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TITLE OF THE INVENTION

5 METHOD FOR THE MICROBIAL PRODUCTION OF SPECIFIC NATURAL
CAPSAICINOIDS

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims priority to U.S. Provisional Application No. 62/363,951,
filed on July 19, 2016, the contents of which are incorporated by reference herein in their
10 entirety.

INCORPORATION OF SEQUENCE LISTING

[002] The sequence listing contained in the file named " C1497.70006WO00.txt",
which was created on June 27, 2016, is contemporaneously filed by electronic submission
15 (using the United States Patent Office EFS-Web filing system) and incorporated herein by
reference in its entirety.

FIELD OF THE INVENTION

[003] The field of the invention relates to methods and processes useful in the
20 microbial production of capsaicinoids. More specifically, in some embodiments, the current
invention provides a method to produce nonivamide and other specific capsaicinoids in yields
not otherwise possible in plant-based systems. The present invention provides, *inter alia*, a
genetically modified *E. coli*. strain that has been modified to biosynthesize a desired
capsaicinoid, a method of producing the genetically modified microorganism to produce the
25 desired capsaicinoid, and a production method for the production of desirable capsaicinoid
products in high yield and purity levels starting from a specific starting material.

BACKGROUND OF THE INVENTION

[004] The present invention, in some embodiments, is directed to a method for modifying a microbial strain to produce a desirable capsaicinoid compound in high yield using a specific feeding source for the microbial production strain. Capsaicinoid compounds (including without limitation: capsaicin (CP), dihydrocapsaicin (DHCP), nonivamide (NV), etc.), are the “spicy” components of hot peppers, and may be effective for preventing a number of pathologies by promoting energy metabolism, stimulating the central nervous system and activating lipolytic enzymes. In addition, these compounds sterilize the gastrointestinal tract, improve immunity when consumed and are also effective for activating immunity and relieving fatigue.

Capsaicinoid Chemistry

[005] “Spicyness” or pungency is a unique characteristic of hot peppers in the *Capsicum* genus that produce alkaloids called capsaicinoids. In nature, it is believed that capsaicinoids are present in plants to deter mammals from consuming their fruits and destroying seeds. Humans can sense capsaicinoids *via* a receptor structurally related to members of the transient receptor potential channels (or “TRP” family of ion channels). Similar to other receptors important in sensory neurons, the capsaicin receptor (“TRPV1” also referred to as the vanilloid TRP, vanilloid 1 Receptor or TRPV1) mediates the pungent odor and pain/hot sensations associated with capsaicin and piperine by reversibly losing sensitivity to capsaicin as well as other pain and heat stimuli when it is under prolonged exposure to the same stimulus (Caterina et al., 1997). This phenomenon may partially explain why humans can tolerate and even enjoy spicy foods, as pain sensors may be turned off.

[006] Capsaicin (“CP”), 8-methyl-N-vanillyl-trans-6-nonenamide, and dihydrocapsaicin (“DHCP”), 8-methyl-N-vanillylnonanamide, are the two major capsaicinoids

in peppers and are collectively responsible for up to 90% of the pungency of chili peppers, including ghost chilies (Garcés-Claver et al., 2007). These two compounds and the more minor capsaicinoid compounds represent important ingredients in foods and other uses consumed throughout the world (European Commission Scientific Committee on Food, 2002). Speaking generally, the capsaicin content of chili peppers ranges from 0.1 to 1% w/w (Govindarajan and Sathyanarayana 1991) and for many uses requires that methods be used to concentrate the CP and/or DHCP compounds. Due to the wide use of capsaicinoids in food, medicine and other industrial and self-defense uses, there has been an increasing demand for the production and concentration of capsaicinoid compounds.

10 [007] The *Capsicum* genus includes over 20 species of peppers, from which *C. annuum*, *C. frutescens*, *C. chinense*, *C. baccatum*, and *C. pubescens* have been domesticated (Walsh and Hoot, 2001). There is wide genetic variation in pungency or spicyness levels across the various *Capsicum* species due to varying levels and types of capsaicinoids present. For example, the non-pungent sweet bell pepper from *C. annuum* scored 0.0 SHU (Scoville Heat Unit; a scale that indicates the amount of capsaicin), while the “Bhut Jolokia” or ghost chili is a hybrid between *C. chinense* and *C. frutescens* from Northeastern India, scored up to 1,001,304 SHUs (Bosland and Baral, 2007).

15 [008] Pure CP extracted from plants typically rates at approximately 16,000,000 Scoville units on the heat index and in this concentration can sell for over \$5000 USD per gram (Batchelor, 2000). However, the content of capsaicinoids in hot peppers is generally very low and can be greatly affected by environmental and growth conditions leading to problems of sustainability and consistency.

20 [009] When extracted from plants, typically solid-liquid extraction using solvents like hexane, chloroform, and ethanol are commonly employed for capsaicinoid recovery (Catchpole et al., 2003). However, solvent extraction is itself energy intensive, leads to problems of toxic

waste disposal, requires extensive acreage for the plants themselves to be grown and yields a product that requires further purification for minor constituents to be recovered. Thus, new production methods are needed to reduce costs of pure capsaicin and/or other capsaicinoids and lessen the environmental impact of large scale cultivation and processing (Yao et al., 5 1994). Genetic manipulation of selected microbial strains has the potential to address these needed improvements and increase the selectivity, abundance and purity of desired capsaicinoid varieties.

[0010] In addition to the above, while consumers approve and actively seek natural and biological sources for food, fragrance, flavor or medicinal components they are also concerned 10 about sourcing, consistent potency and environmentally sustainable production. Into this situation the microbial fermentation and production methods of the current invention provide, in some embodiments, compounds produced by modified microbial strains that have the capability to produce products biologically in quantities useful for a variety of industries and research while doing so in a more natural fashion than inorganic chemical synthesis.

[0011] Accordingly, a need exists for the development of a novel method of producing 15 CP, NV and DHCP and other capsaicinoids through the development of a specific biologic pathway using modified microbial strains that can stably produce desirable capsaicin compounds. Specifically, the current invention provides, *inter alia*, methods to produce CP, NV and DHCP from a fermentation process in commercially relevant amounts.

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SUMMARY OF THE INVENTION

[0012] The present invention encompasses improved methods of producing capsaicinoids. The current invention provides a method to produce capsaicinoids in modified microbes that comprise a cellular system, such as yeast or bacteria. Applicants have isolated 25 the genes for an acyl-CoA synthetase (“ACS”)(SEQ ID NO: 1) and capsaicin synthase

(“CS”)(SEQ ID NO: 2) and inserted them into a cellular system of interest, in a preferred embodiment this cellular system is in *E. coli*. These genes, when expressed, allow the production of capsaicinoids in that system. According to the current invention, in some embodiments, this system produces the CP, DHCP and NV capsaicinoids in substantial amounts.

[0013] According to the current invention, in some embodiments, a biosynthetic method of making carboxyl CoAs from medium/long-chain carboxylic acid including expressing an ACS and CS in a cellular system is provided. In some embodiments, said system requires long-chain carboxylic acids, growing the cellular system transformed by ACS and CS in a medium, and producing carboxyl CoAs. These cultures then produce certain capsaicinoids when the modified microbial strains are fed from natural precursors. Accordingly, nonanoic acid can be used as a feeding precursor and can be obtained, for example, from the oil of pelargonium or European olive (*Olea europaea*). Other feeding precursors of the current invention include octanoic acid, decanoic acid, vanillin and vanillylamine. Octanoic acid can also be used according to the current invention and is naturally found, for example, in palm and coconut oil. Decanoic acid is found in the seeds of *Cuphea* species. Natural vanillin can also be produced by microbial fermentation (Zhou and Yu, 2014). In addition, natural vanillylamine could be obtained from vanillin by pAMT (Weber et al., 2014).

[0014] The current method provides an approach for the development of a bacterial strain that is capable of producing significant volumes of capsaicinoids by genetic modification and targeted feeding of specific starting molecules that are more cost effective and easier to obtain.

[0015] In certain embodiments of the current invention, the target capsaicinoid produced is selected from the group consisting of NV, CP and DHCP.

[0016] An embodiment of the present disclosure is a biosynthetic method of developing capsaicinoids by making carboxyl CoAs from medium to long-chain carboxylic acids comprising expressing an ACS in a cellular system, feeding medium to long-chain carboxylic acids to the cellular system, growing the cellular system in a medium, and
5 producing carboxyl CoAs.

[0017] A further embodiment is that the ACS is expressed from ACS1 cloned from ghost chili pepper. (For reference see Table 1.)

[0018] An alternative embodiment is that the ACS is expressed from *Arabidopsis* based on LCAS4 or LCAS5. In another embodiment, the ACS is expressed from ACS2 cloned from
10 *Capsicum spp.* Further, in some embodiments, the ACS used in the invention is an ACS that shares a sequence identity of at least 66% (e.g., at least 70%, at least 80%, at least 90%, at least 95%, at least 99% or 100%) with the ACS1 cloned from ghost chili pepper. In another embodiment, the ACS is an ACS that shares a sequence similarity of at least 92% (e.g., 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) with the ACS1 cloned from ghost chili
15 pepper at the protein level.

[0019] A further embodiment is that the medium or long-chain carboxylic acid is 8-methyl-trans-6-nonenoic acid. Long chain carboxylic acids generally have 14 to 18 carbons, while medium-chain carboxylic acids generally have 8 to 13 carbons.

[0020] In one embodiment of the current invention, the feeding of medium to long-
20 chain carboxylic acids to the cellular system comprises adding the medium long-chain carboxylic acid to the cellular system for use by the selected strains.

[0021] In an alternative embodiment, the feeding of medium to long-chain carboxylic acid to the cellular system comprises expressing the medium to long-chain carboxylic acid from a biosynthetic pathway in the cellular system.

[0022] In terms of product/commercial utility there are several dozen products containing capsaicin on the market in the United States and can be used in everything from analgesics to pest repellents as well as in foods and as a dietary supplements. Products containing capsaicin can be, for example, aerosols, liquids, or granular formulations.

5 [0023] As for the cellular system in the embodiment, it is selected from the group consisting of bacteria, yeast, and a combination thereof, or any cellular system that would allow the genetic transformation with the selected genes and thereafter the biosynthetic production of the desired capsaicinoids from the alternative feeding precursors. In a most preferred microbial system, *E. coli* are used to produce the desired capsaicinoid compounds.

10 [0024] An embodiment of the present disclosure is a biosynthetic method of making 8-methylnonenoyl-CoA comprising expressing an ACS in a cellular system, feeding 8-methyl-trans-6-nonenic acid to the cellular system, growing the cellular system in a medium, and producing 8-methylnonenoyl-CoA. The ACS of the current invention, in some embodiments, is expressed from ACS1 cloned from ghost chili pepper. Alternatively, the ACS can be expressed
15 from LCAS4 or LCAS5 cloned from *Arabidopsis*.

[0025] In another embodiment, the ACS of the invention is expressed from ACS2 cloned from *Capsicum* spp. Further, in some embodiments, the ACS is an ACS that shares a sequence identity of at least 66% (e.g., at least 70%, at least 80%, at least 90%, at least 95%, at least 99% or 100%) with the ACS1 cloned from ghost chili pepper. In another embodiment, the
20 ACS is an ACS that shares a sequence similarity of at least 92% (e.g., 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100%) at the protein level with the ACS1 cloned from ghost chili pepper.

[0026] In some aspects, the invention provides a biosynthetic method of making a capsaicinoid of interest comprising: expressing an ACS and CS in a transformed cellular
25 system; growing the cellular system in a medium; and producing the capsaicinoid of interest.

[0027] In some embodiments, the ACS is cloned from a plant of the Capsicum genus. In some embodiments, the Capsicum genus plant is the ghost chili. In some embodiments, the ACS expressed is derived from a gene cloned from Arabidopsis based on LCAS4 or LCAS5.

In some embodiments, the CS is cloned from a plant of the Capsicum genus. In some
5 embodiments, the Capsicum genus plant is the ghost chili.

[0028] In some embodiments, the method further comprises feeding a source material to said cellular system in addition to culture media. In some embodiments, the source material is selected from the group consisting of: nonanoic acid; oil of pelargonium; octanoic acid; decanoic acid; vanillin; and, vanillyamine. In some embodiments, the source material is a
10 mixture of two or more of the following: nonanoic; oil of pelargonium; octanoic acid; decanoic acid; vanillin; and, vanillyamine. In some embodiments, the source material is nonanoic acid. In some embodiments, said source material is selected from the group consisting of: C6 to C12 hydrocarbons that are medium to long chain fatty acids. In some
15 embodiments, the source material is a mixture of two or more C6 to C12 hydrocarbons that are medium to long chain fatty acids.

[0029] In some embodiments, the transformed cellular system is selected from the group including yeast, non-capsaicinoid producing plants, algae and bacteria. In some embodiments, the cellular system is E. Coli.

[0030] In some embodiments, the capsaicinoid of interest is capsaicin. In some
20 embodiments, the capsaicinoid of interest is NV. In some embodiments, the capsaicinoid of interest is DHCP. In some embodiments, the capsaicinoids produced are a mixture comprising CP, NV and DHCP. In some embodiments, the capsaicinoid of interest is nordihydrocapsaicin.

[0031] In some embodiments, the ACS utilized is an ACS that shares a DNA sequence similarity of at least 75% (e.g., at least 75%, at least 80%, at least 85%, at least 90%, at least
25 95%, at least 99% or 100%) with SEQ ID NO: 3. In some embodiments, the ACS utilized is

an ACS that shares a protein sequence similarity of at least 90% (e.g., at least 90%, at least 95%, at least 99% or 100%) with SEQ ID NO: 1. In some embodiments, the CS utilized is an CS that shares a DNA sequence similarity of at least 75% (e.g., at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99% or 100%) with SEQ ID NO: 4. In some
5 embodiments, the CS utilized is an CS that shares a protein sequence similarity of at least 90% (e.g., at least 90%, at least 95%, at least 99% or 100%) with SEQ ID NO: 2.

[0032] In some embodiments, the cellular system is selected from the group consisting of bacteria, yeast, plant cells, animal cells, an in vitro translation system and a combination thereof.

10 [0033] In some embodiments, producing the capsaicinoid of interest comprises: i) purifying crude product; and, ii) removing solvents under vacuum to provide a concentrated capsaicinoid product. In some embodiments, the crude product is purified by column chromatography. In some embodiments, the crude product is purified by acid-base extraction. In some
15 embodiments, the crude product is purified by vacuum distillation. In some embodiments, said the product is purified by semi-preparative HPLC.

[0034] In some embodiments, the cellular system further comprises an aminotransferase which is capable of catalyzing the conversion of vanillin to vanillylamine.

[0035] In some embodiments, the transformed cellular system includes a CaUGT2 from *C. roseus*. In some embodiments, the transformed cellular system includes a CaUGT2
20 that shares a DNA sequence similarity of at least 75% (e.g., at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99% or 100%) with SEQ ID NO: 5. In some embodiments, the transformed cellular system includes a CaUGT2 that shares a protein sequence similarity of at least 90% (e.g., at least 90%, at least 95%, at least 99% or 100%) with SEQ ID NO: 6.

