Natural Abundance ^{2}H Nuclear Magnetic Resonance Study of the Origin of (*Z*)-3-Hexenol

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The origin of 11 samples of (*Z*)-3-hexenol was studied by SNIF-NMR measurements. The results show a possible distinction between synthetic and extractive materials and those biogenerated from linolenic acid by enzymic fragmentation at position 13 of the carbon framework of the 13-hydroperoxide obtained by the action of oxygen in the presence of lipoxygenase, followed by baker's yeast-mediated reduction of the intermediate (*Z*)-3-hexenal. Particularly significant to this end result are the deuterium contents at relevant positions of (*Z*)-3-hexenol, such as 3+4 and 1. The results suggest that one sample might be a mixture of materials of extractive origin with those obtained by biodegradation from linolenic acid.

Keywords: *(Z)-3-Hexenol; naturality; SNIF-NMR; biogeneration*

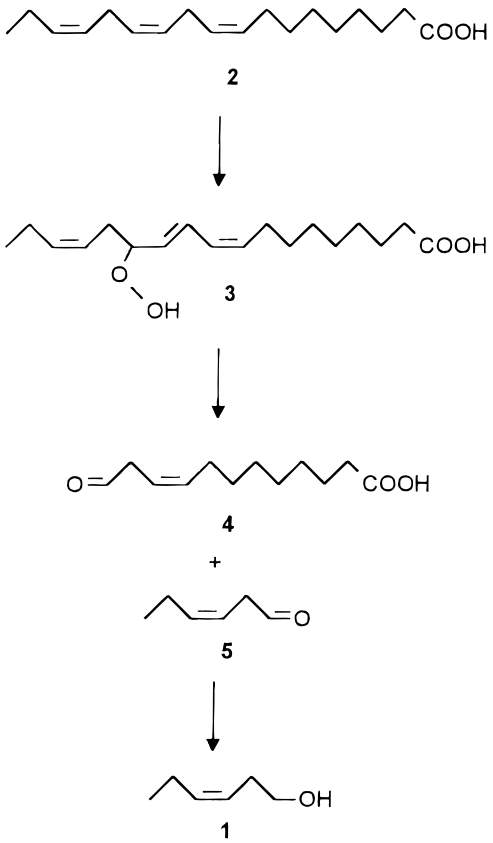
INTRODUCTION

(*Z*)-3-Hexenol (**1**), the so-called leaf alcohol for the typical "green odor", is a key compound in aroma formulations and is produced in the synthetic modification at up to 200 ton/year (Hatanaka, 1993). It is quite widespread in nature, but in minute amounts because it is a compound whose production is stimulated when the plant is mechanically damaged, thus allowing a more intense contact with the oxygen of the air. Indeed, labeling expriments support the derivation in green leaves of *Thea sinensis* of the C-6 framework of **1** from carbon atoms 13−18 of linolenic acid (**2**) through the steps in Scheme 1 (Hatanaka, 1993). Three enzymic activities are apparently involved in the process: (i) the production of the 13-hydroperoxide (**3**), which requires oxygen; (ii) the fragmentation of the carbon skeleton of the latter to provide the C-12 and C-6 moieties **4** and **5**, respectively; and (iii) the reduction of the C-6 aldehyde **5** to **1**.

Recently, due to the legislative discrimination between chemically identical aroma constituents of extractive and synthetic origin (US Code of Federal Regulations, 1985), there has been interest in obtaining substantial quantities of natural **1**. Because of the consumers' preferences for natural products, this requirement could be hardly met completely by the product obtained by extractive manipulation of botanical sources. Accordingly, bioconversion processes mimicking nature have been set up enabling the efficent conversion of **2** into **1** (Muller and Gautier, 1994; Brunerie, 1991). Under these circumstances, the lipoxygenase, the hydroperoxide lyase, and, eventually, the alcohol dehydrogenase activities are required for the execution of the sequence of Scheme 1. These enzymes are provided by soy flour, a plant homogenate particularly rich in lyase acticity and fermenting baker's yeast, respectively, whereas natural **2** used as substrate is obtained from a botanical source.

