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Phytochemistry 65 (2004) 2815–2831
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

The fellowship of natural abundance ^{2}H-isotopomers of monoterpenes

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Received 25 March 2004; received in revised form 7 July 2004

Abstract

Site-specific natural abundance hydrogen isotope ratios have been measured by deuterium-NMR in a wide variety of monoterpenes from numerous kinds of plants grown in different environments. Once the NMR signals have been assigned to the whole sets of isotopomers in the different molecules and schemes of connections to the parent isotopomers in the geranyl diphosphate (GPP) precursor have been defined, a very consistent set of isotopic profiles is evidenced. The results, which are incompatible with the mevalonate pathway, can be satisfactorily interpreted by considering the deoxyxylulose pathway (DOXP), which is now recognized as the usual route for monoterpene biosynthesis in plants. Strong deuterium depletion at ex-site 2 of GPP, accompanied by high isotope ratio values at site ex-6, are consistent with synthesis of GPP from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) molecules independently produced by the DOXP pathway. However, for a given molecular species, significant differences are observed as a function of the plant source, in particular at site ex-6 of GPP. Thus, monoterpenes from plants with a C3 metabolism are mostly characterized by relatively high values of $(D/H)_6$, whereas C4 plants tend to show much lower values. This behavior may be attributed to more or less significant contributions of GPP resulting from the condensation of IPP with DMAPP produced by isomerization.

The isotopic profile therefore enables the role of physiological and environmental factors on the relative importance of the ''independent'' and ''isomerized'' model to be estimated. More generally, isotope ratios at individual sites in geraniol can be traced back to the corresponding sites in GPP, then to sites of the IPP and DMAPP building blocks, then to the pyruvate and glyceraldehyde 3-phosphate DOXP active molecules, and finally to the carbohydrate photosynthetic precursor.

Furthermore, the methylenic hydrogen atoms, which are enantiotopic in geraniol, become diastereotopic in chiral, and more specially in cyclic, monoterpenes. This provides an isotopic verification for the complete stereochemical chain of affiliation, and a way of estimating enantiomeric purity and whether intermolecular exchanges have taken place.

Keywords: C3 and C4 metabolism; Deuterium; Isotopic fractionation; Monoterpene biosynthesis; SNIF-NMR

Abbreviations: DMAPP, dimethylallyl diphosphate; DOXP, deoxyxylulose phosphate pathway; GPP, geranyl diphosphate; G3P, glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate; KIE, kinetic isotope effect; SNIF-NMR, site-specific natural isotope fractionation studied by nuclear magnetic resonance.

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doi:10.1016/j.phytochem.2004.07.015

1. Introduction

The direct access to site-specific natural isotope fractionation by ^{2}H-NMR (SNIF-NMR), initiated in the Eighties (Martin and Martin, 1981), provides a powerful complementary approach to the elucidation of hydrogen genealogy in natural or synthetic molecules (Martin et al., 1986a; Martin and Martin, 1990) and the method is now recognized as a rich source of information on the biochemical history of a product (for recent reviews see (Robins et al., 2003; Schmidt et al., 2003). The method has the advantage of providing isotopic information without the need for deuterium labeling. When based solely on isotopic parameters of extracted natural products, this approach characterizes the result of many successive events that the individual hydrogen atoms have undergone. It is therefore obviously not expected to be sufficient for a detailed elucidation of individual steps of a complex biochemical mechanism and it has not been presented, as supposed by Arigoni et al. (1993) as a general substitute for appropriate labeling experiments. However, as illustrated in the case of the fermentation reaction (Zhang et al., 1995), the analysis of isotopic natural distributions can provide quantitative information on how individual hydrogen atoms in the starting materials are connected to those in the end products, whatever the complexity of the reaction. This affiliation sheds light on many features of a biosynthetic pathway, which can then be used to rule out inconsistent mechanistic hypotheses. Moreover the method is a unique source of information on possible hydrogen exchanges and on environmental effects occurring in undisturbed natural media. This NMR approach was applied in the Eighties to the study of monoterpenes (Martin et al., 1986a). We showed that α-pinene exhibits surprisingly large deviations from a statistical distribution of deuterium and that exploitable differences exist in the isotope contents of enantiomers from a given plant origin. The method has also been used to estimate kinetic isotope effects occurring during the formation of β-pinene (Pascal et al., 1986) and limonene (Leopold et al., 1988). In fact the results obtained on α-pinene were not consistent with the mevalonate mechanism that was generally accepted at that time for explaining monoterpene biosynthesis in plants. In particular a very strong impoverishment in the isotopomer connected to site ex-2 of the geranyl diphosphate (GPP) precursor was highlighted and the results did not exhibit the alternate regularity expected from the isoprene building model. Unfortunately our own experience in the particular field of terpene biosynthesis was insufficient to question such a widely accepted mechanism and specialists of the day tended to suspect the reliability of the new isotopic approach rather than the pathway itself (Arigoni et al., 1993). In 1993, a new mechanism, based on glyceraldehyde-3-phosphate (G3P) and pyruvate precursors was proposed by (Rohmer et al., 1993) to explain the biosynthesis of hopanoids in certain bacteria. This non-mevalonate pathway, which reconciles various cases of anomalous behavior, is now recognized as an ubiquitous mechanism in higher plants. Most of the steps of this DOXP-pathway, which leads from G3P and pyruvate to the GPP precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), through 1-deoxy-D-xylulose-5-phosphate and 2-*C*-methyl-D-erythritol-4-phosphate, have now been identified (Duvold et al., 1997; Eisenreich et al., 2001; Lange et al., 2001; Lichtenthaler, 1999; Rohdich et al., 2001, 2003; Rohmer, 1998) but some points are still not fully elucidated. The mevalonate pathway, which occurs in the cytoplasm, supplies precursors of triterpenes and sterols, whereas the DOXP-pathway, which operates in plastids, supplies many terpenoids and in particular monoterpenes (Arigoni et al., 1997; Eisenreich et al., 1997; Lichtenthaler et al., 1997; Rieder et al., 2000). However there is no absolute compartmental separation of the two pathways and complex cross-talks between the two terpenoid routes have been observed (Piel et al., 1998; Schuhr et al., 2003; Wanke et al., 2001). We have already published partial isotopic results on monoterpenes, in particular linalool (Hanneguelle et al., 1992; Martin, 1995), which we proposed as authentication criteria for the natural status of aromas. In the present work we have carried out a comprehensive investigation of isotopomer distributions in a large number of monoterpene samples biosynthesized in different organs of plants with different photosynthetic metabolisms and grown in different environments. The isotopic parameters are analyzed, in the light of the new DOXP-pathway, to the aim of establishing the entire schemes of individual hydrogen affiliations going back from the monoterpene through the key intermediates, GPP, IPP/DMAPP, pyruvate/G3P, to the carbohydrate precursors.

2. Results

Twelve monoterpenes were extracted from a large number of plants characterized by C3 or C4 metabolic pathways (Table 1). The C3 and C4 origins were confirmed by measurement of the carbon isotopic deviation, $\delta\,^{13}C(‰)$ Eq. (1) (Table 2). To take into account the dispersion associated with both the nature of the plant and the diversity of climatic environments, we collected about 120 samples from different plant materials (leaves, fruit, essential oils ...) and different plant species and varieties (experimental section). Since extraction and purification procedures may induce significant isotope fractionation (Martin and Martin, 1990), experimental conditions were defined which optimize the yield of the

Table 1
Origin of the investigated monoterpene samples: G, M and P refer respectively to the type of skeleton considered, geranilane, *p*-menthane and pinane (Fig. 1a–c)

