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Chili Pepper Fruits: Presumed Precursors of Fatty Acids Characteristic for Capsaicinoids

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Capsaicin is a molecule unique to fruits from the genus *Capsicum*. It is responsible for the pungent sensation and displays valuable pharmacological properties. Despite the fruits' economic importance and decades of research, the regulation of the content of capsaicinoids in individual fruits is not completely elucidated, and no agricultural cultivation of chili of defined pungency is assured. Precursor candidates of the fatty acid moiety of the capsaicinoids, especially for the unique 8-methyl-*trans*-6-nonenoic acid, were examined. Thioesters, acyl-ACP and acyl-CoA, were isolated from the placenta of *Capsicum* fruits by means of DEAE-Sepharose chromatography, selectively converted to the corresponding *N*-butylamides, and analyzed by GC-MS. Fatty acid moieties characteristic for capsaicinoids were identified. In two different varieties (*Capsicum chinense* var. Habanero orange and *Capsicum annuum* var. Jalapeno) it was shown that the fatty acid pattern corresponds to the distribution pattern of the capsaicinoids formed up to this time. The acyl-thioester fractions contained already the 8-methyl-*trans*-6-nonenoic acid.

KEYWORDS: Capsaicinoids; biosynthesis; acyl-ACP; acyl-CoA; 8-methyl-6-nonenoic acid

INTRODUCTION

Plants of the genus *Capsicum* (e.g., bell pepper and chili pepper), belonging to the botanical family of Solanaceae, are among the oldest cultivated plants. Their fruits have been used as spice for over 6000 years (*1*). Pungency, a quality criterion in chili, is caused by a group of vanillylamides, the capsaicinoids (*2*). They are a unique category of alkaloids restricted to fruits of this genus (**Figure 1**).

More than 20 capsaicinoids, differing only in the fatty acid structures, have been described. Branched fatty acids of medium chain length occur also as sucrose esters on leaves of other solanaceous plants, e.g. *Nicotiana*, *Lycopersicon*, and *Petunia* (*3*), but 8-methyl-6-nonenoic acid, a *trans*-monounsaturated branched-chain fatty acid, has been found nowhere else.

Capsaicinoids act on a receptor sensing noxious stimuli, the vanilloid type 1 receptor TRPV1, being a member of the transient receptor potential family of cation channels (*4, 5*). They display analgesic properties due to desensitization and are of interest in elucidating pharmacological pathways (*6*).

Capsaicinoids are synthesized in the placenta of the fruits by an enzymatic condensation of vanillylamine and medium chain length fatty acids. The vanillylamine is derived from the phenylpropanoid pathway. The formation of the characteristic methyl-branched, saturated, or *trans*-monounsaturated fatty acids proceeds from valine and leucine. The transamination of those amino acids to 2-oxocarboxylic acids followed by decarboxylation leads to isovalerate and isobutyrate (*2*). Chain elongation occurs on intermediates bound to the acyl carrier protein ACP, through the addition of two carbon atoms by the fatty acid synthase II (*7*). cDNA clones were isolated from differentially expressed genes for participants in this reaction, for example, ACP, ketoacyl synthase KAS, and thioesterase FAT (*8, 9*).

The condensing enzyme capsaicinoid synthase (CS) acts specifically on medium chain length fatty acids requiring Mg^{2+}, ATP, and coenzyme A (CoA). CS operates with a 6.5-fold higher speed on the acyl-CoA derivative in comparison to free fatty acids (*10*). The heterologous expression of its gene (*cys1*) in *Escherichia coli* yielded a protein with a higher specific activity for 8-methyl-*trans*-6-nonenoic acid than measured for the native enzyme isolated from the *Capsicum* placenta (*11*). This is an indication that it displays both functions (i) esterification of the acid to the CoA derivative and (ii) subsequent condensation to the amide. It is conceivable that hydrolysis of the acyl-ACP precursor by a specific thioesterase and condensation of the acyl chain with vanillylamine occur in a coordinated/integrated reaction in a complex as it is discussed for the biosynthesis of medium-chain fatty acids in *Cuphea lanceolata* (*12*). It might require only very low concentrations of intermediates. Because the specificity of the condensing enzyme analyzed in competition experiments cannot account for the fatty acid pattern of the capsaicinoids (*10*), the acyl-ACP pool is of special interest, as it may determine the pattern in the final product, the capsaicinoids.