Accordingly, at present, the request for **1** by the flavor industry can be formally met by the materials obtained by chemical synthesis and, as far as the *natural* modification is concerned, by the extractive product or by the educt obtained from **2** by the enzymic operations of Scheme 1. However, the latter products are reproduced outside the plant. This circumstance raises the question of the origin of the marketed samples of **1**, to guarantee the customer against adulterations. In many instances up to now, the measurement of the natural abundance of the deuterium content of a molecule was an effective means to determine its origin (Martin, 1982). The successful application of this analytical tool to the determination of the origin of three samples of **1**, obtained by the possible modes of synthesis, extraction,

Scheme 1. Steps in the Biogeneration of (*Z*)-3-Hexenol (1) from Linolenic Acid (2)



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and bioconversion, has been reported in a short communication (Muller and Gautier, 1994). We present here the results of a comparison of the deuterium content of **1** obtained from **2** through the method just outlined (Brunerie, 1991) with that of a whole set of specimens of different origin.

MATERIALS AND METHODS

Deuterium NMR data (46.076 MHz) were recorded at 302 K on a Brucker 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. Samples of **1** were prepared by weighing ~3 g of pure (>97% as determined by gas chromatography) material, 150 mg of hexafluorobenzene for the ^{19}F lock (E. Merck, 64271 Darmstadt, Germany), and 400 mg of tetramethylurea (TMU) as internal (D/H) standard (Fluka Chemie AG, CH-9470 Buchs, Switzerland). The TMU was previously tested by isotope ratio mass spectrometry (IRMS) and showed an average standard isotope ratio (D/H; absolute values of the site-specific D/H isotopic ratio) value of 133.45 ppm. NMR measurement of a sample of ethanol using, alternatively, Fluka TMU and official TMU (Community Bureau of References, BRC EC10; D/H = 136.67 ppm) gave Fluka TMU values consistent with those of IRMS. Ten spectra were run for each sample, 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, and 15 μs (90°) pulse length. Each FID (free induction decay) was Fourier transformed, with no zero filling (0.15 Hz/point digital resolution) and line broadening of 2 Hz, manually phased, and integrated. The signal-to-noise ratio (S/N) was >180 (methyl peak).

Molar fractions, f_i, were calculated from the integrated areas as

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

where S_j is the area of the ith peak. The corresponding statistical molar fractions are 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 of the sample, respectively; MW_L and MW_{WS} are the corresponding respective molecular weights; S_i and S_{WS} are the areas of the i^{th} peak and of the standard, respectively; P_L is the purity of the sample; and $(D/H)_{WS}$ is the working standard isotope ratio as determined by IRMS on the SMOW (standard mean ocean water) scale (Gonfiantini, 1978).

Samples 1−11 of **1** had the following origins: samples 1−5 are synthetic commercial samples from Berje, Oxford, Treatt, Bedoukian, and BBE, respectively; samples 6 and 7 are commericial products sold as natural; and samples 8−11 are materials biogenerated from **2** according to a patented procedure with baker's yeast-mediated reduction of the intermediate (*Z*)-3-hexenal (Brunerie, 1991).

RESULTS AND DISCUSSION

The assignment of the signals appearing in the ^2H NMR spectrum of synthetic **1** (Figure 1) is reported in Table 1. Samples 7 and 10 are reported in Figures 2 and 3, respectively. The analyzed samples include the following: (i) five synthetic commercial samples (samples 1−5); (ii) two natural commercial samples (6 and 7); (iii) four natural samples obtained by biodegradation of **2** (8−11; Brunerie, 1991).

The ^2H NMR spectrum of **1** has been divided into six regions defining different groups of isotopomers (Table 1). The $(D/H)_i$ values of the examined samples are reported in Table 2, whereas the f_i values, without taking into account the mobile hydrogen atom of the hydroxyl group, are reported in Table 3.