No.	Molecule	Type	Formula	CAS No.	Origin
1	Geraniol	G	$C_{10}H_{18}O$	106-24-1	*Citronella*, palmarosa, monarde
2	*S*-(+)-Linalool	G	$C_{10}H_{18}O$	126-90-9	Spike, bergamot, bois de rose, petitgrain, lavender
3	*R*-(−)-Linalool	G	$C_{10}H_{18}O$	126-91-0	Coriander
4	*S*-(−)-Linalyl acetate	G	$C_{11}H_{20}O_2$	115-95-7	*Salvia*, bergamot, petitgrain
5	*R*-(+)-Citronellol	G	$C_{10}H_{18}O$	1117-61-9	*Citronella*, *Eucalyptus citriodora*
6	*S*-(−)-Citronellol	G	$C_{10}H_{18}O$	7540-51-4	*Geranium*, rose
7	R-(+)-Citronellal	G	$C_{10}H_{18}O$	2385-77-5	*Citronella*
8	*S*-(−)-Citronellal	G	$C_{10}H_{18}O$	5949-05-3	*Eucalyptus citriodora*
9	Geranial (citral *E*)	G	$C_{10}H_{16}O$	141-27-5	Lemon grass, lemon, *Litsea cubeba*, *verbena*
10	Neral (citral *Z*)	G	$C_{10}H_{16}O$	106-26-3	Lemon grass, lemon, *Litsea cubeba*, *verbena*
11	Citral (*E*/*Z*)	G	$C_{10}H_{16}O$	5392-40-5	Lemon grass, lemon, *Litsea cubeba*, *verbena*
12	*R*-(+)-Limonene	M	$C_{10}H_{16}$	5989-27-5	Orange, lemon, grapefruit, tangerine, bergamot, carraway, dill
13	*S*-(−)-Limonene	M	$C_{10}H_{16}$	5989-54-8	Commercial
14	*S*-(+)-Carvone	M	$C_{10}H_{14}O$	2244-16-8	Carraway, dill
15	*R*-(−)-Carvone	M	$C_{10}H_{14}O$	6485-40-1	*Mentha dulcis*, *Mentha spicata* (spearmint)
16	1,8-Cineole	M	$C_{10}H_{18}O$	470-82-6	*Eucalyptus globulus*, E. *Citriodora*, laurel, rosemary, aspic, cajeput
17	1*R*,5*R*-(+)-α-Pinene	P[a]	$C_{10}H_{16}$	7785-70-8	Cypress, turpentine oil (N. America),
18	1*S*,5*S*-(−)-α-Pinene	P[a]	$C_{10}H_{16}$	7785-26-4	Turpentine oil (Europa)
19	1*S*,5*S*-(−)-β-Pinene	P[a]	$C_{10}H_{16}$	18172-67-3	Turpentine oil (Europa)
20	1*R*,4*R*-(+)-Camphor	P[a]	$C_{10}H_{16}O$	76-22-2	Camphor tree, spike, rosemary
21	1*S*,4*R*-(−)-Camphor	P[a]	$C_{10}H_{16}O$	464-49-3	*Salvia*

[a] The configuration is defined according to the conventional numbering of carbon atoms in monoterpenes.

successive steps, and the isotopic balance was checked at every step by isotope ratio mass spectrometry (IRMS). Although the resulting isotope ratios are not completely free from fractionation effects, it was verified that, for a given monoterpene, any influence of the technical treatment on the isotopic values remained relatively small and well below variations associated with different plant species.

The site specific hydrogen isotope ratios, $(D/H)_i$, of every purified sample were determined by the SNIF-NMR method (Martin and Martin, 1990). It was first necessary to assign the deuterium signals, labeled in increasing order from high to low chemical shifts, to the corresponding isotopomers (Fig. 1). This was achieved by complete analyses of proton NMR spectra based on one- and two-dimensional experiments and taking into account possible variations due to solvent effects. The molar fractions, f_i, of isotopomers, i, were obtained by quantitative analysis of the ^2H NMR spectrum. For each monoterpene, the isotope ratios, $(D/H)_i$, were first determined by an internal referencing method using an official standard (tetramethylurea) of known isotope content. The results were then compared with those of an external referencing method that combines the isotopomer molar ratios determined by NMR with the value of the overall hydrogen isotope ratio of the molecule measured by IRMS Eqs. (2), (3). The agreement between the results of both methods was satisfactory and so, in order to obtain a homogenous set of data, all the samples were analyzed using the more sensitive external referencing method.

To facilitate comparisons within the terpene family the hydrogen atoms are not numbered according to the IUPAC rules but according to the numbering of the geranylphosphate precursor. In Fig. 1, the molecules being investigated are classified into the three structural families, geranilane, menthane and pinane (camphane) (Erman, 1988). This figure gives the relation between the NMR signal number and the ex-GPP number of the atom, which will be used in the following dicussion to identify hydrogen positions in the monoterpenes. This correspondence is established on the basis of the biosynthetic routes elucidated by thorough biochemical studies of every monoterpenic species (Banthorpe et al., 1972; Croteau, 1987; Croteau et al., 1994; Hiraga et al., 1993; Pyun et al., 1993; Schwab et al., 2001; Suga et al., 1992; Wagschal et al., 1991, 1994). Inasmuch as they are consistent with these schemes of hydrogen connections, our results may provide a further verification of the whole set of accepted pathways. In this respect, it should be emphasized that, in the NMR spectra of enantiomeric pairs of monoterpenes, the affiliation of diastereotopic methylenic hydrogen atoms to the corresponding hydrogen atoms in GPP must be inverted.

Values of the site specific isotope ratios, $(D/H)_i$, averaged over the specified numbers of samples are given in Table 2. As previously discussed, the nature of the plant source and the environmental conditions of its growth may significantly influence the isotopic profile (Hanneguelle et al., 1992; Martin and Martin, 2003). The resulting dispersion in the isotopic ratios has been illustrated in the case of linalool. However, since it is not

Table 2
Site specific hydrogen isotope ratios, $(D/H)_i$ (in ppm), and overall isotopic deviations, $\delta^{13}C$ (in ‰), of monoterpenes extracted from plants with C3 or C4 metabolism

Geraniol	$\delta^{13}C$	C1	C2	C4	C5	C6	C8	C9	C10	OH	
NMR site	‰	4	1	6	5	2	9	8	7	3	
C3	−28.5	148.0	33.4	104.0	142.6	115.3	97.1	113.9	95.5	201.5	
2	0.1	4.0	2.3	4.4	0.7	2.9	1.5	6.8	0.2	12.1	
C4	−12.6	155.6	28.5	114.3	153.9	53.2	105.5	132.0	104.2	207.0	
5	1.2	8.8	6.8	3.9	6.1	4.7	6.7	6.4	4.2	31.3	
Linalool		C1[a]	C1′[a]	C2	C4, 4′	C5, 5′	C6	C8	C9	C10	OH
NMR site		2	4	1	9	6	3	8	7	10	5
R-(−),*S*-(+)[b]	−27.6	128.6	135.0	31.1	136.6	174.0	118.3	121.3	106.8	109.2	177.2
22	1.6	7.3	8.7	9.3	12.8	15.4	18.6	7.2	8.2	10.3	25.0
Linalyl acetate		C1, 1′, C6	C2	C4,4′,C8,C9	C5,5′ CH_3CO	C10					
NMR site		2, 3, 4	1	7, 8, 9	5, 6	10					
Natural	−28.1	131.4	40.2	112.7	163.2	104.3					
4	0.6	4.2	7.4	10.1	7.3	5.0					
Citronellol		C1, 1′	C2′, 3, 8, 9	C2, 4′	C4	C5	C5′	C6	C10	OH	
NMR site		3	6,7,8	9	10	5	4	1	11	2	
C3 *S*-(−)[c]	−28.1	112.0	113.1	93.7	124.2	141.6	141.5	60.2	100.6	158.3	
2	1.1	5.4	4.9	14.4	21.0	3.9	3.7	17.1	1.5	16.7	
		C1	C2, 3, 8, 9	C2′, 4	C4′	C5	C5′	C6	C10	OH	
NMR site		3	6,7,8	9	10	4	5	1	11	2	
C4 *R*-(+)[c]	−11.9	103.6	120.3	113.3	110.9	154.2	154.3	48.7	107.2	151.2	
2	0.6	11.8	6.6	0.7	13.8	4.4	4.2	7.2	4.5	35.9	
Citronellal		C1	C2	C2′	C3, C5, C5′	C4	C4′	C6	C8	C9	C10
NMR site		1	3	4	5,6,7	10	11	2	9	8	12
C3 (*d, l*)[c]	−28.1	111.7	75.2	81.7	151.7	96.6	100.7	71.3	123.3	108.0	117.4
5	0.4	8.7	9.8	8.6	6.5	6.5	5.4	5.0	6.1	7.7	6.1
NMR site		1	4	3	5,6,7	11	10	2	9	8	12
C4 *R*-(+)[c]	−11.4	96.7	101.7	96.4	156.3	108.9	92.1	51.9	132.9	109.5	116.4
4	0.5	5.0	7.3	8.9	8.1	8.1	4.0	4.7	6.0	2.9	4.9
Citral[d]		C1(*E Z*)	C2(*E Z*)	C4(*Z*)	C4E, C5, C10E	C6(*E Z*)	C8, C9	C10(*Z*)			
NMR site		1,2	3	6	7,8,9	4,5	11,12,13	10			
C3	−26.0	132.0	110.8	141.0	145.9	74.8	102.6	133.5			
7	0.7	11.2	21.9	23.6	5.7	13.9	5.2	11.7			
C4	−9.0	144.5	118.4	133.9	143.3	45.9	116.7	144.6			
1											
Limonene		C1 C4	C1′, C4′, C6	C2	C5	C5′	C8(*E Z*)[e]	C9[e]	C10		
NMR site		6,7	3,4,5	1	8	11	2	9	10		
R-(+)	−27.6	142.7	122.9	45.1	179.5	160.9	147.5	91.9	111.7		
8	0.9	13.5	11.1	8.9	9.3	8.3	5.2	4.8	4.2		
		C1′, C4′	C1, C4, C6	C2	C5	C5′	C8(*E Z*)	C9	C10		
NMR site		6,7	3,4,5	1	11	8	2	9	10		
S-(−)	−28.3	121.2	146.9	32.3	165.9	154.8	123.7	99.2	114.1		
4	0.6	4.5	8.2	8.7	3.9	8.9	24.7	7.0	2.1		