After the condensation, no further modification of the fatty acid chain takes place (*13*). Contents of capsaicinoids in ripe fruits depend on genotype and environment (*14, 15*). They vary even between individual fruits from one plant (*16*). Ongoing research aims to provide chili breeders with *Capsicum* varieties

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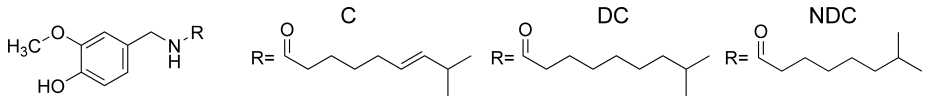

Figure 1. Vanillylamine and the fatty acid moieties of the three major capsaicinoids capsaicin (C), dihydrocapsaicin (DC), and nordihydrocapsaicin (NDC).

producing defined and consistent pungency levels. Quantitative trait loci (QTL) analysis mapped markers on three chromosomes having an influence on capsaicinoid contents (*17*). The presence/absence of pungency is coded in gene locus *Pun1* (*18*), which is not the structural gene for the condensing enzyme CS (*11*).

Analysis of in vivo levels of enzymes and pathway intermediates is a classical approach to the study of metabolic regulation.

The phenylpropanoid pathway, which leads to vanillylamine, also provides precursors for the lignin biosynthesis, vital to the plant (*19*). The special fatty acid moieties, however, are found only in the capsaicinoids. Their precursors seemed to be more likely candidates for regulatory or control points. Indeed, lower capsaicin levels were observed in cell culture experiments with *Capsicum annuum* and *Capsicum frutescens* when the chain elongation step was inhibited by cerulenin, known to act on the KAS enzyme. In contrast, the vanillylamine pool was not the limiting factor in capsaicinoid biosynthesis (*20*). The free 8-methylnonenoic acid, suggested as precursor, was determined in a petroleum ether extract; sodium hydroxide followed by boron trifluoride treatment yielded the methyl esters for quantification by GC-MS. Petroleum ether as solvent also extracted major parts of the capsaicinoids (*21, 22*). By the saponification of the extracted capsaicinoids, the analysis of the free fatty acids present before saponification is confounded, and thereby only a pseudocorrelation between the free fatty acids as intermediates and capsaicinoids as end products of the biosynthesis is shown. In cell cultures of *C. frutescens*, elicitors were applied to enhance capsaicinoid production (*23*). A concomitant increase in free 8-methylnonanoic acid content, determined by the same artifact-prone procedure, implicated this free acid to be the substrate for the enzyme 8-methylnonanoic acid dehydrogenase (8-MNAD). This will then provide the free 8-methyl-*trans*-6-nonenoic acid.

It should be a priority to search for precursors of the free fatty acids that are bound to ACP. Pools of acyl-ACPs have been analyzed in spinach leaves by an immunoblotting technique (*24*) and by a selective formation of acyl-butylamides amenable to GC-MS analysis (*25*). The formation of the *N*-butylamides occurs with both groups of thioesters, the acyl-CoA and acyl-ACP derivatives. To avoid interference by other lipids, for example, glycerides, the focus was on an elaborate extraction and purification procedure. The advantage of this method is its sensitivity and the lack of interference by multiple isoforms of ACP (*25*).

According to this methodology we compared fatty acid patterns in acyl-ACP and acyl-CoA fractions in placental tissue of young fruits with the pattern of capsaicinoids already formed at this time of development.

MATERIALS AND METHODS

Reagents. Capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide, C, natural, ~65% capsaicin and ~35% dihydrocapsaicin) and *N*-vanillylnonanamide (PSVA, purum) were supplied by Roth (Karlsruhe, Germany). 7-Methyloctanoic acid (97%) was obtained from Narchem (Chicago, IL), and 8-methyl-*trans*-6-nonenoic acid (97%) was obtained from Maybridge (Titangel, U.K.). DEAE-Sepharose was purchased from Pharmacia (Uppsala, Sweden). Dihydrocapsaicin (8-methyl-*N*-vanillylnonanamide, DC, 90%), 2-methylhexanoic acid (95%), 3-morpholineethanesulfonic acid (MES, 99%), 3-morpholinepropanesulfonic acid (MOPS, 98%), 8-methylnonanoic acid (98%), *n*-butylamine (99.5%), decanoyl chloride (98%), heptanoyl chloride (99%), hexanoyl chloride (99%), *N*-ethylmaleimide (NEM, 99%), nonanoyl chloride (97%), octanoyl chloride (99%), oleoyl chloride (99%), methyl tetradecanoate (99.0%), and *n*-octanoyl coenzyme A (98%) and other reagents were purchased from Sigma-Aldrich (Steinheim, Germany).