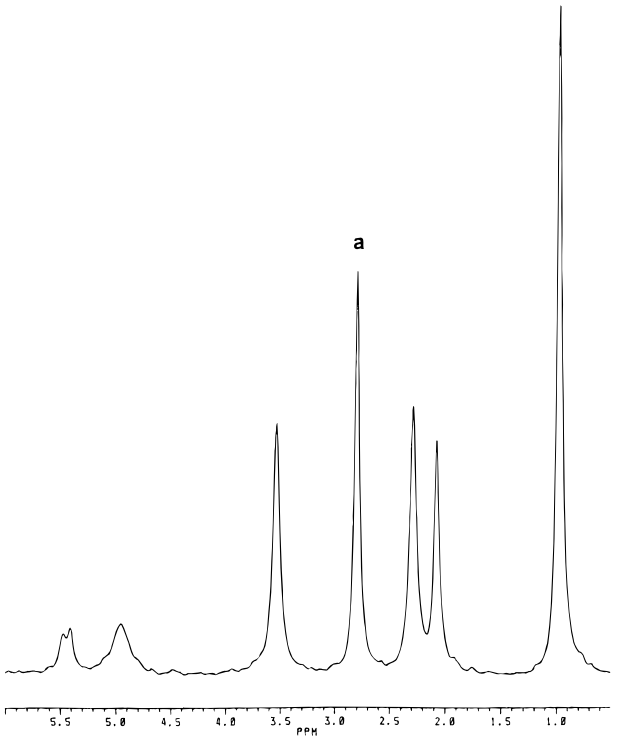


Figure 1. Natural abundance ^2H NMR spectrum of synthetic (*Z*)-3-hexenol: (a) signal of TMU used as internal standard.

Table 1. Assignment of the Deuterium Spectrum of (*Z*)-3-Hexenol (1)[a]

peak[b]	assignment	ppm
1	CH=CH	5.5
2	OH	5.0
3	CH_2O	3.5
4	CH_2(a)	2.3
5	CH_2(b)	2.1
6	CH_3	1.0

[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 in Tables 2 and 3 for the isotopic parameters.

The f_1 and f_3 values in Table 3 allow a clear-cut differentiation between the synthetic (samples 1−5) and the biosynthetic (samples 8−11) materials from **2**. Indeed, the variations of the values of f_1 are in the range 0.047−0.057 and 0.141−0.152, respectively, for the two sets. Similarly, f_3 are between 0.208 and 0.252 in the former instance, whereas in the second, the individual figures are 0.209, 0.100, 0.111, and 0.099, respectively.

When we consider the positions on the framework of **1** to which the aforementioned data are referred, the observed variations can be particularly diagnostic. Indeed, f_1 and f_3 are relative to positions 3+4 and 1, respectively, of **1**. In the synthetic materials (samples 1−5), the two hydrogen atoms at positions 3 and 4 are expected to arise from hydrogen gas (conceivably produced from methane), delivered by *syn* catalytic partial hydrogenation of the triple bond of the late C-6 acetylenic intermediate **7**, obtained, in turn, from **6** (Scheme 2; Stoll and Rouve', 1938). The low deuterium content at those positions reflects the paucity of deuterium of the starting material of petrochemical origin (Fronza et al., 1993). Conversely, the hydrogen atoms at the corresponding positions in the biogenerated products (samples 8−11) arise from completely different origin. They were originally present at positions 15 and 16 of the framework of **2** (Scheme 1). The extent of deuterium labeling at those particular positions of the C-18 fatty

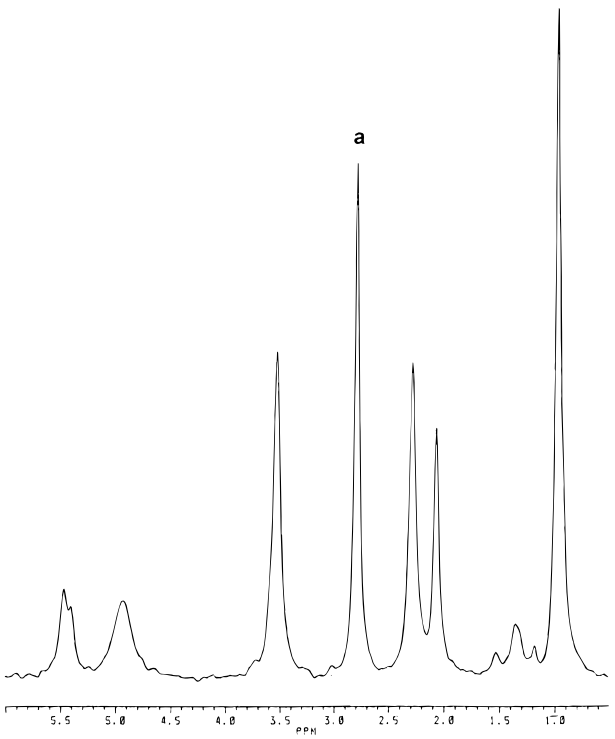


Figure 2. Natural abundance ^{2}H NMR spectrum of natural (*Z*)-3-hexenol (sample 7): (a) signal of TMU used as internal standard.

