%): ^{1}H NMR of **6** (CH_3CN) δ 7.20 (2H, d, J = 8.9 Hz, H_{ortho}), 7.00 (2H, d, J = 8.9 Hz, H_{meta}), 6.74 (1H, s br, NH), 4.62 (1H, td, J = 5.9 and 7.7 Hz, CH-2), 3.64 (3H, s, OCH_3), 3.09 (1H, dd, J = 5.9 and 13.6 Hz, H_{3R}), 2.95 (1H, dd, J = 7.7 and 13.6 Hz, H_{3S}), 2.21 (3H, s, $COCH_3$), 1.84 (3H, s, $COCH_3$).

Baker's Yeast Conversion of Tyrosine (1) into Tyrosol (7). To a mixture made up with Baker's yeast (Distillerie Italiane, San Pancrazio, Italy) (2 kg) and D-glucose (200 g), in tap water (3 L), under stirring at ∼30 °C was added within 3 h a solution of tyrosine **1** (3 g; 0.016 mol) (6 g in the case of the D,L compound], in tap water (600 mL), treated with enough 10% aqueous NaOH to obtain complete dissolution. After 48 h of stirring, acetone (2 L) was added, and the mixture was filtered by suction through a large Büchner funnel using a Celite pad. The cake was repeatedly washed with acetone. The filtrate was concentrated under vacuum to ∼1 L volume. The aqueous phase was extracted with ethyl acetate (300 mL × 3). The residue obtained upon evaporation of the solvent was chromatographed on a silica column with increasing amounts of ethyl acetate in hexane, tyrosol (**7**) (45%) eluting with a ∼1:1 mixture, which was crystallized from hexane−ethyl acetate (*3*): ^{1}H NMR of **7** (acetone) δ 7.98 (1H, s br, OH), 6.95 (2H, d, J = 8.4 Hz, H_{ortho}), 6.64 (2H, d, J = 8.4 Hz, H_{meta}), 3.60 (2H, t, J = 7.1 Hz, CH_2OH), 2.62 (2H, t, J = 7.1 Hz, CH_2).

NMR Experiments. The ^{2}H NMR experiments were performed on a Bruker Avance 500 spectrometer equipped with a 10 mm probehead and a ^{19}F lock (C_6F_6) channel, under CPD (Waltz 16 sequence) proton decoupling conditions. The spectra were recorded at 298 K for phenylalanine and tyrosine derivatives **4** and **6** and at 328 K for the acids **5** and **7**. 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 0.4−0.7 g of material in 2.5−3.0 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 CH_2Cl_2 for **4**, CH_3CN for **6**, DMSO for **5**, and acetone for **7**.

At least five spectra were run for each sample, collecting 4000−8000 scans to reach an S/N > 100 (methyl signals) and using the following parameters: 6.8 s acquisition time, 1200 Hz spectral width, and 16K memory size. 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 NMR software using a Lorentzian line shape.

Internal isotopic ratios R_{ij} are defined as

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

where S_i is the area of the ith site, S_j is the area of a reference peak,

and n_j is the number of isochronous hydrogens at the j site. A statistical distribution of deuterium among the n molecular sites would originate R_{ij} factors equal to the number of hydrogens of the corresponding ith sites.

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

$$(D/H)_i = n_{WS} g_{WS} (MW)_L S_i (D/H)_{WS} / (n_i g_L (MW_{WS}) S_{WS}) \quad (2)$$

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 ith 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; and S_i and S_{WS} are the areas of the ith peak and of the standard, respectively.

^{13}C Isotopic Mass Determination. The mass spectrometry determination of the $^{13}C/^{12}C$ isotope ratios (IRMS) was determined using a Sira II-VG Fisons (Rodano, Italy) mass spectrometer interfaced with a Carlo Erba (Rodano, Italy) NA 1500 elemental analyzer for sample combustion. The measurements are expressed in per mil (‰) versus an international standard (the carbonate Pee Dee Belemnite, PDB) according to the equation

$$\delta^{13}C\ (‰) = (R_{sample}/R_{standard} - 1) \times 10^3 \quad (3)$$

where R is the $^{13}C/^{12}C$ ratio.

