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(54) **Title**: METHODS AND COMPOSITIONS FOR MAKING EPHEDRINE AND RELATED ALKALOID COMPOUNDS

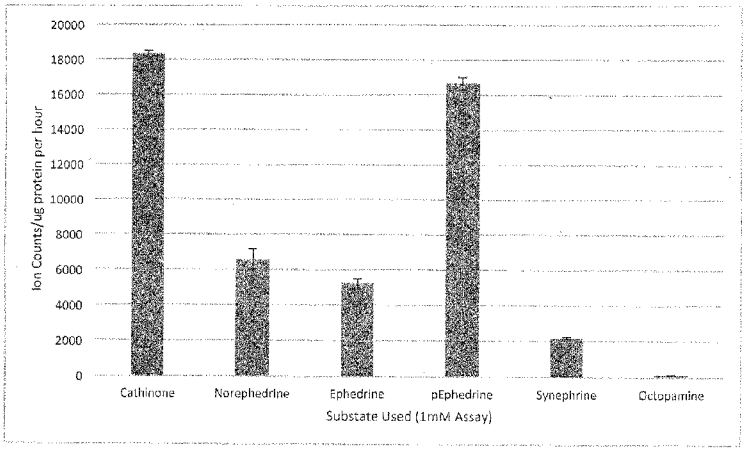


FIGURE 3

(57) **Abstract**: Methods for making alkaloid compounds, including ephedrine and derivatives thereof. The methods involve the performance of an N-methyltransferase catalyzed chemical reaction.

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TITLE: METHODS AND COMPOSITIONS FOR MAKING EPHEDRINE AND RELATED ALKALOID COMPOUNDS

RELATED APPLICATION

[0001] This Patent Cooperation Treaty Application claims benefit under 35 USC §119(e) from U.S. Provisional Patent Application No. 62/183,372 filed on June 23, 2015 which is incorporated by reference herein in its entirety.

FIELD OF THE DISCLOSURE

[0002] The present disclosure relates to methods for making alkaloid compounds, notably ephedrine.

BACKGROUND OF THE DISCLOSURE

[0003] The following paragraphs are provided by way of background to the present disclosure. They are not however an admission that anything discussed therein is prior art or part of the knowledge of persons skilled in the art.

[0004] The pharmacological properties of ephedrine and related alkaloid compounds have long been recognized. Thus ephedrine may be used *inter alia* as a decongestant, stimulant, concentration aid, and appetite suppressant. In order to prepare pharmaceutical formulations, ephedrine may be extracted from natural sources, including plant species belonging to the genus *Catha*, *Catha edulis*, for example, and plant species belonging to the genus *Ephedra*, *Ephedra sinica*, for example. However yields of plant-extracted ephedrine are typically modest (Shukla *et al.*, 2000, World J. Biotechn. 16: 499-506). In practice, plant extracts are commonly used for the preparation of herbal formulations and supplements containing ephedrine. Plant extraction processes, due to their limited efficiency, are less suitable for the large-scale manufacture of substantially pure ephedrine. Ephedrine may also be produced chemically for example by condensing 1-phenyl-1,2-propanedione with methylamine, providing racemic mixtures of ephedrine (Manske and Johnson, 1929, Am. Chem. Soc. 51: 580-582), or from propionic acid (Feldman *et al.*, 1962, J. Appl. Chem. 35, 1309-1311). In general, chemical production of ephedrine is cumbersome as it involves the use of several substantially pure chemical compounds, which are not necessarily available on suitably economic terms and multistep preparation processes. Moreover only limited enantiomeric purity is attainable through chemical synthesis, i.e. the chemical synthesis processes yield a mixture of (*R*)- and (*S*)-enantiomers. It is

noted in this regard that different ephedrine enantiomers exhibit different pharmacological properties. Thus the currently most commonly used process for commercial bulk manufacturing of ephedrine consists of two separate steps, an initial biosynthetic production step, followed by a chemical synthesis step. Notably, this process involves fermentation of sugars in yeast in the presence of benzaldehyde, an inexpensive additive, resulting in the production of (*R*)-phenylacetylcarbinol, also known as (*R*)-PAC. This precursor compound is subsequently used to produce ephedrine by the performance of a chemical reductive amination reaction.

