Natural Abundance ^{2}H Nuclear Magnetic Resonance Study of the Origin of *n*-Hexanol

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The site-specific natural abundance deuterium distribution of hexanol (**1**) obtained through a variety of methods has been determined through ^{2}H NMR spectroscopy. This study, actually performed on the acetate ester **3**, provides a means of distinguishing between "natural" materials isolated from natural sources or obtained by *Colletotrichum gloeosporioides* mediated reduction of natural hexanoic acid and other products of synthetic origin or produced from hexanoic acid by $LiAlH_4$ reduction. Particularly significant were the variations of the molar fraction values at position 1 with respect to positions 3, 4, and 5.

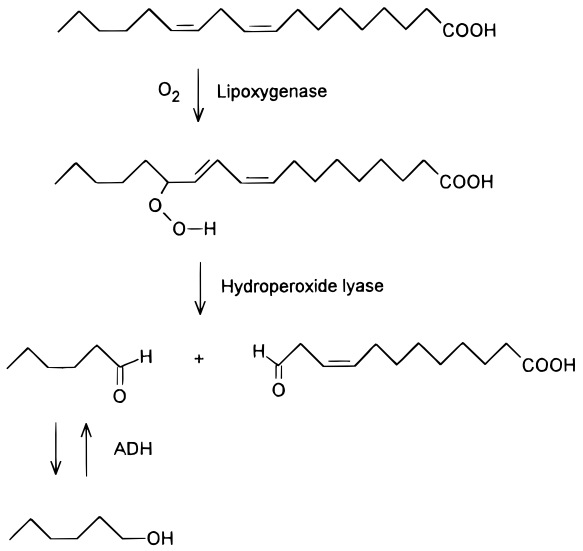
Keywords: *SNIF-NMR; aromas; n-hexanol; authentication*

Hexanol (**1**) and a whole set of its esters are industrially used as "key" ingredients in several fruit flavors. The first report on the occurrence of free and esterified hexanol (**1**) in plant extracts goes back to the beginning of phytochemistry (Frachimont and Zincke, 1872). In green plants, hexanol (**1**) biosynthetically derives by reduction of hexanal formed, in turn, by fragmentation of the carbon skeleton of linoleic acid 13-hydroperoxide (Scheme 1) (Hatanaka, 1993). *cis*-3-Hexenol (**2**) similarly arises from linolenic acid. Products **1**, **2**, *trans*-2-hexenol, and the corresponding aldehydes, due to their nature as "injury" products, the formation of which is particularly accelerated in plants when the damaged tissues are submitted to increased oxygenation, are present in a number of green plants but in minute amounts. Accordingly, the large request by the flavor industry for the above C_6 "green" notes has been traditionally met by chemical synthesis from starting materials of petrochemical origin (Hatanaka, 1993), as occurs for the majority of the flavor chemicals (Sell, 1988).

However, the recent advent of legislative discrimination (*U.S. Code Fed. Regul.*, 1985) between chemically identical food aroma constituents of synthetic origin and those derived from natural sources and the preference of the consumers for products labeled "natural" renewed the industry's interest in the recovery of flavor materials from natural sources. When this approach is unsatisfactory due to the extreme dilution of the principle(s) in the botanical sources, the production of precious flavor components is often accomplished from readily available natural precursors through enzymic procedures. Indeed this is the case, for example, of the above-mentioned C_6 "green" notes (Muller and Gautier, 1994), accessible from abundant linoleic and linolenic acids, respectively, through the intervention of vegetal and microbial enzymes.

A major problem faced in the dual market of the natural and natural-identical flavor components is the manner in which one can guarantee the "naturalness" of the product. Among the several criteria proposed (Fuganti et al., 1993), the determination of the site-specific deuterium distribution data is one of the most effective (Martin et al., 1982, 1983, 1986; Grant et al., 1982; Toulemonde et al., 1983; Hagedorn, 1992; Fronza et al., 1993, 1995a,b). This procedure (SNIF-NMR, Laboratories Eurofins, Nantes, France) has been recently shown (Muller and Gautier, 1994) to be effective in distinguishing between the three studied forms of *cis*-3-hexenol (**2**) obtained by (i) chemical synthesis, (ii) extraction from natural sources, and (iii) biogeneration in three steps from natural linolenic acid by lipoxygenase-mediated oxidation.