[0036] In other aspects, the invention provides a capsaicinoid of interest produced by a transformed cellular system growing within a medium. In some embodiments, the transformed cellular system is selected from the group consisting of: yeast, non-capsaicinoid producing plants, algae and bacteria. In some embodiments, the transformed cellular system is transformed by the presence of an ACS and a CS derived from a plant of the Capsicum genus. In some embodiments, the ACS transformed into the cellular system is an ACS derived from Arabidopsis based on LCAS4 or LCAS5. In some embodiments, the transformed cellular system is transformed by the presence of an ACS and a CS derived from a plant of the Capsicum genus. In some embodiments, the transformed cellular system is transformed by the presence of an ACS and a CS derived the ghost chili.

[0037] In some embodiments, the method of producing further comprises feeding the transformed cellular system a specific source material. In some embodiments, the source material is selected from a group consisting of: nonanoic; oil of pelargonium; octanoic acid; decanoic acid; vanillin; and, vanillyamine. In some embodiments, the source material is selected from a mixture containing two or more of the following: nonanoic; oil of pelargonium; octanoic acid; decanoic acid; vanillin; and, vanillyamine.

[0038] In some embodiments, the capsaicinoid is CP. In some embodiments, said capsaicinoid is NV. In some embodiments, the capsaicinoid is DHCP. In some embodiments, the capsaicinoids produced are a mixture of CP, NV and DHCP. In some embodiments, the capsaicinoid is greater than 70% (e.g., greater than 70%, greater than 80%, greater than 90%, greater than 95%, greater than 98% or greater than 99%) pure. In some embodiments, the capsaicinoid content is greater than about 85% (e.g., greater than about 85%, greater than about 90%, greater than about 95%, greater than about 98%, or greater than about 99%) by weight on a dry basis. In some embodiments, the capsaicinoid is purified capsaicin.

[0039] In yet other aspects, the invention provides a method of producing a capsaicinoid of interest comprising feeding a transformed microbe culture a specific fatty acid precursor. In some embodiments, the capsaicinoid of interest is Capsaicin and the fatty acid precursor is 6E-8-methyl-6-nonenoic acid. In some embodiments, the capsaicinoid of interest is Dihydrocapsaicin and the fatty acid precursor is 8-Methyl nonanoic acid. In some 5 embodiments, the capsaicinoid of interest is Nonivamide and the fatty acid precursor is nonanoic acid. In some embodiments, the capsaicinoid of interest is N-Vanillyl octamide and the fatty acid precursor is octanoic acid. In some embodiments, the capsaicinoid of interest is N-vanilyl decanamide and the fatty acid precursor is decanoic acid. In some embodiments, the 10 transformed culture is a microbe culture of yeast or bacteria.

[0040] In some embodiments, the microbe culture expresses an ACS that shares a DNA sequence similarity of at least 75% (e.g., at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99% or 100%) with SEQ ID NO: 3. In some embodiments, the microbe culture expresses an ACS that shares a protein sequence similarity of at least 90% (e.g., at least 15 90%, at least 95%, at least 99% or 100%) with SEQ ID NO: 1. In some embodiments, the microbe culture expresses a CS that shares a DNA sequence similarity of at least 75% (e.g., at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99% or 100%) with SEQ ID NO: 4. In some embodiments, the microbe culture expresses a CS that shares a protein sequence similarity of at least 90% (e.g., at least 90%, at least 95%, at least 99% or 20 100%) with SEQ ID NO: 2. In some embodiments, the microbe culture expresses a CaUGT2 that shares a DNA sequence similarity of at least 75% (e.g., at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99% or 100%) with SEQ ID NO: 5. In some embodiments, the microbe culture expresses a CaUGT2 that shares a protein sequence similarity of at least 90% (e.g., at least 90%, at least 95%, at least 99% or 100%) with SEQ ID 25 NO: 6.

[0041] In other aspects, the invention provides an analgesic composition for the topical treatment of pain in which the composition contains an effective amount of a capsaicinoid(s), wherein said analgesic composition is produced by any of the methods described herein wherein the improvement comprises producing said capsaicinoid in a microbial cellular system. In some embodiments, the effective amount is at least 0.0125 percent of a capsaicinoid of interest. In some embodiments, the capsaicinoid is selected from the group comprising: nonivamide, N-vanillylnonanamides, N-vanillylsulfonamides, N-vanillylureas, N-vanillylcarbamates, N[(substituted phenyl)methyl]alkylamides, methylene substituted N[(substituted phenyl)methyl]alkanamides, N[(substituted phenyl)methyl]-cis-monosaturated alkenamides, N[(substituted phenyl)methyl]diunsaturated amides, 3-hydroxyacetanilide, hydroxyphenylacetamides, pseudocapsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin I, anandamide, piperine, zingerone, warburganal, polygodial, aframodial, cinnamodial, cinnamosmolide, cinnamolide, civamide, nonivamide, olvanil, N-oleyl-homovanillamide, isovelleral, scalaradial, ancistrodial, and any combinations or mixtures thereof.

[0042] In other aspects, the invention provides a flavor and fragrance composition, wherein the flavor and fragrance composition has at least one capsaicinoid produced according to any of the methods described herein. In some embodiments, the capsaicinoid is selected from the group comprising: nonivamide, N-vanillylnonanamides, N-vanillylsulfonamides, N-vanillylureas, N-vanillylcarbamates, N[(substituted phenyl)methyl]alkylamides, methylene substituted N[(substituted phenyl)methyl]alkanamides, N[(substituted phenyl)methyl]-cis-monosaturated alkenamides, N[(substituted phenyl)methyl]diunsaturated amides, 3-hydroxyacetanilide, hydroxyphenylacetamides, pseudocapsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin I, anandamide, piperine, zingerone, warburganal, polygodial, aframodial, cinnamodial, cinnamosmolide, cinnamolide,

civamide, nonivamide, olvanil, N-oleyl-homovanillamide, isovelleral, scalarial, ancistrodial, and any combinations or mixtures thereof.

[0043] While the disclosure is susceptible to various modifications and alternative forms, specific embodiments thereof are shown by way of example in the drawings and will herein be described in detail. It should be understood, however, that the drawings and detailed description presented herein are not intended to limit the disclosure to the particular embodiment disclosed, but on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the present disclosure as defined by the appended claims.

[0044] Other features and advantages of this invention will become apparent in the following detailed description of preferred embodiments of this invention, taken with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0045] **FIG. 1.** shows the Capsaicinoid Biosynthetic Pathway. The aminotransferase (pAMT) catalyzes the formation of vanillyamine from vanillin.

[0046] **FIG. 2** shows the production levels of specific Capsaicinoid compounds according to an embodiment of the transformed cellular system of the invention. Production of CP (capsaicin), CP8 (N-vanilly octamide), CP9 (nonivamide) or CP10 (N-vanillyl decanamide) from 6E (6E-8-methyl nonenoic acid), C8:0 (octanoic acid or caprylic acid), C9:0 (nonanoic acid or pelargonic acid) and C10:0 (decanoic acid or capric acid), respectively. The experiment was performed in triplicate. Left bar in each pair is 1d. Right bar in each pair is 2d.

[0047] **FIG. 3** shows HPLC Profiles of the preferred Cellular System, in particular HPLC profiles of crude ethyl acetate extracts from *E. coli* cultures fed with C8:0, C9:0 and C10:0, respectively. CP8 (N-vanilly octamide), CP9 (nonivamide) and CP10 (N-vanillyl

decanamide) had retention times of 10.9, 11.3 and 11.8 min, respectively. The inserted pictures are their UV-Vis spectra. FIG. 3 is a reading of substrate and products, peak areas were measured at 280 nm.

[0048] FIG. 4 shows a HPLC profile of the Nonivamide standard to compare with an embodiment of the cellular system of the invention.

[0049] FIG. 5 shows production of Capsaicin from vanillin with the presence of the pAMT synthase.

[0050] FIG. 6 shows Ni-NTA affinity chromatography of CaUGT2. Pre, pre-column; post, post-column; E1, E2, E3, fractions collected from column. His-tagged CaUGT2 has a molecular weight ca. 58 kD.

[0051] FIG. 7 shows production of capsaicin-glu and nonivamide-glu by CaUGT2 in vitro.

[0052] FIG. 8 shows the structure of nordihydrocapsaicin (7M-CP). Nordihydrocapsaicin (7M-CP) is the third most abundant CP in hot peppers, which occupies ca. 7% of total capsaicinoids.

[0053] FIG. 9 shows HPLC profiles of 7M-CP and C9-CP production.

[0054] FIG. 10 shows a comparison of C9-CP and 7M-CP production in the same culture system. The experiment was performed in duplicate.

20 DESCRIPTION OF THE PREFERRED EMBODIMENT

The following abbreviations have designated meanings in the specification:

Explanation of Terms Used Herein:

25 [0055] Capsaicin or CP is a colorless irritant phenolic amide $C_{18}H_{27}NO_3$ and is one of a series of phenolic amides found in various *Capsicum* species and hybrids thereof that gives hot peppers their hotness or pungency and that is used for

food, medicine, and security applications. Pure CP is a volatile, hydrophobic, colorless, odorless, crystalline to waxy compound.

[0056] Capsaicinoid as used herein this refers to a class of irritant compounds, related to Capsaicin, that are responsible for the heat of chili peppers. They are irritants for mammals, including humans, and can produce a sensation of burning in any tissue with which they come into contact. The capsaicinoids are produced as secondary metabolites by chili peppers, probably as deterrents against certain mammals and fungi. Exemplary capsaicinoids include, but are not limited to: nonivamide, N-vanillylnonanamides, N-vanillylsulfonamides, N-vanillylureas, N-vanillylcarbamates, N[(substituted phenyl)methyl]alkylamides, methylene substituted N[(substituted phenyl)methyl]alkanamides, N[(substituted phenyl)methyl]-cis-monosaturated alkenamides, N[(substituted phenyl)methyl]diunsaturated amides, 3-hydroxyacetanilide, hydroxyphenylacetamides, pseudocapsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin I, anandamide, piperine, zingerone, warburganal, polygodial, aframodial, cinnamodial, cinnamosmolide, cinnamolide, civamide, nonivamide, olvanil, N-oleyl-homovanillamidia, isovelleral, scalaradial, ancistrodial, and any combinations or mixtures thereof. Certain capsaicinoids are further described in Table 2.

[0057] Cellular system is any cells that provide for the expression of ectopic proteins. It includes bacteria, yeast, plant cells and animal cells. It includes both prokaryotic and eukaryotic cells. It also includes the *in vitro* expression of proteins based on cellular components, such as ribosomes.

[0058] Fatty Acids, C6-C12. According to the current invention a variety of fatty acids can be used as starting source materials. The source materials include vanillin, vanillylamine or their derivatives with modifications at the aromatic ring such as methylation, ethylation, or glycosylation; and more particularly 6-12 carbon straight chain or branched chain fatty acids or their derivatives such as hydroxy fatty acids (Ex: Hexanoic acid; Heptanoic acid; Octanoic acid; Nonanoic acid; Decanoic acid; Undecanoic acid; and, Dodecanoic acid) can be straight chain fatty acids or branched chain fatty acids and be used to make the capsaicinoids of the current invention.

[0059] Growing the Cellular System. Growing includes providing an appropriate medium that would allow cells to multiply and divide. It also includes providing resources so that cells or cellular components can translate and make recombinant proteins.

[0060] Protein Expression. Protein production can occur after gene expression. It consists of the stages after DNA has been transcribed to messenger RNA (mRNA). The mRNA is then translated into polypeptide chains, which are ultimately folded into proteins. DNA may be present in the cells through transfection - a process of deliberately introducing nucleic acids into cells. The term is often used for non-viral methods in eukaryotic cells. It may also refer to other methods and cell types, although other terms are preferred: "transformation" is more often used to describe non-viral DNA transfer in bacteria, non-animal eukaryotic cells, including plant cells. In animal cells,

transfection is the preferred term as transformation is also used to refer to progression to a cancerous state (carcinogenesis) in these cells. Transduction is often used to describe virus-mediated DNA transfer. Transformation, transduction, and viral infection are included under the definition of transfection for this application.

[0061] Yeast. According to the current invention yeast as claimed herein are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom. Yeast are unicellular organisms which evolved from multicellular ancestors but with some species useful for the current invention being those that have the ability to develop multicellular characteristics by forming strings of connected budding cells known as pseudohyphae or false hyphae.

Acronyms:

15 Pal, phenylalanine ammonia lyase;
Ca4H, cinnamic acid 4-hydroxylase;
4CL, 4-coumarate CoA ligase;
HCT, hydroxycinnamoyl transferase;
C3H, coumaroyl shikimate/quinic acid 3-hydroxylase;
20 COMT, caffeic acid O-methyltransferase; pAMT, aminotransferase;
BCAT, branched-chain amino acid transferase;
Kas, 3-keto-acyl ACP synthase;
ACL, acyl carrier protein;
FatA, acyl-ACP thioesterase;
25 ACS, acyl-CoA synthetase; and,
CS, capsaicin synthase.

DETAILED DESCRIPTION

[0062] The present invention relates, in some embodiments, to a system for an improved production method of CP, DHCP and NV as developed from specific feeding precursors.

[0063] Nonivamide, also called pelargonic acid vanillylamide or PAVA (here "NV"), is one of trace capsaicinoids identified in hot peppers (FIG. 2; Constant et al., 1996). It is used as a food additive for pungent flavors. It is also used the pharmaceutical industry as an alternative to capsaicin. However, due to the extremely low content of nonivamide in hot peppers, using current plant extraction methods is not commercially viable so it has been exclusively made by chemical synthesis. Although chemically-synthesized nonivamide is readily available and cheap, non-natural compounds are not well perceived by consumers whose demand is in favor of natural products. To be natural, the chemicals have to be derived from the extraction of natural materials or to be transformed by enzymes or microbes from natural precursors, as provided in the methods of the current invention.

[0064] One aspect of the present invention are the DNA and corresponding protein sequences of the ACS and CS of the current invention. The DNA sequences for the ACS and CS enzymes were identified and removed from a ghost chili hybrid and inserted into plasmids for use in the current invention. Such sequences are provided herein as SEQ ID NO: 3 and SEQ ID NO: 4, respectively.

[0065] The present invention includes nucleic acid molecules and uses thereof in methods described herein, the nucleic acid molecules having nucleic acid sequences that hybridize to SEQ ID NO: 1 and SEQ ID NO: 2, respectively, or any complements thereof, or any cis elements thereof. The present invention also provides nucleic acid molecules and uses thereof in methods described herein, the nucleic acid molecules comprising a nucleic acid sequence selected from the group consisting of SEQ ID NO: 3 through SEQ ID NO: 4 for the

ACS and CS sequences of the invention, any complements thereof, or any cis elements thereof, or any fragments thereof. (See Table 1, SEQ ID NOs: 3 and 4).

[0066] Additional embodiments include the use of ACS1 to modify the levels of capsaicinoids in pepper plants by overexpressing ACS1 utilizing standard known techniques for overexpression of genes in transformed plants. Another embodiment includes the use of ACS1 to modulate the levels of capsaicinoids in pepper plants by knocking out or knocking down ACS1 utilizing standard known techniques for knocking out or knocking down expression of genes. Again, the overexpression or the knock out/knock down is by standard molecular cellular strategies and techniques known by a person of ordinary skill in the art. Another embodiment includes the use of ACS1 to generate acyl-CoAs and their downstream metabolites including fatty acids involving the expression or overexpression of ACS1. Another variation is the use of ACS1 to modulate the levels of acyl-CoAs and their downstream metabolites including fatty acids comprising knocking out or knocking down ACS1. Different specific capsaicinoids produced are determined by different fatty acids fed into the culture. (See Table 3).

