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Synthesis of Stable Isotope-Labeled Precursors for the Biosyntheses of Capsaicinoids, Capsinoids, and Capsiconinoids

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Stable isotope-labeled precursors were synthesized for an analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS) to elucidate the biosynthetic flow of capsaicinoids, capsinoids, and capsiconinoids. $[1'\text{-}^{13}\text{C}][5\text{-}^2\text{H}]$-Vanillin was prepared by the condensation of guaiacol with $[^{13}\text{C}]$-chloroform and a D_2O treatment. Labeled vanillylamine, vanillyl alcohol, ferulic acid, and coniferyl alcohol were prepared from the labeled vanillin. The labeled vanillylamine was converted to labeled capsaicinoid in a crude enzyme solution extracted from pungent *Capsicum* fruits.

Key words: stable isotope; capsaicinoid; capsinoid; capsaicin synthase; liquid chromatography-tandem mass spectrometry (LC-MS/MS)

The substances responsible for the pungency of *Capsicum* peppers are a group of lipophilic alkaloids knows as capsaicinoid(s). The fundamental structure of capsaicinoids comprises a fatty acid amide of vanillyl-amine. Early studies in which classical *in vivo* tracer experiments were performed with radioactive precursors revealed the outline of the biosynthetic pathway for capsaicinoids (Fig. 1).[1] The aromatic moiety of capsaicinoids is derived from phenylalanine *via* the phenyl-propanoid pathway. However, details of the downstream events on the pathway, *i.e.*, the conversion of vanillin to vanillylamine, have not yet been elucidated. Recent molecular biological approaches suggest that the gene of a putative aminotransferase (*pAMT*) encodes an enzyme that catalyzes the conversion of vanillin to vanillylamine.[2] A putative acyltransferase encoded in *Pun1*, namely capsaicin synthase (CS), is also considered a candidate enzyme that catalyzes the condensation of vanillylamine with a fatty acid to produce capsaicinoids.[3]

Recent studies have revealed that many kinds of pungent and non-pungent *Capsicum* cultivars contained a novel group of non-pungent capsaicinoid-like sub-stances named capsinoid(s).[4] The fundamental structure of capsinoids comprises a fatty acid ester of vanillyl alcohol. We performed in our previous study an *in vivo* tracer experiment using radioactive precursors and found that the aromatic moiety of capsinoids, *i.e.*, vanillyl alcohol, was derived from vanillin that was
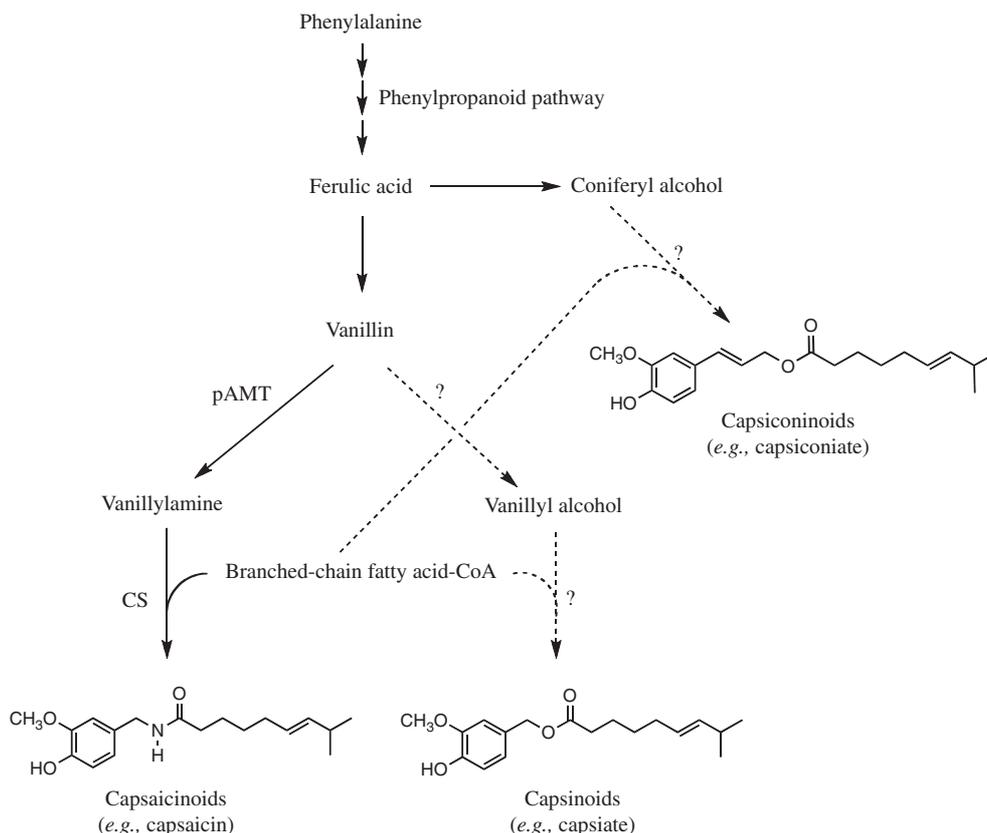
produced *via* the phenylpropanoid pathway (Fig. 1).[5] Although the factor for the conversion of vanillin to vanillyl alcohol seems to be crucial for capsinoid biosynthesis, it has not yet been elucidated. It is also not clear whether CS participates in the condensation of the vanillyl alcohol with a fatty acid in the final stage of capsinoid biosynthesis. A new class of coniferyl esters, namely capsiconinoids, has recently been found in several cultivars of pepper.[6] One of the possible biosynthetic precursors of capsiconinoids is coniferyl alcohol from the viewpoint of its chemical structure.

