Natural Deuterium Distribution in Branched-Chain Medium-Length Fatty Acids is Nonstatistical: A Site-Specific Study by Quantitative ^{2}H NMR Spectroscopy of the Fatty Acids of Capsaicinoids

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*Quantitative ^{2}H NMR spectroscopy has been used to determine the natural abundance site-specific ^{2}H isotopic content of 6,7-dihydrocapsaicin (**1**) and capsaicin (**2**). Prior to analysis, the fatty acyl moieties were released as methyl 8-methylnonanoate (**3**) and methyl* E*-8-methylnon-6-enoate (**4**), respectively. A marked and similar nonstatisitical isotopic distribution of ^{2}H is observed for both fatty acids. Notably, it can be seen that: 1) the isobutyl portion of **3** is more impoverished in ^{2}H than the methylenic portion; 2) the isobutyl portion of **4** is more impoverished than that of **3**; 3) an alternating pattern occurs in the $(^2H/^1H)_i$ between the C3 to C7 positions; and 4) the ethylenic hydrogens at C6 and C7 of **4** are, respectively, impoverished and unchanged relative to these positions in **3**. These observations are compatible with the proposed biosynthetic origins of the different parts of **1** and **2**, and with the view that **1** is a proximal precursor of **2**. Furthermore, it can be suggested that, firstly, the hydrogen atoms at C3 to C7 originate alternatively from the substrate and from the environment and, secondly, that the Δ^6-*E desaturation is introduced by a mechanism closely mimicking that of the* Z *desaturation of higher plants.*

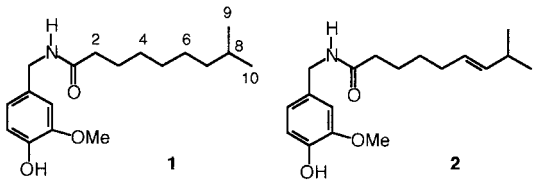
KEYWORDS:

biosynthesis · deuterium · fatty acids · isotope effects · NMR spectroscopy

Introduction

The use of quantitative ^{2}H NMR at natural abundance is proving to be a powerful technique to examine the nonstatisitical distribution of deuterium in natural products.[1–4] A particular interest is to relate the observed variation in site-specific ^{2}H/^{1}H ratios to intrinsic kinetic isotope effects (KIEs). These arise due to the differing physico-chemical properties, reaction velocities and binding constants for the isotopes of hydrogen—^{1}H and ^{2}H (D).[4] Recently, we have been able to show that the introduction of desaturation into the long-chain fatty acids oleic acid and linoleic acid is accompanied by a strong secondary isotope effect.[5] Of particular interest is that this effect is observed at only one of the two carbon centres involved in the reaction, an observation compatible with a mechanism proposed on the basis of experiments with ^{2}H-enriched substrates.[6, 7] In addition, an alternating pattern of the ^{2}H/^{1}H ratio along the length of the chain was observed, which could be related to the source of the hydrogens introduced (acetate or NAD(P)H) during the activity of the fatty acid synthase (FAS).[5]

To date, such isotope effects have only been observed for the *Z* desaturation of the long-chain fatty acids found in plant oils. In order to extend our knowledge of the generality of these phenomena, we have elected to examine the medium-length branched-chain fatty acids (C_{10}) found in capsaicinoids, natural products occurring in the oleoresin extracted from ripe fruits of *Capsicum frutescens*. The principle capsaicinoids present are 6,7-dihydrocapsaicin (**1**) and capsaicin (**2**), amides between a unit of



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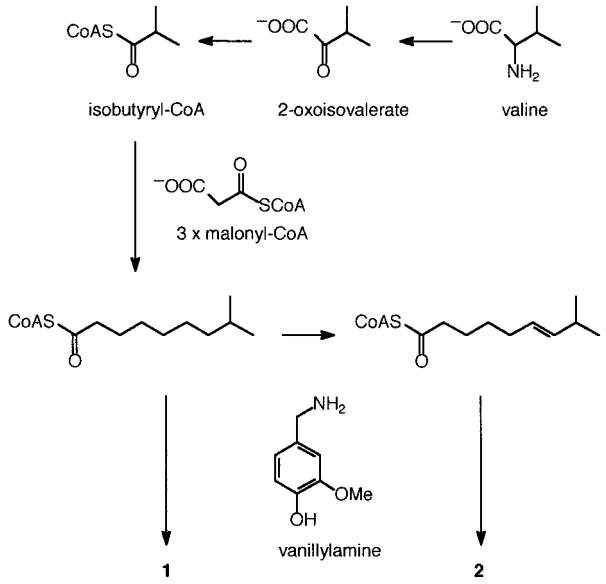
vanillylamine and a C_{10} branched-chain carboxylic acid. These compounds, which are responsible for the hot pungent taste, typically represent about 90% of the capsaicinoid content of the fruits and the oleoresins derived therefrom, with the remaining 10% being a mixture of various homologues.[8, 9]

These compounds provide three particular points of interest. First, **2** contains a Δ^6-*E* desaturation, a configuration rare in plant desaturated fatty acids. Secondly, the presence of the terminal isopropyl group allows the hydrogens at positions 6 and 7 to be distinguished in the NMR spectrum, making it possible to observe their intrinsic $^2H/^1H$ ratios without lengthy degradations. Thirdly, the presence of both **1** and **2** in sufficient quantities in extracts makes it possible to examine their substrate/product relationship.