[0067] The acyl CoAs that are made by the methods hereof could be utilized to make capsaicinoids of interest, and they would generally be of the medium chain variety. Again, although ACS1 can mediate the conversion of both medium chain- and long chain-carboxylic acids to acyl-CoAs, the medium chain activity is far more important than long chain activity as medium chain activity is the essential component in today's biofuel industry. The other importance as mentioned above for ACS1 is that it can be used to modify the capsaicinoid levels in plants through transgenic technology. However, ACS1 is not precluded from usage in regards to long chain acyl-CoAs. In an embodiment, a cellular system, such as a bacterial based system or a yeast based system can be modified to express ACS. The ACS could be ACS1 cloned from ghost pepper. Other ACSs suitable are one based on LCAS4 and LCAS5

from *Arabidopsis*. Other known ACS1 and ACS2 could also be expressed in the cellular systems. Appropriate substrate, such as 8-methyl-trans-6-nonenic acid and 8-methylnonanoic acid, can then be fed to the cellular system. The substrates could also be expressed as part of a biosynthetic pathway within the cellular system. The cellular system is then incubated allowing
5 for the biosynthetic production of 8-methyl-trans-6-nonenoyl-CoA or 8-methyl nonanoyl-CoA.

[0068] According to another embodiment of the current invention the efficiency of heterologous protein production in a microbial system can be enhanced by codon changes that alter the DNA sequences to one that may be preferred by the cellular system being used for expression but that varies from the original gene source organism without changing the
10 eventual polypeptide produced. Approaches normally used to overcome this problem include targeted mutagenesis to remove rare codons or the addition of rare codon tRNAs in specific cell lines to move towards a codon sequence preferred by a host organism that will produce the polypeptide of interest. Recently, improvements in such “codon optimization” technology have enabled cost-effective production of synthetic genes, making this a feasible alternative and
15 potentially useful for the current invention.

Identity and Similarity

[0069] Identity is the fraction of amino acids that are the same between a pair of sequences after an alignment of the sequences (which can be done using only sequence
20 information or structural information or some other information, but usually it is based on sequence information alone), and similarity is the score assigned based on an alignment using some similarity matrix. The similarity index can be any one of the following BLOSUM62, PAM250, or GONNET, or any matrix used by one skilled in the art for the sequence alignment of proteins.

[0070] Identity is the degree of correspondence between two sub-sequences (no gaps between the sequences). An identity of 25% or higher implies similarity of function, while 18-25% implies similarity of structure or function. Keep in mind that two completely unrelated or random sequences (that are greater than 100 residues) can have higher than 20% identity.

5 Similarity is the degree of resemblance between two sequences when they are compared. This is dependent on their identity.

Determination of Sequence Similarity Using Hybridization Techniques

[0071] Nucleic acid hybridization is a technique well known to those of skill in the art of DNA manipulation. The hybridization properties of a given pair of nucleic acids are an
10 indication of their similarity or identity.

[0072] The term "hybridization" refers generally to the ability of nucleic acid molecules to join *via* complementary base strand pairing. Such hybridization may occur when nucleic acid molecules are contacted under appropriate conditions. "Specifically hybridizes" refers to the ability of two nucleic acid molecules to form an anti-parallel, double-stranded
15 nucleic acid structure. A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit "complete complementarity," i.e., each nucleotide in one sequence is complementary to its base pairing partner nucleotide in another sequence. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional
20 "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Nucleic acid molecules that hybridize to other nucleic acid molecules, e.g., at least under low stringency conditions are said to be "hybridizable cognates" of the other nucleic acid molecules. Conventional low
25 stringency and high stringency conditions are described herein and by Sambrook et al.,

MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, New York (1989) and by Haymes et al., NUCLEIC ACID HYBRIDIZATION, A PRACTICAL APPROACH, IRL Press, Washington, DC (1985). Departures from complete complementarity are permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure.

[0073] Low stringency conditions may be used to select nucleic acid sequences with lower sequence identities to a target nucleic acid sequence. One may wish to employ conditions such as about 0.15 M to about 0.9 M sodium chloride, at temperatures ranging from about 20°C to about 55°C. High stringency conditions may be used to select for nucleic acid sequences with higher degrees of identity to the disclosed nucleic acid sequences (Sambrook et al., 1989). High stringency conditions typically involve nucleic acid hybridization in about 2X to about 10X SSC (diluted from a 20X SSC stock solution containing 3 M sodium chloride and 0.3 M sodium citrate, pH 7.0 in distilled water), about 2.5X to about 5X Denhardt's solution (diluted from a 50X stock solution containing 1% (w/v) bovine serum albumin, 1% (w/v) ficoll, and 1% (w/v) polyvinylpyrrolidone in distilled water), about 10 mg/mL to about 100 mg/mL fish sperm DNA, and about 0.02% (w/v) to about 0.1% (w/v) SDS, with an incubation at about 50°C to about 70°C for several hours to overnight. High stringency conditions are preferably provided by 6X SSC, 5X Denhardt's solution, 100 mg/mL fish sperm DNA, and 0.1% (w/v) SDS, with an incubation at 55°C for several hours. Hybridization is generally followed by several wash steps. The wash compositions generally comprise 0.5X to about 10X SSC, and 0.01% (w/v) to about 0.5% (w/v) SDS with a 15 minute incubation at about 20°C to about 70°C. Preferably, the nucleic acid segments remain hybridized after washing at least one time in 0.1X SSC at 65°C.

[0074] A nucleic acid molecule preferably comprises a nucleic acid sequence that hybridizes, under low or high stringency conditions, with SEQ ID NO: 3 and SEQ ID NO: 4,

any complements thereof, or any fragments thereof, or any cis elements thereof. A nucleic acid molecule most preferably comprises a nucleic acid sequence that hybridizes under high stringency conditions with SEQ ID NO: 3 and SEQ ID NO: 4, any complements thereof, or any fragments thereof, or any cis elements thereof.

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Analysis of Sequence Similarity Using Identity Scoring

[0075] As used herein "sequence identity" refers to the extent to which two optimally aligned polynucleotide or peptide sequences are invariant throughout a window of alignment of components, e.g., nucleotides or amino acids. An "identity fraction" for aligned segments of a test sequence and a reference sequence is the number of identical components which are shared by the two aligned sequences divided by the total number of components in reference sequence segment, i.e., the entire reference sequence or a smaller defined part of the reference sequence.

[0076] As used herein, the term "percent sequence identity" or "percent identity" refers to the percentage of identical nucleotides in a linear polynucleotide sequence of a reference ("query") polynucleotide molecule (or its complementary strand) as compared to a test ("subject") polynucleotide molecule (or its complementary strand) when the two sequences are optimally aligned (with appropriate nucleotide insertions, deletions, or gaps totaling less than 20 percent of the reference sequence over the window of comparison). Optimal alignment of sequences for aligning a comparison window are well known to those skilled in the art and may be conducted by tools such as the local homology algorithm of Smith and Waterman, the homology alignment algorithm of Needleman and Wunsch, the search for similarity method of Pearson and Lipman, and preferably by computerized implementations of these algorithms such as GAP, BESTFIT, FASTA, and TFASTA available as part of the GCG® Wisconsin Package® (Accelrys Inc., Burlington, MA). An "identity fraction" for aligned segments of a test sequence and a reference sequence is the number of identical components which are shared

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by the two aligned sequences divided by the total number of components in the reference sequence segment, i.e., the entire reference sequence or a smaller defined part of the reference sequence. Percent sequence identity is represented as the identity fraction multiplied by 100.

The comparison of one or more polynucleotide sequences may be to a full-length

5 polynucleotide sequence or a portion thereof, or to a longer polynucleotide sequence. For purposes of this invention "percent identity" may also be determined using BLASTX version 2.0 for translated nucleotide sequences and BLASTN version 2.0 for polynucleotide sequences.

[0077] The percent of sequence identity is preferably determined using the "Best Fit" or "Gap" program of the Sequence Analysis Software Package™ (Version 10; Genetics
10 Computer Group, Inc., Madison, WI). "Gap" utilizes the algorithm of Needleman and Wunsch (Needleman and Wunsch, JOURNAL OF MOLECULAR BIOLOGY 48:443-453, 1970) to find the alignment of two sequences that maximizes the number of matches and minimizes the number of gaps. "BestFit" performs an optimal alignment of the best segment of similarity between two sequences and inserts gaps to maximize the number of matches using the local homology
15 algorithm of Smith and Waterman (Smith and Waterman, ADVANCES IN APPLIED MATHEMATICS, 2:482-489, 1981, Smith et al., NUCLEIC ACIDS RESEARCH 11:2205-2220, 1983). The percent identity is most preferably determined using the "Best Fit" program.

[0078] Useful methods for determining sequence identity are also disclosed in the Basic Local Alignment Search Tool (BLAST) programs which are publicly available from
20 National Center Biotechnology Information (NCBI) at the National Library of Medicine, National Institute of Health, Bethesda, Md. 20894; see BLAST Manual, Altschul et al., NCBI, NLM, NIH; Altschul et al., J. MOL. BIOL. 215:403-410 (1990); version 2.0 or higher of BLAST programs allows the introduction of gaps (deletions and insertions) into alignments; for peptide sequence BLASTX can be used to determine sequence identity; and, for polynucleotide
25 sequence BLASTN can be used to determine sequence identity.

[0079] As used herein, the term "substantial percent sequence identity" refers to a percent sequence identity of at least about 70% sequence identity, at least about 80% sequence identity, at least about 85% identity, at least about 90% sequence identity, or even greater sequence identity, such as about 98% or about 99% sequence identity. Thus, one embodiment of the invention is a polynucleotide molecule that has at least about 70% sequence identity, at least about 80% sequence identity, at least about 85% identity, at least about 90% sequence identity, or even greater sequence identity, such as about 98% or about 99% sequence identity with a polynucleotide sequence described herein. Polynucleotide molecules that have the activity of the ACS and CS genes of the current invention are capable of directing the production of a variety of capsaicinoids and have a substantial percent sequence identity to the polynucleotide sequences provided herein and are encompassed within the scope of this invention.

[0080] "Homology" refers to the level of similarity between two or more nucleic acid or amino acid sequences in terms of percent of positional identity (i.e., sequence similarity or identity). Homology also refers to the concept of similar functional properties among different nucleic acids or proteins.

[0081] In an alternative embodiment, the nucleic acid molecule comprises a nucleic acid sequence that exhibits 70% or greater identity, and more preferably at least 80 or greater, 85 or greater, 87 or greater, 88 or greater, 89 or greater, 90 or greater, 91 or greater, 92 or greater, 93 or greater, 94 or greater, 95 or greater, 96 or greater, 97 or greater, 98 or greater, or 99% or greater identity to a nucleic acid molecule selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, any complements thereof, any fragments thereof, or any cis elements thereof. The nucleic acid molecule preferably comprises a nucleic acid sequence that exhibits a 75% or greater sequence identity with a polynucleotide selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, any complements thereof, any fragments

thereof, or any cis elements thereof. The nucleic acid molecule more preferably comprises a nucleic acid sequence that exhibits an 80% or greater sequence identity with a polynucleotide selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, any complements thereof, any fragments thereof, or any cis elements thereof. The nucleic acid molecule most
5 preferably comprises a nucleic acid sequence that exhibits an 85% or greater sequence identity with a polynucleotide selected from the group consisting of SEQ ID NO: 3 and SEQ ID NO: 4, any complements thereof, any fragments thereof, or any cis elements thereof.

[0082] For purposes of this invention "percent identity" may also be determined using BLASTX version 2.0 for translated nucleotide sequences and BLASTN version 2.0 for
10 polynucleotide sequences. In a preferred embodiment of the present invention, the presently disclosed corn genomic promoter sequences comprise nucleic acid molecules or fragments having a BLAST score of more than 200, preferably a BLAST score of more than 300, and even more preferably a BLAST score of more than 400 with their respective homologues.

[0083] As is evident from the foregoing description, certain aspects of the present
15 disclosure are not limited by the particular details of the examples illustrated herein, and it is therefore contemplated that other modifications and applications, or equivalents thereof, will occur to those skilled in the art. It is accordingly intended that the claims shall cover all such modifications and applications that do not depart from the spirit and scope of the present disclosure.

20 [0084] Moreover, unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure belongs. Although any methods and materials similar to or equivalent to or those described herein can be used in the practice or testing of the present disclosure, the preferred methods and materials are described above.

[0085] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of understanding, it will be apparent to those skilled in the art that certain changes and modifications may be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

[0086] Accordingly, it is to be understood that the embodiments of the invention herein providing for the production of specific capsaicinoids are merely illustrative of the application of the principles of the invention. It will be evident from the foregoing description that changes in the form, methods of use, and applications of the elements of the disclosed production methods and selected microbial strains may be resorted to without departing from the spirit of the invention, or the scope of the appended claims.

Example 1

[0087] 500 mg/L of vanillylamine and 500 mg/L of individual fatty acid were fed into *E. coli* culture overexpressing ghost chili ACS1 and CS genes. Production samples were taken one (1) day and two (2) days after the substrate feeding and analyzed by high-performance liquid chromatography (HPLC) (see FIG. 2).

[0088] As seen in FIGs 1 and 2 nonivamide and other capsaicinoids were produced as a result of this experiment. NV is an amide of pelargonic acid (n-nonanoic acid) and vanillylamine. Nonivamide is widely used as a food additive for spicy flavors and a medicine for relieving arthritis and muscle pain.

[0089] The CP, DHCP and NV capsaicinoids produced herein were synthesized in modified *E. coli* cultures that were modified to carry the ACS and CS genes from ghost chili peppers. These genes allowed the properly fed selected strains to synthesize the capsaicinoids *via* the inserted acyltransferase CS that transfers the 8-methylnonenoyl moiety from 8-

methylnonenoyl-CoA to vanillylamine to form an amide conjugate. Vanillylamine is formed from the phenylpropanoid pathway whereas the branched-chain fatty acid is derived from a branched-chain amino acid, e.g., valine (Curry, et al., 1999; Mazourek, et al., et al., 2009). The aminotransferase (pAMT) catalyzes the formation of vanillylamine from vanillin (FIG 1).

5 Glycerol stock of BL21(DE3) culture co-overexpressing ACS1 and CS was used to inoculate 5 ml of TB (Terrific Broth) medium containing 100 mg/L of carbenicillin and 100 mg/L of spectinomycin and the culture was shaken at 37°C overnight. Then the overnight culture was used to inoculate 20 ml of TB medium containing 100 mg/L of carbenicillin and 100 mg/L of spectinomycin. The culture was first grown at 37°C to an OD600 of 0.4 and cooled down to
10 25°C. Then 1 mM IPTG was added to induce the expression of ACS1 and CS. After 3h of incubation at 25°C, 500 mg/L of VN and 500 mg/L of each individual fatty acid were added to the culture and the culture was continued to be incubated at 25°C. Samples were taken at 24h and 48h after the feeding of substrates. Capsaicinoids were extracted from the culture by ethyl acetate and the ethyl acetate phase was analyzed by HPLC. The experiment was performed in
15 triplicates.