Plant Material. Capsicum plants (*C. annuum* var. Jalapeno and *C. chinense* var. Habanero orange, Ruehlemanns, Horstedt, Germany) were grown in a greenhouse at 25 °C, 12 h photoperiod (Osram Vialux NAV-T 400 W Plantastar lamps) with an irrigation period of 2 min (three times per day). The fruits were harvested and cut into halves. The placental tissue was separated from the pericarp, and both tissues were immediately frozen in liquid nitrogen. The frozen tissue was ground to a powder and immediately used for further purification.

***N*-Butylamide Standards.** Ten *N*-butylamides varying in fatty acid moiety were synthesized from *n*-butylamine and fatty acid chlorides in diisopropyl ether. The chloride derivatives were obtained by reaction with oxalyl chloride. The synthesis products were purified by preparative column chromatography (silica gel 60, 40–60 μm, diisopropyl ether) and were submitted to an authenticity control by means of ^{1}H NMR, ^{13}C NMR, and mass spectrometry.

Isolation of Acyl-CoA and Acyl-ACP from Chili Fruits. The frozen fruit tissue (~20 g of fresh weight) was homogenized in 70 mL of trichloroacetic acid (50 g/l, at 2 °C) using an Ultra-Turrax (Janke & Kunkel, Staufen, Germany). After centrifugation at 5000*g* for 20 min at 2 °C, the supernatant was discarded. The precipitate was washed with 50 mL of trichloroacetic acid (10 g/L, at 2 °C) and the precipitated acyl-thioesters were dissolved in 50 mL of MOPS buffer (50 mM, pH 7.6, containing 10 mM *N*-ethylmaleimide). The insoluble material (pellet) contains the capsaicinoids. The dissolved acyl-thioesters were loaded onto a 10 mL DEAE-Sepharose column, which had been equilibrated with 50 mL of MES buffer (10 mM, pH 6.1). The column was subsequently washed with 50 mL of MES buffer (10 mM, pH 6.1) followed by 250 mL of 2-propanol/MES buffer mixture (80% 2-propanol/20% MES buffer (v/v) to remove capsaicinoids, lipids, and free fatty acids. Thereafter, the column was washed with 50 mL of MES buffer. Then the first acyl-thioester fraction (acyl-CoAs) was eluted from DEAE-Sepharose with 50 mL of MES buffer [10 mM, pH 6.1, containing 25% 2-propanol (v/v) and 0.15 M LiCl]. The column was again washed with 50 mL of MES buffer. Subsequently, the acyl-ACP fraction can be eluted from DEAE-Sepharose with 50 mL of MES buffer [10 mM, pH 6.1, containing 25% 2-propanol (v/v) and 0.5 M LiCl].

Aminolysis Reaction of Plant Acyl-thioesters. The thioester fractions were transferred into a 100 mL Erlenmeyer flask, and 5 mL of butylamine (99.5%) was added. The flask was strongly shaken for 20 s, which was repeated after reaction times of 5 and 10 min. Precisely after 15 min, the reaction was stopped by the addition of 12.5 mL of HCl (4 M) to achieve a neutral pH. The solution was transferred into a separatory funnel and extracted twice with 20 mL of diisopropyl ether. The pooled organic phases were dried by anhydrous sodium sulfate and filtered, and the solvent was evaporated to a remaining volume of 3 mL by rotary evaporation. After transfer into a 10 mL conical flask, the remaining solvent was completely removed. The residue was dissolved in 200 μL of diisopropyl ether and transferred into a microinsert of a 2 mL vial, evaporated, and dissolved in exactly 20 μL of diisopropyl ether (containing 67 μg/mL methyl tetradecanoate as internal standard for the adjustment of injection variabilities). Two microliters of the sample was injected for GC-MS analysis.