RESULTS AND DISCUSSION

Origin of the Samples Examined. Concerning the origin of the amino acids, it has to be pointed out that tyrosine (**1**) is rather difficult to obtain by chemical synthesis. Accordingly, its world demand (100 tons/year) is apparently completely satisfied by the product obtained by acid hydrolysis of keratin-rich materials of animal origin. Currently, human hairs and hen feathers constitute the sources of tyrosine-rich proteins, as a substitute for those previously obtained from other animals. The commercially available D,L-form is manufactured by racemization of the L-enantiomer. In the study on the origin of tyrosine (**1**) based on the determination of the $\delta^{18}O$ values of the phenolic oxygen, a sample of tyrosol (**7**) (**Figure 2**) extracted from *Ligustrum ovalifolium* was also included. This material is a direct metabolite of **1**, and in the above context it formally represents the equivalent of an authentic sample of "plant" tyrosine. To compare the ^2H NMR properties of the samples of tyrosine (**1**) here examined with those of plant origin **7**, selected samples of **1** were converted into tyrosol (**7**) by fermentation with baker's yeast. The biotransformation, which implies decarboxylation and deamination of **1** with the introduction of one or two new hydrogen atoms in position 1 of **7**, depending on the mechanism of the reaction (*10*, *11*), leaves the atoms in position 2 untouched. However, the deuterium enrichments at position 1 of the samples of tyrosol (**7**) generated from **1** were not considered in the present study as an element of differentiation.

Quite different from that of tyrosine (**1**) is the manufacturing source of L-phenylalanine (**2**). Before the advent of aspartame (α-L-aspartyl-L-phenylalanine methyl ester) as an artificial sweetener, **2** was required by the market in amounts similar to those of tyrosine **1**. The L-material was obtained by extraction from protein sources or by enzymic hydrolysis of *N*-acyl derivatives of the racemic form produced by chemical synthesis using benzaldehyde as a source of the C-6−C-1 part of the molecule. The sudden requirement for L-phenylalanine (**2**) (~3000 tons in 1995) for the manufacture of aspartame necessarily required a modification of the manufacturing process, which is now mainly based on microbial production with genetically modified organisms using carbohydrates as the carbon source.

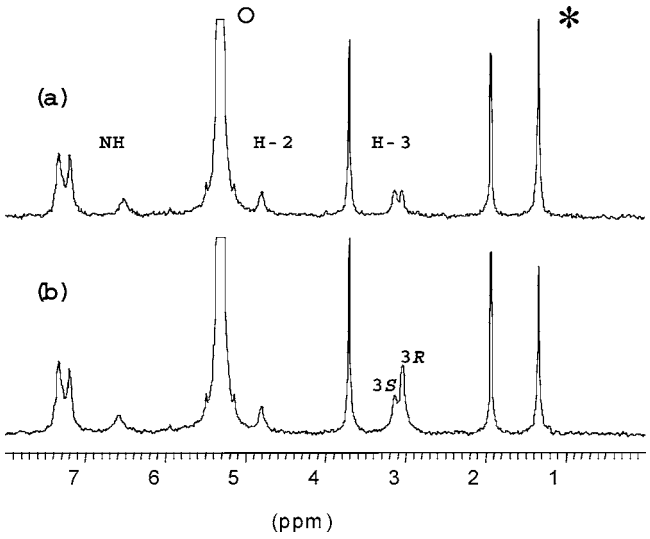

Figure 3. Natural abundance ^2H NMR spectra of phenylalanine derivative **4**: (a) commercial sample 2; (b) natural sample 1; (*) signal of calibrated *tert*-butyl disulfide used as internal standard; (○) CH_2Cl_2 solvent signal.

Benzoic Acids 5. The ^2H NMR spectrum of the phenylalanine derivatives **4** provides an unresolved signal for the aromatic nuclei (**Figure 3**). Accordingly, the phenylalanine samples were converted into the corresponding benzoic acids **5**, which show much more dispersed signals allowing the differences occurring in the aromatic part of the molecule to be revealed.