[0090] In our previous application, we described a process for the production of CP and DHCP in *E. coli* cultures overexpressing ACS1 and CS genes from ghost chili pepper upon the feeding corresponding substrates (Table 1; Chen et al., 2015). According to the current invention we report the production of NV. According to the current invention the
20 transformed cultures are fed specific fatty acids so that the product produced by such cultures is a single species of capsaicinoid (see Table 3). Of course this is an improvement in efficiency relative to plant based production where a mixture of various capsaicinoids with CP and DHCP are produced and extracted.

[0091] Although nonivamide has been identified as a naturally occurring capsaicinoid in *Capsicum* species (Constant et al. 1996), the content is so low that no natural nonivamide has been commercially used.

[0092] Inorganic or non-biological processes for the synthesis of capsaicin and analogues thereof have been reported, for example, by Crombie et al., (J. CHEM SOC., 1025-27 (1955)) describes an unambiguous synthesis of capsaicin, N-(4-hydroxy-3-methoxybenzyl)-8-methylnon-trans-6-enamide, the active principle in red pepper. Other organic pathways have been shown in U.S. Pat. No. 4,493,848 issued to LaHann et al., and U.S. Pat. No. 5,094,782 issued to Chen et al.

[0093] Capsaicinoids, have long been used as an experimental tool because of their selective action on the small diameter afferent nerve fibers C-fibers and A-delta fibers that are believed to signal pain. From studies in animals, capsaicinoids appears to trigger C-fiber membrane depolarization by opening cation channels permeable to calcium and sodium. Recently one of the receptors for capsaicinoid effects has been cloned.

[0094] In most chili peppers, vanillylamine is formed from phenylalanine *via* ferulic acid, vanillin and related compounds, and capsaicinoid is produced from vanillylamine and branched chain fatty acid by capsaicin synthase (FIG. 1).

Synthetic Biology

[0095] Genetically engineered microbes have become an increasingly important platform for the production of drugs, chemicals, and biofuels from renewable resources (Du et al., 2011). These biotechnological products, when used in food, can be labeled 'natural' in food sector according to current regulations (Häusler and Münch, 1997).

[0096] Exemplary capsaicinoids include, but are not limited to: nonivamide, N-vanillylnonanamides, N-vanillylsulfonamides, N-vanillylureas, N-vanillylcarbamates,

N[(substituted phenyl)methyl]alkylamides, methylene substituted N[(substituted phenyl)methyl]alkanamides, N[(substituted phenyl)methyl]-cis-monosaturated alkenamides, N[(substituted phenyl)methyl]diunsaturated amides, 3-hydroxyacetanilide, hydroxyphenylacetamides, pseudocapsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin I, anandamide, piperine, zingerone, warburganal, polygodial, aframodial, cinnamodial, cinnamosmolide, cinnamolide, civamide, nonivamide, olvanil, N-oleyl-homovanillamidia, isovelleral, scalaradial, ancistrodial, and any combinations or mixtures thereof.

[0097] Previously, an *E. coli* fermentation platform was developed where various capsaicinoids could be produced upon the feeding of fatty acids and vanillylamine/vanillin (Chen et al. 2015). As described herein, the production of nonivamide by this system is also possible (FIG. 2).

Producing Acyl-CoAs: Cloning

[0098] Applicants amplified ACS1 gene from the cDNA of the green fruits of the ghost chili pepper using the primers of ACS1-sumo-F: CGC GAA CAG ATT GGA GGT GCAACAGATAAATTTATTATTG and ACS1-sumo-R: GTG GCG GCC GCT CTA TTA TCACTTGGTACCCTTGACAT. The resulting PCR product was purified on 1% agarose gel and mixed with linear pETite N-His SUMO Kan expression vector (Lucigen, Middleton, WI). The DNA mixture was used to transform HI-control 10G chemically competent cells by heat shock (Lucigen). The gene insertion was fully sequenced and the encoded amino acid sequence was aligned with that of ACS1. As shown previously (Chen et al., 2015), these two sequences are new due to a replacement mutation in which Ile476 in the known *Capsicum* sequence is replaced by a valine residue in ghost pepper ACS1 (SEQ ID NO: 1 provided herein). The sequence of ghost pepper ACS1 was used to blast the *Arabidopsis* database (<http://www.arabidopsis.org>) and identified LCAS4 and LCAS5 as homologues. As previously

shown, these three sequences share a sequence identity of 66% and a sequence similarity of 92%. Both LCAS4 and LCAS5 have been biochemically characterized as long chain acyl-CoA synthetases that participate in fatty acid and glycerolipid metabolism (Shockey et al., 2003). Recently, LCAS4 is demonstrated to be required for the formation of pollen coat lipids in
5 *Arabidopsis* (Belza and Jessen, 2005).

Expression

[0099] Applicants used pETite N-His SUMO-ghost pepper ACS1 to transform HI-Control BL21(DE3) cells (Lucigen) and the expression of His-SUMO-ACS1 was induced by
10 0.5 mM IPTG at 16°C for 20 hrs. The fusion protein was purified by Ni-NTA column. ACS 1 has a molecular weight of ca. 73.5 Kd and the size of His-SUMO tag is ca. 12 Kd. The His-SUMO-ghost pepper ACS1 fusion protein on SDS-PAGE migrated close to the predicted size.

Products

15 [00100] Applicants used an HPLC-based method to measure the activity of ghost pepper ACS1 (Chen et al., 2011). Briefly, reaction mixtures (400 µE) contained 0.1 M Tris-HCl, pH 7.5, 2 mM DTT, 5 mM ATP, 10 mM MgCl₂, 0.5 mM CoA, 0.1% Triton and 200 µM carboxylic acids. The reaction was initiated by adding 20 µl of purified enzyme and stopped after 30 minutes by addition of 20 micromolar acetic acid. HPLC was performed with Dionex -
20 UltiMate© 3000 LC Systems (Thermo Scientific) using an Acclaim® 120 CI 8 reversed-phase column (Thermo Scientific; 3 µ, 120 A, 150 x 3 mm). The mobile phase consisted of solvent A (0.1% trifluoroacetic acid) and solvent B (acetonitrile). The gradient elution procedure was as follows: 0 to 5 min, 5% of B; 5 to 9 min, a linear gradient from 5 to 80% of B; 9 to 11 min, 80% of B; 11 to 12 min, 5% of B. The flow rate was 0.6 ml/min. The diode array detector

collected data in the 200- to 400-nm range. For detection and quantification of substrate and products, peak areas were measured at 257 nm.

[00101] As shown in FIG. 8, ACS1 had activities in various medium against long chain carboxylic acids with the highest activity against capric acid (C10). In contrast, ACS1
5 did not show any activity against acetic acid (C2) or butyric acid (C4) - short chain carboxylic acid.

[00102] Applicants then used 8-methyl-trans-6-nonenic acid (6E), the endogenous intermediate in the capsaicinoid biosynthetic pathway or its reduced product, 8-methylnonanoic acid (8M), as a substrate to assay ACS1 activity. As shown in FIG. 2, ACS1
10 showed activities with both substrates with a higher activity for 6E. Applicants collected the corresponding HPLC fractions for the product peaks and dried them over a SpeedVac Concentrator for further MS/MS identification.

Confirmation of Product

[00103] Methanol: Water: Acetonitrile buffer. 10 μ E was used for direct infusion
15 using the TriVersa Nanomate® (Advion, Ithaca, NY). The mass spectrometer, LTQ-Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA), was operated in negative ionization mode. The MS survey scan was performed in the FT cell from a mass range of 300 to 2,000 m/z. The resolution was set to 60,000 @ 400 m/z. CID fragmentation was used for MS/MS, and
20 detection was done in the ion trap with an isolation window of 1.5 m/z Fragmentation was performed with normalized collision energies of 35%. As shown previously the MS data matched the molecular weight of 8-methyl-trans-6-nonenoyl-CoA and 8-methyl nonanoyl-CoA, respectively.

[00104] The pH optimal of ACS1 against 8-methylnonanoic acid was also
25 studied. Acetate, phosphate, Tris and glycine/NaOH buffers were used to provide a pH range

from 4.0 to 10.5. The optical pH of ACS1 is ca. 9.5. Accordingly, applicants have identified a novel medium/long chain acyl-CoA synthetase in ghost hot pepper which provides the substrate for capsaicin synthase. In addition, the novel enzyme may also have applications in biofuel industry for making medium-chain fatty acid derivatives.

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Production of Nordihydrocapsaicin with Addition of CaUGT2 from *C. roseus*

[00105] According to the current invention a variety of selected capsaicinoids can be produced through the use of selected starting materials. The current invention also provides for the production of specific capsaicinoid compounds through the use of specific enzymes.

10 This process allows applicants to produce specific and desirable capsaicinoids in quantities not previously possible so that the medicinal, food and fragrances uses can be fully determined and modifications can be made to assist in solubilization and dosing.

[00106] According to the current invention, glycosylation of capsaicin and 8-nordihydrocapsaicin by cultured cells of *Catharanthus roseus* has been reported (Shimoda et al. 2007). However, the gene that is responsible for this specific activity has not been previously identified. Other workers (Kaminaga et al. (2004)) identified two genes encoding UDP-glucosyltransferases, CaUGT1 and CaUGT2 from *C. roseus* and demonstrated that they catalyzed the formation of curcumin monoglucoside from curcumin and also conversion of curcumin monoglucoside to curcumin diglucoside without mention of capsaicinoid activity. In fact, these authors also tested the activity of CaUGT2 against capsaicin but found it could not glycosylate capsaicin (Kaminaga et al., 2004). However, according to the current invention we demonstrated that CaUGT2 can catalyze the formation of capsaicins glucoside both *in vitro* and *in vivo*.

[00107] According to the current invention the *Catharanthus roseus* CaUGT2 gene (GenBank: AB159213.1) was synthesized and codon-optimized for *E. coli* and cloned

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into a pDEST17 vector (see Table 5). The resulting pDEST17-CaUGT2 plasmid was used to transform BL21 Star (DE3) competent cells. The transformed culture was first grown at 37°C in LB(AMP+) medium until OD₆₀₀=0.4 and then cool down to 16°C and 1mM IPTG was added to induce the expression of CaUGT2 protein. Cells were harvested 16h after induction by centrifugation and soluble protein was extracted by B-PER™ Bacterial Protein Extraction Reagent (Thermo Fisher Scientific) according to the manufacture's instruction and further purified by Ni-NTA affinity chromatography (FIG. 6). It was determined that the use of this modified enzyme could produce capsaicinoids of interest (FIGs. 7-10).

[00108] In addition, in vivo biotransformation of capsaicin and nonivamide was performed for the production of capsaicin-glu and nonivamide-glu, respectively using BL21 Star (DE3) culture overexpressing CaUGT2 with a titer of ca. 5 mg/L. Further, the fatty acid chain of 7M-CP is derived from the fatty acid, 7-methyloctanoic acid (7M) or isopelargonic acid. When 7M was fed into this culture system, 7M-CP was produced (FIGs. 9 and 10). As shown in FIG. 9, the retention time of 7M-CP (10.077 min) is slightly different from that of C9-CP (10.190 min) and their UV spectra are almost identical (see inserted picture in FIG. 9). The levels of 7M-CP and C9-CP in the cultures were also quantified (FIG. 10). The titer of 7M-CP was slightly lower than that of C9-CP.

Production of Capsaicinoids

[00109] As shown in FIG. 2, our culture system had a CP9 titer similar to that of CP although the titers for CP 8 and CP10 were slightly lower. On HPLC, CP8 (N-vanillyl octamide), CP9 (nonivamide) or CP10 (N-vanillyl decanamide) had retention times of 10.9, 11.3 and 11.8 min, respectively. However, their UV spectra are very similar to each other (FIG. 3). The retention time and UV spectrum of CP9 match those of nonivamide standard very well (FIG. 4).

[00110] HPLC was performed with Dionex – UltiMate® 3000 LC Systems (Thermo Scientific) using an Acclaim® 120 C18 reversed-phase column (Thermo Scientific; 3 μ, 120 Å, 150 × 3 mm). The mobile phase consisted of solvent A (0.1% trifluoroacetic acid) and solvent B (acetonitrile). The gradient elution procedure was as follows: 0 to 5 min, 5% of B; 5 to 9 min, a linear gradient from 5 to 80% of B; 9 to 11 min, 80% of B; 11 to 12 min, 5% of B. The flow rate was 0.6 ml/min. The diode array detector collected data in the 200- to 400-nm range. For detection and quantification of substrate and products, peak areas were measured at 280 nm (FIGs. 3 and 4).

10 STATEMENT OF INDUSTRIAL APPLICABILITY / TECHNICAL FIELD

[00111] This disclosure has applicability in the food, medicinal, and pharmacological industries. This disclosure relates generally to a method for the biosynthetic production of capsaicinoids *via* a modified microbial strain.

15 TABLES REFERENCED IN THE SPECIFICATION

Table 1. Amino acid sequences of ACS1 and CS cloned from ghost chili pepper.

ACS1	MATDKFIIIEVESAKPAKDGRPSMGPVYRSIFAKHGFPPPIPGLDSCWDIFRMSVEKYPPNN RMLGRREIVDVGKPGKYVWMSYKEYDIVIKVGNRSIRSIGVDVGDKCGIYGANCPWEIIS MEACNAHGLYCVPLYDTLGAGAVEFIISHAEVTIAFVEEKKLPELLKTFPNASKYLKTIV SFGKVTPEQKKELEEFVVLVSWDEFLQLGSGKQFDLPVKKKEDICTIMYTSGTTGDPK GVLISNTSIVTLIAGVRRFLGSVDES LNVDVYLSYLPLAHIFDRVIEECFIHHGASIGFW RGDVKLLTEDIGELKPTVFCVPRVLDRIYSGLQQKIAAGGFLKSTLFNLAYAYKHHNL KKGRKHFEASPLSDKVVFSKVKEGLGGRVRLILSGAAPLAHVAFRLRVVACCHVLQG YGLTETCAGTFVSLPNRYDMLGTVGPPVNVVCLVPEMSYDALSSTPRGEVVCVRG DVLFSGYKREDLTKEVMIDGWFHTGDVGEWQPNGSLKIHDRKKNIFKLSQGEYVAVE NLENIYGNPNIIDSIWIYGNSFESFLVAVINPNQRAVEQWAEVNGLSGDFASLCEKPEVK EYILRELTKTGKEKLLKGFELKAVHLDPVVDFMERDLLTPTFKKKRPQLLYYKDVVID SMYKGTK
CS	MAFALPSSLVSVCDKSFIPSSLTPSKLRFHKLSFIDQSLSNMYIPCAFFYPKVQQRLEDS KNSDELSHIAHLLQTSLSQTLVSYYPYAGKLDNATVDCNDMGAEFLSVRIKCSMSEIL DHPHASLAESIVLPKDLPWANNCEGGNLLVVQVSKFDCGGIAISVCFSHKIGDGCSSLNF LNDWSSVTRDHTTTALVPSPRFVGDVSTKKYGSLITPQILSDLNECVQKRLIFPTDKL DALRAKVAEESGVKNPTRAEVVSALLFKCATKASSSMLPSKLVHFLNIRTMIKPRLPRN

	AIGNLSSIFSIEATNMQDMELPTLVRNLRKEVEVAYKKDQVEQNELILEVVESMREGKL PFENMDGYENVYTCNLCKYPYTVTDFGWGRPERVCLGNGPSKNAFFLKDYKAGQGV EARVMLHKQQMSEFERNEELLEFIA
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In the above protein sequences ACS shall be considered SEQ ID NO 1 and CS shall be considered SEQ ID NO 2.