GC-MS of *N*-Butylamides. Instrumentation used was a HP 5890 series II gas chromatograph equipped with a HP 5989 MS engine. *N*-Butylamides were separated on a J&W DB-5-MS capillary column (30 m × 0.25 mm × 0.25 μm). The gas chromatograph was operated

Table 1. *N*-Butylamides

	mol formula	mol mass (g/mol)	retention time (min)	ratio of intensity *m/z* 115 to *m/z* 128
N-butylhexanamide	$C_{10}H_{21}NO$	171	14.7	4.2:1
N-butyl-2-methylhexanamide (ASTD)	$C_{11}H_{23}NO$	185	14.8	1.6:1
N-butylheptanamide	$C_{11}H_{23}NO$	185	16.4	4.0:1
N-butyloctanamide	$C_{12}H_{25}NO$	199	17.9	4.4:1
N-butyl-7-methyloctanamide	$C_{13}H_{27}NO$	213	18.9	4.3:1
N-butylnonanamide	$C_{13}H_{27}NO$	213	19.5	4.1:1
N-butyl-8-methyl-*trans*-6-nonenamide	$C_{14}H_{27}NO$	225	20.0	**1:1**
N-butyl-8-methylnonanamide	$C_{14}H_{29}NO$	227	20.4	3.9:1
N-butyldecanamide	$C_{14}H_{29}NO$	227	21.0	4.1:1
N-butyl-9-oleoylamide	$C_{22}H_{43}NO$	337	33.1	**1:1**

with helium carrier gas (1.2 mL/min; injector temperature, 250 °C; transfer line temperature, 250 °C). After an initial temperature of 35 °C, the oven temperature was immediately raised to 60 °C at 40 °C/min, held for 1 min, and subsequently raised to 250 °C at 8 °C/min and held for 17 min. The mass range of *m/z* 35–350 was used to obtain total ion chromatograms (TIC). Selective ion chromatograms (SIM) were obtained using the masses *m/z* 115 and 128.

Liquid Chromatography of Capsaicinoids. Capsaicinoids in the pellet remaining after the MOPS buffer solubilization of the TCA precipitate were analyzed by HPLC−fluorescence according to the method of Kirschbaum-Titze et al. (*26*).

RESULTS

***N*-Butylamides.** A spectral database was compiled for 10 authentic *N*-butylamides (**Table 1**). All mass spectra of the *N*-butylamides show a distinct molecular ion accompanied by the formation of a characteristic M − 72 ion type R-C≡O^+. A series of fragments results from the cleavage at each bond of the fatty acid carbon chain and charge retention at the amide bond. A diagnostically valuable ion is the fragment with *m/z* 128 and as a McLafferty rearrangement product the ion with *m/z* 115. **Figure 2a** shows the mass spectrum of *N*-butyldecanamide as a representative for *N*-butylamides with an unbranched saturated fatty acid moiety.

Figure 2b shows the mass spectrum of *N*-butyl-8-methyl-*trans*-6-nonenamide as a representative for *N*-butylamides with a branched, monounsaturated fatty acid moiety. The presence of the *trans*-monounsaturated structure in *N*-butyl-8-methyl-*trans*-6-nonenamide has a profound effect on the fragmentation pattern. The major peak at *m/z* 128 is a fragment presumably energetically favored by the double bond of the fatty acid moiety. Both mass tracks *m/z* 115 and 128 are used as bases for the sensitive measurement in the SIM mode. The ratios of intensities are different between the *N*-butylamides with saturated and unsaturated fatty acid chains (**Table 1**). The ion with the mass 129 was recorded additionally, which allows the measurement of the main fragment of the *N*-butyl-2-methylhexanamide (ASTD, analytical standard for recovery). Hence, measurements in SIM mode consist of *m/z* 115, 128, and 129. For quantification in TIC mode the mass range *m/z* 35–350 was recorded. Response factors were determined in SIM mode for *N*-butyl-7-methyloctanamide (0.65) and *N*-butyl-8-methylnonanamide (0.72) expected as reaction products from capsaicinoid precursors relative to the most characteristic *N*-butyl-8-methyl-*trans*-6-nonenamide (1.00). These were used to obtain the fatty acid pattern from peak areas in GC-MS chromatograms. The limit of detection for *N*-butyloctanamide was 0.2 μg/mL, determined according to the calibration curve procedure of DIN 32645 in a linear range up to 4 μg/mL (*27*). This corresponds to 2 pmol in the injection volume of 2 μL, representing the end product of the analytical procedure applied to ∼2 g of plant material (fresh weight). To achieve a feasible signal-to-noise ratio it is necessary to pool at least 10 fruits.