[0005] Despite the well-understood chemistry relating to the synthesis of ephedrine and related alkaloid compounds, it was heretofore unknown whether and how *de novo* biosynthetic production of ephedrine may be achieved. Such biosynthetic production system is desirable as it represents a large scale economical production process for substantially pure ephedrine and related alkaloid compounds using a one-step process, obviating the need for a chemical synthesis step converting (*R*)-PAC to ephedrine, as is required to operate the currently used commercial production systems for ephedrine.

[0006] There exists therefore a need in the art for improved methods for the production of ephedrine and related alkaloid compounds.

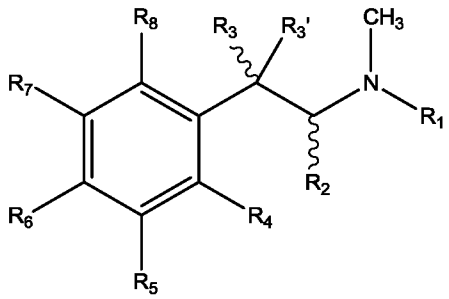
SUMMARY OF THE DISCLOSURE

[0007] The following paragraphs are intended to introduce the reader to the more detailed description that follows and not to define or limit the claimed subject matter of the present disclosure.

[0008] In one aspect, the present disclosure relates to ephedrine and related alkaloid compounds.

[0009] In one aspect, the present disclosure relates to N-methyltransferases.

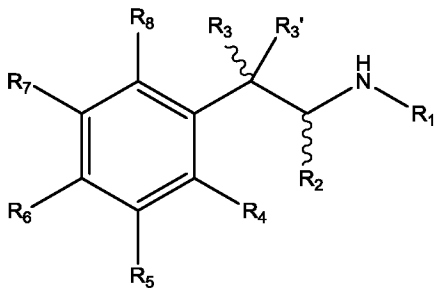
[00010] Accordingly, the present disclosure provides, in at least one implementation, a method of making an alkaloid compound having a chemical formula (II):



(II),

the method comprising

providing an alkaloid compound having a chemical formula (I):



(I),

contacting the alkaloid compound having a chemical formula (I) with sufficient quantities of a methyl-donor and a catalytic quantity of an N-methyl-transferase under reaction conditions permitting an enzyme catalyzed conversion of the alkaloid compound having chemical formula (I) to an alkaloid compound having chemical formula (II).

[00011] In some implementations, in the alkaloid compound having chemical formula (I) R_1 is a hydrogen atom, a methyl group or a halogen; R_2 is a hydrogen atom, a methyl group or a methoxy group; R_3 is a hydroxyl group, or a halogen , R_3' is a hydrogen atom, or taken together R_3 and R_3' form a carbonyl group (C=O); R_4 – R_8 are each independently or simultaneously a hydrogen atom, methyl group, hydroxyl group, halogen or methoxy group; and each R_1 to R_8 and R_3' in the alkaloid compound having chemical formula (I) are identical to R_1 to R_8 and R_3' in the alkaloid compound having chemical formula (II).

[00012] In some implementations, in both the alkaloid compound having chemical formula (I) and the alkaloid compound having chemical formula (II), R_1 is a hydrogen atom, R_2 is a methyl group, R_3 is a hydroxyl group, R_3' is a hydrogen atom and R_4 – R_8 are hydrogen atoms. It is noted that in such implementations, the alkaloid compound having chemical formula (I) is also referred to as norephedrine

and the alkaloid compound having chemical formula (II) is also referred to as ephedrine.

[00013] In some implementations, in both the alkaloid compound having chemical formula (I) and the alkaloid compound having chemical formula (II) R_1 is a methyl group, R_2 is a methyl group, R_3 is a hydroxyl group, R_3' is a hydrogen atom and R_4 – R_8 are hydrogen atoms. It is noted that in such implementations, the alkaloid compound having chemical formula (I) is also referred to as ephedrine and the alkaloid compound having chemical formula (II) is also referred to as N-methylephedrine.