The properties of the deuterium isotopic distribution for the three different positions (i.e., ortho, meta, and para) of the phenyl ring present in natural compounds are well-known from previous studies carried out on important food flavors such as phenylacetic acid (*9*) and natural benzaldehyde (*12*−*14*). It was shown that for such natural material the isotopic enrichment follows the trend para > ortho > meta. In contrast, samples of synthetic origin show very similar deuterium content in all positions. Such different behavior can be shown also by using appropriate indicators such as the ratio between the molar fraction at the ortho position and the sum of the molar fractions at the meta and para positions $[f_{ortho}/(f_{meta} + f_{para})]$ (*12*) or the internal isotopic ratios R_{ij} (*2*). In **Table 1** are reported the (D/H) values, the ratio of the molar fractions and the internal $R_{meta/para}$ ratio for the examined samples 1−8 of benzoic acids obtained by degradation of the corresponding phenylalanines. As expected, the synthetic samples 2 and 4 show quite similar deuterium contents in the three positions; the indicators $f_{ortho}/(f_{meta} + f_{para})$ and $R_{meta/para}$ display values near 0.667 and 2, respectively, characteristic of a statistical distribution of deuterium nuclei among the five positions of the phenyl ring. Very similar results were obtained previously for synthetic benzoic acids and benzaldehydes (*12*, *13*).

All other samples in **Table 1** show deuterium contents following the trend para > ortho > meta, except for sample **1** in which the ortho and meta positions are nearly equally populated. The indicators $f_{ortho}/(f_{meta} + f_{para})$ and $R_{meta/para}$ are very different with respect to those of synthetic samples. Very marked changes are shown by the ratio $R_{meta/para}$ ranging from 1.2 to 1.58. The ratio $f_{ortho}/(f_{meta} + f_{para})$ display values in the range of 0.60−0.65 (less than the statistical value) for samples 1, 3, 5, and 7, or ~0.72 (greater than the statistical value) for samples 6 and 8. Interestingly, similar values were found also

Table 1. Origin, $(D/H)_i$ Isotopic Ratios (ppm), Deuterium Molar Fraction Ratios, Deuterium Internal Isotopic Ratios, and $\delta^{13}C$ (‰) Global Isotope Ratios of Benzoic Acids **5**[a] from Phenylalanine (**2**)

sample	origin	$(D/H)_{total}$	$(D/H)_{ortho}$	$(D/H)_{para}$	$(D/H)_{meta}$	$f_o/(f_m + f_p)$	$R_{m/p}$	$\delta^{13}C$ (‰)
1	natural (certified)	141.8	132.6	169.1	134.3	0.61	1.59	–25.4
2	D,L Fluka	137.4	139.2	130.6	139.1	0.68	2.13	–27.3
3	anonymous	143.0	141.0	162.8	134.0	0.65	1.65	–26.3
4	D,L Aldrich	137.8	138.9	128.8	141.2	0.67	2.19	–29.4
5	aspartame HSC	128.3	125.4	153.7	119.1	0.64	1.55	–26.7
6	aspartame Miwon	134.1	141.5	175.4	106.1	0.73	1.21	–13.8
7	aspartame Ajinomoto	150.1	144.4	180.2	140.9	0.62	1.56	–25.8
8	aspartame Caremoli	129.1	134.3	152.3	112.3	0.71	1.47	–12.4

[a] Mean calculated standard deviation of $(D/H)_i$ values is 2.7.

Table 2. Origin and $(D/H)_i$ Isotopic Ratios (ppm) of Phenylalanine Derivatives 4[a]

sample	origin	$(D/H)_{Ph}$	$(D/H)_2$	$(D/H)_3$	$(D/H)_{H-3S}$	$(D/H)_{H-3R}$	$(D/H)_{total}$[b]
1	natural (certified)	144.0	128.0	283.0	167.4	374.8	185.0
2	D,L Fluka	135.0	98.5	118.0	114.7	113.9	117.2
3	anonymous	145.0	75.0	323.0	254.4	393.5	181.0
4	D,L Aldrich	130.7	102.5	90.6	88.7	92.6	107.9
5	aspartame HSC	134.8	127.5	352.7	326.3	391.3	205.0
6	aspartame Miwon	141.1	118.4	141.2	119.7	159.3	133.6
7	aspartame Ajinomoto	148.1	110.6	118.0	112.8	127.4	125.6
8	aspartame Caremoli	136.0	122.1	131.1	105.0	142.4	129.7

[a] Mean calculated standard deviation of $(D/H)_i$ values is 2.5. [b] $(D/H)_{total}$ values are calculated from the individual positional deuterium contents.

for natural benzaldehyde samples obtained from bitter almond oil (0.58) and from cassia oil (0.75) (*12*).