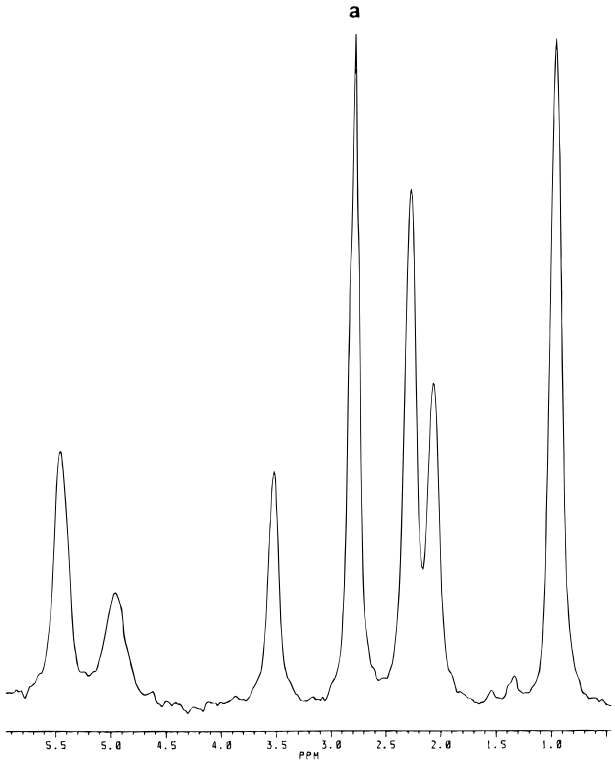


Figure 3. Natural abundance ^{2}H NMR spectrum of natural (*Z*)-3-hexenol (sample 10): (a) signal of TMU used as internal standard.

acid precursor **2** depends, in turn, upon the deuterium content of the saturated intermediate and upon the deuterium kinetic isotope effect exerted by the enzymatic system presiding over the desaturation process leading to **2** during its biosynthesis.

Similarly, the values of f_3 in the synthetic materials 1−5 reflect the extent of the deuterium content of the ethylene oxide used to elongate the C-4 framework of but-1-yne (**6**). For the examined samples, the values range between 0.208 and 0.252, respectively. In the biogenerated products 8−11, the C-1 methylene group to which f_3 is referring is generated at the latest stage by enzymatic reduction of the intermediate aldehyde **5**. In the alcohol dehydrogenase-mediated aldehyde reduction, the hydrogen atom ending up in the *pro-S* position of the carbinol arises from the reduced form of the nicotine cofactor. This hydrogen atom is derived, in turn, from the oxidation of the pool of the alcoholic materials supplied in the fermentation medium as *fuel* in the cofactor regenerating cycle. However, the occurence of exchange in baker's yeast of this hydrogen atom located in position 4 of the reduced nicotine cofactor for the solvent hydrogens is a known phenomenon, catalyzed by a diaphorase, and its operation in the case of 1-hexanol has been demonstrated recently (Fronza et al., 1995a). Accordingly, the extent of the deuterium labeling of the methylene carbinol of the alcohol **1** incubated with baker's yeast might change from one experiment to another. The deuterium substitution of the two hydrogen atoms present at position 1 of **1** thus depends on the original content of deuterium of the precursor aldehyde **5** generated from **2**, which provides one of the two hydrogen atoms. The second hydrogen depends on the nature of the alcohol used as *fuel* in the cofactor regeneration and on the extent of the equilibration with the water protons of the hydrogen of the reduced cofactor delivered in the reduction. As a consequence of the multiple operations of a kinetic isotope effect, the f_3 values of the natural samples 9−11 are considerably lower (∼0.100 in all instances) than those of the synthetic materials. In the case of sample 8, however, the f_3 of 0.209 is nearly identical to the lower figure (0.208) measured in the synthetic materials. The data observed for sample 8 could be tentatively explained supposing a limited diaphorase activity in the baker's yeast used in the final step of the bioconversion and/or that the **1** of sample 8 is a mixture of the material biogenerated from **2** and of that originally present to a certain extent in the green plant homogenate, expressing the aldehydolyase activity and added in quantity to the incubation mixture. Indeed, many D/H and *f* values of this sample are positioned in an intermediate range between those of sample 7 (discussed later) and of samples 9−11.