[00014] In some implementations, R_2 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 2R-enantiomer.

[00015] In some implementations, R_2 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 2S-enantiomer.

[00016] In some implementations, R_3 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 1R-enantiomer.

[00017] In some implementations, R_3 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 1S-enantiomer.

[00018] In some implementations, the alkaloid compound having chemical formula (I) is (1S, 2S)-pseudonorephedrine and the alkaloid compound having chemical formula (II) is (1S, 2S)-pseudoephedrine.

[00019] In some implementations, the alkaloid compound having chemical formula (I) is (1R, 2R)-norephedrine and the alkaloid compound having chemical formula (II) is (1R, 2R)-ephedrine.

[00020] In some implementations, the alkaloid compound having chemical formula (I) is (1R, 2S)-norephedrine and the alkaloid compound having chemical formula (II) is (1R, 2S)-ephedrine.

[00021] In some implementations, the alkaloid compound having chemical formula (I) is (1S, 2R)-pseudonorephedrine and the alkaloid compound having chemical formula (II) is (1S, 2R)-pseudoephedrine.

[00022] In some implementations, the alkaloid compound having chemical formula (I) is (1S, 2S)-pseudoephedrine and the alkaloid compound having chemical formula (II) is (1S, 2S)-N-methyl-pseudoephedrine.

[00023] In some implementations, the alkaloid compound having chemical formula (I) is (1R, 2R)-ephedrine and the alkaloid compound having chemical formula (II) is (1R, 2R)-N-methyl-ephedrine.

[00024] In some implementations, the alkaloid compound having chemical formula (I) is (1R, 2S)-ephedrine and the alkaloid compound having chemical formula (II) is (1R, 2S)-N-methyl-ephedrine.

[00025] In some implementations, the alkaloid compound having chemical formula (I) is (1S, 2R)-pseudoephedrine and the alkaloid compound having chemical formula (II) is (1S, 2R)-N-methyl-pseudoephedrine.

[00026] In some implementations, in both the alkaloid compound having chemical formula (I) and (II) R_1, and R_4-R_8 are each hydrogen atoms, R_2 is a methyl group, and, taken together, R_3 and R_3' form a carbonyl group (C=O). It is noted that the compound having chemical formula (I) is known as cathinone, and the compound having chemical formula (II) is known as methcathinone.

[00027] In some implementations, in both the alkaloid compound having chemical formula (I) and (II), R_1, R_2, R_3', R_4, R_5, R_7 and R_8 are each hydrogen atoms, and R_3 and R_6 are hydroxyl groups. It is noted that the compound having chemical formula (I) is known as p-octopamine, and the compound having chemical formula (II) is known as synephrine.

[00028] In some implementations, in both the alkaloid compound having chemical formula (I) and (II), R_1 is a methyl group, R_2, R_3', R_4, R_5, R_6 and R_8 are each hydrogen atoms, and R_3 and R_7 are hydroxyl groups. It is noted that the compound having chemical formula (I) is known as N-desmethylphenylephrine, and the compound having chemical formula (II) is known as phenylephrine.

[00029] In some implementations, in both the alkaloid compound having chemical formula (I) and (II), R_1 is a methyl group, R_2, R_3', R_4, R_5, R_7 and R_8 are each hydrogen atoms, and R_3 and R_6 are hydroxyl groups. It is noted that the compound having chemical formula (I) is known as synephrine, and the compound having chemical formula (II) is known as N-methyl-synephrine.

[00030] In some implementations, the N-methyl-transferase is obtained or obtainable from a plant species belonging to the genus *Catha* or from a plant belonging to the genus *Ephedra.*

[00031] In some implementations, the methods disclosed herein are conducted *in vivo*.