The biosynthesis of **1** and **2** is intriguing (Scheme 1). The vanillylamine is derived entirely from phenylalanine via vanillin.[10] The fatty acyl moiety has two biosynthetic origins. The four terminal carbons have been shown by ^{14}C labelling to derive



Scheme 1. *Schematic biosynthesis of the capsaicinoids.*

from valine and not, as might be equally plausible, from leucine.[10] 2-Oxo-isovalerate, isobutyrate and 8-methylnonanoic acid were all labelled from valine.[11] Thus, it is probably 2-oxo-isovaleric acid that is the natural precursor, this readily being produced from valine by transamination. The other six carbon atoms appear to originate by the condensation of three units of malonyl-CoA—an origin analogous either to that of straight-chain fatty acids[12] or the flavanoid A-ring.[13] Branched-chain fatty acids isolated from tobacco leaves also show the same labelling from valine.[14] The consistency of ^{14}C-labelling into **1** and **2** indicates a close relationship between these compounds.[10] The origin of the Δ^6-*E* desaturation of **2** is, however, not established. While theoretically this could arise from the incomplete activity of enoyl reductase following the condensation of the isobutyl-ACP unit with the first malonyl-CoA during the formation of the 6-carbon chain,[12] such a mechanism is unknown (ACP = acyl carrier protein). Thus, it is most probable that a fully formed C_{10} fatty acid unit is synthesised, followed by desaturation. Evidence obtained from studies of the condensation of acyl-CoA units and vanillylamine in cell-free extracts supports this route.[15] The *E*-Δ^6 desaturation could then be introduced either by *Z* desaturation followed by epimerisation or by the direct action of an Δ^6-*E*-desaturase.

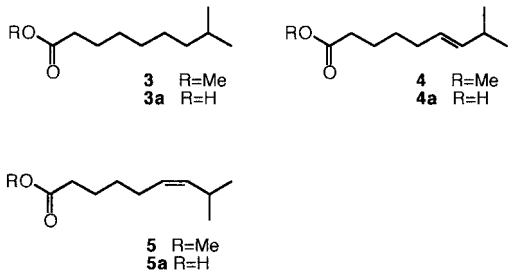
An analysis of the site-specific distribution of deuterium by quantitative ^2H NMR at natural abundance of the methyl esters **3** and **4**—derived respectively from **1** and **2**—allows the direct observation of the $^2H/^1H$ ratios in the fatty acyl moieties of **1** and **2**. A strongly nonstatisitical distribution is found that can be putatively related to various aspects of the biosynthesis of these compounds. In particular, it indicates that the biosynthesis is closely related to that of long-chain fatty acids.

Results

Chemical modification and analytical conditions

Capsaicinoids are complex molecules with substituants of widely differing polarity and have proved difficult to study directly by ^2H NMR spectroscopy. They are not soluble in classical solvents at the high concentration required for quantitative ^2H NMR spectroscopy at natural abundance and give broad peaks. Furthermore, the isolation of pure **1** and **2**, particularly under the stringent conditions required for isotopic analysis, was not practical. Therefore, the strategy employed was to release the fatty acyl moieties as methyl esters from a capsaicinoid preparation by cleaving the amide bond and to study the fatty acyl preparation by ^2H NMR spectroscopy. The advantages of this approach were that, firstly, fatty acyl methyl esters are known to have good ^2H NMR spectroscopy properties[16] and, secondly, that the risk of introducing isotopic fractionation during sample preparation was diminished.

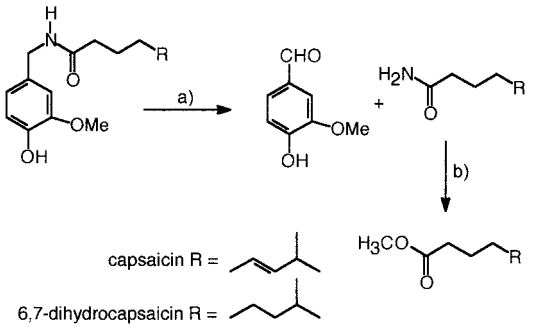
In the first instance, the resolution in the NMR spectra of the resonances due to Me-**3a** and Me-**4a**—methyl esters prepared from synthetic 8-methylnonanoic acid (**3a**) and *E*-8-methylnon-6-enoic acid (**4a**)—was examined. Methyl *Z*-8-methylnon-6-enoic acid (Me-**5a**), the nonnatural isomer of the capsaicin C_{10} fatty acid, was also examined. (In order to distinguish natural and



synthetic products, the synthetic product is designated "a". Thus, **3** and Me-**3a** are chemically equivalent but differ in origin.) Identity of the ^1H and ^{13}C spectra was performed by two-dimensional (2D) heteronuclear NMR spectroscopy. ^1H and ^2H NMR spectroscopic analysis confirmed that the hydrogens

on positions C6 and C7 of Me-**4a** are sufficiently resolved in the NMR spectrum, with the α-isopropyl group deshielding the C7 resonance relative to the C6 resonance sufficiently to create an upfield displacement of 24 Hz ($\Delta\delta = 0.05$). In Me-**3a**, the magnetic environment is also sufficiently affected by the α-isopropyl group to cause the C7 atom to resonate about 60 Hz ($\Delta\delta = 0.12$) upfield of the C6 atom. However, in Me-**3a** the C6 resonance is not resolved from those of the C4 and C5 atoms. Certain positions, notably C2 and C3, show coincident resonances, making it impossible to measure individually the $^2H/^1H$ isotopic ratios at these positions in a mixed preparation. Sufficient resolution is achieved, however, to obtain the desired data from a mixed preparation. Furthermore, the spectral differences between Me-**4a** and Me-**5a** could be used to confirm that no significant configurational change occurred during chemical release of the fatty acyl methyl esters from **1** and **2**.