The data in **Table 1** allow the synthetic or natural origin of the examined benzoic acids and consequently also the origin of the starting phenylalanines to be assigned with confidence. In particular, the values of the benzoic acids 5−8 suggest that the phenylalanines used for the synthesis of the corresponding aspartame samples are of natural derivation.

Phenylalanine. The $(D/H)_i$ values of the eight phenylalanine samples examined as derivatives **4** are reported in **Table 2**. It is noteworthy that the $(D/H)_{Ph}$ ratios are very similar to those of the derived benzoic acids, definitely proving that the chemical degradation did not alter substantially the deuterium enrichment of the target molecule. Inspection of **Table 2** highlights as the main element of differentiation the deuterium content at position 3 of the CH_2CH moiety, well exemplified in the ^{2}H NMR spectra of samples 1 and 2 (**Figure 3**) obtained from the natural and racemic commercial **1**. Samples 1, 3, and 5 form a group characterized by an extremely high deuterium enrichment at C-3 with $(D/H)_3$ values in the range of 283−351 ppm. The other samples show more modest $(D/H)_3$ ratios going from 90.6 ppm of sample 4 to 141.2 ppm of sample 6. It may be of interest in this context to examine the global (D/H) values for the reported samples, which span from 107.9 to 205 ppm (**Table 2**). For natural aromatic amino acids derived from C-3 plants the global (D/H) ratios should occur at ∼140 ppm (*15*). In our case, apart the racemic synthetic samples 2 and 4, which show quite low global enrichments of 117.2 and 107.9 ppm, respectively, all other samples are of natural derivation as established on the basis of isotopic analysis of the derived benzoic acids discussed above. However, only samples 6−8 show global (D/H) in the range of 125−133 ppm near the values characteristic of natural samples. Samples 1, 3, and 5 are peculiar, showing very high $(D/H)_{total}$ (181−205 ppm) as a direct consequence of the abnormal deuterium content at position 3. Nevertheless, other examples are reported to show particularly high $(D/H)_{total}$. In a study carried out on the amino acids alanine, aspartic acid, glutamic acid, proline, and lysine a very wide distribution of the global (D/H) ratios determined by the IRMS technique was found (*7*). Among them in particular several samples of alanine and proline displayed global enrichments in the range 165−180 ppm, not too far from the values found here for phenylalanine samples 1, 3, and 5.

In addition the methylene nuclei of the phenylalanine framework are diastereotopic and give rise to two separate NMR signals, which show strong relative intensity variations (**Table 2** and **Figure 3**). The resonances can be assigned on the basis of the vicinal coupling constants with the proton in position 2. It was shown for derivative **4** dissolved in acetonitrile that the signal at low field (3.08 ppm, $J_{vic} = 5.9$ Hz) belongs to the *pro-S* hydrogen, whereas the signal at high field (2.93 ppm, $J_{vic} = 8.3$ Hz) is due to the *pro-R* hydrogen (*16*). The assignment can be easily transferred to the spectra taken in other solvents. The $(D/H)_{H-3R}$ and $(D/H)_{H-3S}$ values, reported in **Table 2**, are substantially identical for the synthetic samples 2 and 4, whereas for the other samples $(D/H)_{H-3S}$ is always smaller than $(D/H)_{H-3R}$, such a difference being dramatic for the natural sample 1 (167.4 vs 374.8 ppm, 69% of the 3*R*-isotopomer). Imbalances of the deuterium enrichment at the diastereotopic positions of the methylene group, although not as strong as in the present case, were observed previously for aspartic and glutamic acids (*6*, *7*). Because such differences were detected only for natural samples the method was proposed as a tool for recognizing, in an absolute and simple way, the biological origin of a compound (*6*). By applying this criterion of naturalness to phenylalanine samples 1−8, clearly samples 2 and 4 are confirmed to be synthetic and all other samples are natural, in agreement with the conclusion based on the isotopic analysis of the benzoic acids.