Table 1. cont'd. Nucleic Acid sequences of ACS1 and CS cloned from ghost chili pepper.

ACSI	<p>ATGGCTACGGACAAATTTATTATTGAAGTTGAATCAGCAAACCGGCAAAGATG GTCGCCCGAGCATGGGCCCGGTCTATCGTTCGATCTTTGCGAAACATGGCTTCCG CCGCCGATCCCGGGTCTGGATTCATGCTGGGACATTTTTCGTATGTCGGTGGAAA AATATCCGAACAATCGCATGCTGGGCCGTCGCGAAATTGTTGATGGCAAACCGGG TAAATACGTTTGGATGAGCTACAAAGAAGTCTACGACATCGTTATCAAAGTCGGT AACAGTATTCGTTCCATCGGCCGTGGATGTTGGTGACAAATGCGGCATTTATGGTG CAAACGTGTCGGAATGGATTATCAGCATGGAAGCATGCAATGCTCATGGCCTGTA TTGTGTCCCGCTGTACGATACCCTGGGCGCAGGTGCTGTGGAATTTATTATCTCTC ACGCGGAAGTGACCATCGCCTTCGTTGAAGAGAAAAAACTGCCGGAAGTCTGTA AAACCTTCCGAATGCGAGCAAATATCTGAAAACCATTTGCTCTTTTCGGCAAAGT GACGCCGGAACAGAAGAAAGAACTGGAAGAATTTGGTGTGGTTCTGTACAGTTG GGATGAATTTCTGCAGCTGGGCTCCGGTAAACAATTCGATCTGCCGGTGAAAAAG AAAGAAGATATTTGCACCATCATGTATACGAGCGGCACCACGGGTGATCCGAAA GGTGTGCTGATTTCAAACACCTCGATTGTGACGCTGATCGCCGGTGTTCGTCGCTT TCTGGGCTCAGTTGATGAATCGCTGAATGTGGATGACGTTTATCTGTCATACCTGC CGCTGGCACATATTTTGACCGTGTGATCGAAGAATGCTTCATTCATCACGGCGCT TCGATCGGTTTTTTGGCGCGGCGATGTGAAACTGCTGACCGAAGACATTGGCGAAC TGAAACCGACGGTTTTCTGTGCGGTCCCGCGTGTGCTGGATCGCATCTATTCAGGT CTGCAGCAAAAAATTGCGGCCGGCGGTTTTCTGAAATCGACCCTGTTCAACCTGG CGTATGCCTACAAACATCACAATCTGAAGAAAGGCCGCAAACACTTTGAAGCCA GCCCCGTGTCTGATAAAGTCGTGTTTCAAGTAAAGTGAAGAAGGCCTGGGCGGTTCG TGTTTCGCTGATTCTGTCCGGTGCAGGCTCCGCTGGCCGCACATGTGGAAGCGTTTC TGCGTGTGTCGCTGCTGTCACGTTCTGCAGGGCTATGGTCTGACCGAAACGTGC GCAGGCACCTTCGTGAGTCTGCCGAACCGCTACGATATGCTGGGCACGGTTGGTC CGCCGGTCCCGAATGTGATGTGTGCCTGGAAAGCGTGCCGGAATGTCTTATGA CGCTCTGAGCTTACCCCGCGTGGTGAAGTTTGTGTCCGCGGCGATGTTCTGTTTT CCGTTATTACAAACGTGAAGACCTGACCAAAGAAGTTATGATTGATGGCTGGTT CCATACGGGCGACGTCCGTGAATGGCAGCCGAACGGTAGCCTGAAAATCATCGA TCGTAAGAAAAACATCTTCAAACGTCTCAAGGCGAATATGTGGCCGTTGAAAAC CTGGAATAATTTACGGCAACAATCCGATTATCGACAGCATTGGATCTATGGTA ACAGTTTTGAATCCTTCCTGGTTCGCG GTGATCAACCCGAATCAGCGTGCAGTCAACAATGGGCTGAAGTGAATGGCCTG AGTGGTGATTTTCGCTCCCTGTGTGAAAAACCGGAAGTGAAGAATACATTCTGC GCGAACTGACCAAAACGGGCAAAGAGAAAAAACTGAAAGTTTTCGAATTTCTGA AAGCAGTTCATCTGGACCCGGTGCCGTTTGTATGGAACGTGACCTGCTGACCCC GACGTTCAAGAAAAACGTCCGCAACTGCTGAAATACTATAAAGATGTGATCGA CTCAATGTATAAAGGCACGAAATAA</p>
CS	<p>ATGGCGTTCGCACTGCCGTGAGTCTGGTCTCTGTGTGTGATAAATCCTTCATCAA GCCGAGTTCGCTGACCCCGAGCAAACCTGCGTTTTTCATAAACTGAGCTTCATTGAT CAGTCTCTGAGTAATATGTATATCCCGTGCGCCTTTTTCTACCCGAAAGTGCAGCA ACGTCTGGAAGATTCTAAGAACAGTGACGAACTGTCTCATATTGCACACCTGCTG CAGACGTCCCTGTCACAAACGTGGTTAGCTATTACCCGTATGCTGGCAAACCTGA AGGATAACGCGACCGTGGATTGCAATGACATGGGTGCCGAATTTCTGAGCGTTTCG CATTAAATGTTTCGATGAGCGAAATCCTGGATCATCCGCACGCGTCGCTGGCCGAA</p>

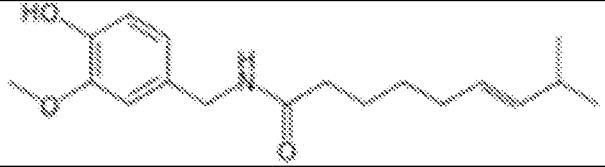
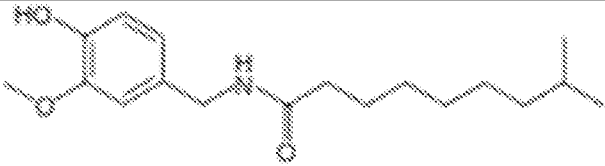
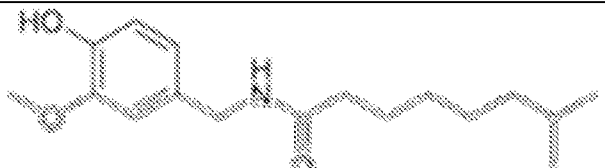

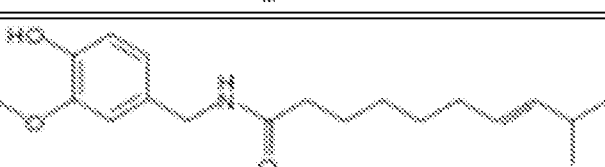
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CCTGAAGGATTACAAGGCCGGCCAGGGTGTGTAAGCACGTGTCATGCTGCACAA
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In the above nucleic acid sequences ACS shall be considered SEQ ID NO: 3 and CS shall be considered SEQ ID NO: 4.

Table 2.

Capsaicinoid Name	Abbrev.	Typical relative amount	Scoville heat units	Chemical structure
Capsaicin	C	69%	16,000,000	
Dihydrocapsaicin	DHCP	22%	15,000,000	
Nordihydrocapsaicin	NDHC	7%	9,100,000	
Homodihydrocapsaicin	HDHC	1%	8,600,000	
Homocapsaicin	HC	1%	8,600,000	

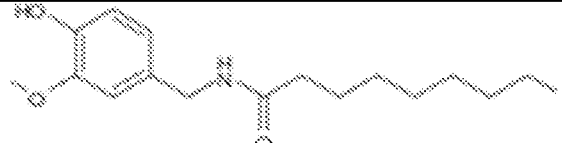
Nonivamide	NV		9,200,000	
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Table 3. Feeding Schedule for the Production of Specific Capsaicinoids

Fatty Acid Fed Into the Microbial Culture	Specific Capsaicinoid Produced
6E-8-methyl-6-nonenoic acid	Capsaicin (CP)
8-Methyl nonanoic acid	Dihydrocapsaicin (DHCP)
Pelargonic Acid (nonanoic acid)	Nonivamide (NV)
Caprylic Acid (octanoic acid)	N-Vanillyl octamide
Capric Acid (decanoic acid)	N-vanillyl decanamide

5 Table 4. ¹³C Isotope Analysis of Capsaicinoid Samples

<u>Sample</u>	<u>δ¹³C</u> <u>(‰)</u>
CP	-30.1
DHCP	-28.0
NV	-28.8
NV standard	-30.2
Natural CP	-31.5
<u>In Planta capsaicinoid*</u>	
<u>mixture</u>	<u>-31.9</u>

*taken from a Chinese fern used in experimentation

Table 5. Sequence of codon-optimized CaUGT2

CaUGT2 DNA	ATGGTTAATCAATTACACATCTTCAATTTCCCCTTTATGGCGCAGGGTCACATGTTACCGGCGTTGGATATGG CGAATCTGTTCACTTCAAGAGGCGTAAAAGTTACTGATTACCACTCATCAACACGTGCCAATGTTTACGAA GAGCATCGAACGCTCGCGTAATTCTGGTTTCGATATCTCAATCCAAAGCATTAAATTTCCCGGCGAGCGAAGT AGGCTTACCTGAAGGTATCGAATCATTGGATCAGGTTAGCGGCGATGACGAAATGCTGCCGAAATTCATGC GCGGTGTTAACCTGTTACAACAGCCATTAGAACAATTGCTGCAAGAATCACGTCCGATTGTTATTGAGCG ATATGTTTTCCCTTGGACAACGGAAAGTGCTGCAAAATTCGGCATTCCGCGCCTGTTATTTACGGTTCTTG CTCATTGCTCTGTCGGCCGCGGAATCTGTGCGTAGAAAATAAGCCTTTTAAAACGTCTCCACCGATACTGA AGAATTTGTTGTGCCAGACTTCCGCGATCAAATTAAGTGAACAAGACGCAGATCAGTACCTATGAACGCGA AAACATCGAATCCGATTTCACTAAGATGCTGAAGAAAGTTCGTGACAGCGAAAGTACATCCTACGGTGTCTGT AGTTAATTTCTTCTACGAATTAGAACCAGATTACGCAGACTATTACATTAACGTTTTGGGCCGTAAGCCTGG CATATCGGTCCGTTTTGCTGTGTAACAACTGCAAGCAGAAGATAAGGCCAGAGAGGTAAGGTAAGGTCAGC AATTGATGCCGACGAATGTCTGAATTGGTTAGACAGTAAACAACCTAACTCCGTGATCTATTTGTGCTTTGGC TCGATGGCGAATCTGAATCTGCTCAATTGCACGAAATGCTACTGCACTGGAATCTCAGGCCAGAAATTTCA TCTGGGTGGTCAGAAAATGCGTCGATGAAGAAAACAGCAGTAAGTGGTTTCTGAAGGTTTCAAGAACGC ACCAAAGAAAAGGGCCTGATTATCAAAGGTTGGCCACCACAGACTTTAATTTTGAACATGAATCGGTGGG CGCCTTTGTACACACTGTGGTTGGAATCTACGTTGGAAGGCATCTGCGCGGGTGTACCTCTGGTTACATG GCCATTTTTGCTGAACAATTTTTCAACGAAAACTGATCACCGAAGTATTAAGACTGGTTACGGCGTTGGT GCCCGTCAGTGGAGTAGAGTGTCCACGGAAATTATCAAAGGCGAAGCCATTGCGAATGCTATCAACCGCGT
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	GATGGTCGGCGATGAAGCAGTCGAAATGAGAAATCGCGCCAAAGACCTGAAAGAAAAGGCGCGTAAGGCT TTAGAAGAAGATGGCTCCTCGTATAGAGACTTGACCGCGCTGATTGAAGAACTGGGTGCTTACCGCTCTCAG GTGGAACGTAAACAACAGGATTAA
CaUGT2 Amino acid	MVNQLHIFNFPFMAQGHMLPALDMANLFTRSGVKVTLITTHQHVPMTFKSIERSRNSGFDISIQSIKFPASEVGL PEGIESLDQVSGDDEMLPKFMRGVNLLQQPLEQLLQESRPHCLLSDMFFPWTTESA AKFGIPRLLFHGSCSFALS AAESVRRNKPFENVSTDEEFVVPDLPHQIKLRTQISTYERENIESDFTKMLKKVRDSESTSYGVVVNSFYELEPDY ADYYINVLGRKAWHIGPFLLCNKLAEDKAQRGKKS AIDADECLNWLDSKQPNSVIYLCFGSMANLNSAQLHEIA TALESSGQNFIVVVRKCVDEENSSKWFPEGFEEERTKEKGLIIGWAPQTLILEHESVGFVTHCGWNSTLEGICA GVPLVTWPFPAEQFFNEKLITEVLKTGYGVGARQWSRVSTEIIGGEAIANAINRVMVGDEAVEMRNRRAKDLKEK ARKALEEDGSSYRDLTALIEELGAYRSQVERKQQD

In the above nucleic acid and amino acid sequences for CaUGT2 the DNA shall be considered SEQ ID NO: 5 and the protein sequence shall be considered SEQ ID NO: 6.

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CLAIMSWhat is claimed is:

1. A biosynthetic method of making a capsaicinoid of interest comprising:
 - a) expressing an ACS and CS in a transformed cellular system;
 - b) growing the cellular system in a medium; and,
 - c) producing the capsaicinoid of interest.
2. The biosynthetic method of claim 1, wherein the ACS is cloned from a plant of the *Capsicum* genus.
3. The biosynthetic method of claim 2, wherein the *Capsicum* genus plant is the ghost chili.
4. The biosynthetic method of claim 1, wherein the ACS expressed is derived from a gene cloned from *Arabidopsis* based on LCAS4 or LCAS5.
5. The biosynthetic method of claim 1, wherein the CS is cloned from a plant of the *Capsicum* genus.
6. The biosynthetic method of claim 5, wherein the *Capsicum* genus plant is the ghost chili.
7. The biosynthetic method of claim 1, further comprising feeding a source material to said cellular system in addition to culture media.
8. The biosynthetic method of claim 7, wherein said source material is selected from the group consisting of: nonanoic acid; oil of pelargonium; octanoic acid; decanoic acid; vanillin; and, vanillyamine.
9. The biosynthetic method of claim 7, wherein the source material is a mixture of two or more of the following: nonanoic; oil of pelargonium; octanoic acid; decanoic acid; vanillin; and, vanillyamine.