Carvone		C1, C5′	C1′, C5	C2	C6	C8(*E Z*)	C9	C10					
NMR site		5	4	1	3	2	6	7					
4*S*-(+)	−26.8	152.7	165.8	28.6	103.0	160.0	102.1	122.0					
3	1.1	9.6	4.7	8.2	18.3	7.0	1.1	1.8					
NMR site		4	5	1	3	2	6	7					
4*R*-(−)	−26.0	155.5	150.0	29.3	127.1	148.0	93.7	109.3					
2	1.5	6.8	0.1	2.2	2.2	4.2	7.6	3.7					
1,8-Cineole		C1, C5	C1′, C5′, C2′, C4′	C2, C4	C6	C8, C9	C10						
NMR site		1	3[f]	2	4	5	6						
Eucalyptus globulus	−28.0	149.9	106.5	129.2	136.8	102.4	112.7						
10	1	6.1	5.4	6.8	23.3	4.4	5.0						
Other[g]	−26.9	157.8	102.2	137.5	103.0	110.2	122.1						
11	2	7.8	4.4	12.3	22.3	4.8	5.8						
α-*Pinene*		C1	C1′	C2	C4	C5	C5′	C6	C8 (C9)	C9 (C8)	C10		
NMR site		2	9	6	1	3	4	5	10	8	7		
1*R*,5*R*-(+)	−26.6	153.3	149.1	40.8	124.2	165.5	148.1	140.1	110.6	91.6	115.8		
4	1.9	2.3	4.6	4.1	10.4	6.5	4.9	6.5	2.9	4.1	1.7		
NMR site		9	2	6	1	3	4	5	10	8	7		
1*S*,5*S*-(−)	−25.8	159.1	151.0	37.7	93.3	162.9	157.3	152.9	111.9	90.5	119.5		
4	1.9	3.3	4.0	7.7	1.2	7.9	7.0	5.1	3.8	4.8	5.9		
β-*Pinene*		C1	C1′	C2	C4	C4′	C5	C5′	C6	C8 (C9)	C9 (C8)	C10	C10′
NMR site		10	2	4	3	6	8	9	7	12	11	1	2
1*S*,5*S*-(−)	−28.4	153.4	141.7	33.8	172.7	107.9	173.1	139.4	164.2	97.9	81. 8	130.4	121.1
4	1.7	3.6	3.9	5.1	6.7	5.6	8.4	4.7	9.4	1.9	1.3	3.6	3.9
Camphor		C1	C1′	C4	C4′	C5	C5′	C6	C8 (C9)	C9 (C8)	C10		
NMR site		1	4	5	6	7	3	2	8	10	9		
1*R*-(+)	−25.3	147.7	149.5	130.2	94.8	152.1	140.6	97.4	111.5	93.6	111.5		
7	1.9	7.6	9.6	12.9	10.9	12.0	10.2	17.0	5.3	7.4	5.9		
NMR site		4	1	6	5	3	7	2	8	10	9		
1*S*-(−)	−27.2	145.7	148.5	116.6	108.1	151.3	147.6	125.0	110.9	87.7	113. 7		
2	0.5	9.1	15.8	16.1	20.1	6.5	3.8	30.4	0.1	5.4	5.0		

The reported values of $(D/H)_i$ and $\delta^{13}C$ data are the means and standard deviations over different samples. The numbers of samples investigated are given in the first column and the origins are specified in Table 1. C_i refers to hydrogen atom at carbon site i in the GPP precursor (Fig. 1). When two diastereotopic hydrogen atoms at site i are distinguished they are denoted C_i and C_i'. "NMR site" denotes the numbering of the signal in the ^{2}H-NMR spectrum (in the direction of increasing screening constants). The investigated samples frequently exhibit different enantiomeric enrichments.

[a] H-1 pro-*R* and H-1 pro-*S* of GPP correspond respectively to positions trans and cis with respect to H-2 in *R* -(−)-linalool.

[b] The reported values are the means over 20 *R*-(−) samples (10 lavender, 2 spike lavender, 2 bergamot, 3 bois de rose, 3 petitgrain) and two *S*-(−) samples (coriander).

[c] The average enantiomeric enrichment is low.

[d] Since geranial (*E*) and neral (*Z*) are not separated, computations require in addition the knowledge of the relative concentrations.

[e] The elimination is 100% regiospecific with limonene cyclase in citrus. The regiospecificity is poorer with pinene cyclases (Pyun et al., 1993).

[f] The two signals corresponding to sites-1′,5′ and -2′,4′ are too close to be integrated with precision; however it may be concluded that the signal with the smallest chemical shift, which pertains to ^{2}H-2′,4′, is strongly depleted with respect to ^{2}H-1′,5′.

[g] mainly rosemary.

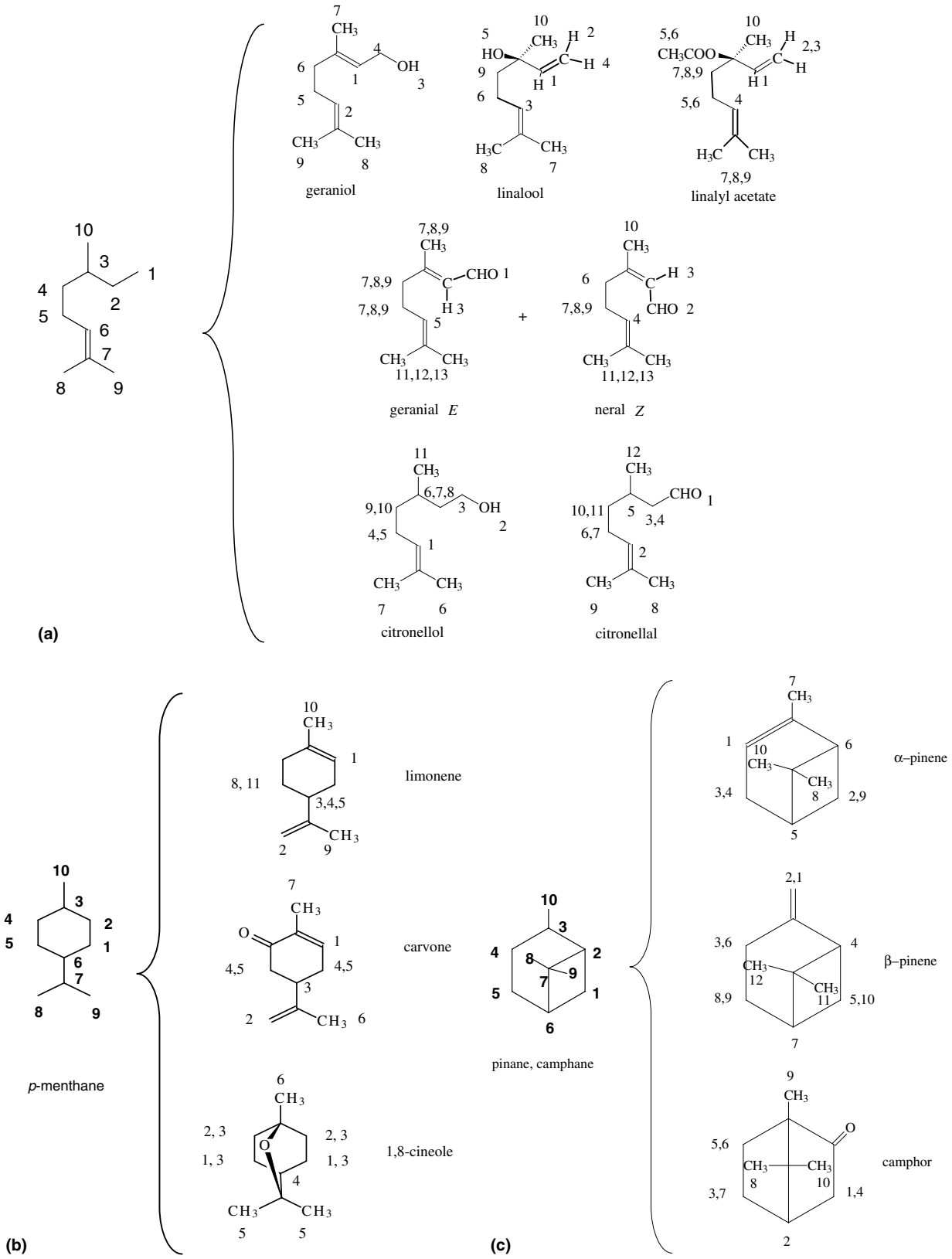

Fig. 1. Correspondence between the carbon atoms of the three investigated monoterpenes families and those of the geranyl diphosphate precursor, and connection with the number of the isotopomer signal in the NMR spectrum. (a) geranilane (b) *p*-menthane (c) pinane familly. The terpene nomenclature is given in the left-hand side column. The numbering of the NMR signal, increases from low to high screening constants. The main NMR signal numbers are reported on the terpene formulae. However, in chiral molecules, the NMR spectrum is more complex since hydrogen atoms borne by the same carbon are frequently differentiated. More detailed assignments are given in Table 2.