Scheme 1. Steps in the Biosynthesis of Hexanol (1) from Linoleic Acid



In this context, we present now the results of a SNIF-NMR study on the origin of hexanol (**1**). We became interested in the problem of the differentiation of the origin of hexanol after the recent observation (Fronza, et al., 1995a) of the ability of the fungus *Colletotrichum gloeosporioides* CBS 193.82 to aerobically reduce to the corresponding alcohols a whole set of carboxylic acids, including hexanoic acid. When the biotransformation

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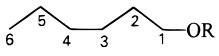
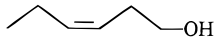


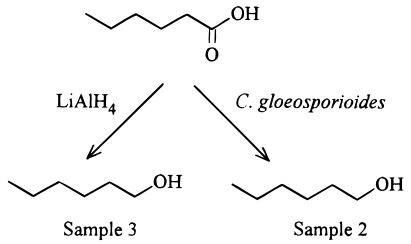
(**1**) R = H
(**3**) R = $COCH_3$



(**2**)

is applied to natural hexanoic acid, the yield of hexanol (**1**) reaches 2 g/L after 24 h of incubation. The material obtained in this way is *natural* by any means and legislation. We therefore desired to perform a comparison of the mode of labeling of **1** obtained from the natural C_6 precursor by carboxyl group bioreduction with that of different commercial samples of natural and synthetic hexanol and with that of the product obtained from natural haxanoic acid upon $LiAlH_4$ reduction (Scheme 2). For experimental simplification, the measurements were performed on the acetate ester (**3**), obtained from the carbinol **1** according to standard procedure by the action of acetic anhydride in pyridine.

Scheme 2. Steps in the Conversion of Hexanoic Acid into Hexanol of Samples 2 and 3



EXPERIMENTAL PROCEDURES

Deuterium NMR data (46.076 MHz) were recorded at 302 K on a Bruker AC300 spectrometer equipped with a process controller, a 10 mm selective deuterium probehead, and a ^{19}F lock channel, under broad-band proton decoupling conditions.

Hexyl acetate samples were prepared by carefully weighing pure (>97% GC) hexyl acetate (about 3 g) and hexafluorobenzene for ^{19}F lock (150 mg, Merck), with tetramethylurea (TMU) as internal (D/H) standard (400 mg, Fluka). TMU was previously tested by isotope ratio mass spectrometry (IRMS) and showed an averaged (D/H) value of 133.45 ppm. NMR measurement of a sample of ethanol using alternatively Fluka TMU and official TMU (Community Bureau of References, BCR EC010, (D/H) = 136.67 ppm) gave for Fluka TMU values consistent with the IRMS ones.

Ten spectra were run for each sample by collecting 1024 scans and using the following parameters: 6.8 s acquisition time; 0.05 s relaxation delay; 1200 Hz spectral width; 16K memory size; 15 μs (90°) pulse length. Each FID was Fourier transformed with no zero filling (0.15 Hz/point digital resolution) and line broadening of 2 Hz, manually phased, and integrated. S/N was >180 (methyl peak of the hexyl group).

Molar fractions f_i were calculated from the integrated areas.

$$f_i = S_i/\sum_i S_i \quad (1)$$

S_i is the area of the ith peak. The corresponding statistical molar fractions are

$$f_i = n_i/\sum_i n_i \quad (2)$$

where n_i is the number of equivalent or isochronous deuterium nuclei of the ith peak.

Internal isotopic ratios are

$$R_{ij} = n_j S_i/S_j \quad (3)$$

The absolute values of the site-specific (D/H) ratios were calculated according to the formula (Martin et al., 1985)

$$(D/H)_i = n_{WS} g_{WS} (MW)_L S_i (D/H)_{WS}/n_i g_L (MW_{WS}) S_{WS} P_L \quad (4)$$

where WS stands for the working standard (TMU) with a known isotope ratio $(D/H)_{WS}$; L represents the product under examination; n_{WS} and n_i are the number of equivalent deuterium atoms of TMU and of the ith peak, respectively; g_{WS} and g_L are the weights of the standard and the sample, respectively; MW_L and MW_{WS} are the corresponding molecular weights, respectively; S_i and S_{WS} are the areas of the ith peak and of the standard, respectively; and P_L is the purity of the sample. $(D/H)_{WS}$ is the working standard isotope ratio as determined by isotope ratio mass spectrometry on the SMOW scale (Gonfiantini, 1978).