10. The biosynthetic method of claim 1, wherein said transformed cellular system is selected from the group including yeast, non-capsaicinoid producing plants, algae and bacteria.
11. The biosynthetic method of claim 10, wherein said cellular system is *E. Coli*.
12. The biosynthetic method of claim 1, wherein the source material is nonanoic acid.
13. The biosynthetic method of claim 1, wherein the capsaicinoid of interest is capsaicin.
14. The biosynthetic method of claim 1, wherein the capsaicinoid of interest is NV.
15. The biosynthetic method of claim 1, wherein the capsaicinoid of interest is DHCP.
16. The biosynthetic method of claim 1, wherein the capsaicinoids produced are a mixture comprising CP, NV and DHCP.
17. A capsaicinoid of interest produced by a transformed cellular system growing within a medium.
18. The capsaicinoid of interest of claim 17, wherein said transformed cellular system is selected from the group consisting of: yeast, non-capsaicinoid producing plants, algae and bacteria.
19. The capsaicinoid of interest of claim 18, wherein said transformed cellular system is transformed by the presence of an ACS and a CS derived from a plant of the *Capsicum* genus.
20. The capsaicinoid of interest of claim 17, wherein the method of producing further comprises feeding the transformed cellular system a specific source material.
21. The capsaicinoid of interest of claim 20, wherein said source material is selected from a group consisting of: nonanoic; oil of pelargonium; octanoic acid; decanoic acid; vanillin; and, vanillyamine.
22. The capsaicinoid of interest of claim 20, wherein said source material is selected from a mixture containing two or more of the following: nonanoic; oil of pelargonium; octanoic acid; decanoic acid; vanillin; and, vanillyamine.

23. The capsaicinoid of interest of claim 18, wherein said transformed cellular system is transformed by the presence of an ACS and a CS derived from a plant of the *Capsicum* genus.
24. The capsaicinoid of interest of claim 18, wherein said ACS transformed into the cellular system is an ACS derived from *Arabidopsis* based on LCAS4 or LCAS5.
25. The capsaicinoid of interest of claim 17, wherein said capsaicinoid is CP.
26. The capsaicinoid of interest of claim 17, wherein said capsaicinoid is NV.
27. The capsaicinoid of interest of claim 17, wherein said capsaicinoid is DHCP.
28. The capsaicinoid of interest of claim 17, wherein the capsaicinoids produced are a mixture of CP, NV and DHCP.
29. The capsaicinoid of interest of claim 19, wherein said transformed cellular system is transformed by the presence of an ACS and a CS derived from a plant of the *Capsicum* genus.
30. The capsaicinoid of interest of claim 29, wherein said transformed cellular system is transformed by the presence of an ACS and a CS derived the ghost chili.
31. The biosynthetic method of claim 1, wherein the ACS utilized is an ACS that shares a DNA sequence similarity of at least 75% with SEQ ID NO: 3.
32. The biosynthetic method of claim 1, wherein the ACS utilized is an ACS that shares a protein sequence similarity of at least 90% with SEQ ID NO: 1.
33. The biosynthetic method of claim 1, wherein the CS utilized is an CS that shares a DNA sequence similarity of at least 75% with SEQ ID NO: 4.
34. The biosynthetic method of claim 1, wherein the CS utilized is an CS that shares a protein sequence similarity of at least 90% with SEQ ID NO: 2.
35. The biosynthetic method of any one of claims 1 to 34, wherein the cellular system is selected from the group consisting of bacteria, yeast, plant cells, animal cells, an *in vitro* translation system and a combination thereof.
36. The capsaicinoid of interest of claim 17, wherein said capsaicinoid is purified capsaicin.

37. The capsaicinoid of interest of claim 36, wherein said capsaicinoid is greater than 70% pure.
38. The capsaicinoid of interest of claim 17, wherein said capsaicinoid content is greater than about 85% by weight on a dry basis.
39. The biosynthetic method of claim 1, wherein step c) comprises: i) purifying crude product; and, ii) removing solvents under vacuum to provide a concentrated capsaicinoid product.
40. The biosynthetic method of claim 39, wherein said crude product is purified by column chromatography.
41. The biosynthetic method of claim 39, wherein said crude product is purified by acid-base extraction.
42. The biosynthetic method of claim 39, wherein said crude product is purified by vacuum distillation.
43. The biosynthetic method of claim 39, wherein said crude product is purified by semi-preparative HPLC.
44. A method of producing a capsaicinoid of interest comprising feeding a transformed microbe culture a specific fatty acid precursor.
45. The method of claim 44, wherein the capsaicinoid of interest is Capsaicin and the fatty acid precursor is 6E-8-methyl-6-nonenic acid.
46. The method of claim 44, wherein the capsaicinoid of interest is Dihydrocapsaicin and the fatty acid precursor is 8-Methyl nonanoic acid.
47. The method of claim 44, wherein the capsaicinoid of interest is Nonivamide and the fatty acid precursor is nonanoic acid.
48. The method of claim 44, wherein the capsaicinoid of interest is N-Vanillyl octamide and the fatty acid precursor is octanoic acid.
49. The method of claim 44, wherein the capsaicinoid of interest is N-vanillyl decanamide and the fatty acid precursor is decanoic acid.

50. The method of claim 44, wherein the transformed culture is a microbe culture of yeast or bacteria.
51. The biosynthetic method of claim 10, wherein the cellular system further comprises an aminotransferase which is capable of catalyzing the conversion of vanillin to vanillylamine.
52. The biosynthetic method of claim 7, wherein said source material is selected from the group consisting of: C6 to C12 hydrocarbons that are medium to long chain fatty acids.
53. The biosynthetic method of claim 7, wherein the source material is a mixture of two or more C6 to C12 hydrocarbons that are medium to long chain fatty acids.
54. The biosynthetic method of claim 1, wherein the capsaicinoid of interest is nordihydrocapsaicin.
55. The biosynthetic method of claim 1, wherein said transformed cellular system includes a CaUGT2 from *C. roseus*.
56. The biosynthetic method of any one of claims 1 to 16, 31 to 35, and 51-55 wherein said transformed cellular system includes a CaUGT2 that shares a DNA sequence similarity of at least 75% with SEQ ID NO: 5.
57. The method of any one of claims 1 to 16, 31 to 35, and 51-55 wherein said transformed cellular system includes a CaUGT2 that shares a protein sequence similarity of at least 90% with SEQ ID NO: 6.
58. The method of claim 44, wherein the microbe culture expresses an ACS that shares a DNA sequence similarity of at least 75% with SEQ ID NO: 3.
59. The method of claim 44, wherein the microbe culture expresses an ACS that shares a protein sequence similarity of at least 90% with SEQ ID NO: 1.
60. The method of claim 44, wherein the microbe culture expresses a CS that shares a DNA sequence similarity of at least 75% with SEQ ID NO: 4.
61. The method of claim 44, wherein the microbe culture expresses a CS that shares a protein sequence similarity of at least 90% with SEQ ID NO: 2.

62. The method of any one of claims 44 and 58-61, wherein the microbe culture expresses a CaUGT2 that shares a DNA sequence similarity of at least 75% with SEQ ID NO: 5.
63. The biosynthetic method of claim 44, wherein said microbe culture expresses a CaUGT2 that shares a protein sequence similarity of at least 90% with SEQ ID NO: 6.
64. An analgesic composition for the topical treatment of pain in which said composition contains an effective amount of a capsaicinoid(s), wherein said analgesic composition is produced by the method of any one of claims 1-16, 31-35, and 39-63 wherein the improvement comprises producing said capsaicinoid in a microbial cellular system.
65. The analgesic composition of claim 64, where said effective amount is at least 0.0125 percent of a capsaicinoid of interest.
66. A flavor and fragrance composition, wherein the flavor and fragrance composition has at least one capsaicinoid produced according to the method of any one of claims 1-16, 31-35, and 39-63.
67. The flavor and fragrance composition according to claim 66, wherein the capsaicinoid is selected from the group comprising: nonivamide, N-vanillylnonanamides, N-vanillylsulfonamides, N-vanillylureas, N-vanillylcarbamates, N[(substituted phenyl)methyl]alkylamides, methylene substituted N[(substituted phenyl)methyl]alkanamides, N[(substituted phenyl)methyl]-cis-monosaturated alkenamides, N[(substituted phenyl)methyl]diunsaturated amides, 3-hydroxyacetanilide, hydroxyphenylacetamides, pseudocapsaicin, dihydrocapsaicin, nordihydrocapsaicin, homocapsaicin, homodihydrocapsaicin I, anandamide, piperine, zingerone, warburganal, polygodial, aframodial, cinnamodial, cinnamosmolide, cinnamolide, civamide, nonivamide, olvanil, N-oley-homovanillamidia, isovelleral, scalaradial, ancistrodial, and any combinations or mixtures thereof.