Table 3
Relative deuterium enrichments (or impoverishments), e_i%, at sites i of monoterpenes Eq. (4)

Terpene	e_i (%)											
Geraniol	C1	C2	C4	C5	C6	C8	C9	C10	OH			
C3	29.4	−70.8	−9.1	24.7	0.8	−15.1	−0.4	−16.5	76.1			
C4	29.6	−76.2	−4.8	28.2	−55.7	−12.2	9.9	−13.2	72.4			
Linalool	C1	C1′	C2	C4	C5	C6	C8	C9	C10	OH		
R-(−),*S*-(+)	4.2	9.4	−74.9	10.6	41.0	−4.2	−1.8	−13.6	−11.6	43.4		
Linalyl acetate	C1, 1′, C6	C2	C4, 4′, C8, C9	C5, 5′, CH_3CO	C10							
S-(−)	6.7	−67.5	−8.6	32.5	−15.3							
Citronellol	C1, C1′	C2′, 3, 8, 9	C2,4′	C4	C5	C5′	C6	C10	OH			
C3 *S*-(−)	0.0	0.8	−16.7	11.1	26.2	26.1	−46.6	−10.3	40.9			
	C1, C1′	C2, 3, 8, 9	C2′, C4	C4′	C5	C5′	C6	C10	OH			
C4 *R*-(+)	−11.5	2.9	−3.0	−4.7	32.2	32.3	−58.4	−8.2	28.9			
Citronellal	C1	C2	C2′	C3, C5, C5′	C4	C4′	C6	C8	C9	C10		
C3 (*d l*)	−1.4	−33.6	−27.8	33.9	−14.7	−11.1	−37.0	8.8	−4.6	3.7		
C4 *R*-(+)	−16.8	−12.5	−17.0	34.4	−6.4	−20.8	−55.5	14.3	−5.8	0.1		
Citral	C1(*E Z*)	C2(*E Z*)	C4(*Z*)	C4E, C5, C10*E*	C6(*E Z*)	C8, C9	C10(*Z*)					
C3	9.1	−8.6	16.5	20.6	−38.2	−15.1	10.3					
C4	15.2	−5.6	6.8	14.2	−63.4	−7.0	15.3					
Limonene	C1, C4	C1′, C4′, C6	C2	C5	C5′	C8(*E Z*)	C9	C10				
R-(+)	17.8	1.5	−62.8	48.2	32.8	21.7	−24.2	−7.8				
	C1′, C4′	C1, C4, C6	C2	C5	C5′	C8(*E Z*)	C9	C10				
S-(−)	0.8	22.2	−73.2	38.0	28.8	2.8	−17.4	−5.1				
Carvone	C1, C5′	C1′, C5	C2	C6	C8(E Z)	C9	C10					
S-(+)	21.3	31.8	−77.1	−18.3	27.2	−18.8	−2.9					
R-(−)	30.2	25.7	−75.5	6.4	23.9	−21.6	−8.5					
1,8-Cineole	C1, C5	C1′, C5′, C2′, C4′	C2, C4	C6	C8, C9	C10						
Eucalyptus globulus	29.4	−8.0	11.5	27.4	−11.5	−2.7						
other	33.4	−13.6	16.1	−12.8	−6.9	3.1						
α-*Pinene*	C1	C1′	C2	C4	C5	C5′	C6	C8 (C9)	C9 (C8)	C10		
1*R*,5*R*-(+)	30.8	27.2	−65.2	6.0	41.2	26.4	19.6	−5.6	−21.9	−1.2		
1*S*,5*S*-(−)	35.5	28.5	−68.0	−20.5	38.7	33.9	30.1	−4.7	−23.0	1.7		
β-*Pinene*	C1	C1′	C2	C4	C4′	C5	C5′	C6	C8 (C9)	C9 (C8)	C10	C10′
1*S*,5*S*-(−)	30.8	20.8	−71.2	47.2	−8.0	47.6	18.8	40.0	−16.5	−30.3	11.2	3.2
Camphor	C1	C1′	C4	C4′	C5	C5′	C6	C8 (C9)	C9 (C8)	C10		
1*R*-(+)	27.1	28.5	11.8	−18.5	31.0	20.9	−16.3	−4.2	−19.6	−4.2		
1*S*-(−)	24.0	26.4	−0.8	−8.0	28.8	25.6	6.4	−5.6	−25.3	−3.2		

Signal assignments and complementary information are given in Table 2.

possible to extract significant quantities (≈200–500 mg) of all the molecules to be studied from the same plant, we have considered this dispersion as a second order effect and the reported mean values are only distinguished according to the metabolic type. The maximum relative magnitudes of the dispersion corresponding to individual sites may be appraised from the values of the standard deviations given in Table 2.

It is also convenient, in order to compare the different isotope profiles, to compute the deviations of the isotopomeric molar fractions, f_i, with respect to the statistical distribution, F_i. The excess parameters, e_i, defined in Eq. (4) are gathered in Table 3.

3. Discussion

From the isotopic point of view, reactions occurring in Nature are remarkably reproducible in given environmental conditions. As a consequence, for a given molecular species the isotopic fingerprint can be analyzed in terms – of the nature of the raw materials – of individual connections between hydrogen atoms of reactants and products – of stereospecificity, regioselectivity and branching of certain transformations and – of possible occurrence of partial or total intermolecular hydrogen exchanges (Martin et al., 1986b; Zhang et al., 1995, 2000). The isotopic distribution in monoterpenes is the result of complex chains of reactions starting from carbohydrates. Many steps are kinetically invisible in vivo because, in the absence of branching, they may be considered as taking place in a closed system and are therefore devoid of isotopic fractionation whatever the extent of the possible kinetic isotope effects may be. The final isotopic profile is dictated by the genealogy and is mainly governed by the site-specific isotope contents of the precursors, including water, and by active fractionation effects introduced at isotopically sensitive branching points.

3.1. Characterization of the origin of GPP: influence of plant metabolism

In principle, the isotopic profile of monoterpenes is related to that of geranyl diphosphate (GPP), which is generally considered as their common intermediate precursor. As evoked in Section 1, recent studies have questioned the role of the mevalonate pathway that had been traditionally accepted as the terpene biosynthesis pathway. Although atypical behavior is always possible, this mevalonate interpretation has now been largely ruled out for monoterpene biosynthesis in plants, to the benefit of the newly identified DOXP mechanism, in which the precursors of GPP are glyceraldehyde 3-phosphate (G3P) and pyruvate. Examined in the light of this new mechanistic route (Fig. 2) the isotopic results given in Tables 2 and 3 demonstrate several striking features.

The strong deuterium depletion previously determined for site-2 of pinene (Martin et al., 1986b) is also observed in site-2 of geraniol, linalool, linalyl acetate, limonene and carvone. This depletion, associated in pinene with a much higher isotope ratio at site ex-6 of GPP could not be reconciled with the mevalonate mechanism in which both atoms have the same origin (Arigoni et al., 1993). It has been recognized recently that these isotopic results offered in fact a good argument for rejecting the mevalonate pathway and that they are in favor of the new DOXP pathway (Rieder et al., 2000; Schmidt et al., 2003) (Fig. 2). In this scheme, G3P and pyruvate lead to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) the building blocks of GPP. It has been proven that IPP may isomerize into DMAPP, in a reversible reaction, and the biosynthesis of GPP was previously thought to result from the assembling of these two dependent entities (Bentley, 1970; Erman, 1988). However there is now increasing evidence that IPP and DMAPP may be synthesized independently from a common precursor intervening at the end of the DOXP sequence (Rieder et al., 2000; Rodriguez-Concepcion et al., 2000). Our results are particularly informative in this respect. In the independent synthesis of IPP and DMAPP the hydrogen at site-2 of GPP is the ex- pro-*S* hydrogen at site-2 of IPP which was introduced in the end step (Rohdich et al., 2001; Schuhr et al., 2003), with, according to our results, a high isotope effect. The observed depletion is much higher than that which usually characterizes direct hydride transfer from NADPH (cf. fractionation effect at site-4 discussed later on). According to recent investigations (Adam et al., 2002; Altincicek et al., 2002; Rohdich et al., 2002, 2003; Wolff et al., 2002), H-2, has been introduced in the form of a proton in an ispH-catalyzed (1-hydroxy-2-methyl-butenyl 4-diphosphate reductase) reaction, which requires auxiliary flavoprotein enzymes. The reproducible fractionation factor, of the order of 5 with respect to water, determined in a great variety of samples from the monoterpene family (Table 2) therefore provides a reliable criterion for such a proton transfer, and consequently for rapidly characterizing a DOXP origin of terpenoid precursors.