Clarifying details about the flow of metabolites is a relatively direct approach to elucidating the biosynthetic pathway, although a molecular biological approach would also be helpful in understanding the pathway. We chemically prepared stable isotopes of the putative precursors of capsaicinoids, capsinoids, and capsiconinoids in the present study as analytical tools to elucidate details of their biosynthesis (Fig. 2). The natural abundance of the $[M + 2]$ molecule to $[M]$ molecule of these compounds is about 1%, although that of $[M + 1]$ to $[M]$ is about 9%. We therefore synthesized doubly labeled compounds to get higher sensitivity, and then detected the enzyme activity of CS by liquid chromatography-tandem mass spectrometry (LC-MS/MS), using the labeled precursor.
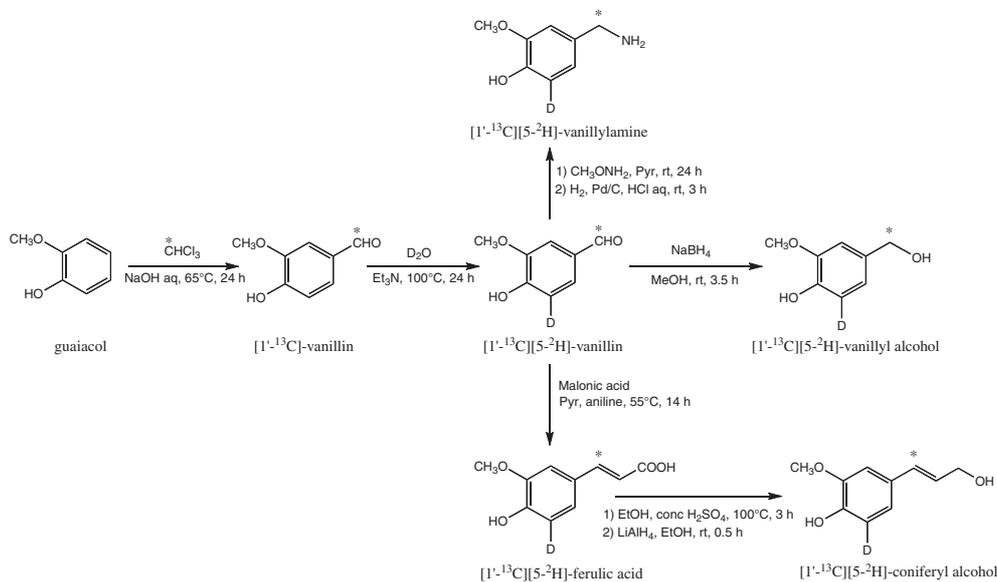
$[1'\text{-}^{13}\text{C}]$-Vanillin was prepared according to the method of Ohara and Fukuda[7] with a slight modification. Briefly, a mixture of 4 mL of 5 M guaiacol (20 mmol) in MeOH, 390 μL of $[^{13}\text{C}]$-chloroform (4.9 mmol; Taiyo Nippon Sanso Co., Tokyo, Japan), and 11 mL of 2.8 M NaOH *aq.* was heated at 65 °C for 24 h. An ethyl acetate extract from the reaction mixture was purified by silica gel and subsequently by successive octadecylsilyl silica gel column chromatography to afford 111.9 mg of $[1'\text{-}^{13}\text{C}]$-vanillin (0.73 mmol, 15% yield from $[^{13}\text{C}]$-chloroform). The ^{13}C-nuclear magnetic resonance (NMR) spectrum[8] of the compound showed an extremely intense signal at 191 ppm indicating a carbonyl carbon containing the ^{13}C isotope. The ^1H-NMR spectrum[8] showed a set of doublet signals coupled with 173 Hz at 9.82 ppm, indicating the presence of an aldehyde proton attached to the ^{13}C carbonyl carbon. The other signals in both NMR spectra of the compound were completely coincident with those of standard vanillin. The positive