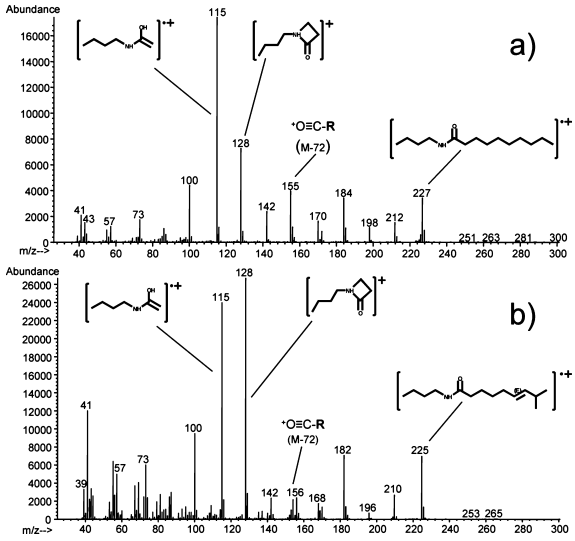

Figure 2. Mass spectra of (**a**) *N*-butyldecanamide and (**b**) *N*-butyl-8-methyl-*trans*-6-nonenamide.

To check the steps after the chromatography of the plant thioesters on DEAE-Sepharose and the amide formation, *N*-butyl-2-methylhexanamide (ASTD) was used. This special amide is not expected as a reaction product from intermediates in capsaicinoid biosynthesis, because no branched-chain fatty acid with the methyl group in the C-2 position has been detected. It is not present as an impurity in the reagents used. Therefore, it is suitable for monitoring isolation and analysis of the branched-chain *N*-butylamides.

Control Experiments. The capsaicinoids (vanillylamides) may be present in the plant material in concentrations up to 3000 mg/kg (10 mmol/kg, 20 μmol in 2 g), 7 orders of magnitude higher than the detection limit for the *N*-butylamides mentioned above. It is therefore very important to demonstrate that no interference, for example, false-positive results, occurs. The major part of the capsaicinoids is found in the pellet of the centrifugation step after the MOPS buffer solubilization. Additionally, washing the DEAE-Sepharose with 2-propanol/buffer will remove lipophilic compounds. Traces may still be found in the acyl-CoA and acyl-ACP fractions. Therefore, 10 mg of capsaicin was subjected to aminolysis with *n*-butylamine to check for a transamination reaction. No *N*-butylamides with characteristic fatty acids were detected; the original unchanged vanillylamides were seen at later retention times, t_r > 30 min.

Free fatty acids, discussed as precursors for capsaicinoids (*10, 20*), are not eluted from DEAE-cellulose in the relevant fractions (*25*); nevertheless, their interference with the thioester determination was tested. Twelve milligrams of 8-methyl-*trans*-

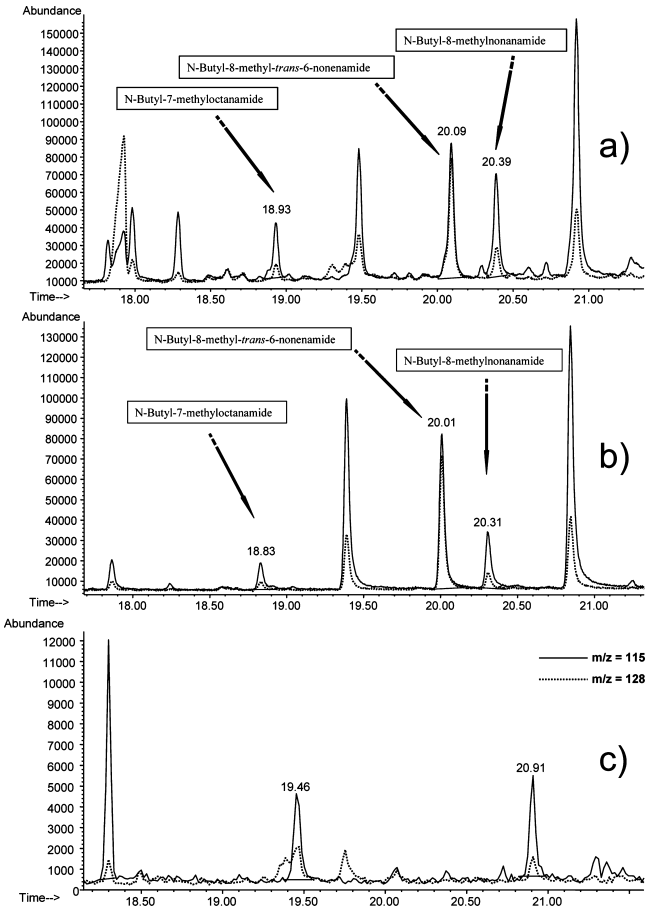

Figure 3. *C. chinense* var. Habanero orange: chromatograms (SIM mode *m*/*z* 115, 128) of (**a**) the *N*-butylamides originating from the acyl-CoA fraction obtained from placenta tissue; (**b**) the *N*-butylamides originating from the acyl-ACP fraction obtained from placenta; and (**c**) the *N*-butylamides originating from the 0.5 M LiCl fraction obtained from pericarp.