Considering together the data relative to derivatives **4** and **5**, obtained from the eight samples of phenylalanine (**Tables 1** and **2**), it is possible to make a representation of the main differences of phenylalanine samples combined with those present in the derived benzoic acids by a multivariate data analysis (*17*). Starting from the $(D/H)_i$ values of benzoic acid and phenylalanine, it is observed that two principal components C_1 and C_2 explain 84.1% of the overall variance. Thus, **Figure 4**, relative to the principal component analysis (PCA), indicates

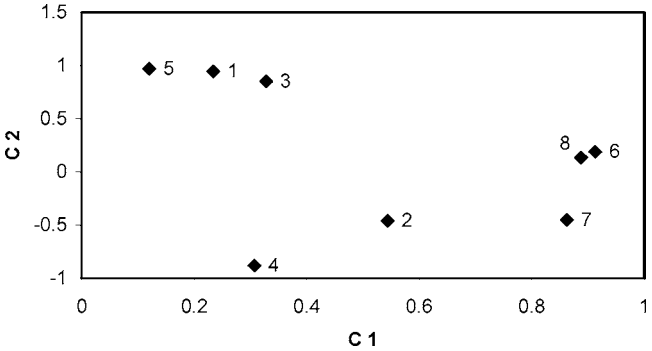

Figure 4. Graphical representation of the distribution of samples 1–8 of phenylalanine derivative **4** and benzoic acid (**5**) by PCA. Principal components C1 and C2 explain >80% of the overall variance. Samples can be divided into three groups: natural 1 (samples 1, 3, and 5), natural 2 (samples 6–8), and synthetic (samples 2 and 4).

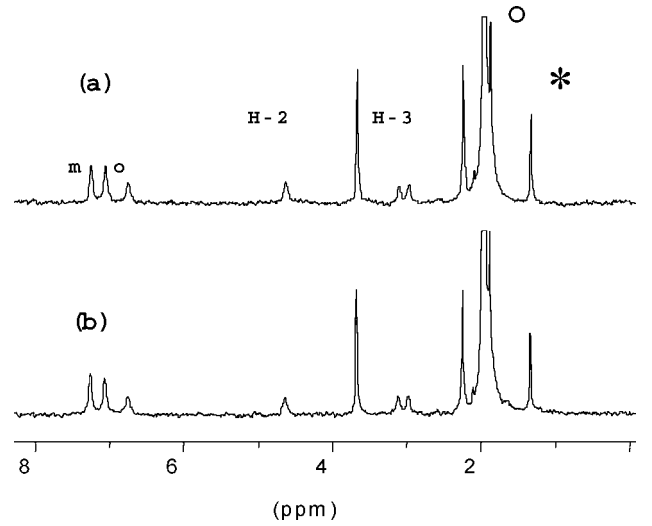

Figure 5. Natural abundance ^{2}H NMR spectra of tyrosine derivative **6**: (a) commercial sample 1; (b) natural sample 2 (hen feathers); (o) signal of nuclei ortho to the side chain; (m) signal of nuclei meta to the side chain; (∗) signal of calibrated *tert*-butyl disulfide used as internal standard; (○) CH_3CN solvent signal.

the presence of a group of three samples (5, 1, and 3, named "natural 1"), quite distinct from a second group (samples 6−8, named "natural 2"), and, finally, a third set (samples 2 and 4). The first two groups are certainly of natural origin due to the fingerprint of naturalness shown by the labeling pattern of benzoic acid. Benzoic acid produced by oxidation of the components of the third group shows the typical statistical labeling pattern of products of petrochemical origin. An additional element of differentiation of the latter material from the products of natural origin is the equal deuterium content at positions 3*R* and 3*S* of the phenylalanine framework.