[00032] In some implementations, the methods disclosed herein are conducted *in vitro.*

[00033] Other features and advantages of the present disclosure will become apparent from the detailed description. It should be understood, however, that the detailed description, while indicating preferred implementations of the disclosure, are given by way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those of skill in the art of the detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[00034] The disclosure is in the hereinafter provided paragraphs described in relation to its Figures. The Figures provided herein are provided for illustration purposes and are not intended to limit the present disclosure.

[00035] **FIGURE 1** depicts the chemical structures of certain alkaloid chemical compounds having the general chemical formula (I) or (II).

[00036] **FIGURE 2** depicts the chemical structures of various stereoisomers of norephedrine (**FIG. 2A - FIG. 2D**), ephedrine (**FIG. 2E - FIG. 2H**), and N-methylephedrine (**FIG. 2I – FIG. 2L**).

[00037] **FIGURE 3** depicts a bar graph reflecting certain results obtained in the performance of the experiments described in Example 1, notably, the production of N-methylated alkaloids using a variety of substrates under standard assay conditions. Substrates used are listed along the x-axis, and the N-methylated product is quantified as MS ion counts per μg protein per hour. Error bars represent standard deviation (SD) calculated using 4-6 replicates. It is noted that pEphedrine denotes pseudoephedrine.

[00038] **FIGURE 4** depicts another bar graph reflecting certain results obtained in the performance of the experiments described in Example 1, notably, the production of N-methylated alkaloids using norephedrine, ephedrine, and pseudoephedrine (pEphedrine) substrates under standard assay conditions. Substrates used are listed along the x-axis, and the N-methylated product is

quantified as ng alkaloid per μg protein per hour. Error bars represent standard deviation (SD) calculated using 4-6 replicates

[00039] The figures together with the following detailed description make apparent to those skilled in the art how the disclosure may be implemented in practice.

DETAILED DESCRIPTION OF THE DISCLOSURE

[00040] Various compositions and methods will be described below to provide an example of an embodiment of each claimed subject matter. No embodiment described below limits any claimed subject matter and any claimed subject matter may cover methods, processes, compositions or systems that differ from those described below. The claimed subject matter is not limited to compositions or methods having all of the features of any one composition, method, system or process described below or to features common to multiple or all of the compositions, systems or methods described below. It is possible that a composition, system, method or process described below is not an embodiment of any claimed subject matter. Any subject matter disclosed in a composition, system, method or process described below that is not claimed in this document may be the subject matter of another protective instrument, for example, a continuing patent application, and the applicants, inventors or owners do not intend to abandon, disclaim or dedicate to the public any such subject matter by its disclosure in this document.

[00041] As hereinbefore mentioned, the present disclosure relates to processes for the production of ephedrine and certain related alkaloid compounds. The herein provided processes are novel and provide, for the first time, a means permitting *de novo* biosynthetic production at commercial scale of ephedrine and related alkaloid compounds, obviating the need for a chemical synthetic step using a precursor compound. The obtained compounds of present disclosure are useful *inter alia* in the manufacture of pharmaceutical compositions.

Terms and Definitions

[00042] The term “norephedrine”, as used herein, refers to the compound shown in **FIG. 1A.** It is noted that the term norephedrine, unless specifically used in conjunction with stereoisomer notation (e.g. (1R, 2S)-norephedrine), is intended to include all 4 stereoisomers of ephedrine, i.e. (1R, 2S)-norephedrine,

(1S, 2R)-pseudonorephedrine, (1R, 2R)-norephedrine and (1S, 2S)-pseudonorephedrine.

[00043] The term "ephedrine", as used herein, refers to the compound shown in **FIG. 1B.** It is noted that the term ephedrine, unless specifically used in conjunction with stereoisomer notation (e.g. (1R, 2S)-ephedrine), is intended to include all 4 stereoisomers of ephedrine, i.e. (1R, 2S)-ephedrine, (1S, 2R)-pseudoephedrine, (1R, 2R)- ephedrine and (1S, 2S)-pseudoephedrine.