The fatty acid methyl esters were released in two steps (Scheme 2) with an overall yield of 80–85%. Treatment of a crude capsaicinoid preparation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)/H_2O effectively hydrolysed the amide



Scheme 2. *Chemical modification of capsaicinoids **1** and **2** to release the fatty acyl moieties as their methyl esters. a) DDQ, CH_2Cl_2/H_2O (10:1 v/v); b) Amberlite IR-120 resin, MeOH, 80 °C, 30 h.*

bond,[17, 18] with the released amide being recovered free of phenolic compounds after extraction with ethyl acetate. Without further isolation, the amide was then converted into the methyl ester in a one step deaminative methylation by refluxing with MeOH in the presence of Amberlite IR-120 resin.[19] ^1H and ^{13}C spectral data from the released methyl esters (Table 1) coincided with those obtained from the synthetic samples (see Experimental Section).

^2H NMR analysis of the fatty acyl moieties of capsaicinoids

As the surface below the ^2H NMR signal is directly proportional to the number of monodeuterated isotopomers present,[5] the molar concentration of hydrogen at each signal (P_i, see Experimental Section) must be taken into account in calculating the site-specific $(^2H/^1H)_i$ ratio. Cleavage of commercial crude capsaicin extracts yielded preparations of mixed fatty acyl methyl esters consisting principally of **3** and **4**, but also

Table 1. *NMR spectral data for **3** and **4**.*

Carbon atom	$^{13}C\ \delta$		$^1H\ \delta$[a]		$^2H\ \delta$[b]	
	3	**4**	**3**	**4**	**3**	**4**
1	175.5		–	–	–	–
2	34.2	34.1	2.30		2.30	
3	25.1	24.4	1.58		1.60	
4	29.3	29.2	1.35		1.37	
5	29.5	32.2	1.2–1.3	1.96	1.24–1.31	2.00
6	–	138.2	1.2–1.3	5.30	1.24–1.31	5.35
7	39.1	138.2	1.10	5.36	1.15	5.40
8	28.2	31.1	1.48	2.20	1.50	2.20
9 + 10	14.2		0.83	0.92	0.86	0.95
OCH_3	51.6		3.60		3.60	

[a] ^1H multiplicity and integration gave values identical to synthetic compounds Me-**3a** and Me-**4a**. ^1H displacements are given with respect to tetramethylsilane (TMS) at $\delta = 0.00$. [b] ^2H displacements with respect to tetramethylurea (TMU) at $\delta = 2.80$ relative to TMS at $\delta = 0.00$.

containing a number of minor compounds (Table 2). The majority of these are saturated or nonsaturated homologues of **3** or **4** and co-resonate with peaks from **3** or **4** respectively, as can be seen from the ^2H NMR spectrum (Figure 1). The small contribution of these compounds to P_i has been taken into account in the calculation of $(^2H/^1H)_i$.

Table 2. *Compositional analysis of the samples of capsaicinoids used for isotopic analysis.*

Compound	Methyl ester of:[a]	Sample composition [%][b]	
		cap5	cap6
nordihydrocapsaicin	7-methylheptanoate	3.1	2.1
nonanoyl vanillylamide	nonanoate[c]	1.2	0.9
dihydrocapsaicin	8-methylnonanoate[c]	28.4	28.0
Z-capsaicin	*Z*-8-methylnon-6-enoate[c]	0.0	2.1
capsaicin	*E*-8-methylnon-6-enoate[c]	60.7	60.0
decanoyl vanillylamide	decanoate[c]	1.6	1.6
homodihydrocapsaicin	9-methyldecanoate	1.3	1.5
homocapsaicin II	*E*-8-methyldec-6-enoate	2.6	2.9
homocapsaicin I	*E*-9-methyldec-6-enoate	0.6	0.7

[a] The identity of the peaks was confirmed by GC/MS under identical conditions. [b] A number of trace compounds (< 0.5%) are not included. [c] Compounds verified against authentic standards.

The nonstatisitical site-specific natural isotopic distributions of ^2H in **3** and **4**, derived from **1** and **2** respectively, are reported in Table 3. Two samples, cap5 and cap6, representing independent treatments of separate samples are given. The overall precision of the analysis is satisfactory, with most $(^2H/^1H)_i$ values being determined with an accuracy of < 5%. Furthermore, the repeatability is acceptable, with the two samples having values for $(^2H/^1H)_i$ at all positions in acceptable agreement.

As anticipated, a degree of overlap in the signals is found. The resonances due to the positions C2 and C3 of **3** and **4** are coincident. However, the $(^2H/^1H)$ values for the C2 atom must be treated with caution, as there is a risk of exchange with the solvent during work-up. Otherwise, however, all the resonances of **3** are resolved from the same positions of **4**. Within **4**, all the positions C4–C9 + C10 can be resolved, while in **3** the resonances of the C4, C5 and C6 hydrogens are coincident.