When considering the $(D/H)_1$ values of the two aforementioned sets of materials, it appears that the former is in the range 29.32−35.72 for the synthetic materials 1−5 and in the range 83.6−96.4 for products 8−11 derived from **2**. Conversely, $(D/H)_3$ is high in the case of the synthetic materials (141.7−156.4) and low for the natural samples 9−11 (54.9−64.8), but higher (142.3) for product 8 (see sample 7). Conversely, the $(D/H)_4$ values are quite uniform (133.9−147.1) for the biogenerated materials 8−11, and, in general, higher than those of the synthetic products 1−5 (88.2−100.1), whereas sample 5 displays the value of 140.3.

As far as the remaining *natural* commercial samples 6 and 7 are concerned, it appears that sample 7 displays a deuterium content at site 1 with the $(D/H)_1$ value of 65.7, which is in between those of the synthetic and of the biogenerated materials just mentioned, whereas the $(D/H)_3$ of 167.3 is much higher than in the latter case. Comparison of the spectrum of sample 7 (Figure 2) with that of sample 10 (Figure 3) and comparison of these spectra with the reported spectra of natural samples of **1** from mint and obtained by biogeneration, respectively

Table 2. Specific Isotope Ratios for (*Z*)-3-Hexenol Samples[a]

sample	$(D/H)_1$	$(D/H)_2$	$(D/H)_3$	$(D/H)_4$	$(D/H)_5$	$(D/H)_6$	$(D/H)_{av}$[b]
1	29.3 (1.9)	109.4 (4.5)	152.5 (2.8)	94.7 (1.8)	88.0 (3.6)	161.0 (2.8)	96.7
2	34.1 (1.7)	102.6 (6.2)	141.7 (4.0)	95.3 (2.6)	44.3 (3.6)	185.9 (4.7)	92.1
3	33.5 (3.0)	110.0 (8.4)	155.7 (3.9)	88.2 (1.5)	39.2 (1.4)	204.8 (2.9)	96.1
4	32.8 (1.8)	119.2 (8.0)	144.6 (3.8)	100.1 (1.9)	65.3 (1.7)	190.6 (2.8)	98.9
5	35.7 (2.1)	108.7 (6.6)	156.4 (3.2)	140.3 (2.0)	110.4 (2.0)	204.9 (3.3)	117.0
6	93.5 (2.5)	124.3 (10.9)	63.3 (2.1)	112.3 (3.2)	40.9 (3.7)	142.8 (4.4)	85.9
7	65.7 (4.0)	178.2 (6.5)	167.3 (3.8)	98.9 (2.6)	50.8 (2.7)	155.3 (3.1)	104.5
8	96.4 (6.0)	203.8 (11.9)	142.3 (5.6)	133.9 (2.6)	64.6 (2.1)	162.1 (3.0)	116.9
9	83.6 (3.0)	114.9 (3.8)	54.9 (2.6)	139.8 (2.8)	86.7 (1.2)	123.5 (3.1)	91.0
10	84.3 (3.6)	87.5 (6.0)	64.8 (3.1)	147.1 (2.4)	92.5 (3.6)	128.5 (2.4)	93.5
11	86.9 (1.6)	114.9 (4.6)	57.6 (2.0)	145.1 (2.3)	100.6 (2.8)	129.2 (3.1)	96.1

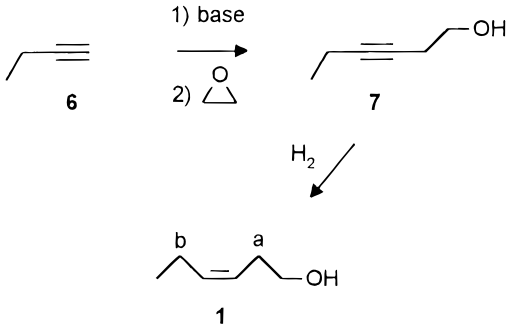
[a] The values of the D/H ratios are averaged over 10 determinations; the corresponding standard deviations are reported in parentheses. [b] The averaged isotope ratio is obtained from the formula $(D/H)_{av} = \sum [n_i (D/H)_i]/\sum n_i$, where n_i is the number of equivalents or isochronous deuterium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