Tyrosine and Tyrosols. Figure 5 shows the NMR spectra of derivative **6** of samples 1 and 2 (racemic material from Merck and the L-isomer from hen feathers) of tyrosine (**1**), whereas in **Table 3** are reported the $(D/H)_i$ (ppm) values relative to the nine examined samples. A direct comparison of the data of **Tables 3** and **2**, relative to the derivatives of tyrosine (**1**) and phenylalanine (**2**), shows that the values greatly differentiating the two amino acids are those of the $CHCH_2$ moiety, that is, the $(D/H)_3$ and $(D/H)_2$ ratios. The $(D/H)_2$ values for tyrosine (**1**) are, in general, higher than those of phenylalanine (**2**) (119.0−195.6 vs 75.0−128.8). Significantly high is the value 195.6 ppm of sample 1 (D,L-tyrosine from Merck), possibly due to the fact that racemic tyrosine is prepared from the L-enantiomer via azlactone, which is hydrolyzed with aqueous acid causing the introduction at position 2 of a hydrogen atom from water in substitution of that originally present in the parent L-amino acid (*18*). Conversely, the $(D/H)_3$ values of **1** do not show the extremely strong variations observed for **2**. In addition, all tyrosine samples display practically equal deuterium contents at the diastereotopic positions 3*R* and 3*S*, a fact that is quite puzzling because **1** presumably is obtained from natural sources, that is, keratin-rich animal material. This implies that the amino acid has been synthesized directly by plants or partially by animal hydroxylation of plant-synthesized phenylalanine (*3*), both following the shikimate pathway. Due to the probable natural derivation of the samples a more or less evident imbalance of $(D/H)_{3R}$ and $(D/H)_{3S}$ values should be expected, a fact that does not occur. Moreover, the global $(D/H)_{total}$ values of C-3 plant products from the shikimate pathway should range from 125 to 145 ppm (*15*). The global values of **1** reported in **Table 3** are well within the predicted range for samples 1, 3, 5, and 9 (∼130 ppm), whereas for the other samples they are somewhat lower than expected (102.6−119.7 ppm). In any case they cannot be used as an element of differentiation among the samples.

From the data in **Table 3** it appears that the nine samples of tyrosine (**1**) are poorly differentiable also on the basis of their site-specific deuterium content. The (D/H) values of the aromatic hydrogen atoms of samples 2 (hen feathers), 3 (D,L Fluka), 5 (L Fluka), and 9 (animal origin) are quite similar and, in general, higher than those of the remaining samples. The three batches of tyrosine (**1**) from human hairs (samples 6−8) are characterized by similar low values of the aromatic moiety and by side-chain values consistent with those of the other materials. In addition, it should be noted that the majority of tyrosine samples follow the trend $(D/H)_{ortho} > (D/H)_{meta}$ (where meta and ortho are defined with respect to the side chain), although generally the reverse occurs for para-hydroxylated natural compounds (*15*). Possibly this behavior may be due to the fact that tyrosine is produced from protein hydrolysates in strong acidic conditions. It is reported that in these conditions the tyrosine meta hydrogen atoms exchange with those of water (*19*). We have verified that when a sample of the tyrosine is left in acidic D_2O/H_2O medium, the deuterium spectrum shows a strong enhancement of the meta signal (ortho to the OH group), whereas all other signals remain unchanged. Thus, the deuterium content of the tyrosine meta position reflects the ^{2}H abundances of hydrolysis water medium rather than the result of the metabolic pathway. For this reason the tyrosine samples, although certainly of natural origin, cannot be compared easily with other natural substances sharing the common derivation from **3**.

In **Table 4** are included the $(D/H)_i$ values relative to tyrosol (**7**) of plant extraction (sample 1) and to the five samples produced in baker's yeast from tyrosine (**1**) (samples 2−4 and 6 from samples 1, 5, 2, and 6 of tyrosine; sample 5 from L-tyrosine from Aldrich). Because during the bioconversion new hydrogen atoms are introduced in position 1 of **7** (*10*), the (D/H) values of the biogenerated CH_2OH moiety and consequently the global (D/H) ratios were not taken into account as an element of comparison with respect to the parent tyrosine samples. On the contrary, the aromatic and the benzylic portions of tyrosine do not undergo variations during the biotransformation.