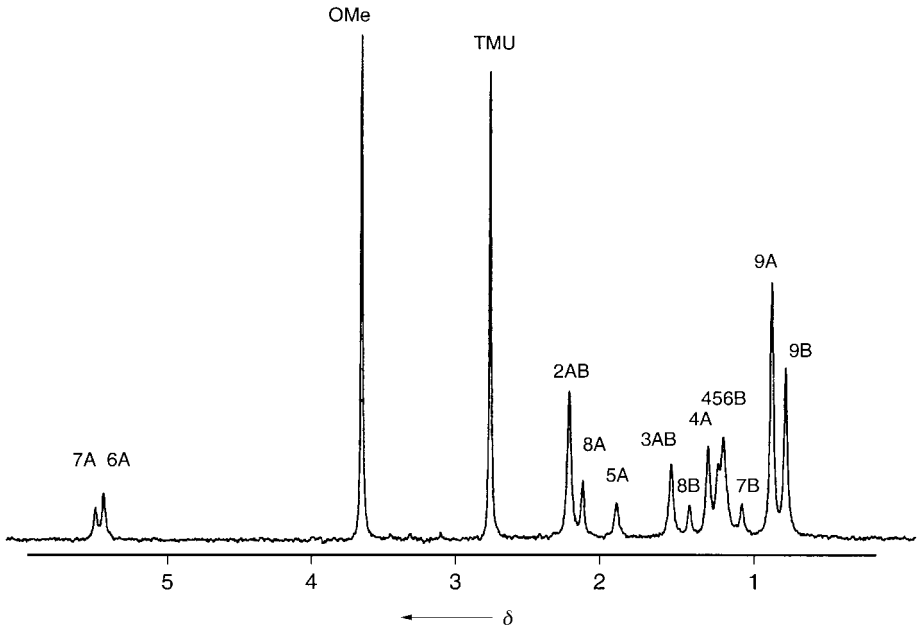


Figure 1. *^{2}H NMR spectrum (61.4 MHz) of the mixed methyl esters **4** and **3** derived from a commercial extract of chilli pepper containing both capsaicin (**2**) and 6,7-dihydrocapsaicin (**1**) by the protocol described in the Experimental Section. Peaks designated "A" are derived from **4**; peaks designated "B" are derived from **3**.*

^{2}H NMR analysis of methyl esters

The site-specific isotopic distribution of ^{2}H in the methyl esters Me-**3a**, Me-**4a** and Me-**5a**, prepared from synthetic 8-methylnonanoic acid (**3a**), *E*-8-methylnon-6-enoic acid (**4a**) and *Z*-8-methylnon-6-enoic acid (**5a**), respectively, are reported in Table 4.

Discussion

The distribution of the $(^2H/^1H)_i$ values in **3** and **4**, obtained from the natural products **1** and **2**, respectively, (Table 3; Figure 2A) is found to be non-statisitical. Three aspects of the biosynthesis of these compounds could potentially play a role in creating this variation: the biosynthetic origin of the different parts of the molecule, the biosynthetic origin of the individual hydrogen atoms and the biosynthetic relationship between **1** and **2**.

The acyl moieties of **1** (studied as **3**) and **2** (studied as **4**) are considered to originate from two separate origins: the carbons C2 – C6 from acetate and the carbons C7 – C10 from valine. An analysis of the mean ^{2}H/^{1}H isotopic ratios in the C3 – C6 and the C7 – C10 positions of **3** indicates that the overall mean molar ^{2}H/^{1}H value of the acetate-derived part is 136.8 ppm, while that of the valine-derived portion is 110.2 ppm. A similar calculation cannot be performed for **4** due to the influence of the desaturation (see below). The difference of 26 ppm between these portions of the molecule is significant. Relative impoverishment is typically associated with longer pathways.[2, 3] The greater impoverishment in the C7 – C10 portion is compatible with its proposed incorporation from

Table 3. *$(^2H/^1H)_i$ values (ppm) of methyl esters obtained by ^{2}H NMR spectroscopy from a crude mixture of capsaicinoids.*

Capsaicinoid sample[c]	Derived compd	Composition [%][a]	Carbon atom number 2[b]	3[b]	4	5	6	7	8	9 + 10	OCH_3[b]	global
cap5	**3**	35.3 {28.4}	132.6	72.0	148.2 [d]	148.2 [d]	148.2 [d]	73.6	147.7	111.8	130.1	114.8
			(2.4)	(0.5)	(5.3)	(5.3)	(5.3)	(0.7)	(5.1)	(0.6)	(0.4)	
	4	63.3 {60.7}	132.6	72.0	134.5	65.3	102.1	76.6	120.2	97.4	130.1	105.8
			(2.4)	(0.5)	(4.6)	(1.3)	(2.6)	(2.6)	(12.6)	(0.4)	(0.4)	
cap6	**3**	34.1 {28.0}	138.4	75.8	167.2 [d]	167.2 [d]	167.2 [d]	74.1	159.4	118.3	131.7	121.6
			(3.1)	(1.7)	(21.8)	(21.8)	(21.8)	(5.6)	(25.4)	(4.5)	(0.3)	
	4	64.9 {60.0}	138.4	75.8	133.7	65.5	112.9	79.8	127.8	99.1	131.7	108.6
			(3.1)	(1.7)	(0.5)	(3.7)	(1.0)	(1.0)	(8.0)	(2.4)	(0.3)	

[a] The percentage composition is for the methyl esters of the capsaisinoids that contribute to the respective peaks with the percentage due to **3** and **4**, respectively, given in parentheses. [b] These positions resonate at the same frequency in **3** and **4**. [c] cap5 = Fluka lot no.: 346725/1 and cap6 = Fluka lot no.: 372701/1. [d] These positions resonate at the same frequency in **3**.

Table 4. *$^2H/^1H$ ratios (ppm) obtained by ^{2}H NMR spectroscopy for the methyl esters of compounds **3a**, **4a** and **5a**.*

Compound	Carbon atom number 2	3	4	5	6	7	8	9	OCH_3	global
Me-**3a**	138.9	130.4	117.7[c]	117.7[c]	117.7[c]	123.7	160.3	136.8	134.1	130.7
	(1.5)	(1.7)	(0.8)	(0.8)	(0.8)	(4.6)	(1.9)	(2.1)	(2.0)	(1.3)
Me-**4a**[a]	144.1	134.2	114.0	109.3	86.9	108.2	161.4	139.6	133.0	129.8
	(3.0)	(4.3)	(1.8)	(3.3)	(3.7)	(5.2)	(3.2)	(2.0)	(2.2)	(1.6)
Me-**5a**[b]	143.9	127.1	113.5	123.3	121.1[d]	121.1[d]	157.4	142.5	132.0	129.1
	(2.5)	(1.5)	(2.4)	(1.9)	(2.0)	(2.0)	(6.5)	(1.4)	(2.3)	(0.4)

[a] Contains 12% *Z* isomer. [b] Contains 13% *E* isomer. [c], [d] These positions resonate at the same frequency in the given compound.