Samples 1, 5, 6, 11, 12, 14, 15, and 16 are synthetic commercial hexyl acetate from H&R, Oxford, PCAS, Treatt, Grau, Merck, Oxford (different lot), and Grau (different lot), respectively. Sample 2 is hexyl acetate obtained upon acetylation of hexanol produced in *C. gloeosporioides* (Fronza et al., 1995a) by reduction of natural hexanoic acid (Henkel). To this end, the carbinol (5 g) in dichloromethane (10 mL) is treated with acetic anhydride (5 mL) and pyridine (5 mL) at 0 °C overnight. The reaction mixture is treated with ice−water, and the separated organic layer is washed with cold 3% $NaHCO_3$ solution, 3% HCl, and water. The residue obtained upon evaporation of the dried (Na_2SO_4) solution is bulb-to-bulb distilled under vacuum to provide the desired ester in 90% yield. Sample 3 is hexyl acetate obtained upon acetylation of the carbinol produced from natural hexanoic acid upon $LiAlH_4$ reduction. To this end, hexanoic acid (5 g) in ether (10 mL) is added dropwise under stirring to $LiAlH_4$ (3 g) in ether (100 mL) at room temperature. The mixture is then refluxed for 1 h. The excess hydride is destroyed by careful addition of ethanol, followed by a saturated solution of potassium−sodium tartrate (50 mL). The hexanol obtained upon evaporation of the dried ether layer is purified by bulb-to-bulb vacuum distillation and then acetylated. Sample 7 is hexyl acetate prepared from synthetic hexanol (Aldrich) upon acetylation. Samples 8 and 9 are the acetate esters obtained upon acetylation of natural hexanol samples from Daniel and Aldrich, respectively. Sample 10 is natural hexyl acetate from Daniel. Sample 13 is hexyl acetate obtained upon acetylation of a sample of synthetic hexanol (sample 4, Merck) submitted to the action of fermenting bakers' yeast. To this end, to a mixture composed of commercial moist bakers' yeast (100 g) and D-glucose (50 g) in 1 L tap water at 38 °C was added hexanol (10 mL). After 20 h, the incubation mixture was submitted to hydrodistillation and the hexanol recovered by extraction (ether) from the distillate was purified by vacuum distillation.

RESULTS AND DISCUSSION

The assignment of the signals appearing in the ^2H NMR spectrum (Figure 1) of hexyl acetate (**3**) is reported in Table 1. Inspection of Figure 1 indicates the presence of separate signals for all methylene groups present in the framework of hexyl acetate, with the exception of those at positions 3, 4, and 5, which appear together. The samples of **3** included the following sets: (i) synthetic commercial samples from different suppliers and lots (samples 1, 4−7, and 11−16; (ii) natural commercial samples (samples 8−10); (iii) the material produced in *C. gloeosporioides* from natural hexanoic acid (sample 2); and (iv) the material produced from the same acid upon $LiAlH_4$ reduction (sample 3).

The ^2H NMR spectrum of **3** has been divided into five regions defining different groups of isotopomers (Table 1). Accurate integration of the signals allows one to calculate the molar fractions f_i of each group and the absolute site-specific isotope ratios $(D/H)_i$. The data are reported in Tables 2 and 3, respectively.

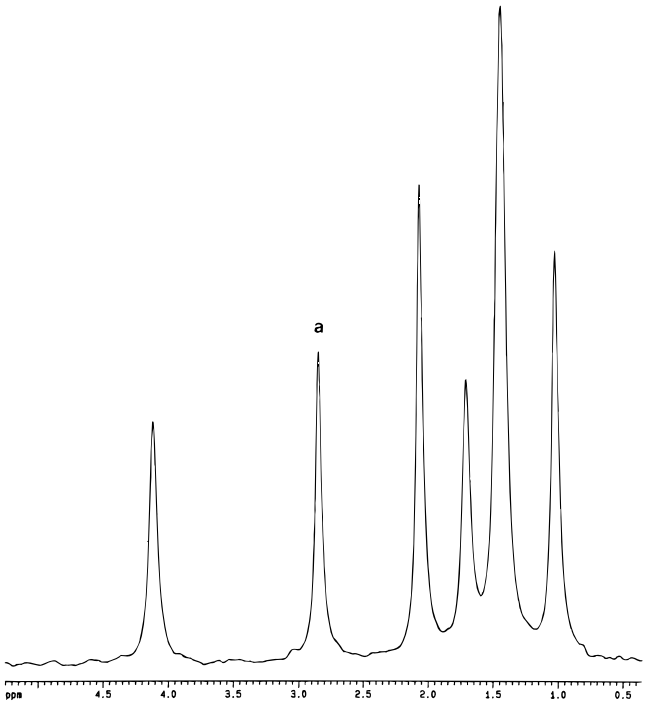


Figure 1. Natural abundance ^2H NMR spectrum of hexyl acetate (a) signal of TMU used as internal standard.