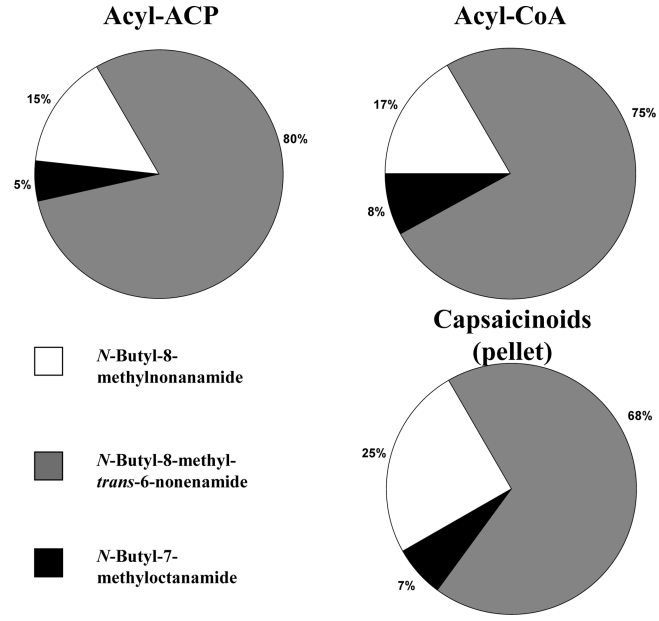

Figure 4. *C. chinense* var. Habanero orange: fatty acid distribution in both thioester fractions, acyl-ACP and acyl-CoA, and in the capsaicinoids found in the MOPS buffer pellet.

6-nonenoic acid was subjected to the aminolysis reaction; <0.4% of the free acid was converted to the corresponding *N*-butylamide.

The selective stepwise elution of thioesters from DEAE-Sepharose was checked with octanoyl-CoA carried through the purification procedure and aminolysis reaction. Ten micrograms of octanoyl-CoA, in 50 mL of MOPS buffer, was loaded onto the DEAE-Sepharose column; the elution protocol was as described above for the chili fruit material. *N*-Butyl-2-methylhexanamide (ASTD; 5.4 μg) was added to both relevant thioester fractions and used as reference peak for quantification in GC-MS TIC mode. *N*-Butyloctanamide as reaction product after aminolysis was detected predominantly in the first thioester fraction (acyl-CoA). Small amounts, about 5%, were eluted with higher LiCl concentrations (0.5 M), seen in wash fractions and also in reagent blanks. An acyl-ACP derivative was not available to us. Kopka et al. (*25*) have shown with [^{14}C]oleoyl-thioesters that 75% of the ACP derivative is recovered in the second thioester fraction with no interference by the CoA derivative or free fatty acid. We therefore conclude that *N*-butylamides detected after aminolysis of the second fraction, which is eluted with 0.5 M LiCl, originate from acyl-ACPs.

Fruits from *C. chinense* var. Habanero orange. The variety Habanero was selected because it is known to produce the most pungent chili fruits. It also shows a high biosynthetic activity (*8*).

The total of 27.8 g of placenta from 31 green, unripe (up to 15 days after flowering) fruits was analyzed according to the described procedure. *N*-Butylamides with the characteristic acid side chains of the capsaicinoids were detected in the reaction products from the acyl-CoA fraction from the DEAE-Sepharose chromatography (**Figure 3a**). A similar chromatogram was obtained from the acyl-ACP fraction eluted with 0.5 M LiCl (**Figure 3b**). The almost identical peak area for both monitored ions at 20.09 min is a first immediate indication for the fatty acid moiety of capsaicin, the most characteristic capsaicinoid analogue. The final identification took place by comparing retention times and mass spectra obtained in TIC mode.