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Fig. 1. Proposed Biosynthetic Pathway for Capsaicinoids, Capsinoids, and Capsiconinoids in *Capsicum* Plants.
pAMT, putative aminotransferase; CS, capsaicin synthase.

Fig. 2. Synthetic Scheme for the Stable Isotope-Labeled Precursors.
*, ^{13}C; D, ^{2}H.

atmospheric pressure chemical ionization-mass spectrometric (APCI-MS) spectrum[9] showed a protonated molecular ion peak at m/z 154.

[1′-^{13}C][5-^{2}H]-Vanillin was prepared according to the method of Krings *et al.*[10] with a slight modification. Briefly, a mixture of 53.7 mg of [1′-^{13}C]-vanillin (0.37 mmol), 290 μL of deuterium oxide (14.5 mmol), and 52 μL of triethylamine was heated at 100 °C for 24 h under nitrogen gas. The ethyl acetate extracted from the reaction mixture gave 55.2 mg (0.36 mmol) of [1′-^{13}C][5-^{2}H]-vanillin. The ^{1}H-NMR spectrum of the compound showed signal attenuation at 7.04 ppm, this being assigned to the 5-position of the benzene ring of vanillin. The rate of substitution of a proton with deuterium at the 5-position of vanillin was calculated as 89% from the area of the attenuated proton signal. The positive APCI-MS spectrum showed a protonated molecular ion peak at m/z 155.

[1′-^{13}C][5-^{2}H]-Vanillylamine was prepared from [1′-^{13}C][5-^{2}H]-vanillin *via* an oxime according to the

method of Gannett *et al.*[11] with a slight modification. Briefly, a mixture of 100.8 mg of [1'-^{13}C][5-^{2}H]-vanillin (0.65 mmol), 60.0 mg of methoxyamine hydrochloride (0.78 mmol), and 18 mL of dry pyridine was agitated for 24 h at room temperature. The reaction mixture was then subjected to rotary evaporation *in vacuo* to eliminate pyridine. CHCl$_3$ extracted from the residue gave 109.5 mg of [1'-^{13}C][5-^{2}H]-4-hydroxy-3-methoxyben-zaldehyde *O*-methyloxime (0.60 mmol, 92% yield). A mixture of 20.2 mg of the oxime (0.11 mmol), 42 mg of palladium carbon, and 79 mL of 12 M HCl was agitated at room temperature for 3 h in 150 cm^3 of hydrogen gas. The reaction mixture was passed through Celite to remove the palladium carbon, and was then subjected to rotary evaporation *in vacuo* to afford 21.7 mg of [1'-^{13}C][5-^{2}H]-vanillylamine (51% purity by HPLC, 60% total yield from [1'-^{13}C][5-^{2}H]-vanillin). The ^{1}H-NMR spectrum showed a set of doublet signals of 2 protons coupled with 142 Hz at 4.01 ppm, indicating the presence of a labeled methylene group together with amine and phenyl groups. Labeled methylene was observed as an extremely intense signal at 44 ppm in the ^{13}C-NMR spectrum. The other signals in these spectra were similar to those of standard vanillylamine, except for the signal indicating deficiency at the 5-position in the labeled compound in its ^{1}H-NMR spectrum. The positive APCI-MS data for standard vanillylamine shows a typical fragment ion peak predominantly at *m/z* 137 caused by the elimination of an amine group. Labeled vanillyl-amine showed a fragment ion peak at *m/z* 139, indicating that 2 labels remained in the fragment.