Table 1. Chemical Shift Values for Hexyl Acetate Deuterium NMR Spectrum[a]

peak[b]	assignment	ppm
1	CH_2-1	4.07
2	$COCH_3$	2.01
3	CH_2-2	1.66
4	CH_2-3,4,5	1.39
5	CH_3	0.98

[a] The chemical shifts are referred to the internal standard (TMU) peak taken at 2.80 ppm. [b] Numbering of the different groups of nuclei used in Tables 2 and 3 for the isotopic parameters.

From the numerical values collected in Table 2 it can be seen that the molar fraction f_1 (relative to the deuterium atoms located on the oxygen-bearing carbon atom at position 1) shows strong changes depending upon the origin of the samples. In particular, sample 2, obtained by microbial reduction of the carboxyl group of natural hexanoic acid, displays the highest value (0.260), much superior to that of sample 3 (0.045), formed from the same precursor upon metal hydride reduction. At variance with sets 2 and 3, the value f_1 for the synthetic samples (samples 1, 4−7, and 11−16) and for the natural ones (samples 8−10) is not too different, ranging from 0.127 to 0.145 for the former and from 0.151 to 0.154 for the latter. However, these values are quite different from those of samples 2 and 3 reported above. In particular, the f_1 of sample 13, obtained by submitting the hexanol of sample 4 (f_1 = 0.142) to incubation with fermenting bakers' yeast, is 0.156. We have shown previously (Fronza et al., 1995a) that the H_S proton of hexanol in the presence of bakers' yeast undergoes a partial equilibration with the solvent protons. Thus, the modest increase of f_1 and the parallel increase of the $(D/H)_1$ values from 138.9 to 151.5 on going from sample 4 to sample 13 might be an indication of such proton exchange.

However, a definite differentiation between the commercial synthetic and natural materials can be made by comparison of the f_4 values. These range from 0.407 (sample 5) to 0.374 (sample 11) for the first set and from 0.312 (sample 8) to 0.324 (sample 10) for the second. Thus, the natural (extractive) samples with respect to the synthetic materials show higher f_1 and lower f_4 values. The same is true for the specific isotope ratios $(D/H)_4$, which are smaller for the natural (samples 8, 9, and 10 showing ratios 87.6, 80.3, and 87.6, respectively) than for the synthetic samples [ranging from 115.2 (sample 12) to 126.9 (sample 13)]. The $(D/H)_4$ ratio for samples 2 (83.1) and 3 (95.1) obtained from the same batch of natural hexanoic acid by microbial and metal hydride reduction, respectively, is also approximately in line with the deuterium content characteristic for the natural hexanol samples 8−10. Concerning samples 2 and 3, the reason for the increase of $(D/H)_4$ on going from sample 2 to sample 3 is obscure since the deuterium content of positions 3, 4, and 5, the "*core*" of **1**, conceivably is not susceptible to enzymic or purely chemically induced changes in the isotopic composition during the manipulations occurring at the carboxyl group. Similarly intriguing is the strong variation of the $(D/H)_3$ values on going from sample 2 (78.7) to sample 3 (112.5). Possibly this variation may be due to the occurrence of some ketoenolic equilibrium during the long-lasting microbial reduction of hexanoic acid in water leading to a loss of deuterium in position 2; however, such hypothesis is in contrast with the results of some reduction experiments carried out in deuterated water (Fronza et al., 1995a) showing that any incorporation of deuterium atoms occurs at position 2.