[00044] The term "N-methylephedrine" as used herein refers to the compound shown in **FIG. 1C.** It is noted that the term N-methylephedrine, unless specifically used in conjunction with stereoisomer notation (e.g. (1R, 2S)-N-methylephedrine), is intended to include all 4 stereoisomers of ephedrine, i.e. (1R, 2S)-N-methylephedrine, (1S, 2R)-N-methylpseudoephedrine, (1R, 2R)-N-methylephedrine and (1S, 2S)-N-methylpseudoephedrine.

[00045] The term "(1R, 2S)-norephedrine", as used herein refers to the chemical compound shown in **FIG. 2A**.

[00046] The term "(1S, 2R)-norpseudoephedrine", as used herein refers to the chemical compound shown in **FIG. 2B**.

[00047] The term "(1R, 2R)-norephedrine" as used herein refers to the chemical compound shown in **FIG. 2C**.

[00048] The term "(1S, 2S)-pseudonorephedrine", as used herein refers to the chemical compound shown in **FIG. 2D**.

[00049] The term "(1R, 2S)-ephedrine", as used herein refers to the chemical compound shown in **FIG. 2E**.

[00050] The term "(1S, 2R)-pseudoephedrine", as used herein refers to the chemical compound shown in **FIG. 2F**.

[00051] The term "(1R, 2R)-ephedrine", as used herein refers to the chemical compound shown in **FIG. 2G**.

[00052] The term "(1S, 2S)-pseudoephedrine", as used herein refers to the chemical compound shown in **FIG. 2H**.

[00053] The term "(1R, 2S)-N-methylephedrine", as used herein refers to the chemical compound shown in **FIG. 2I**.

[00054] The term "(1S, 2R)-N-methylpseudoephedrine", as used herein refers to the chemical compound shown in **FIG. 2J**.

[00055] The term "(1R, 2R)-N-methylephedrine", as used herein refers to the chemical compound shown in **FIG. 2K**.

[00056] The term "(1S, 2S)-N-methylpseudoephedrine" as used herein refers to the chemical compound shown in **FIG. 2L**.

[00057] The terms "N-methyltransferase" and "NMT" which may be used interchangeably herein refer to any and all enzymes comprising a sequence of amino acid residues which is (i) substantially identical to the amino acid sequences constituting any N-methyltransferase polypeptide set forth herein, including, for example, SEQ.ID NO: 3, or (ii) encoded by a nucleic acid sequence capable of hybridizing under at least moderately stringent conditions to any nucleic acid sequence encoding any N-methyltransferase polypeptide set forth herein, but for the use of synonymous codons.

[00058] The term "nucleic acid sequence" as used herein refers to a sequence of nucleoside or nucleotide monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term also includes modified or substituted sequences comprising non-naturally occurring monomers or portions thereof. The nucleic acid sequences of the present disclosure may be deoxyribonucleic acid sequences (DNA) or ribonucleic acid sequences (RNA) and may include naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The sequences may also contain modified bases. Examples of such modified bases include aza and deaza adenine, guanine, cytosine, thymidine and uracil, and xanthine and hypoxanthine.

[00059] The term "nucleic acid sequence encoding N-methyltransferase", "nucleic acid sequence encoding a N-methyltransferase polypeptide", refer to any and all nucleic acid sequences encoding a N-methyltransferase polypeptide, including, for example, SEQ.ID NO: 1 and SEQ.ID NO 2. Nucleic acid sequences encoding a N-methyltransferase polypeptide further include any and all nucleic acid sequences which (i) encode polypeptides that are substantially identical to the N-methyltransferase polypeptide sequences set forth herein; or (ii) hybridize to any N-methyltransferase nucleic acid sequences set forth herein under at least moderately stringent hybridization conditions or which would hybridize thereto under at least moderately stringent conditions but for the use of synonymous codons.