Hydrogen at site-6 is the ethylenic hydrogen of the independently synthesized DMAPP molecule. It previously occupied site-2 of G3P but may have been exchanged with water in the step of glyceraldehyde-dihydroxyacetone phosphate isomerization (Zhang et al., 1995). Relatively high values of the (*D*/*H*) ratio are expected for this kind of exchangeable hydrogen as effectively observed in pinenes and to a large extent in samples of geraniol, linalool, citronellol, (−)-limonene, extracted from plants with C3 metabolism. Interestingly a somewhat different behavior is observed in monoterpe-

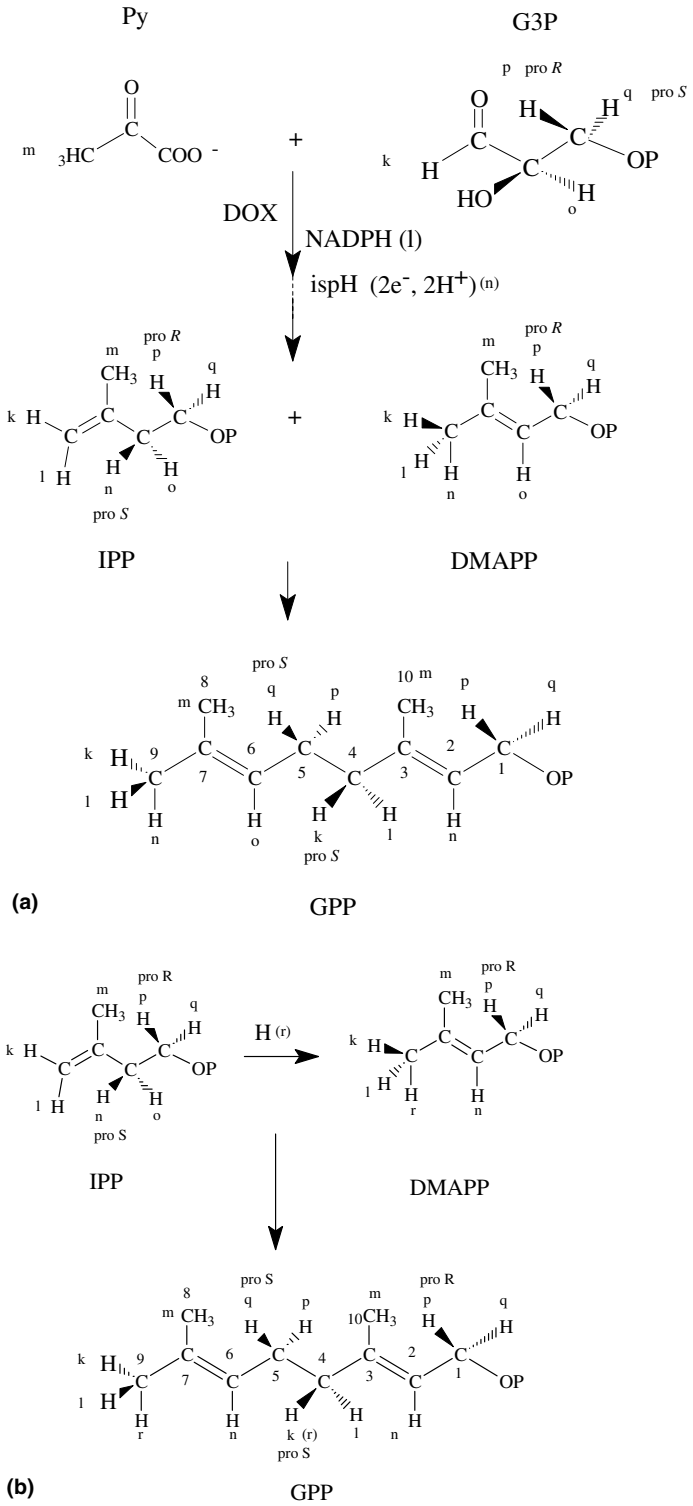

Fig. 2. Hydrogen affiliation of geranyl diphosphate (GPP) to its isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) building blocks and formerly to the pyruvate (Py) and glyceraldehyde phosphate (G3P) precursors. In Fig. 2(a) condensation involves IPP and DMAPP molecules biosynthesized independently by the DOXP pathway. In Fig. 2(b) GPP results from the condensation of IPP directly produced in the DOXP mechanism and of DMAPP obtained by isomerization of IPP. Hydrogen atoms l and n are introduced early and lately respectively in the course of the DOXP pathway (Rieder et al., 2000; Rohdich et al., 2001, 2003).

nes extracted from plants with a C4 metabolism. In C4 geraniol for instance, whereas $(D/H)_2$ is still very low, site ex-6 is also strongly depleted. Similarly, in citronellol, citronellal, geranial, neral the $(D/H)_6$ ratio is much smaller for C4 plants than for C3 plants. Since hydrogen-6 of GPP has been introduced from the DMAPP

precursor without cleavage, its origin must be quite different in geraniol from the C3 and C4 plants being studied. A double depletion at sites-2 and -6 of GPP may be explained by the assembling of DOXP-synthesized IPP with DMAPP derived from the isomerization of IPP (Fig. 2). According to the mechanistic routes recognized for isomerization by bacterial or yeast enzymes (Bentley, 1970; Erman, 1988) the pro-*R* hydrogen at site-2 of IPP, issued from G3P, is eliminated whereas the depleted pro-*S* hydrogen is preserved. The strong impoverishment determined at site-6 of many C4 samples is in favor of a similar isomerization mechanism with plant enzymes. However, in this hypothesis, similarly low values of $(D/H)_2$ and $(D/H)_6$ are expected. In fact, in C4 geraniol samples, the deuterium content at site-6 remains higher than that at site-2 by about 20 ppm. This behavior can be explained by contributions of both DMAPP from isomerization and direct DMAPP. The high $(D/H)_6$ values determined in C3 pinenes, in particular, are probably typical of GPP synthesized purely from independently produced IPP and DMAPP molecules. However the smaller $(D/H)_6$ values and the large standard deviations observed in certain C3 monoterpenes, in particular citronellol, are indicative of a partial contribution of DMAPP resulting from isomerization. A third situation could be considered in which DOXP-biosynthesized DMAPP is condensed with IPP resulting from isomerization. Both site-2 and -6 of GPP would then be characterized by relatively high deuterium contents. Contribution of this situation is certainly small in the majority of the investigated samples but it is not excluded in cases, such as those of some limonene samples, where $(D/H)_2$ is noticeably higher than the mean value.

On the basis of the $(D/H)_2/(D/H)_6$, pattern, the SNIF-NMR method may therefore be used for directly estimating the in vivo pools of primary and isomerized IPP-DMAPP molecules and the role of plant physiological characteristics and environmental parameters in biosyntheses involving different plant species and taking place either in laboratory or in field conditions.

3.2. Genealogy of the isotopic distribution in geraniol

From a more general point of view, we can examine to what extent the entire sets of isotopic parameters of the monoterpene family can be rationalized and connected to those of their photosynthetic precursors. We have already shown that glucose, the key product of the photosynthesis, is characterized by a wide range of isotopic values and that the isotopic profile of the carbon-bound hydrogen atoms is very different for sugars derived from plants with C3 and C4 metabolisms (Zhang et al., 2002). Moreover individual connections between natural abundance isotope ratios of sugars and glycolytic products have been precisely characterized (Zhang et al., 1995). In this context, site-specific isotope ratios of geraniol, the most immediate descendant of GPP, may be connected to source carbohydrates. Thus, in the main glycolytic pathway, both methyl groups 8 and 10, which come from pyruvate in the DOXP-pathway, are derived – from the two hydrogen atoms at site-6 of glucose – to a lesser extent from sites -1 and -2 of glucose, and – from water for more than one third (Fig. 3). These methyl groups are both expected to exhibit $(D/H)_i$ values close to those of the methyl group of fermentation ethanol which has a similar pyruvate origin. Indeed, values measured in geraniol from C3 plants (95–97 ppm) are in good agreement with those of C3 ethanol (90–100 ppm) (Martin et al., 1986b). In