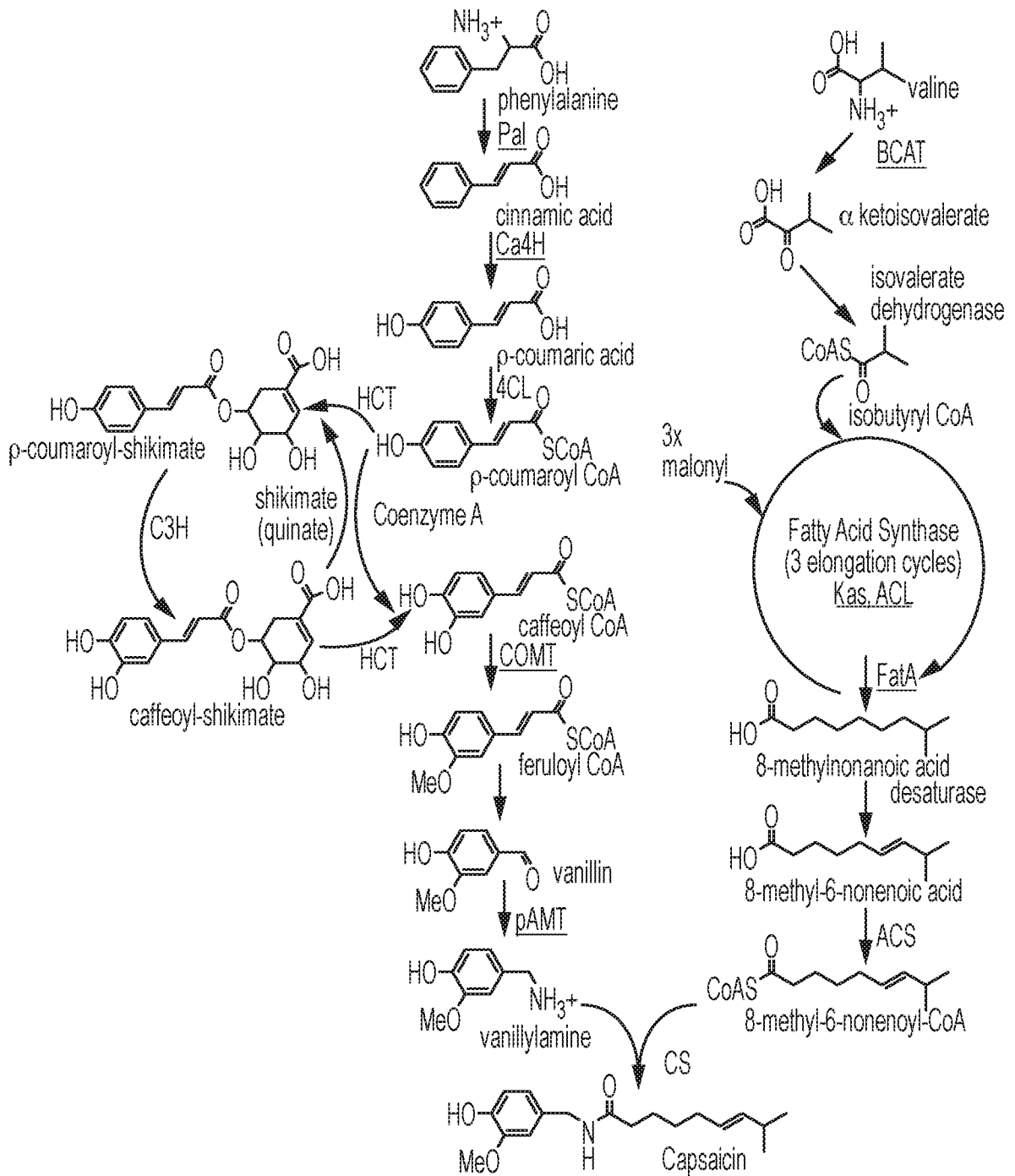


FIG. 1

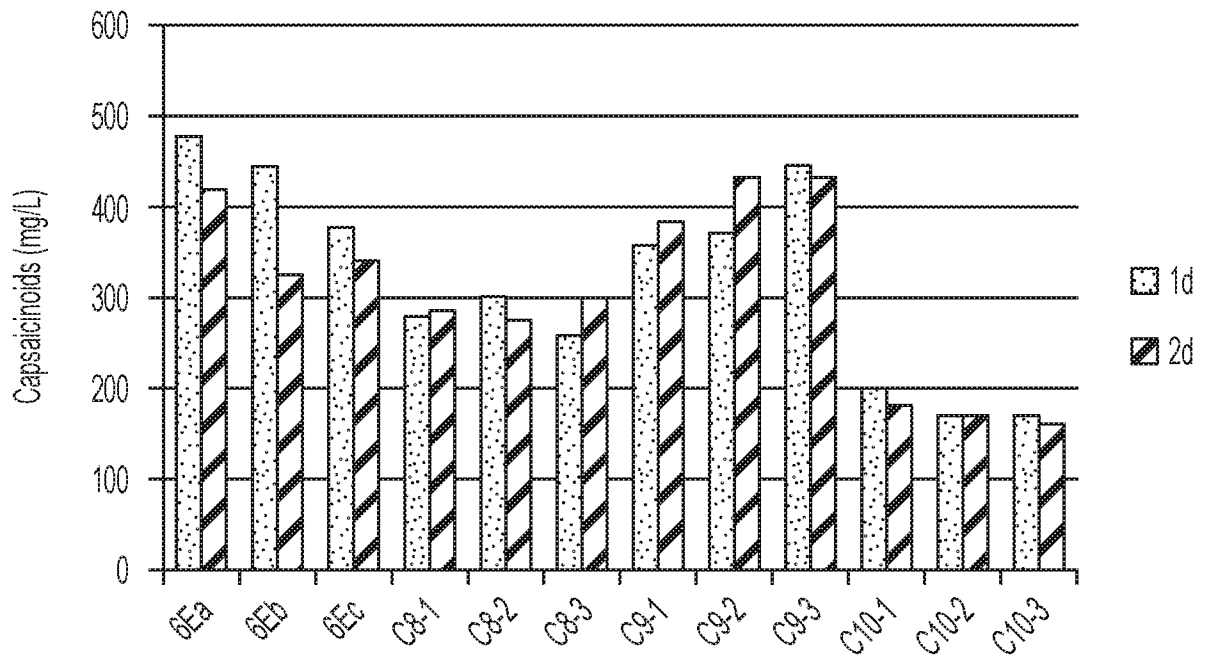


FIG. 2

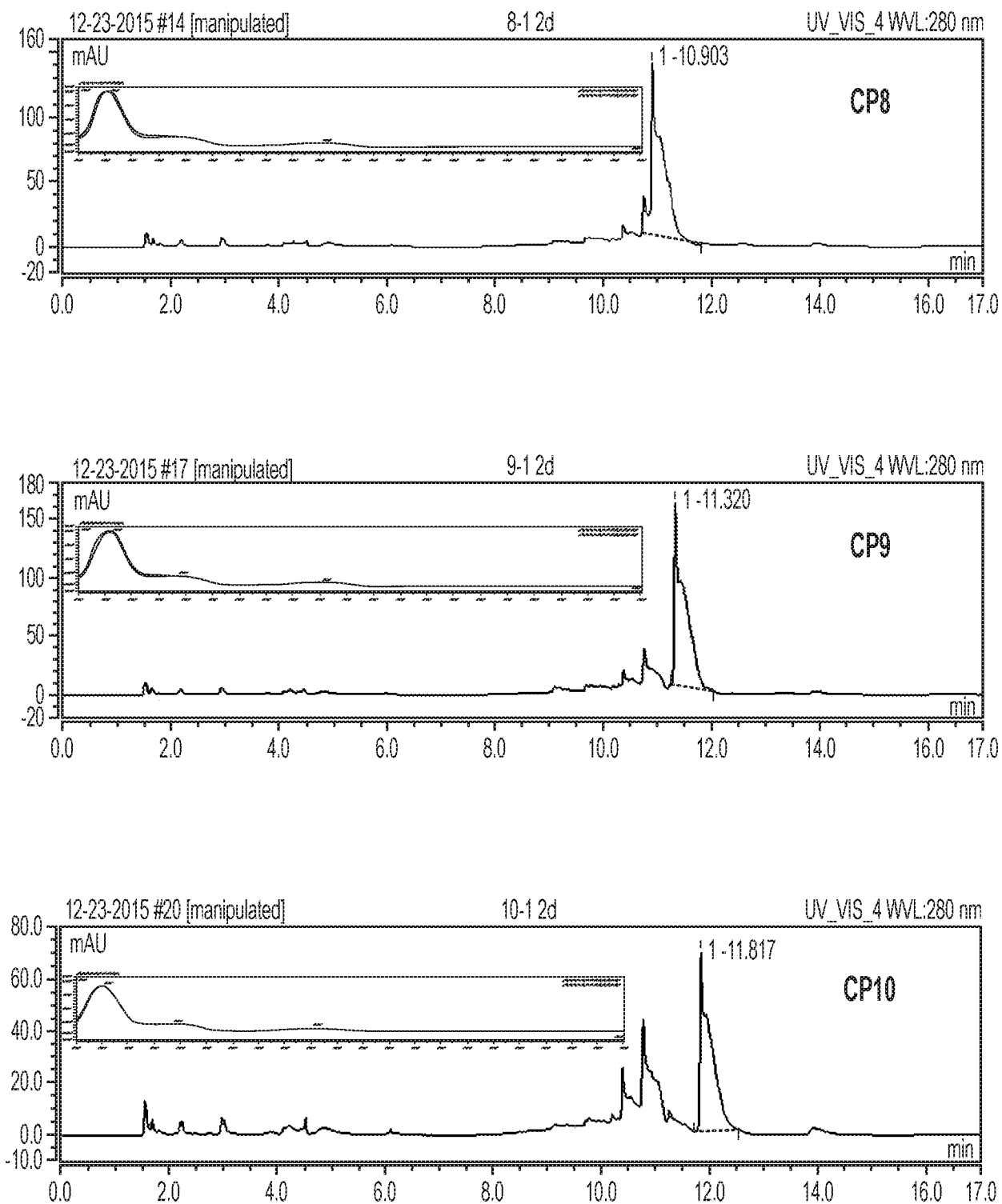


FIG. 3

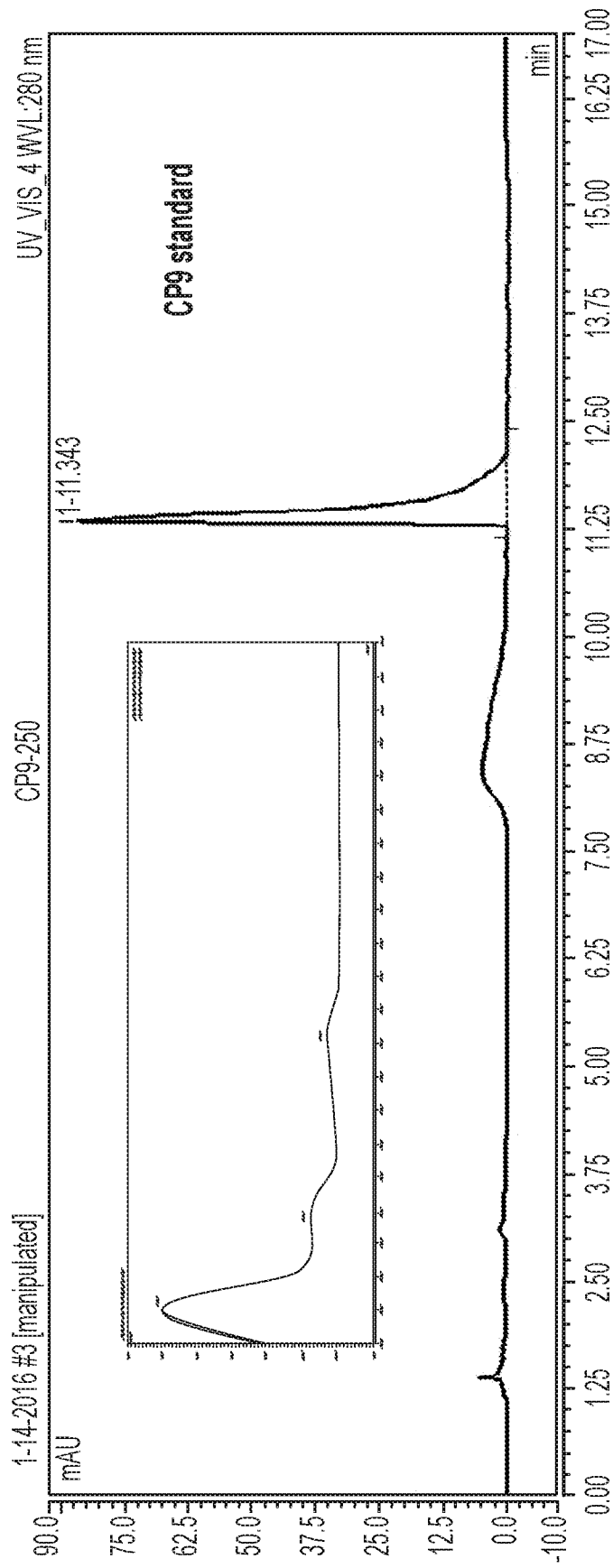


FIG. 4

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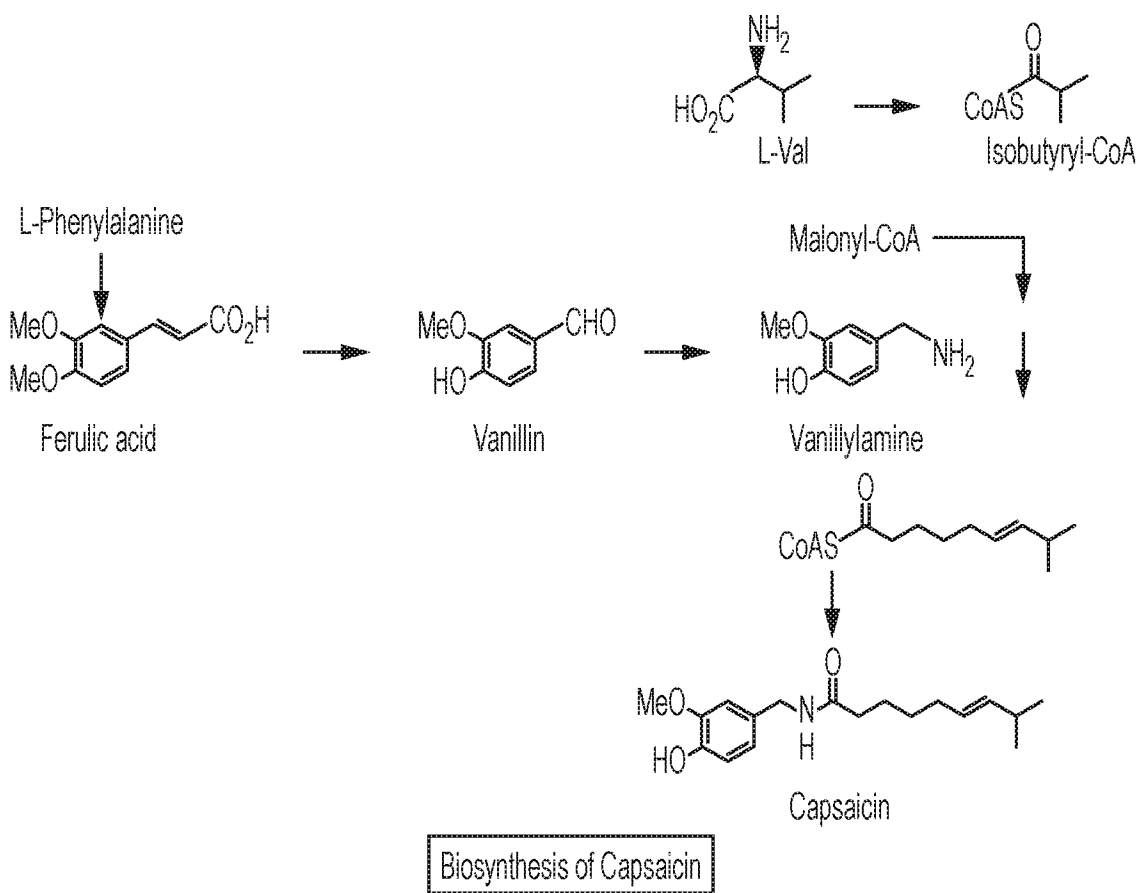


FIG. 5

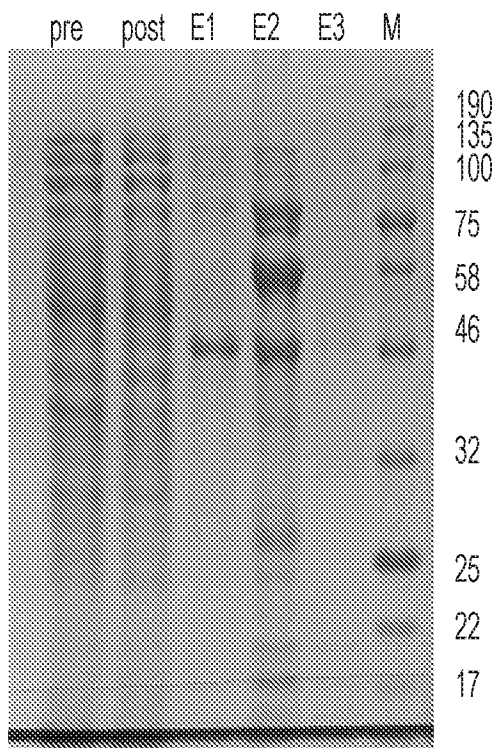


FIG. 6

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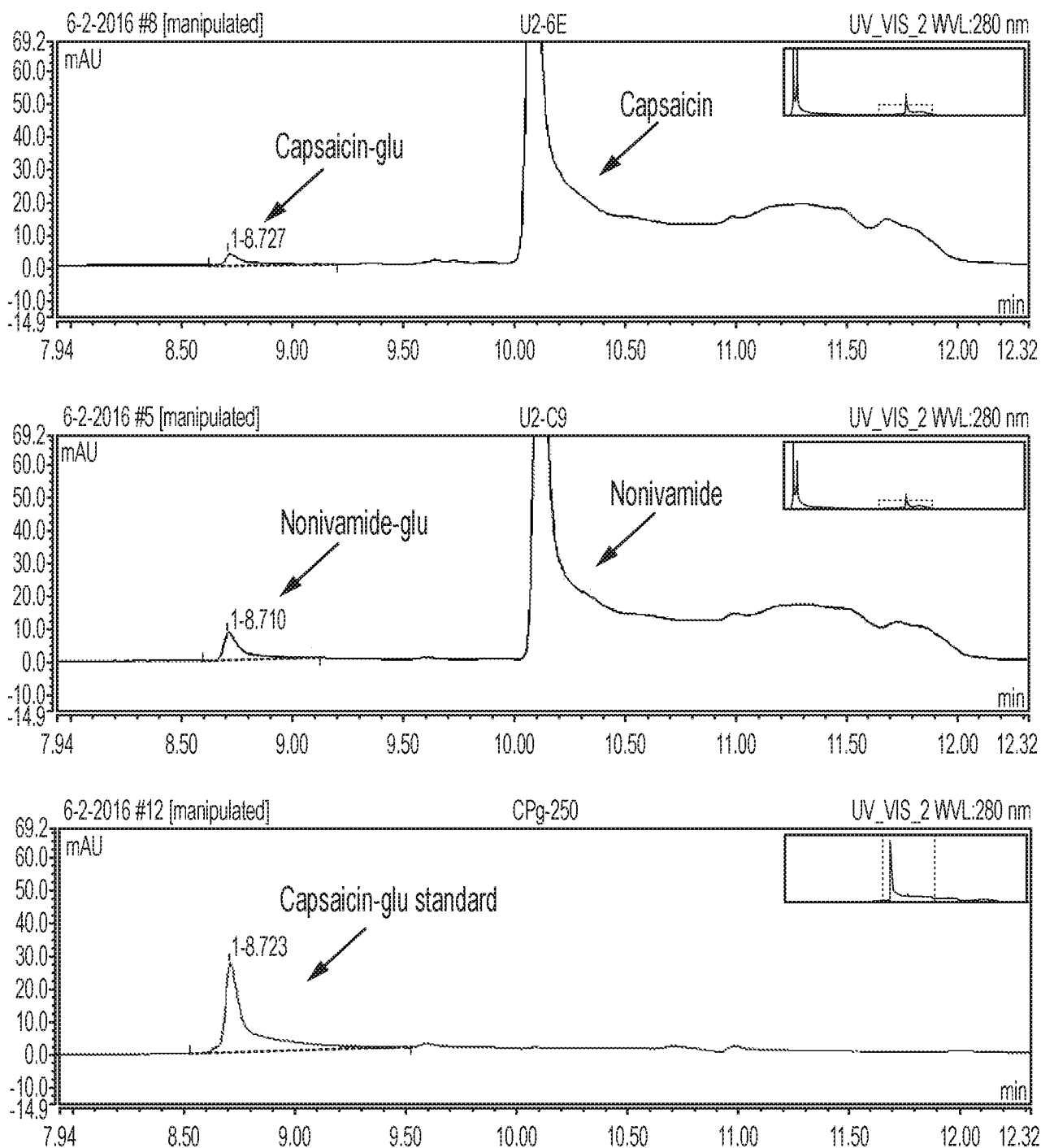