As expected the deuterium distribution of tyrosol samples 2−6 is in agreement with that of the tyrosine from which they derive. Consequently, tyrosols such as tyrosines are poorly differentiable. Apart from the deuterium content at position 1

Table 3. Origin and $(D/H)_i$ Isotopic Ratios (ppm) of Tyrosine Derivative Samples 6[a]

sample	origin	$(D/H)_{ortho}$[b]	$(D/H)_{meta}$[b]	$(D/H)_2$	$(D/H)_3$	$(D/H)_{total}$[c]
1	D,L Merck	104.4	106.3	195.6	108.7	128.7
2	hen feathers	115.2	106.4	118.9	108.1	112.1
3	D,L Fluka	128.4	124.1	151.1	119.6	130.8
4	Fluka (old)	108.1	93.9	135.3	111.4	112.2
5	L Fluka	134.4	126.0	150.8	109.2	130.1
6	hair (primary sample)	97.9	92.6	134.2	115.9	110.1
7	hair (primary sample)	103.4	96.9	159.0	119.8	119.8
8	hair (primary sample)	96.7	79.1	125.7	108.9	102.6
9	unknown animal sample	134.4	126.8	136.9	116.9	128.7

[a] Mean calculated standard deviation of $(D/H)_i$ values is 2.5. [b] Positions ortho and meta refer to the side chain. [c] $(D/H)_{total}$ values are calculated from the individual positional deuterium contents.

Table 4. Origin and $(D/H)_i$ Isotopic Ratios (ppm) of Tyrosol Samples 7[a]

sample	origin	$(D/H)_{ortho}$[b]	$(D/H)_{meta}$[b]	$(D/H)_2$	$(D/H)_1$
1	ligustrum	100.0	103.0	94.1	95.1
2	Merck	98.8	98.3	107.6	71.0
3	Fluka	106.7	102.1	102.0	54.7
4	hen feathers	116.4	109.8	114.6	62.2
5	Aldrich	111.9	103.7	100.8	61.7
6	hair	97.8	106.6	110.0	47.5

[a] Mean calculated standard deviation of $(D/H)_i$ values is 2.9. Tyrosol samples 2–4 and 6 were obtained from tyrosine samples 1, 5, 2, and 6, respectively, and sample 5 was from L-tyrosine from Aldrich. [b] Positions ortho and meta refer to the side chain.

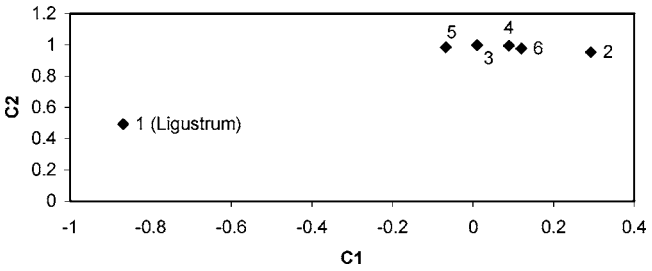

Figure 6. Discrimination of tyrosols through PCA. Principal components C1 and C2 explain >84% of the overall variance.

the deuterium pattern of plant tyrosol **7** (sample 1) is quite similar to that of the other samples. This observation corroborates the hypothesis that the commercial tyrosine is produced entirely from natural sources (animal keratin). Considering as variables the site-specific deuterium enrichments of all tyrosol samples including that of the CH_2 in position 1, the graphical representation of **Figure 6** can be drawn, where the two principal components C1 and C2 account for 84.1% of the overall variance. The graph shows two well-separated regions, the first containing the isolated sample of "plant" tyrosol from *Ligustrum* and the second the other samples grouped together.

Thus, the deuterium isotope analysis is not able to differentiate between tyrosine and tyrosol samples of different origins. On the contrary, for the phenolic oxygen, the same samples show quite distinct $\delta^{18}O$ values: +26.0 and +31.5‰, respectively, for samples 1 and 5, obtained from the parent amino acid of plant origin [tyrosine directly derived from arogenic acid (**3**)] and +18.9 and +17.8‰, respectively, for samples 4 and 6 of animal origin, indicative of the presence in the samples, close to plant tyrosine (**1**) taken up by the animal with the diet, of ca. 46 and 51%, respectively, of the same chemical species, formed in the body by hydroxylation of phenylalanine (**2**) (*3*).