[1'-^{13}C][5-^{2}H]-Vanillyl alcohol was prepared by NaBH$_4$ reduction of [1'-^{13}C][5-^{2}H]-vanillin. We added 12 mg of NaBH$_4$ (0.32 mmol) to 2.4 mL of an MeOH solution containing 23.9 mg of [1'-^{13}C][5-^{2}H]-vanillin (0.16 mmol), and placed the mixture at room temperature for 3.5 h. Excess water was added to the reaction mixture, and subsequent ethyl acetate extraction gave 24.8 mg of [1'-^{13}C][5-^{2}H]-vanillyl alcohol (0.16 mmol). The ^{1}H-NMR spectrum showed a set of doublet signals of 2 protons coupled with 144 Hz at 4.59 ppm, indicating the labeled methylene group together with hydroxy and phenyl groups. The other signals in these spectra were similar to those of standard vanillyl alcohol, except for the signal indicating a deficiency at the 5-position in the labeled compound in its ^{1}H-NMR spectrum. The positive APCI-MS data for the labeled compound showed a typical fragment ion peak predominantly at *m/z* 138 caused by elimination of the aliphatic hydroxy group of vanillyl alcohol.

[1'-^{13}C][5-^{2}H]-Ferulic acid was prepared from [1'-^{13}C][5-^{2}H]-vanillin according to the method of Krings *et al.*[10] with a slight modification. A mixture of 23.0 mg of [1'-^{13}C][5-^{2}H]-vanillin (0.15 mmol), 31.0 mg of malonic acid (0.30 mmol), 46 μL of pyridine, and 5 μL of aniline was heated at 55 °C for 14 h. We then added 200 μL of 5 M HCl and 30 mL of water to the reaction mixture. Subsequent ethyl acetate extraction gave 24.0 mg of [1'-^{13}C][5-^{2}H]-ferulic acid (0.12 mmol, 80.0% yield). The ^{13}C-NMR spectrum of the compound showed an extremely intense signal at 148 ppm indicating a sp^2 ^{13}C isotope of carbon. The ^{1}H-NMR spectrum showed a set of double-doublet signals coupled with 155 and 16 Hz at 7.59 ppm, indicating a conjugated

olefinic proton attached to the ^{13}C isotope with *trans* configuration. The other signals in these spectra were similar to those of standard ferulic acid, except for the signal corresponding to a deficiency at the 5-position in the labeled compound in its ^{1}H-NMR spectrum. The negative APCI-MS data showed a deprotonated molecular ion peak at *m/z* 195.