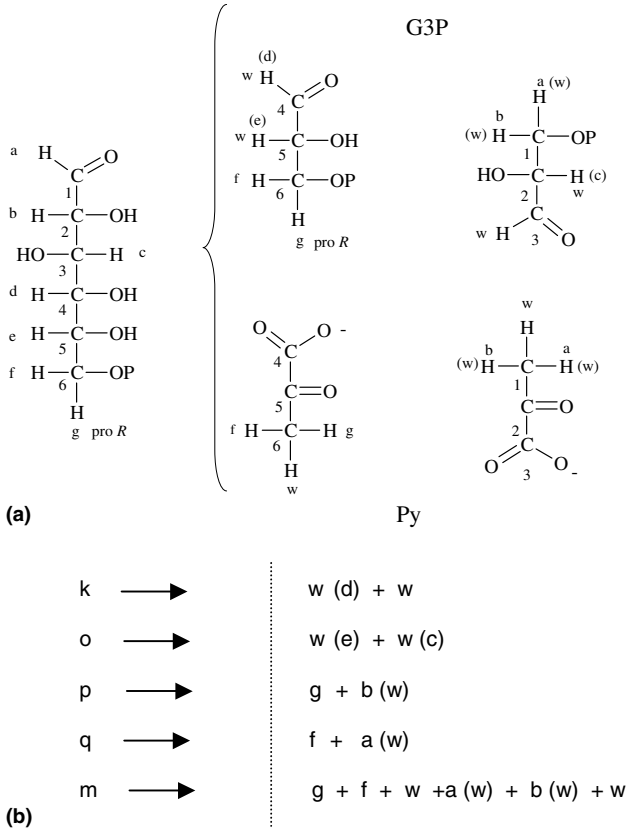

Fig. 3. Hydrogen affiliation of geranyl diphosphate (GPP) with respect to carbohydrates, represented by glucose. (a) Hydrogen affiliation of the pyruvate (Py) and glyceraldehyde phosphate (G3P) precursors of GPP to glucose. (b) Correspondence between labels adopted for denoting hydrogen atoms in G3P/Py on one hand and in GPP (Fig. 2) on the other hand. (Taking into account the multiple origins of the atoms, two sets of letters have been used for clarity of the drawings). Hydrogen atoms introduced from the aqueous medium are denoted by the letter w. When the contribution of water remains relatively small w is inserted within parentheses. Similarly, formal precursor atoms replaced totally or in majority by medium hydrogen atoms in the course of the reaction pathway are denoted by letters within parentheses. For more details see Zhang et al. (1995).

addition, a similar increase is observed for C4 plants (104–110 ppm). Higher deuterium contents in site-6 of glucose from C4 plants as compared to C3 plants may be responsible for this behavior.

Whatever the direct or isomerized origin of IPP and DMAPP, the two methylenic hydrogen atoms at sites-1 and -5 have the same affiliation. They stem from the pro-*R* and pro-*S* hydrogen atoms at site 3 of G3P which are themselves strongly related respectively – to sites-6 pro-*R* of glucose – to site -2 of glucose (largely transferred to site-1 pro-*R* of fructose 6 P) – and to site-6 pro-*S* and site-1 of glucose (Zhang et al., 1995). As expected, nearly equal values of $(D/H)_1$ and $(D/H)_5$ are measured and their order of magnitude is consistent with the considered genealogy. Moreover the higher values observed for C4 as compared to C3 geraniol may be explained on the basis of higher deuterium contents at sites 1, 2 and 6 in C4 sugars (Zhang et al., 2002).

The methylenic hydrogen atoms at site-4 pertained previously to IPP. When GPP is built from IPP and DMAPP that is directly DOXP-synthesized one of these atoms comes from H-1 of G3P and is therefore connected formally to H-4 of glucose and therefore to water through the aldolase reaction step. NADPH introduces the other atom in the second step of the DOXP mechanism (Fig. 2). Since the isotope ratio of water is of the order of 155 ppm, kinetic isotope effects involving the hydrogen transferred from NADPH are probably responsible for the relatively low values of $(D/H)_4$. In this respect we have already observed that hydrogen incorporation from NADH at the methylenic pro-*R* site of fermentation ethanol is associated with a fractionation factor of the order of 1.3 with respect to water (Zhang et al., 1995). It should also be noted that hydrogen exchange with water occurring at site-4 of IPP in the course of the isomerization process has been detected (Bentley, 1970). This phenomenon, which connects site-4 of GPP to the water medium, involves a methyl intermediate and is therefore expected to average the deuterium contents over the geminal positions at methylene-4 of terpenes. In principle, the SNIF-NMR method may therefore be proposed for estimating possible in vivo participation of such an exchange from an analysis of differences in the $(D/H)_4$, and $(D/H)_{4'}$ values that can be observed in enantiomeric molecules biosynthesized from isomerized precursors (see Section 3.3.).

Two of the three hydrogen atoms at site-9 have the same origin as those at site-4. In condensation of independently DOXP-synthesized IPP and DMAPP, which prevails for C3 geraniol, the third atom is introduced into DMAPP at a late step of the DOXP pathway (as the very depleted H-2 pro-*S* of IPP). In contrast, when IPP is condensed with isomerized DMAPP, as prevailing in C4 geraniol, this third atom is introduced from water into DMAPP in the course of the isomerization. The significant deuterium enhancement observed at site 9 of C4 as compared to C3 geraniol is consistent with these different origins of the third atom.

3.3. Isotopic connections in the biosynthesis of monoterpenes

With this relatively rich family of monoterpenes at our disposal, we can examine whether the isotopic parameters are consistent with the mechanistic affiliations for connecting individual sites of the different molecules to those of the geranylpyrophosphate precursor (Fig. 1). Consistency of the results will, on the other hand, attest to the exactitude of the NMR analyses.

Interestingly, in chiral derivatives and especially in cyclic molecules the methylenic positions, which are enantiotopic in geraniol, become diastereotopic and, in principle, can be observed separately in the ^2H NMR spectrum. Therefore the isotope contents at these positions may provide information on the overall stereospecificity of the chain of reaction and on possible scrambling. In this respect, it should be emphasized that, when considering an enantiomeric pair of a given monoterpene, the assignment of the two methylenic ^2H NMR signals to the corresponding positions in the GPP precursor must be inverted. Quantification of methylenic isotopomers therefore provides interesting criteria for characterizing the degree of enantiomeric purity of the sample. For a given monoterpene, the higher the difference between the D/H ratios of geminal hydrogen positions, the higher the optical purity is. In pinenes for instance the deuterium contents in the endo and exo positions corresponding to carbon-5 are inverted, as expected, in the (+) and (−) samples (Table 2) and the difference in the isotope ratios of the two signals may be related to the enantiomeric enrichment of the sample. In addition, when optically pure enantiomers are considered, the SNIF-NMR method provides a mean to selectively trace back the isotope contents in the enantiotopic methylenic positions of the GPP precursor. In cases of incomplete purity, computation of these isotope ratios must take into account the rate of enantiomeric enrichment.

As discussed above, the isotopic parameters of sites-2 and -6 exhibit very typical behavior and they may be used for appraising the origin of the IPP and DMAPP building blocks of GPP. It should be noted that while site-2 is strongly depleted in most molecules it exhibits relatively high mean values in citral and citronellal. In citral $(D/H)_2$ of both the geranial and neral components is of the order of 110 ppm. This behavior may be due to exchange phenomena associated with interconversion of the two stereoisomers at thermodynamic equilibrium (Banthorpe et al., 1983; Potty and Bruemmer, 1970). In citronellal, the second exception, one atom at site-2 is connected to GPP but another one has been

incorporated in the step of reduction of the double bond. Since the two methylenic hydrogen atoms are diastereotopic their isotopic ratios can be measured separately. Taking into account the statistical standard deviations, the computed values are nearly equal. However the average enantiomeric purities of the investigated samples are too low to ensure reliable conclusions. It would be interesting to compare these results with the corresponding values in citronellol. Unfortunately, the $(D/H)_2$ ratio cannot be estimated directly due to signal overlap in the ^{2}H-spectrum.

Although they may have undergone different secondary isotope effects, the individual methylenic hydrogen atoms at positions ex-1 and -5 of GPP should exhibit roughly similar isotopic ratios since they have the same origin (Figs. 2 and 3). The enantiotopic positions are connected to glucose sites-6 pro-*R* and -2 on one hand, and -6-pro-*S* and -1 on the other hand. The isotopic contents of these geminal positions are accessible at the vinylic position of linalool and, more generally, in optically pure monoterpene samples. The differences measured in limonene, pinenes and camphor samples for instance, do not exceed 10–25 ppm, an order of magnitude in agreement with the moderate differences characterizing the two 6-positions and the 1- and 2-positions in glucose from C3 plants (Zhang et al., 2002). In this respect, it should be emphasized that monoterpenes offer new probes for investigating isotopic properties of carbohydrates. These properties, which are not easily accessible directly, have been shown to depend not only on the photosynthetic metabolism but also on physiological features of the plant.