[00060] By the term "substantially identical" it is meant that two polypeptide sequences preferably are at least 70% identical, and more preferably are at least 85% identical and most preferably at least 95% identical, for example 96%, 97%, 98% or 99% identical. In order to determine the percentage of identity between two polypeptide sequences the amino acid sequences of such two sequences are aligned, using for example the alignment method of Needleman and Wunsch (J. Mol. Biol., 1970, 48: 443), as revised by Smith and Waterman (Adv. Appl. Math., 1981, 2: 482) so that the highest order match is obtained between the two sequences and the number of identical amino acids is determined between the two sequences. Methods to calculate the percentage identity between two amino acid sequences are generally art recognized and include, for example, those described by Carillo and Lipton (SIAM J. Applied Math., 1988, 48:1073) and those described in Computational Molecular Biology, Lesk, e.d. Oxford University Press, New York, 1988, Biocomputing: Informatics and Genomics Projects. Generally, computer programs will be employed for such calculations. Computer programs that may be used in this regard include, but are not limited to, GCG (Devereux *et al.*, Nucleic Acids Res., 1984, 12: 387) BLASTP, BLASTN and FASTA (Altschul *et al.*, J. Molec. Biol., 1990:215:403). A particularly preferred method for determining the percentage identity between two polypeptides involves the Clustal W algorithm (Thompson, J D, Higgines, D G and Gibson T J, 1994, Nucleic Acid Res 22(22): 4673-4680 together with the BLOSUM 62 scoring matrix (Henikoff S & Henikoff, J G, 1992, Proc. Natl. Acad. Sci. USA 89: 10915-10919 using a gap opening penalty of 10 and a gap extension penalty of 0.1, so that the highest order match obtained between two sequences wherein at least 50% of the total length of one of the two sequences is involved in the alignment.

[00061] By "at least moderately stringent hybridization conditions" it is meant that conditions are selected which promote selective hybridization between two complementary nucleic acid molecules in solution. Hybridization may occur to all or a portion of a nucleic acid sequence molecule. The hybridizing portion is typically at least 15 (e.g. 20, 25, 30, 40 or 50) nucleotides in length. Those skilled in the art will recognize that the stability of a nucleic acid duplex, or hybrids, is determined by the Tm, which in sodium containing buffers is a function of the sodium ion concentration and temperature (Tm=81.5° C.−16.6 (Log10

[Na+])+0.41(% (G+C)−600/l), or similar equation). Accordingly, the parameters in the wash conditions that determine hybrid stability are sodium ion concentration and temperature. In order to identify molecules that are similar, but not identical, to a known nucleic acid molecule a 1% mismatch may be assumed to result in about a 1° C. decrease in Tm, for example if nucleic acid molecules are sought that have a >95% identity, the final wash temperature will be reduced by about 5° C. Based on these considerations those skilled in the art will be able to readily select appropriate hybridization conditions. In preferred embodiments, stringent hybridization conditions are selected. By way of example the following conditions may be employed to achieve stringent hybridization: hybridization at 5× sodium chloride/sodium citrate (SSC)/5×Denhardt's solution/1.0% SDS at Tm (based on the above equation) −5° C, followed by a wash of 0.2×SSC/0.1% SDS at 60° C. Moderately stringent hybridization conditions include a washing step in 3×SSC at 42° C. It is understood however that equivalent stringencies may be achieved using alternative buffers, salts and temperatures. Additional guidance regarding hybridization conditions may be found in: Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 1989, 6.3.1.-6.3.6 and in: Sambrook *et al.*, Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989, Vol. 3.

[00062] The term “chimeric” as used herein in the context of nucleic acid sequences refers to at least two linked nucleic acid sequences which are not naturally linked. Chimeric nucleic acid sequences include linked nucleic acid sequences of different natural origins. For example a nucleic acid sequences constituting a yeast promoter linked to a nucleic acid sequence encoding a N-methyltransferase polypeptide is considered chimeric. Chimeric nucleic acid sequences also may comprise nucleic acid sequences of the same natural origin, provided they are not naturally linked. For example a nucleic acid sequence constituting a promoter obtained from a particular cell-type may be linked to a nucleic acid sequence encoding a polypeptide obtained from that same cell-type, but not normally linked to the nucleic acid sequence constituting the promoter. Chimeric nucleic acid sequences also include nucleic acid sequences comprising any naturally occurring nucleic acid sequences linked to any non-naturally occurring nucleic acid sequence.