[1'-^{13}C][5-^{2}H]-Coniferyl alcohol was prepared from [1'-^{13}C][5-^{2}H]-ferulic acid *via* its ethyl ester according to the method of Quideau and Ralph[12] with a slight modification. A mixture of 81.0 mg of [1'-^{13}C][5-^{2}H]-ferulic acid (0.41 mmol), 800 μL of conc. HCl, and 10 mL of ethyl alcohol was heated for 3 h at 100 °C. After evaporating the reacted mixture, the resulting residue gave a corresponding amount of an ethyl ester. This ethyl ester was dissolved in 5 mL of dehydrated diethyl ether, and 137 mg of LiAlH$_4$ was then added to the solution. The mixture was allowed to remain at room temperature for 30 min, and then the catalyst was removed by filtration. The ethyl acetate extract from the residue was purified by ODS column chromatography to afford 22 mg of [1'-^{13}C][5-^{2}H]-coniferyl alcohol (0.12 mmol, 29% yield). The ^{1}H-NMR spectrum showed a set of double-doublet signals coupled with 150 and 16 Hz at 6.53 ppm, indicating a shift of the olefinic proton at the 1'-position because of the reduction from carboxylic acid to an alcohol. The other signals in the spectrum were similar to those of standard coniferyl alcohol, except for the signal indicating a deficiency at the 5-position in the labeled compound. The positive APCI-MS data showed a dehydroxy molecular ion peak at *m/z* 165, this being 2 masses larger than that of standard coniferyl alcohol.

A preliminary experiment was carried out for measuring the capsaicin synthase (CS) activity in a crude enzyme solution extracted from the *Capsicum* pepper fruits according to the method of Fujiwake *et al.*[13] with a slight modification. Conversion of the labeled vanillyl-amine into a labeled vanillyl octanamide, a model analog of capsaicin, was measured by an LC-MS/MS system (LC: Nanospace SI-1, Shiseido, Tokyo, Japan; MS/MS: API 2000, Applied Biosystems, Carlsbad, CA, USA). The placenta and dissepiment (1.4 g) were dissociated from 3 pods of fresh fruits of Habanero (*Capsicum chinense*) and were ground at 4 °C with 3 mL of 50 mM Tris–HCl (pH 8.0) containing 1% Triton X-100. After centrifuging at 2000 g and 4 °C for 30 min, the supernatant was used as the crude enzyme solution (8.6 mg/mL in protein content). We incubated 100 μL of a 50 mM Tris–HCl (pH 8.0) solution containing 2 mM [1'-^{13}C][5-^{2}H]-vanillylamine, 1 mM octanoyl-CoA (Sigma), and 10 μL of the enzyme solution at 37 °C for 1 h. The reaction was terminated by adding 6 M HCl to the mixture. The ethyl acetate extract from the mixture was dried and then dissolved in MeOH containing 0.1% acetic acid for an LC-MS/MS analysis. The LC-MS/MS conditions for measuring vanillyl octanamide were as follows: LC column, a reversed-phase Unison UK-C18 silica gel column (2 mm i.d. × 150 mm (Imtakt Co., Kyoto, Japan)); column temperature, 40 °C; solvent, 70–100% MeOH containing 0.1% acetic acid (0–10 min); flow rate, 0.2 mL/min; injection volume, 5 μL; MS/MS ion source, ESI; polarity, positive; detection mode, multiple reaction monitoring (MRM); detected ions,

precursor/product, 280/137 for the natural predominant isotope of vanillyl octanamide [M] and 282/139 for the labeled one [M + 2]. The optimum parameters for detecting vanillyl octanamide were automatically tuned by using an authentic sample with Analyst software (Applied Biosystems). The ions of vanillyl octanamide were observed in the mass chromatogram at 4.38 min.

The enzyme solution from native Habanero contained a trace of vanillyl octanamide (the naturally predominant isotope [M], 3.9 nmol/mg of protein). A negligible amount of the native [M + 2] isotope (50 pmol/mg of protein) was observed as 1.2% of the abundance ratio that coincides with the calculated abundance ratio of the isotope (1.3%). Incubating the enzyme solution with labeled vanillylamine and octanoyl-CoA substantially increased the amount of the [M + 2] isotope of vanillyl octanamide to 370 pmol/mg of protein, and its abundance ratio was 8.8%. No marked increase in the [M + 2] isotope was apparent in the absence of the enzyme or when using its boiled solution.

We synthesized in this study stable isotope-labeled precursors for capsaicinoid, capsinoid, and capsiconinoid biosyntheses and showed that the precursors could be useful tools for biosynthetic studies. Further investigations are in progress using the labeled precursors for information on the flow and properties of enzymes involved in the biosyntheses.

Acknowledgments

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