Table 3. Molar Fractions Values for (*Z*)-3-Hexenol Samples[a]

sample	f_1	f_3	f_4	f_5	f_6
1	0.048 (0.003)	0.252 (0.005)	0.156 (0.004)	0.145 (0.005)	0.398 (0.003)
2	0.057 (0.003)	0.238 (0.005)	0.160 (0.005)	0.075 (0.006)	0.469 (0.007)
3	0.054 (0.005)	0.250 (0.005)	0.141 (0.003)	0.063 (0.003)	0.492 (0.005)
4	0.052 (0.003)	0.230 (0.005)	0.159 (0.003)	0.104 (0.003)	0.455 (0.004)
5	0.048 (0.003)	0.208 (0.002)	0.187 (0.003)	0.147 (0.002)	0.410 (0.002)
6	0.178 (0.004)	0.121 (0.003)	0.214 (0.005)	0.078 (0.005)	0.409 (0.007)
7	0.107 (0.006)	0.272 (0.004)	0.161 (0.003)	0.083 (0.004)	0.378 (0.006)
8	0.142 (0.007)	0.209 (0.007)	0.197 (0.003)	0.095 (0.002)	0.357 (0.007)
9	0.152 (0.004)	0.100 (0.005)	0.254 (0.002)	0.158 (0.002)	0.337 (0.006)
10	0.145 (0.005)	0.111 (0.005)	0.253 (0.005)	0.159 (0.005)	0.331 (0.004)
11	0.149 (0.003)	0.099 (0.003)	0.248 (0.004)	0.172 (0.005)	0.332 (0.005)

[a] The values of the mole fractions f_1 are averaged over 10 determinations; the corresponding standard deviations are reported in parentheses.

Scheme 2. Steps in the Synthesis of (*Z*)-3-Hexenol (1)



(Muller and Gautier, 1994), allows assignment of sample 7 to an extractive origin from mint (looking particularly at the deuterium content at positions 1 and 3). Sample 6 shows $(D/H)_1$ and f_1 values much higher than those of the synthetic samples 1−5 and in the same range of materials 9−11 biogenerated from **2**. Moreover, sample 6 displays a $(D/H)_3$ of 63.3, which is nearly equal to those of biogenerated samples 9−11 (54.9−64.8), indicating that sample 6 it is likely obtained by a biogeneration procedure similar to that of samples 8−11. In Figure 4 a plot of the molar fractions relative to positions 3+4 and 1 of samples 1−11 is presented. It appears that the synthetic and the biosynthetic samples obtained from **2** are well defined in two distinct regions, whereas samples 7 and 8 of extractive and mixed (extractive/biosynthetic) origin, respectively, are unequivocally outside these regions.

Seen together, these results indicate a possible distinction by means of the SNIF-NMR technique (a trademark of Laboratoires Eurofins, Nantes, France) of the synthetic samples 1−5 of **1** from those obtained by biogeneration (8−11, with possible dilution of the former with material of extraction) considering the extent of labeling at sites 1, 3, and 4. There is a differentiation

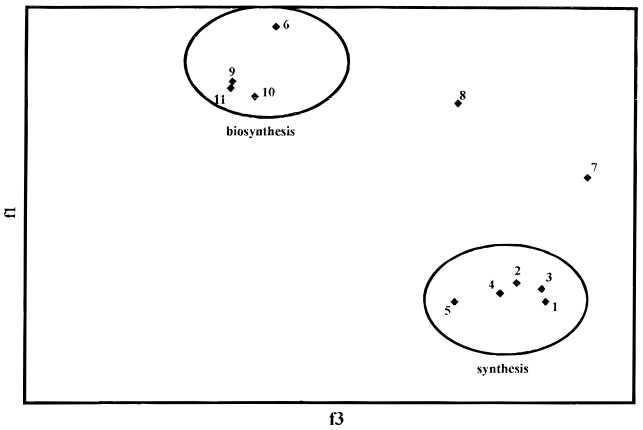


Figure 4. Graphical representation of the molar fractions f_1 vs f_3 showing distinct regions for (i) natural biosynthetic samples 6, 9, 10, and 11 from linolenic acid (**2**); (ii) synthetic commercial samples 1, 2, 3, 4, and 5; (iii) natural extractive sample 7; and (iv) natural extractive/biosynthetic sample 8.

between the aforementioned samples and sample 7, which is conceivably extracted from mint. In this case, the difference with respect to the aforementioned materials arises from the high value of $(D/H)_3$. On the basis of the present experiments, sample 6 has to be considered natural and biogenerated from **2**. Thus, as in the case of 2-phenylethanol (Fronza et al., 1995b) and *n*-hexanol (Fronza, 1996), the origin of samples of **1** can be clearly defined by the present technique, in particular taking account the mechanistic aspects of their biogeneration from defined precursors to explain the extent of deuterium labeling.

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