Table 2. Molar Fraction Values for the Hexyl Acetate Samples[a]

sample	f_1	f_2	f_3	f_4	f_5
1	0.131 (0.004)	0.181 (0.002)	0.125 (0.002)	0.396 (0.002)	0.167 (0.003)
2	0.260 (0.005)	0.195 (0.003)	0.088 (0.009)	0.281 (0.003)	0.176 (0.003)
3	0.045 (0.003)	0.243 (0.003)	0.144 (0.003)	0.365 (0.002)	0.203 (0.003)
4	0.142 (0.002)	0.190 (0.003)	0.101 (0.002)	0.385 (0.002)	0.182 (0.002)
5	0.127 (0.004)	0.161 (0.003)	0.128 (0.001)	0.407 (0.003)	0.177 (0.003)
6	0.140 (0.003)	0.189 (0.003)	0.102 (0.001)	0.383 (0.003)	0.186 (0.003)
7	0.140 (0.003)	0.198 (0.003)	0.103 (0.002)	0.379 (0.003)	0.180 (0.003)
8	0.151 (0.004)	0.229 (0.003)	0.113 (0.002)	0.312 (0.003)	0.195 (0.002)
9	0.153 (0.005)	0.235 (0.002)	0.116 (0.002)	0.314 (0.003)	0.182 (0.003)
10	0.154 (0.003)	0.210 (0.003)	0.117 (0.002)	0.324 (0.002)	0.196 (0.004)
11	0.146 (0.003)	0.193 (0.002)	0.103 (0.002)	0.374 (0.002)	0.184 (0.003)
12	0.144 (0.003)	0.201 (0.001)	0.104 (0.002)	0.376 (0.002)	0.175 (0.003)
13	0.156 (0.003)	0.181 (0.002)	0.088 (0.002)	0.389 (0.002)	0.187 (0.002)
14	0.145 (0.003)	0.190 (0.002)	0.100 (0.001)	0.382 (0.002)	0.183 (0.002)
15	0.146 (0.002)	0.182 (0.002)	0.103 (0.002)	0.384 (0.002)	0.185 (0.003)
16	0.141 (0.003)	0.188 (0.002)	0.103 (0.001)	0.383 (0.003)	0.185 (0.003)

[a] The values of the mole fractions (f_i) are averaged over 10 determinations; the corresponding standard deviations are reported in parentheses.

Table 3. Specific Isotope Ratios and Averaged Isotope Ratios for the Hexyl Acetate Samples[a]

sample	$(D/H)_1$	$(D/H)_2$	$(D/H)_3$	$(D/H)_4$	$(D/H)_5$	$(D/H)_{av}$[b]
1	133.5 (3.4)	123.0 (3.0)	127.1 (4.4)	134.3 (3.4)	113.1 (4.2)	127.2
2	231.3 (10.3)	115.2 (3.9)	78.7 (9.9)	83.1 (2.6)	104.1 (2.8)	111.0
3	35.3 (2.5)	126.6 (3.4)	112.5 (4.8)	95.1 (2.7)	106.0 (3.5)	97.7
4	138.9 (4.4)	123.9 (4.8)	99.4 (3.2)	125.9 (4.0)	118.9 (4.0)	122.5
5	124.1 (4.7)	104.5 (2.7)	125.0 (2.3)	132.6 (2.1)	115.4 (3.0)	122.1
6	127.4 (2.8)	114.7 (2.3)	93.0 (2.2)	116.4 (2.4)	112.7 (2.5)	113.8
7	131.4 (5.1)	123.8 (3.7)	96.6 (3.8)	118.6 (3.8)	112.5 (2.9)	117.3
8	127.3 (5.5)	128.4 (3.4)	94.8 (3.3)	87.6 (2.5)	109.3 (2.7)	105.2
9	117.6 (4.5)	120.2 (2.6)	88.6 (2.5)	80.3 (1.8)	93.0 (2.2)	95.9
10	124.7 (4.5)	113.5 (3.8)	95.3 (3.6)	87.6 (3.0)	106.1 (3.6)	101.5
11	137.0 (3.9)	120.5 (6.6)	96.1 (6.5)	116.7 (5.4)	114.5 (4.5)	117.0
12	132.2 (5.1)	123.1 (4.2)	95.1 (4.3)	115.2 (3.4)	107.0 (4.3)	114.7
13	151.5 (4.7)	117.2 (2.0)	85.2 (2.4)	126.1 (1.9)	121.1 (2.1)	121.6
14	144.5 (3.8)	126.3 (3.7)	100.2 (2.4)	126.9 (2.4)	121.6 (3.2)	124.7
15	139.4 (4.2)	115.9 (3.5)	97.9 (2.6)	122.1 (3.5)	117.5 (4.3)	119.2
16	134.5 (4.9)	119.9 (3.2)	98.9 (1.9)	121.9 (2.1)	118.0 (2.9)	119.5

[a] The values of the (D/H) ratios are averaged over 10 determinations. The corresponding standard deviations are reported in parentheses. [b] The averaged isotope ratio is obtained from the formula $(D/H)_{av} = \Sigma[n_i(D/H)_i]/\Sigma n_i$, where n_i is the number of equivalents or isochronous deuterium atoms of the ith peak.