The basic aim of this study was the search for a fingerprint of the formation pathway of tyrosine samples of animal derivation through a comparison of the deuterium labeling pattern of samples of **1** and **2** of different origins. This primary goal has not been achieved because among the nine examined tyrosine samples no differences in the natural abundance deuterium pattern that might be indicative of different origins were observed. However, the mode of deuterium labeling of the tyrosine and phenylalanine samples shows interesting features that require some comments. The main differences between L-tyrosine (**1**) and L-phenylalanine (**2**) regard the deuterium enrichment at the CH_2 and CH groups of the side chain. First of all, the $(D/H)_2$ values are smaller than the $(D/H)_3$ ratios for **1**, whereas they are greater for **2**. Second, the deuterium enrichments at the two diastereotopic positions of the benzylic methylene group are identical for L-tyrosine (**1**) samples but different for L-phenylalanine (**2**) samples, which show $(D/H)_{3R} > (D/H)_{3S}$. Tentatively, an explanation for this phenomenon can be proposed by considering the biosyntheses of **1** and **2** in light of the recent observations on the mode of deuterium labeling pattern of D-glucose and D-fructose in different plants (*20*). Indeed, Martin et al. (*20*) have elegantly demonstrated that hexoses produced through the three photosynthetic pathways C3, C4, and CAM differ in their deuterium distributions. An important observable difference regards the deuterium enrichment at positions 6*R* and 6*S* of D-glucose. $(D/H)_{6R}$ and $(D/H)_{6S}$ are practically identical for D-glucose from C3 plants, whereas in the case of C4 plants $(D/H)_{6R}$ is greater than $(D/H)_{6S}$ with differences in the range of 10−15 ppm. The reverse is true for CAM plants.

It is well-known (*4*) that in the biosynthesis of **1** and **2** the benzylic CH_2 group incorporates both of the methylene hydrogens at positions 1 and 6 of fructose-1,6-diphosphate, which is formed from glucose at the beginning of the glycolysis process, with conservation of the stereochemical information associated with the biosynthetic mechanism. Thus, the substantial differences here observed in the deuterium labeling of benzylic position in L-tyrosine (**1**) and L-phenylalanine (**2**) might possibly be consequences of the mode of labeling of the parent sugars. That is, the examined L-tyrosine (**1**), in which $(D/H)_{3R} = (D/H)_{3S}$, can seemingly derive from C3 plants, the glucose of which is equally labeled at position 6. Conversely, L-phenylalanine (**2**) isolated from commercial aspartame and obtained on the market can possibly be produced by fermentation processes supplemented with cane sugar molasses and/or corn syrup, which represent the cheapest carbon source for microorganisms. Both sugar cane and maize are C4 plants, shown to provide D-glucose (*20*) in which the deuterium signals $(D/H)_{3R} > (D/H)_{3S}$.

The interpretation above may be reasonable for L-tyrosine samples that are obtained from animal keratin derived originally with high probability from C3 plants. In contrast, the theory is

rather weak as an explanation for the behavior of L-phenylalanine samples because the observed differences of the deuterium population at the methylene group are much stronger than that reported for the hypothesized starting sugar material. To gain more information about this point, the values of $\delta^{13}C$ of the benzoic acids 1−8 obtained by degradation of the corresponding phenylalanine samples have been measured (**Table 1**). The material deriving from C3 plants is expected to have $\delta^{13}C$ of ∼26−30‰, whereas that from C4 plants should have $\delta^{13}C$ of 10−13‰ (*21*). The ^{13}C isotopic ratios in **Table 1** show that only the benzoic acid samples 6 and 8 from the aspartame Miwon (−13.8‰) and aspartame Caremoli (−12.4‰) originate from C4 plants, whereas all remaining samples must derive with certainty from C3 plant material (values from −25.4 to −29.4‰). Thus, the correlation of the asymmetry of the deuterium population at the methylene group of L-phenylalanine (**2**) with that of the starting sugar material fails. Most probably such a strong difference is due to drastic biotechnological effects connected to the kind of microorganism used in the fermentation processes.

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