FIG. 7

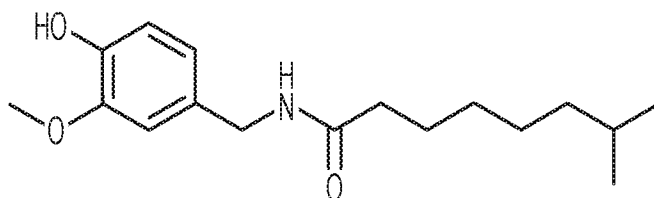


FIG. 8

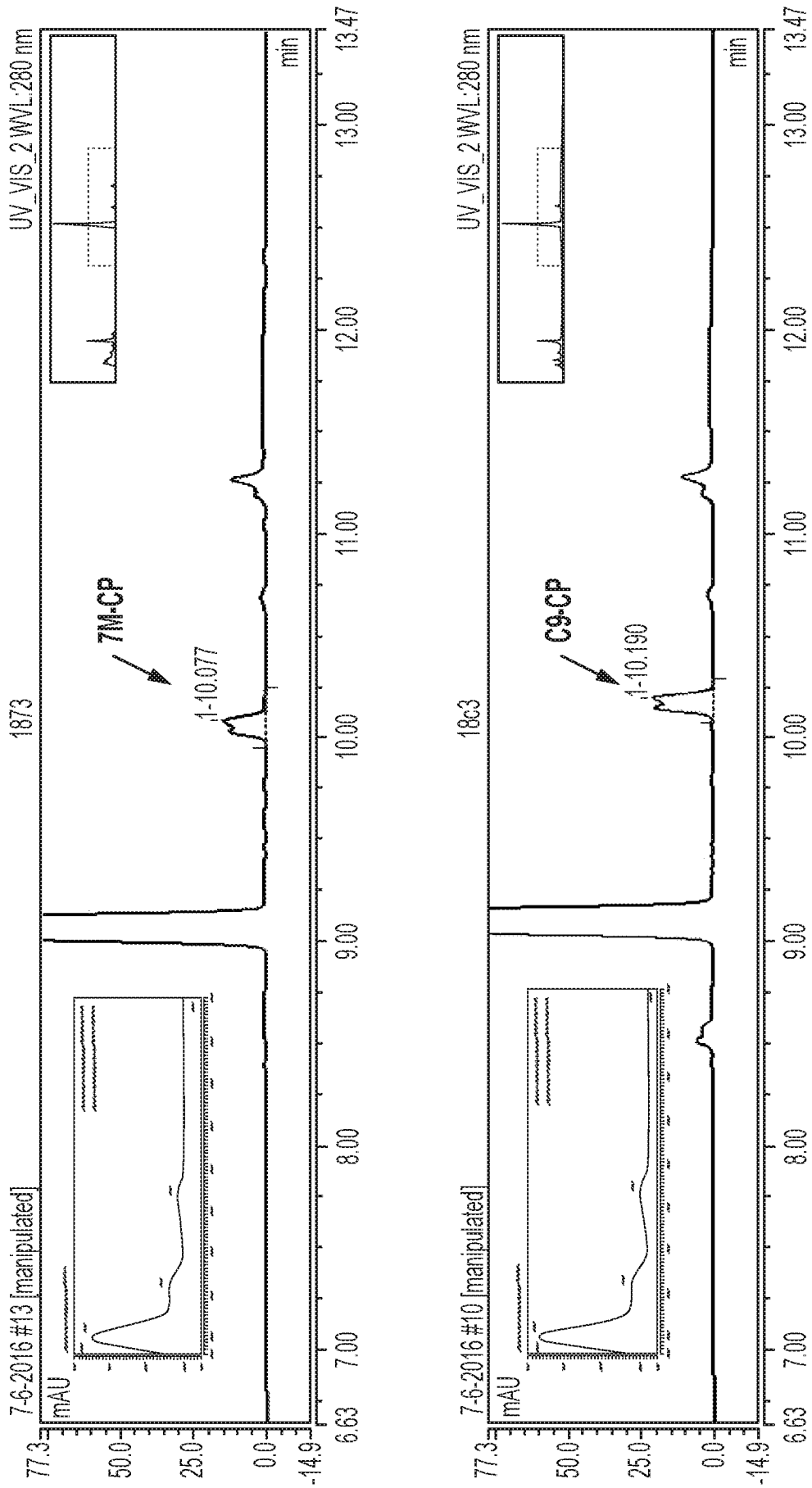


FIG. 9

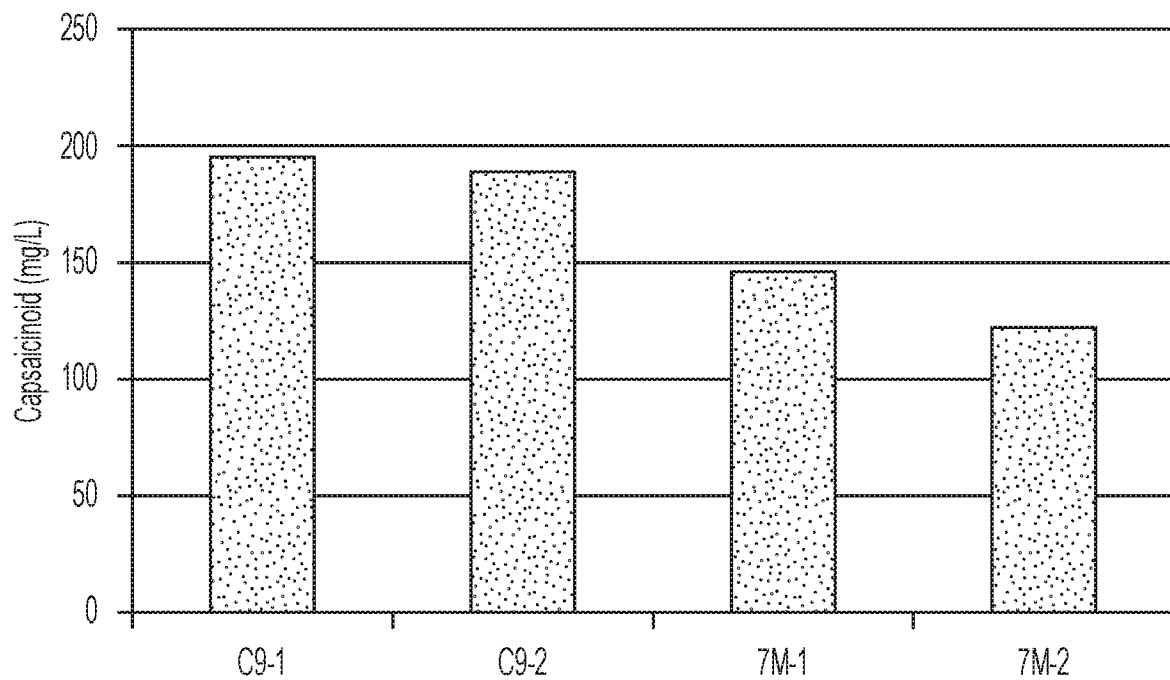


FIG. 10

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/42944

Box No. 1 Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. forming part of the international application as filed:
 - in the form of an Annex C/ST.25 text file.
 - on paper or in the form of an image file.
 - b. furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. furnished subsequent to the international filing date for the purposes of international search only:
 - in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
2. In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.: 56-57, 64-67
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
--please see extra sheet--

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-3, 5-13, 31-34, 35 (in part), 39-45, 50-53, 55, 58-63, limited to ACS cloned from Capsicum, capsaicin as the capsaicinoid of interest, and 6E-8-methyl-6-nonenic acid as the fatty acid precursor

Remark on Protest

- The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
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A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 1/21, C12N 15/70, C12N 9/00 (2017.01)
CPC - C12N 15/8243, C12N 15/52, C12N 9/1029

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y ----- A	WO 2015/109168 A1 (CONAGEN INC.) 23 July 2015 (23.07.2015) para [0002], [0003], [0005], [0011], [0014], [0032], [0033], [0035], [0052]; SEQ ID NO: 1	1-3, 5-13, 35/(1-3, 5-13), 44-45, 50-53, ----- 32, 34, 35/(32,34), 39-43, 55, 59, 61, 63 ----- 31, 33, 35/(31,33), 58, 60, 62
Y ----- A	WO 2015/066615 A2 (CONAGEN INC.) 07 May 2015 (07.05.2015) para [0034] to [0036], [0050]; SEQ ID NO: 5	32, 35/32, 39-43, 59 ----- 31, 35/31, 58
Y ----- A	UniProt Accession No. Q58VT0 "UniProtKB - Q58VT0 (Q58VT0_CAPCH) Acyltransferase, Pun1, Capsicum chinense - protein sequence" 26 April 2005 [located online Nov 16, 2017 at http://www.uniprot.org/uniprot/Q58VT0]	34, 35/34, 61
Y ----- A	US 2014/0371477 A1 (WOOD et al.) 18 December 2014 (18.12.2014) para [0117], [0182]; SEQ ID NO: 91	55 ----- 31, 35/31, 58
Y ----- A	WO 2008/034648 A1 (METANOMICS GMBH) 27 March 2008 (27.03.2008) SEQ ID NOs: 136890 and 136891	63 ----- 62

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

15 November 2017

Date of mailing of the international search report

07 DEC 2017

Name and mailing address of the ISA/US

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Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/42944

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	UniProt accession number B5LAV6 "Putative Long Chain acyl-CoA synthetase" (14 October 2008) [retrieved on 13 November 2017 from http://www.uniprot.org/uniprot/B5LAV6] pg 2, Sequence	31, 35/31, 58
A	CN 10/3173444 A (HUANG et al.) 26 June 2013 (26.06.2013) SEQ ID NO: 1	31, 33, 35/(31,33), 58, 60
A	WO 2006/100680 A2 (HAZERA GENETICS LTD et al.) 28 September 2006 (28.09.2006) SEQ ID NO: 4	33, 35/33, 60
A	US 2014/0248668 A1 (RAGHAVAN et al.) 04 September 2014 (04.09.2014) SEQ ID NO: 13	33, 35/33, 60
A	UniProt accession number Q6F4D5 "Glycosyltransferase" (16 August 2004) [retrieved on 13 November 2017 from http://www.uniprot.org/uniprot/Q6F4D5] pg 2, sequence	62
A	WO 2013/021261 A2 (SHRIRAM et al.) 11 February 2014 (11.02.2014) SEQ ID NO: 13	62

--continuation of Box III: Observations where unity of invention is lacking--

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-16, 31-34, 35 (in part), 39-55, 58-63, drawn to a biosynthetic method of making a capsaicinoid of interest. The method will be searched to the extent that:

- the ACS is cloned from a plant of the Capsicum genus (claims 2, 3)
- the capsaicinoid of interest is capsaicin (claim 13, 45), and
- the fatty acid precursor is 6E-8-methyl-6-nonenic acid (claim 45).

It is believed that claims 1-3, 5-13, 31-34, 35 (in part), 39-45, 50-53, 55, 58-63, limited to ACS cloned from Capsicum, capsaicin as the capsaicinoid of interest, and 6E-8-methyl-6-nonenic acid as the fatty acid precursor, encompass this first named invention, and thus these claims will be searched without fee to the extent that they encompass a biosynthetic method of making a capsaicinoid of interest by expressing an ACS and CS in a transformed cellular system, limited to ACS cloned from Capsicum, capsaicin as the capsaicinoid of interest, and 6E-8-methyl-6-nonenic acid as the fatty acid precursor. [Note that claim 16 will be searched only if additional capsaicinoids of interest are elected for search]. Additional sources of ACS, capsaicinoids of interest, and fatty acid precursors will be searched upon the payment of additional fees. Applicants must specify the claims that encompass any additionally elected source(s) of ACS, capsaicinoid(s) of interest, and fatty acid precursor(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched. An exemplary election would be a method of making a capsaicinoid of interest by expressing an ACS and CS in a transformed cellular system wherein

- the ACS is cloned from Arabidopsis based on LCAS4 or LCAS5 (claim 4)
- the capsaicinoid of interest is NV (Nonivamide) (claim 14), and
- the fatty acid precursor is nonanoic acid (claim 47).

i.e. claims 1, 4-12, 14, 31-34, 35 (in part), 39-44, 47, 50-53, 58-63, limited to ACS cloned from Arabidopsis, NV (Nonivamide) as the capsaicinoid of interest, and nonanoic acid as the fatty acid precursor.

Group II: Claims 17-30, 35 (in part), 36-38, drawn to a capsaicinoid of interest produced by a transformed cellular system

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features

Group I+ requires the method step of expressing an ACS and CS in a transformed cellular system, and feeding a transformed microbe a specific fatty acid precursor, not required by Group II. Further, the technical feature of each of the inventions listed as Group I+ is the specific sources of ACS, capsaicinoids of interest, and fatty acid precursors recited therein. Each invention requires a source of ACS, capsaicinoid of interest, and fatty acid precursor not required by any of the other inventions

Group II requires a composition of matter comprising a capsaicinoid of interest, not required by Group I+.

Common Technical Features

The feature shared by Groups I+ and II is the production of a capsaicinoid of interest by a transformed cellular system growing within a medium.

Other features shared by the inventions listed as Groups I+ are the methods of claims 1 and 44.

However, these shared technical features do not represent a contribution over prior art, because the shared technical features are taught by WO 2015/109168 A1 to Conagen Inc. (hereinafter 'Conagen').

Conagen discloses production of a capsaicinoid of interest by a transformed cellular system growing within a medium (para [0008] "a method of bioconversion making a capsaicinoid including expressing a first gene product of CS/AT3/Pun1 in a cellular system, growing the cellular system in a medium, and collecting the capsaicinoid").

Conagen discloses [claim 1] a biosynthetic method of making a capsaicinoid of interest comprising: a) expressing an ACS and CS in a transformed cellular system; b) growing the cellular system in a medium; and, c) producing the capsaicinoid of interest (para [0002] "This disclosure relates generally to a method for the biosynthetic production of capsaicin and related capsaicinoids, particularly utilizing acyl-CoA synthetase (ACS), . . . and capsaicin synthase (CS)"; para [0011] "Another disclosure is a biosynthetic method of making a capsaicinoid comprising expressing a gene product of CS/AT3/Pun1 in a cellular system, providing fatty acid-CoA (an activated form of fatty acid), providing vanillylamine, growing the cellular system in a medium, and collecting the capsaicinoid"; para [00032] "a method of bioconversion making a capsaicinoid comprising expressing a first gene product of CS/AT3/Pim1 in a mixture"; para [00033] "expressing a second gene product of ACS, particularly ACS1"; para [00035] "In regards to providing 8-methyl-nonanoyl-CoA, it includes expressing a second gene product of ACS, particularly ACS1, in the cellular system and providing 8-methyl nonanoic acid").

Conagen discloses [claim 44] a method of producing a capsaicinoid of interest comprising feeding a transformed microbe culture a specific fatty acid precursor (para [00011] "a biosynthetic method of making a capsaicinoid comprising expressing a gene product of CS/AT3/Pun1 in a cellular system, providing fatty acid-CoA (an activated form of fatty acid), providing vanillylamine, growing the cellular system in a medium, and collecting the capsaicinoid"; para [00033] "Another disclosure includes providing the first substrate to the mixture further comprises by expressing a second gene product of ACS, particularly ACS1, in a mixture and providing a second substrate. . . . The second substrate is a fatty acid selected from the group consisting of 8-methyl-6-nonenic acid, 8-methyl nonanoic acid, octanoic acid, decanoic acid, and a combination thereof"; para [00035] "another embodiment further comprises providing 8-methyl-6-nonenoyl-CoA and providing vanillylamine").

--continued in next supplemental box--

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 17/42944

--continuation of Box III: Observations where unity of invention is lacking--

As the technical features were known in the art at the time of the invention, they cannot be considered special technical features that would otherwise unify the groups.

Groups I+ and II therefore lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.