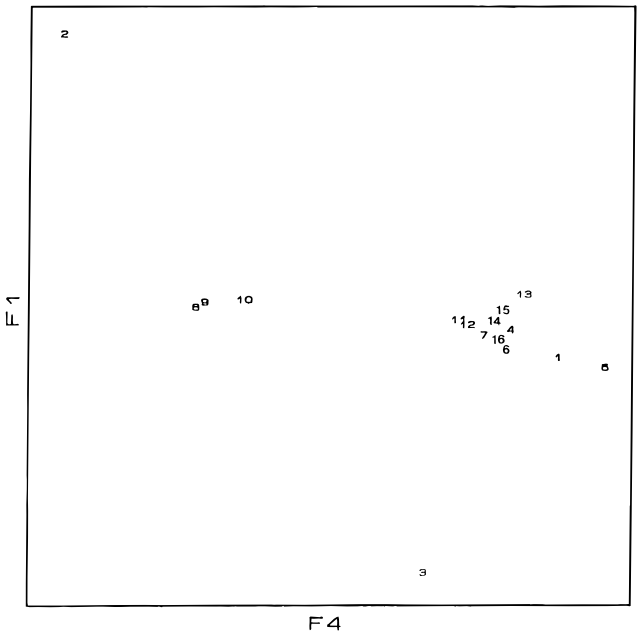


Figure 2. Graphical representation of the molar fractions f_1 vs f_4 showing four distinct regions for the natural commercial products (samples 8−10), the synthetic commercial products (samples 1, 4−7, 11−16), the product from the metal hydride reduction of the natural hexanoic acid (sample 3), and the product from the bioreduction of the natural hexanoic acid (sample 2).

Seen together, the data relative to the relevant deuterium molar fractions and isotope ratios of samples 2 and 3 here outlined indicate a clear-cut difference of the deuterium distribution of the educts obtained from the same natural precursor by enzymic and chemical means, respectively. This would assure against the production of natural hexanol from natural hexanoic acid by the fraudulent chemical metal hydride reduction. Similarly quite evident is the difference between the two sets of natural hexanol samples (extractive and microbially generated from natural hexanoic acid) and the two sets of the synthetic products (from materials of petrochemical origin and from natural hexanoic acid by chemical reduction).

The plot of f_1 against f_4 values (Figure 2), relative to the 16 samples of **3** examined in the present study, allows the unequivocal pictorial identification of four regions relative to (i) the natural commercial products, (ii) the natural material formed by bioreduction of natural hexanoic acid, (iii) the material obtained by natural hexanoic acid by chemical, metal hydride reduction, and (iv) the synthetic commercial products.

These SNIF-NMR data relative to hexanol (**1**) can be compared with those recently reported for *cis*-3-hexenol (**2**) (Muller and Gautier, 1994). The examined samples of **2** included the synthetic product, the natural material extracted from mint, and that biogenerated from linolenic acid through a procedure involving as the latest stage the baker's yeast mediated reduction of *cis*-3-hexenal. The main difference between the last two samples is in the deuterium content at position 1, which is much lower in the yeast-generated product with respect to the extractive one. On the contrary, the synthetic material is differentiated from the two natural products by its much lower deuterium content at the vinylic carbon at position 4. In the case of hexanol (**1**) f_4 (including positions 3−5) is lower in the natural (extractive) series than in the synthetic products, whereas the reverse is true for f_1. Moreover, f_1 is very high in the case of the natural hexanol produced by microbial reduction of hexanoic acid, through a process that involves the stepwise, enzyme-mediated addition of two hydrogen atoms, conceivably arising from reduced nicotine cofactors (Fronza et al., 1995a). In the case of biogenerated *cis*-3-hexenol (**2**) one hydrogen atom is delivered at position 1 by a similar mechanism.

In conclusion, the present work demonstrates the utility of the SNIF-NMR method in determining the origin of the hexanol samples here examined, allowing a clear-cut distinction between natural materials obtained from different precursors through different pathways and other synthetic products. In the meantime, the comparison between the data here obtained for **1** and those reported in the literature for **2** raises subtle mechanistic questions for the different labeling patterns of the two extractive C_6 alcohols, formed in nature by fragmentation of the 13-hydroperoxide of linoleic and linolenic acids, possessing the same skeletal framework.

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