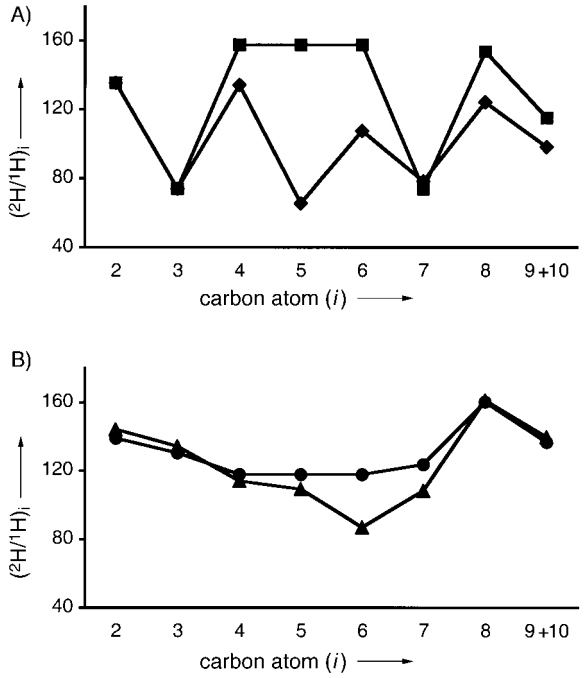


Figure 2. *Alternating pattern of $(^2H/^1H)_i$ values for A) natural methyl 8-methylnonanoate (**3**; ■) and natural methyl 8-methylnon-6-enoate (**4**; ◆), and for B) synthetic methyl 8-methylnonanoate (Me-**3a**; ●) and synthetic methyl 8-methylnon-6-enoate (Me-**4a**; ▲). Note that in **3** and Me-**3a** the values shown for C4, C5 and C6 are the mean for (C4 + C5 + C6) as these positions resonate at the same frequency in the ^2H NMR spectrum.*

valine.[10, 11] The higher value of the C3 – C6 portion is, similarly, compatible with direct incorporation from acetate.

Within the isobutyl moiety of **3**, however, a range of $^2H/^1H$ values between 74 ppm (C7) and 154 ppm (C8) is observed (Figure 2 A). Thus, within this moiety, a considerable nonstatisitical distribution of ^2H is found. The methyl groups at the C9 + C10 positions show a value of 115 ppm, while the C8 and C7 atoms, at 153 and 74 ppm, respectively, appear to be from different origins. The isobutyl moiety of **4** shows a very similar pattern, with the C9 + C10 atoms showing a value of 98 ppm, while the C8 and C7 atoms are at 124 and 78 ppm, respectively. These data are compatible with the methyl groups originating from the methyl group of pyruvic acid without further hydrogen exchange, as occurs during 2-oxo-isovalerate biosynthesis. The C8 hydrogen atom is introduced by a dehydratase (EC 4.2.1.9) and is thus of a more proximal origin.

The C7 atom is also of proximal origin but, in contrast to the C8 and C9 + C10 atoms, is markedly impoverished in both **3** and **4**. During biosynthesis, it is probable that valine is not a normal precursor of capsacinoids, but that these are produced directly from 2-oxo-isovalerate and isobutyrate (or isobutyryl-CoA), both of which are labelled by U-[^{14}C]-valine.[11] Thus, the C7 hydrogen atoms are introduced during the process by which isobutyrate is condensed with the first of the three proposed molecules of malonyl-CoA (Scheme 1), and they therefore originate from the environment. Assuming that the C1 – C6 atoms are indeed derived by condensation of malonyl-CoA, the C3 and C5 hydrogens will also originate from the environment. This proposed origin for the C7 finds support in that the C3 hydrogen atom in **3** and **4** and the C5 atom in **4** (Table 3; Figure 2 A) are also relatively strongly impoverished. (The C5 atom of **4** is inaccessible, as it resonates with the C4 and C6 positions.) This pattern mimics closely that previously reported for the elongation of C_{18} long-chain fatty acids, in which the sites derived from the carboxyl groups of acetyl-CoA are systematically impoverished relative to those derived from the methyl group.[5] This parallel adds support to the proposal that the C_{10} branched-chain fatty acids of capsacinoids are indeed formed by a FAS-type elongation process.[12]

As discussed above, the isobutyl moieties of **3** and **4** show very similar patterns of ^2H distribution, although all positions in **4** are impoverished relative to the equivalent carbon positions in **3** (Table 3; Figure 2 A). Overall, this moiety has a mean molar $^2H/^1H$ value of 99 ppm in **4**, making it about 11 ppm more impoverished than the equivalent moiety in **3** (110 ppm). This relationship is compatible with the view that **1** is the proximal precursor for **2**, based on evidence that saturated acyl-CoAs are the preferred substrates for the vanillylamine:acyl-CoA condensing enzyme.[15] A similar comparison in the acetate-derived moiety cannot satisfactorily be made, due firstly to the effects of the desaturation in **2** and, secondly, to the lack of resolution at the C3 position between **3** and **4**.