Capsaicin, the vanillylamide with the unsaturated fatty acid moiety, is the main capsaicinoid in *C. chinense* var. Habanero. This is also reflected in the chromatogram of the butylamides originating from the thioesters, because the *N*-butyl-8-methyl-*trans*-6-nonenamide (t_r = 20.09 min) shows the highest peak area value among the three precursors. The masses *m*/*z* 115 and 128 are equally represented with this peak. The chromatogram shows furthermore a number of byproducts of the aminolysis reaction. The peaks at 19.4 min (*N*-butylnonanamide) and 20.9 min (*N*-butyldecanamide) are seen in all chromatograms and originate from the reagents.

Pericarp tissue (20.0 g from a total amount of 71.9 g of pericarp) was analyzed on the same day. As expected, no thioesters were found (**Figure 3c**). This represents a matrix blank confirming that the pericarp tissue is not a place of capsaicin biosynthesis.

Capsaicinoids already synthesized at this time of fruit development were extracted from the material insoluble in MOPS buffer and analyzed by HPLC−fluorescence. They showed a fatty acid pattern comparable with that of the presumed precursor acyl-thioester fractions (**Figure 4**).

The analysis of a pool of 15 ripe, orange-colored fruits (≥50 days after flowering) of *C. chinense* var. Habanero orange showed a chromatogram like that obtained from pericarp or from the reagents alone without the signals for the characteristic fatty acid derivatives (**Figure 3c**).

Fruits from *C. annuum* var. Jalapeno. *C. annuum* var. Jalapeno, distinguished by a large placenta with intermediate

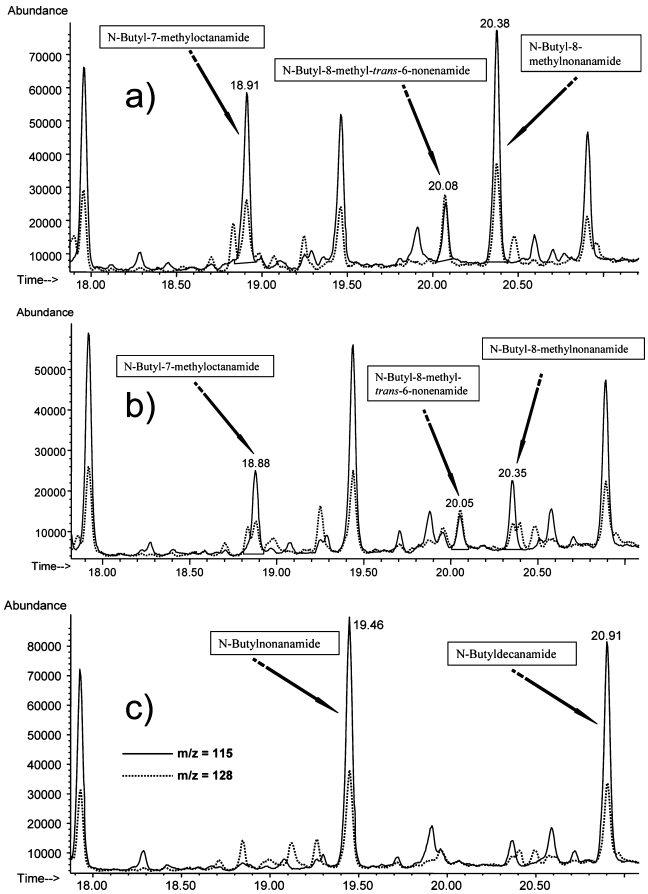

Figure 5. *C. annuum* var. Jalapeno: chromatograms (SIM mode *m*/*z* 115, 128) of (**a**) the *N*-butylamides originating from the acyl-CoA fraction obtained from placenta tissue; (**b**) the *N*-butylamides originating from the acyl-ACP fraction obtained from placenta; and (**c**) the *N*-butylamides originating from the 0.5 M LiCl fraction obtained from pericarp.

pungency, was cultivated as a second *Capsicum* variety. Twenty-five green fruits (up to 40 days after flowering) were harvested, and 18.0 g of placenta was analyzed according to the described procedure. *N*-Butylamides of the characteristic acid side chains of the capsaicinoids were detected in the reaction products from the acyl-CoA fraction (**Figure 5a**) as well as from the acyl-ACP fraction (**Figure 5b**) from the DEAE-Sepharose chromatography.