The hydrogen atoms at ex-site-4 of GPP, which are enantiotopic in geraniol, also become diastereotopic in chiral species and they are well distinguished in β-pinene and camphor. It is therefore possible to check the hypotheses advanced above in the interpretation of their affiliation. Thus significant relative impoverishments are observed at the most screened H-4 position in (−)-β-pinene and the two H-4 positions in (+)- and (−)-camphor exhibit, as expected, inverted behavior. Moreover the remaining hydrogen at site-4 of α-pinene is strongly impoverished in (−) samples with respect to (+) samples. The different origin of hydrogen-4 in the two enantiomers confirms and further explains the relatively high sensitivity of site-4 to the enantiomeric purity of α-pinene (Martin et al., 1986a). More generally, these results confirm that, in the considered compounds, no scrambling has occurred and that the depleted hydrogen is related through a stereochemical chain of affiliation to H-4 pro-*R* of GPP, to the vinylic hydrogen of IPP trans to the methyl group, and back to the NADPH hydrogen introduced into 2-*C*-methyl-D-erythritol 4-phosphate by the DOXP mechanism.

In the Eighties, different routes had been proposed for the biosynthesis of carvone. Our results, which confirm the interpretation of in-depth biochemical studies (Bouwmeester et al., 1995; Croteau, 1993), show that a pathway involving a shift of the endocyclic double bond with respect to limonene (Akhila et al., 1980) can be immediately ruled out on the basis of the SNIF-NMR profile. The very high depletion of the ethylenic hydrogen is not consistent with a parental C-4 origin and this position is necessarily related to site ex-2 of GPP.

The isotopic profile of cineole offers another example of the stereochemical potential of the method. As a result of symmetry, the pairs of geminal positions at sites ex-2, 4 on one hand and ex-1, 5 on the other hand are equivalent. Moreover, due to overlap of the endo ex-2, 4 and ex-1, 5 deuterium signals, the corresponding *D*/*H* ratios are not accurately determined (Table 2). Nevertheless it may be concluded from the very strong ^{2}H depletion affecting the endo ex-2, 4-position that the ex-2 hydrogen of GPP has been preserved and occupies the endo position in cineole. Moreover the isotopic profile provides direct information on the stereochemistry of the biosynthetic route starting from the linalyl diphosphate precursor. The hypothesis of equal involvement of the *R* and *S* linalyl precursors may be excluded since it would imply equal values of the $(D/H)_{endo}$ and $(D/H)_{exo}$ ratios for both the ex-2, 4 and ex-1, 5 atoms. Our results show that the enantioselectivity in favor of the *R* precursor, determined by Croteau et al. (1994) through in vitro biochemical experiments also prevails in natural conditions. On the basis of a $(D/H)_{1,5}$ endo value on the order of 150 ppm, a $(D/H)_{2,4}$ endo value of about 65 ppm is roughly estimated. This order of magnitude is consistent with an affiliation of the endo ex-2, -4 hydrogen atoms of cineole to the ex-4 pro-*R* hydrogen originating from NADPH (about 100 ppm) and to the depleted ex-2 hydrogen originating from DOXP-synthesized IPP (about 30 ppm).

The interest of the SNIF-NMR method for estimating certain active kinetic isotope effects (KIE) (Martin and Martin, 1990) has already been illustrated by two examples in the field of terpene biosynthesis (Leopold et al., 1988; Pascal et al., 1986). As confirmed by our results the deuterium enrichment at the exo-methylene group-10 of β-pinene as compared to the methyl group at C-8 may be attributed to a KIE on the deprotonation step of the pinyl cation and the relative enrichment at C-10 with respect to C-8 of α-pinene is consistent with a partitioning of the cation into the two pinenes. Similarly, although the argument invoked for assigning the methyl group undergoing hydrogen elimination cannot be retained (Pascal et al., 1986) the relative enrichment observed at C-8 of limonene may be used for estimating the intramolecular KIE for the methyl-methylene elimination in limonene biosynthesis. On the basis of the present results, further analyses of the isotopic parameters at sites ex-8 and -9 in terms of plant species may be expected to provide

information on the in vivo regioselectivity of the elimination and therefore on the participation of the different kinds of cyclases (Pyun et al., 1993).

Finally it should be emphasized that the present data represent averages over a great diversity of plant species and of plant organs. Moreover differences in climatic environments are responsible, in particular, for different isotopic contents of photosynthetic water. This diversity is reflected in the values of the statistical standard deviations which are significantly higher than the experimental precision. When devoted to the investigation of monoterpenes derived from a given plant species or from plants grown in the same environment, the isotope profile should enable to detect even more subtle effects due in particular to secondary isotope effects revealed at the various branching points of monoterpene biosyntheses. Thus secondary variations in the isotope ratios of enantiomers (Martin et al., 1986a) could be further exploited in the light of the thorough elucidation of branching effects in the formation of α-pinene, β-pinene and camphene by different types of cyclases (Wagschal et al., 1994). More detailed studies should also consider the variety and concentrations of the different terpenic metabolites biosynthesized in a given plant.

Considering the remarkable consistency of the scheme of specific affiliations going back – from the whole family of monoterpenes, – to the key intermediate GPP, – to the IPP and DMAPP building blocks, – to the G3P/pyruvate couple, and ultimately – to photosynthetic precursors, it may be reaffirmed that the SNIF-NMR method provides a very attractive tool for characterizing biosynthetic pathways. Certainly, not all steps of complex mechanisms can be elucidated from the sole investigation of end products, but the present results show that careful analyses of isotopic profiles are particularly helpful – for eliminating certain mechanistic hypotheses, – for elucidating stereochemical pathways, – for detecting and even quantifying intermolecular exchanges, – for identifying isotopically sensitive branching points – and, in certain circumstances, such as hydride and proton transfers at two steps of the DOXP pathway, for estimating the order of magnitude of isotope effects. Undoubtedly, if adopted earlier, this approach would have spared considerable experimental time in the numerous labeling strategies developed for elucidating terpene biosynthetic pathways. Once the affiliation scheme has been elucidated, the method provides now a unique tool for investigating in vivo metabolic responses to physiological characteristics of the plants and, in particular, for estimating possible contributions of the mevalonate pathway and relative fluxes of DMAPP directly synthesized or resulting from isomerization. It also offers a source of information on the effects of environmental factors and on the role of different enzymic equipments.

4. Experimental

4.1. Materials

25 stereoisomers associated with 12 molecular species belonging to three basic monoterpene skeletons were considered (Table 1). The natural compounds were obtained as pure molecules or extracted from about two hundreds plants and essential oils purchased from the herbs and spices trade or kindly provided by academic institutions or flavor and aroma companies. The raw materials utilized are the following (common name, Latin name, organ of the plant used):

Bergamot (*Citrus bergamia* Riss. et Poit.), fruits peel (expression); cajeput (*Melaleuca leucadendron* L.), leaves; caraway (*Carum carvi* L.), fruits; citronella (*Cymbopogon nardus* L. var. *lenabatu* or var. *mahapengiri*), herbs; clary sage (*Salvia sclarea* L.), leaves; coriander (*Coriandum sativum* L.), fruits; cypress (*Cupressus sempervirens* L.), leaves; dill (*Anethum graveolens* L.), fruits; eucalyptus (*Eucalyptus citriodora* L.), leaves; geranium (*Pelargonium graveolens* L'Herit.), leaves; grapefruit (*Citrus paradisi Macf.*), fruits peel (expression); Ho-wood (camphor tree) (*Cinnamomum camphora* L.), wood; laurel (*Laurus nobilis* L.), leaves; lavandin (*Lavandula hybrida*), flowering stems with leaves; lavender (*Lavandula angustifolia* L.), flowering stems with leaves; lemon (*Citrus lemonum* L.), fruit peel (expression); lemongrass (*Cymbopogon citratus* DC or *Cymbopogon flexuosus* Stapf.), herbs; lime (*Citrus aurantifolia* Swingle), fruit or fruit peel (expression or distillation); listea cubeba (*Listea cubeba* L.), leaves; mandarin (*Citrus madurensis* L. Osb.), fruit peel (expression); monarda (*Monarda fistulosa* L.), flowering stems with leaves; orange (*Citrus sinensis* L.), fruit peel (expression); palmarosa (*Cymbopogon martini* Stapf. var. *motia*), herbs; petitgrain (*Citrus aurantium* L. var. *amara* L.), leaves; pine (*Pinus* ssp. or *Abies* spp.), leaves (needles); rose (*Rosa damascena* L.), flowers; rosemary (*Rosmarinus officinalis* L.), flowering stems with leaves; rosewood (*Aniba rosaeodora* var. *amazonica* A. Ducke), wood; sage (*Salvia officinalis* L.), leaves; shiu (*Cinnamomum camphora* L.), wood; spearmint (*Mentha spicata* L.), herbs; spike (*Lavandula latifolia* L.), flowering stems with leaves; turpentine oil (*Pinus* ssp. or *Abies* spp.), gum; verbena (*Lippia citriodora* Kunth.), leaves.