Capsaicin **2** differs from **1** by the presence of a Δ^6 desaturation. An examination of the natural isotopic fractionation in ^2H in oleic (C18:1, Δ9) and linoleic (C18:2, Δ9,12) acids has shown there to be a strong secondary KIE, leading to impoverishment in the residual hydrogen.[5] Critically, this is found only at the C9 and C13 positions of the products of Δ^9- and Δ^{12}-desaturase activity, respectively, with the C10 and C12 positions showing no impoverishment. At the C7 position of **4**, $k_H/k_D = 0.89$ (range, 0.05) can be estimated by using the measured deuterium content at C7 of **3** as the initial substrate value; this value indicates there to be a slight inverse secondary KIE at this position. At the C6 position, however, $k_H/k_D = 1.88$ (range, 0.04) can be calculated by using the mean deuterium content (in ppm) at the C4 – C6 positions as the initial substrate value for the unresolved C6 position of **3**. This relatively high value could indicate that the pro-*S* hydrogen atom plays a role in the enzyme-catalysed oxidation, as $k_H/k_D = 1.88$ is rather high for a straightforward secondary KIE. It should be noted that similar values were observed for oleic and linoleic acids.[5] However, the value must be treated with caution, as this estimation is based on two approximations. First, that the deuterium distribution between the pro-*R* and pro-*S* sites of **1** is equal. Secondly, that, as the C4, C5 and C6 hydrogen atoms of **3** resonate together, the mean deuterium content (in ppm) used for the estimation is a mean for these three positions. In view of the alternating pattern observed in **4** (see above), it is likely that this mean value is a minimal estimate of $(^2H/^1H)_6$.

In contrast to oleic and linoleic acids, **2** contains an *E* ethylenic linkage. This could be introduced either by an initial *Z* desaturation followed by epimerisation or by direct *E* desaturation. While *E*-desaturated fatty acids are relatively rare compared to the *Z* isomers, the available evidence indicates that they arise

directly by desaturation of a saturated precursor. Labelling studies have shown that Δ^3-*E*-hexadecenoic acid, found in the plastidic membranes of the green alga *Dunaliella salina*, is derived directly from palmitic acid.[20] Similarly, the Δ^4-*E* double bond of ceramide is introduced in hepatic tissues from the dihydroceramide precursor.[21] Recently, this desaturation has also been found to show a strong KIE at the C4 but not the C5 position.[22] The present data, while unable to distinguish between direct Δ^6-*E* desaturation and Δ^6-*Z* desaturation followed by isomerisation, is compatible with the general mechanistic model now proposed for both *Z* and *E* desaturation of fatty acids.[6, 7, 22] It should be noted, however, that ξ-carotene *E*-desaturase activity has also been identified in *C. annuum*.[23] Thus, it is possible that this type of activity is responsible for the desaturation of capsaicinoids. ξ-Carotene desaturase and the fatty acid desaturases are mechanistically quite distinct. The close similarity between the data presented here and that obtained for the plant fatty acid desaturases supports the latter mechanism, rather than the former. However, to date, no isotopic analysis of the carotene desaturases has been undertaken.

The $(^2H/^1H)_i$ ratios in natural **3** and **4**, derived from **1** and **2**, respectively, show marked differences with those observed in the methyl esters Me-**3a** and Me-**4a**, obtained from the synthetic acids **3a** and **4a**, respectivetly (Table 4; Figure 2B). Firstly, the C3, C4, and C5 group of Me-**4a** does not show the alternating pattern observed in **4**. Secondly, in both **3** and **4** the C6 atom is less impoverished than the C7 atom, whereas in Me-**3a** and Me-**4a** the relationship is reversed. These differences could provide useful biomarkers by which synthetic and natural capsaicinoids can be distinguished (Figure 2).

Conclusions

The power of analysis of metabolic relationships through the use of the intramolecular distribution of $(^2H/^1H)_i$ ratios at natural abundance lies in part in its ability to determine simultaneously all resolved sites within a molecule. This method has proved effective, for example, at following the affiliation of hydrogens between glucose and glycerol[24] and at determining isotopic effects in fatty acid desaturases.[5] Although the technique has to date been applied to pure samples, it is now shown that mixtures can effectively be examined, provided: 1) all components of interest are sufficiently soluble, 2) the NMR spectroscopy resolution of the pertinent peaks is sufficient, and 3) the exact molar composition of the mixture can be determined. With complex or structurally closely related molecules, this approach offers the major advantage of not requiring a difficult and tedious isolation, a procedure likely to introduce isotopic fractionation.

The technique has been applied to study the metabolic relationship between the capsaicinoids capsaicin (**2**) and 6,7-dihydrocapsaicin (**1**). While mechanistic data can only be inferred from analysing the nonstatisitical distribution of $(^2H/^1H)_i$ ratios (Figure 2A), the data obtained is compatible with the proposed biosynthetic origins for the different parts of the molecule and with a very proximal relationship between **1** and **2**. Furthermore, for the first time, evidence is obtained that the C1 – C6 positions are derived by a process closely analogous to that of fatty acid biosynthesis and that the mechanism by which the Δ^6-*E* desaturation is introduced appears similar to that responsible for the *Z* desaturations of long-chain fatty acids. The possibility of a direct Δ^6-*E* desaturation merits further attention, particularly as it would appear that the uncharacterised Δ^6-*E*-desaturase of *Capsicum* sp. is closely related to the desaturases responsible for the introduction of the Δ^9 and Δ^{12}-*Z* desaturations commonly found in plant fatty acids.[12]

Experimental Section

Chemicals: Compounds **3a**, **4a** and **5a** were obtained from Maybridge (Tintagel, UK); BF_3/MeOH (12%) from Sigma; capsaicinoid extract and DDQ from Fluka; Amberlite IR-120 cation-exchange resin (ASTM: 20 – 50 mesh; 0.3 – 0.9 mm) was purchased from Merck. Solvents were normally distilled before use ($CHCl_3$, EtOAc, MeOH).