The chromatogram for 20.0 g of pericarp (**Figure 5c**) analyzed on the same day shows only *N*-butylnonanamide, *N*-butyldecanamide, and some byproduct, which are seen also in the reagent blank. The fatty acid pattern of capsaicinoids, already synthesized at this time of fruit development, and from the presumed precursor acyl-thioester fractions (**Figure 6**) show a higher portion of 7-methyloctanamide originating from leucine and a ratio of unsaturated 8-methylnonenamide to saturated 8-methylnonanamide close to 1 in contrast to ≥3 if compared to *C. chinense* var. Habanero orange (**Figure 4**).

Red mature fruits (≥45 days after flowering) were harvested, and 28 g of placental tissue was analyzed. In both thioester fractions the fatty acid moieties of the capsaicinoids were not found.

Fruits (both red ripe and green unripe) of the nonpungent variety *C. annuum* var. Tinkerbell were analyzed; neither thioesters with the characteristic fatty acid structures nor capsaicinoids were detected.

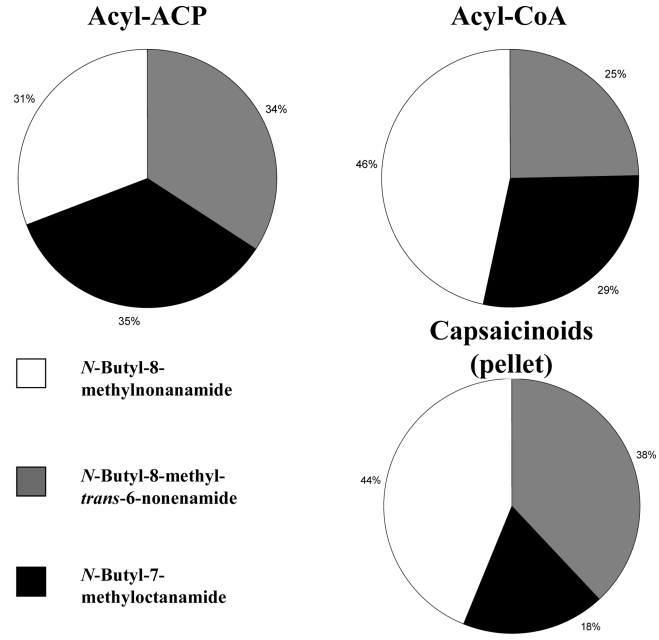

Figure 6. *C. annuum* var. Jalapeno: gatty acid distribution in both thioester fractions, acyl-ACP and acyl-CoA, and in the capsaicinoids found in the MOPS buffer pellet.

DISCUSSION

The detection of the unsaturated 8-methyl-*trans*-6-nonenoic acid in the acyl-ACP fraction isolated from the placenta of developing fruits suggests that the desaturation to the unique *trans* configuration occurs before the thioesterase FAT liberates the free fatty acids characteristic for the capsaicinoids. For the first time it could be shown that the acyl-ACPs include a *trans*-monounsaturated fatty acid. In *Arabidopsis* there is a gene responsible for the desaturation of a straight long-chain fatty acid to the *trans*-monounsaturated configuration, which is formed at a later step when already bound to phosphatidylglycerol (*28*). The desaturation step may constitute an important regulatory point; no hint is yet available for a structural gene for the postulated enzyme.

The acyl-CoA pattern in the first thioester fraction from DEAE-Sepharose matches the pattern found for acyl-ACP. It remains to be investigated whether the acyl-CoA part not precipitated with 5% TCA at the start of the sample preparation shows the same distribution of fatty acids.

In ripe fruits and fruits from nonpungent varieties these precursors are not present. Investigation of single fruits harvested in narrow time intervals will show the change in relation to the maturation stage of fruits. The method by Kopka et al. (*25*), adapted here, is not sensitive enough for this purpose. There are promising results in the area of liquid chromatography. It has been shown that acyl-CoA esters can be derivatized to their fluorescent acyl-etheno-CoA esters, separated by ion-paired reversed phase HPLC, and detected fluorometrically (*29*). Mass spectrometry has increasingly occupied a central position in the methodologies developed for the determination of metabolic states (*30*). In 2007 Gao et al. (*31*) reported a novel method for the quantification of malonyl-CoA and seven other short-chain acyl-CoAs in various rat and mouse tissues using ion-paired reversed phase HPLC-MS with a lower limit of quantification of 0.2 pmol for all acyl-CoAs. If these methodologies can be transferred to plant material and stable isotope marked standards are available, LC-MS could be an approach for the investigation of single fruits.

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