4.2. Extraction and purification

A steam distillation apparatus at atmospheric pressure was used to extract the terpene molecules from plants. The extraction took several hours, depending on the plant type. For some products, (i.e. carvone) extracting the remaining water with a mixture of pentane and ethyl ether increased the yield.

Essential oils and laboratory extracts were analyzed by gas chromatography in order to estimate the concentration of active molecule that is removed from the mixture by preparative low pressure liquid chromatography and eluted on a polar phase (Silica gel Merck 9385) with increasing amounts of diethyl ether in pentane. The solvents were then evaporated carefully to avoid any isotopic fractionation of the residual product. In some cases (pinenes, citral, limonene) a simple distillation with a rotating band gives satisfactory results.

The optical rotation of samples involving enantiomers, which confirms the natural status of the product, was determined in an ethanol solution at 20 °C using a Perkin–Elmer 241 spectropolarimeter.

The overall δ ^{13}C values of the products under study were measured before and after any physical treatment in order to detect possible isotopic fractionation due to the extraction process.

4.3. Isotope ratio mass spectrometry

The overall hydrogen isotope ratios, $(D/H)_{ms}$, were measured off-line on hydrogen gas. The purified products were burnt into carbon anhydride and water in a Carlo Erba micro-analyzer. The nitrogen oxides were reduced on a copper catalyst in order to avoid subsequent poisoning. The combustion water was separated from the carbon anhydride by differential cooling and Zn reduced at 550 °C into hydrogen gas, which was analyzed with a VG SIRA 9 isotope ratio mass spectrometer. The isotope hydrogen ratios measured with respect to the V.SMOW reference are expressed in ppm on the absolute scale by using the value $(D/H)_{V\cdot SMOW} = 155.76$ ppm (Craig, 1961; Hagemann et al., 1970).

The repeatability of the analytical chain is of the order of 0.2 ppm. The standard deviation of the $(D/H)_{ms}$ value for all the individuals of a given group, computed from the weighed variance, is of the order of 2–3 ppm.

The $^{13}C/^{12}C$ isotope ratios were measured on carbon anhydride using a Delta E Thermo-Finnigan spectrometer, on line with a Carlo Erba micro-analyzer. They are reported in ‰ on the conventional δ-scale in which the isotope ratios are referred to the V.PDB reference (Coplen, 1994).

$$\delta = [(R_i/R_{ref}) - 1)]1000, \quad (1)$$

where R_i and R_{ref} are respectively the isotope ratio of the product i and of the reference, V.PDB.

The precision is of the order of 0.2‰ and the mean standard deviation is smaller than 2‰.

4.4. Nuclear magnetic resonance

4.4.1. Proton spectroscopy

Correct assignment of the deuterium signals required the prior determination of the hydrogen chemical shifts from the proton NMR spectra of the 12 investigated molecules. These experiments were performed on Bruker AM 400 and AM 500 NMR spectrometers. Two-dimensional sequences exploiting Overhauser and $^{1}H–^{13}C$ chemical shift correlations were carried out when useful. In most cases, the proton chemical shifts and coupling constants thus obtained are in agreement with values published in the literature (see for instance the 2003 on-line database from the National Institute of Advanced Industrial Science and Technology, Tsukuba, Ibaraki, Japan). When assigning the ^{2}H spectra, it is necessary to take into account possible differential solvent effects resulting from different concentrations of the samples investigated in proton and deuterium NMR. In particular, certain chemical shift inversions of close methyl signals have been detected. Some ambiguities may subsist in the assignment of these methyl signals, which are usually devoid of informative coupling constants in the ^{1}H NMR spectrum.

4.4.2. Deuterium spectroscopy

The ^{2}H NMR spectra were recorded at either 61.4 or 76.7 MHz with dedicated Bruker AM400 or AM500 spectrometers equipped with a fluorine-locking device. The experimental conditions were the following: broad-band proton decoupling, frequency window 1200–4800 Hz, memory size 32 K, exponential multiplication associated with a line broadening of 1 Hz. About 2400–3800 scans were accumulated, depending on the concentration. The samples were dissolved in carbon tetrachloride (1/3) and hexafluorobenzene (50 μl) was added as a locking material.

Signal intensities were determined using the dedicated software Eurospec (derived from the former version Interliss) (Martin, 1994). Two methods were used to determine the values of the isotope ratios. In the purely NMR method, an internal referencing was carried out by adding to the sample a working standard, WS, with a known isotope ratio, $(D/H)_{WS}$. Signal intensities of the isotopomers are then referred to that of WS (Martin and Martin, 1990). The requirements for a good working standard are manifold but this procedure is specially valuable when a great number of samples with the same structure has to be recorded. *N*,*N*-Tetramethyurea (TMU) has been proved to be a suitable internal reference for quantifying the ethanol probe and it is now distributed as a certified standard by the Institute for Reference Materials and Measurements (Joint Research Center, Geel, Belgium). This TMU reference is also convenient for many monoterpenes. However, since we were faced with the study of molecules very different in terms of magnetic properties (relaxation rate, line width, chemical shift dispersion, etc) the method could not be generally optimized. Moreover the use of an internal reference is detrimental to the sensitivity. Therefore we have also treated the whole set of samples

according to the external referencing procedure which combines the NMR determination of the isotopomer molar fractions, f_i, with the determination of the overall isotope ratio, $(D/H)_{ms}$, by IRMS Eq. (2) :

$$(D/H)_i = \frac{f_i (D/H)_{ms}}{F_i}, \quad (2)$$

F_i, the statistical molar fraction of isotopomer i, is computed by Eq. (3)

$$F_i = \frac{p_i}{\sum p_i}, \quad (3)$$

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

In the presence of hydroxyl groups the measured overall $(D/H)_{ms}$ values integrate fractionation effects due to exchange phenomena associated with the extraction and purification steps. However these perturbations may be largely taken into account since they appear in the NMR determination of the relative values of the hydroxyl deuterium contents.

We have checked, for most monoterpene structures, that the internal and external referencing procedures give consistent results.

In order to facilitate the comparison between isotopomer distributions within the monoterpene family we have also computed the relative enrichments (e_i) or depletions ($-e_i$) associated with every molecular site i. For a given molecule, e_i (%) is defined as the increase (decrease) of the molar fraction of isotopomer i with respect to the statistical fraction F_i:

$$e_i = 100[(f_i - F_i)/F_i]. \quad (4)$$

The standard deviation of repeatability, Sr, in the determination of f_i and e_i depends not only on the nature of the investigated molecule but also on the nature of the isotopomer. Usually, isotopomers with relatively long relaxation times, and therefore small line widths, have the smallest standard deviation of repeatability. For example, Sr is of the order of 0.8% for cineole or limonene and 3% for camphor or geraniol. Similarly, relatively large spreads of Sr values may be observed among the different isotopomers of a given species. Thus the two gem methyl isotopomers of camphor exhibit the following line widths and Sr values: 1.4 Hz and 1%; 1.7 Hz and 4.9%.

In general, assuming a normal distribution of the measurements (Martin and Naulet, 1988), the least significant difference, "LSD", between two values of enrichment can be computed from Student statistics in terms of the standard deviations of both measurements. Thus, at the 95% confidence level, and for NE = 5 replications of the spectrum, the LSD value increases from 2 to 11 when Sr changes from 1% to 7%. Moreover, higher the number of replications NE for a given sample, smaller is the LSD value. For instance, with a standard deviation equal to 3%, the LSD value increases from 3.4 to 4.9 and 6.3 when NE decreases from 10 to 5 and 3 respectively. In this context, the difference in enrichment of C6 in cineole from *Eucalyptus globulus* ($e_6 = 27.4$) and from rosemary ($e_6 = -12.8$) is highly significant (>99%). In contrast, due to the relatively high value of the standard deviation (4.1%), the difference observed in sites-2 of C3 ($e_2 = -71$) and C4 ($e_4 = -76$) geraniol is not significant. Partial overlap of deuterium NMR signals is usually responsible for relatively high LSD values. In most of these cases we have not reported separate values of the isotopic parameters but have computed less specific values representing an average over the parameters of the overlapping signals.

Acknowledgements

We are grateful to Dr. Peyron, Dr. G. Collin, (Univ Quebec), A. Zola (Adrian), Dr. H. Boelens (Bordas), Dr. R. Pisano (Citrus and Allied Essences), Dr. D. Joulain (Robertet), M. Stagliano (Roure), Dr. G. Georges (formerly Sanofi Bioindustrie) and several other aromas and perfumes companies (Apaesa, Argeville, Cavallier, Munoz Galvez, Rouvière) for providing us with authentic essential oils. Dr. M. Lees (Eurofins Scientific) is also acknowledged for her critical reading of the manuscript.

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