^{1}H and ^{13}C NMR spectroscopy: ^{1}H and ^{13}C NMR spectra were recorded on a DRX AVANCE 500 MHz NMR spectrometer (Bruker). Attribution of **1** and **2** was performed according to DEPT ^{13}C, COSY ^{1}H-^{1}H and HSQC ^{1}H-^{13}C two-dimensional NMR spectral analysis. All NMR spectra were recorded in $CDCl_3$ with TMS as an internal reference.

Stable isotope determinations: ^{2}H NMR spectra were recorded on a DPX 400 MHz NMR spectrometer (Bruker) operating at 61.4 MHz with an ^{2}H-specific probe (10 mm) fitted with a ^{19}F field-frequency lock (C_6F_6). Samples were prepared in chloroform (approximately 2 mL), and tetramethylurea (approximately 70 mg) of a calibrated ^{2}H/^{1}H ratio (123.38 ppm) was added. Acquisition conditions were: number of scans 14000; sweep width, 1200 Hz; acquisition time 6.8 s; pulse width 90°, 19 μs; $T = 303$ K. Each sample was analysed at least three times and the signal intensities calculated by using dedicated software (INTERLIS, Eurofins Scientific, Nantes, France). Molar ^{2}H/^{1}H ratios for all isotopomers of each compound (A, B...) are calculated by using Equation (1); where $(^2H/^1H)_i^A$ is the isotope ratio at site *i* of compound A; P^R is the hydrogen population of the reference (in this case, TMU); P_i^A is the hydrogen population at site *i* of compound A; m^R and m^A are the masses of the reference and compound A, respectively; M^R and M^A are the molecular masses of the reference and compound A, respectively; t^R and t^A are the molar concentrations of the reference and compound A, respectively (expressed in %w/w); S_i^A is the area of the NMR signal of a given isotopomer *i* of compound A; S^R is the area of the NMR signal of the reference compound; and $(^2H/^1H)^R$ is the known isotopic ratio of the reference compound.

$$\left(\frac{^2H}{^1H}\right)_i^A = \left(\frac{P^R}{P_i^A}\right)\left(\frac{m^R}{m^A}\right)\left(\frac{M^A}{M^R}\right)\left(\frac{t^R}{t^A}\right)\left(\frac{S_i^A}{S^R}\right)\left(\frac{^2H}{^1H}\right)^R \quad (1)$$

Oleoresin extracted from *Capsicum frutescens* contains **1** and **2** as the principle components, accounting for about 61% and 28%, respectively, of the capsaicinoids present (see Table 2). The remaining 11% are composed of at least nine minor components, individually present at a maximum level of about 3%. The contribution of the saturated and nonsaturated hydrogens present in these components has been taken into account for calculating the molar participation in $(^2H/^1H)_i$ as appropriate.

Preparation of the synthetic methyl esters Me-3a, Me-4a and Me-5a: Compound **5a** was prepared by the Wittig reaction of (5-carboxypentenyl)triphenylphosphonium bromide with *iso*-butyral-

dehyde; **4a** by isomerisation of **5a** with sodium nitrite/nitric acid; and **3a** by catalytic hydrogenation (H_2/Pd/C; 2 atm) of **5a**. The methyl esters Me-**3a**, Me-**4a** and Me-**5a** were prepared by treatment with BF_3/MeOH (12% v/v).

Typical procedure for the methylation of 3a, 4a and 5a: BF_3/MeOH (20 mL at 12%) was added slowly to the free acid (500 mg). The mixture was heated at 80 °C with stirring for 3 h then cooled to RT and neutralised with Na_2CO_3 (10%) to pH 7. The methyl ester was extracted with chloroform (5 × 40 mL), the organic phase dried over Na_2SO_4, solvent removed *in vacuo* and the product purified by passage through a short-path silica gel plug eluted with ethyl acetate/cyclohexane (3:7). Solvent removal by evaporation gave the corresponding methyl ester (92–97% yield). ^1H and ^{13}C NMR spectroscopic and gas chromatographic (GC) analysis revealed the presence of Me-**5a**, the *Z* isomer, in Me-**4a** (ratio Me-**4a**/Me-**5a** 88/12) and of Me-**4a**, the *E* isomer, in Me-**5a** (ratio Me-**5a**/Me-**4a** 87/13).

Methyl 8-methylnonanoate (Me-3a): ^1H NMR (500 MHz, $CDCl_3$, 300 K, TMS): $\delta = 3.65$ (s, 3H; O-CH_3), 2.30 (t, $^3J(H,H) = 7.5$ Hz, 2H; CH_2-2), 1.62 (tt, $^3J(H,H) = 7.4$ Hz, 2H; CH_2–3), 1,51 (tsep, $^3J(H,H) = 6.6$, $^3J(H,H) = 6.7$ Hz, 1H; CH-8), 1.35–1.20 (m, 6H; CH_2-4,5,6), 1.15 (td, $^3J(H,H) = 6.6$, $^3J(H,H) = 6.7$ Hz, 2H; CH_2-7), 0.86 (d, $^3J(H,H) = 6.7$ Hz, 6H; CH_3-9,10); ^{13}C NMR (125 MHz, $CDCl_3$, 300 K, TMS): $\delta = 174.3$ (C=O), 51.4 (O-CH_3), 39.0 (CH_2-7), 34.2 (CH_2-2), 29.5 (CH_2-5), 29.2 (CH_2-4), 28.0 (CH-8), 27.2 (CH_2-3), 25.0 (CH_2-6), 22.6 (CH_3-9,10).

Methyl 8-methylnon-6-enoate (Me-4a): ^1H NMR (500 MHz, $CDCl_3$, 300 K, TMS): $\delta = 5.38$ (ddt, $^3J(H,H) = 6.1$, $^3J(H,H) = 15.4$, $^3J(H,H) = 1.1$ Hz, 1H; CH-7), 5.33 (tdd, $^3J(H,H) = 5.7$, $^3J(H,H) = 15.4$, $^3J(H,H) = 0.9$ Hz, 1H; CH-6), 3.60 (s, 3H; O-CH_3), 2.30 (t, $^3J(H,H) = 7,5$ Hz, 2H; CH_2-2), 2.22 (ddsep, $^3J(H,H) = 6.8$, $^3J(H,H) = 6.1$, $^3J(H,H) = 0.9$ Hz, 1H; CH-8), 1.98 (dtd, $^3J(H,H) = 5.7$, $^3J(H,H) = 7.5$, $^3J(H,H) = 1.1$ Hz, 2H; CH_2-5), 1.62 (tt, $^3J(H,H) = 7,5$, $^3J(H,H) = 7.2$ Hz, 2H; CH_2-3), 1.37 (tt, $^3J(H,H) = 7.2$, $^3J(H,H) = 7.5$ Hz, 2H; CH_2-4), 0.95 (d, $^3J(H,H) = 6.8$ Hz, 6H; CH_3-9,10); ^{13}C NMR (125 MHz, $CDCl_3$, 300 K, TMS): $\delta = 174.1$ (C=O), 138.1 (CH=-6), 126.7 (CH=-7), 51.4 (O-CH_3), 34.0 (CH_2-2), 32.1 (CH_2-5), 31.0 (CH-8), 29.1 (CH_2-4), 24.4 (CH_2-3), 22.7 (CH_3-9,10).

Methyl 8-methylnon-6-enoate (Me-5a): ^1H NMR (500 MHz, $CDCl_3$, 300 K, TMS): $\delta = 5.21–5.19$ (m, 2H; CH-7,6), 3.63 (s, 3H; O-CH_3), 2.57 (ddsep, $^3J(H,H) = 6.6$, $^3J(H,H) = 6.6$, $^3J(H,H) = 1.7$ Hz, 1H; CH-8), 2.31 (t, $^3J(H,H) = 7,5$ Hz, 2H; CH_2-2), 2.05 (dtd, $^3J(H,H) = 7.4$, $^3J(H,H) = 7.4$, $^3J(H,H) = 1.7$ Hz, 2H; CH_2-5), 1.64 (tt, $^3J(H,H) = 7.5$, $^3J(H,H) = 7.4$ Hz, 2H; CH_2-3), 1.37 (tt, $^3J(H,H) = 7.4$, $^3J(H,H) = 7.4$ Hz, 2H; CH_2-4), 0.95 (d, $^3J(H,H) = 6.6$ Hz, 6H; CH_3-9,10); ^{13}C NMR (125 MHz, $CDCl_3$, 300 K, TMS): $\delta = 174.1$ (C=O), 138.1 (CH=-6), 126.7 (CH=-7), 51.4 (O-CH_3), 34.0 (CH_2-2), 29.4 (CH_2-5), 26.9 (CH_2-4), 26.5 (CH-8), 24.6 (CH_2-3), 23.2 (CH_3-9,10).

Typical procedure for transformation of the capsaicinoids into fatty acids methyl esters: Water (5 mL) then DDQ (852 mg, 1.1 equiv) were added to a capsaicinoid sample (1 g, 3.3 mmol) in CH_2Cl_2 (50 mL) carefully mixed in a fume hood.[17, 18] The solution was stirred at RT for 30 h then the CH_2Cl_2 removed by evaporation. The aqueous phase was extracted with ethyl acetate (100 mL + 4 × 30 mL) and the organic phases washed separately with Na_2CO_3 (1%, 30 mL). The combined organic phases were dried over Na_2SO_4 and solvent removed in vacuo. Washed Amberlite IR-120 cation exchange resin (15 × w/w) was added to this crude extract in MeOH and the mixture heated with stirring at 80 °C for 30 h in dry conditions.[19] After cooling to RT, the resin residue was removed by filtration, the polymer washed with MeOH (2 × 20 mL) and the solvent evaporated. The organic phase was dried over Na_2SO_4, concentrated in vacuo and purified by passage through a short-path silica gel plug eluted with ethyl acetate/cyclohexane (3:7). Solvent removal by evaporation gave the mixture of the methyl esters, notably **3** and **4**, from **1** and **2**, respectively (80–85% yield from capsaicinoids).

Analytical and spectroscopic data of methylated esters **3** and **4** derived from natural amides **1** and **2** (R_f, R_t (GC), ^1H and ^{13}C NMR) were identical respectively to those of synthetic Me-**3a** and Me-**4a**.

The percentage compositions of the capsaicinoid extracts were determined by GC on the methyl esters. Conditions of GC analysis were: column, HP-INNOWAX, 30 m × 0.32 mm, 0.52 μm coating; carrier gas, He, 1.2 mL min^{-1}; split injection 1:40; injection temperature, 280 °C; flame-ionisation detector temperature, 290 °C; thermal gradient, 80° for 1 min, then 80° to 160° at 2.5° min^{-1}, then 160° to 220° at 5° min^{-1}, then 220 to 250° at 20° min^{-1}, then 250° for 2 min; sample vol.; 0.5 μL.

The identity of the peaks was confirmed by GC/MS under identical conditions. A number of trace compounds (< 0.5%) are not included for the isotopic analysis.

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