[00063] The terms "substantially pure" and "isolated", as may be used interchangeably herein describe a compound, e.g., ephedrine or a polypeptide, which has been separated from components that naturally accompany it. Typically, a compound is substantially pure when at least 60%, more preferably at least 75%, more preferably at least 90%, 95%, 96%, 97%, or 98%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole percent or mole fraction) in a sample is the compound of interest. Purity can be measured by any appropriate method, e.g., in the case of polypeptides, by chromatography, gel electrophoresis or HPLC analysis.

[00064] The term "recovered" as used herein in association with an enzyme or protein or ephedrine, or a related alkaloid compound, refers to a more or less pure form of the enzyme or ephedrine or a related alkaloid compound.

[00065] The term "*in vivo*" as used herein to describe methods of making ephedrine or related alkaloid compounds refers to contacting a first chemical compound with an enzyme capable of catalyzing a conversion of the compound within a living cell, including, for example, a microbial cell or a plant cell, to form ephedrine or a related alkaloid compound.

[00066] The term "*in vitro*" as used herein to describe methods of making ephedrine or related alkaloid compounds refers to contacting a first chemical compound with an enzyme capable of catalyzing conversion of the compound in an environment outside a living cell, including, without limitation, for example, in a microwell plate, a tube, a flask, a beaker, a tank, a reactor and the like, to form ephedrine or a related alkaloid compound.

[00067] The term "halogen", as used herein, refers to an atom of chlorine, bromine, fluorine, or iodine.

[00068] The term "methyl-donor" as used herein refers to a compound or molecule which can transfer a methyl (CH_3) group to an alkaloid compound of formula (I) resulting in an alkaloid compound of formula (II).

[00069] It should be noted that terms of degree such as "substantially", "essentially" "about" and "approximately" as used herein mean a reasonable amount of deviation of the modified term such that the end result is not significantly changed. These terms of degree should be construed as including a

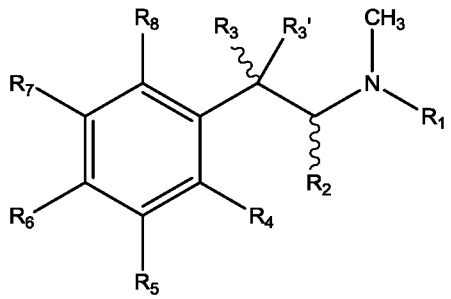
deviation of the modified term if this deviation would not negate the meaning of the term it modifies.

[00070] As used herein, the wording "and/or" is intended to represent an inclusive-or. That is, "X and/or Y" is intended to mean X or Y or both, for example. As a further example, "X, Y, and/or Z" is intended to mean X or Y or Z or any combination thereof.

[00071] All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication.

General implementation

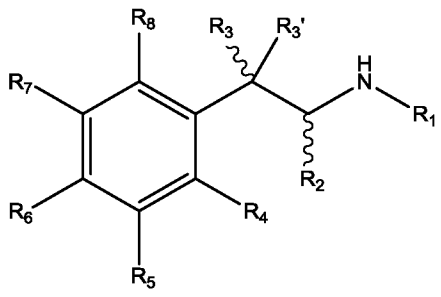
[00072] In one implementation, in accordance herewith there is provided, a method of making an alkaloid compound having a chemical formula (II):



(II),

the method comprising

providing an alkaloid compound having a chemical formula (I):



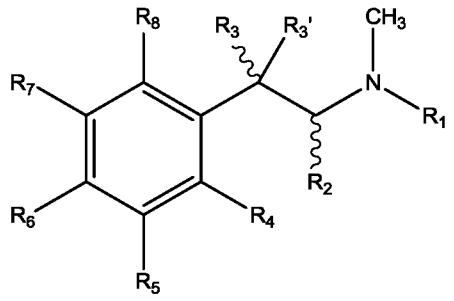
(I),

contacting the alkaloid compound having a chemical formula (I) with sufficient quantities of a methyl-donor and a catalytic quantity of an N-methyl-transferase under reaction conditions permitting an enzyme-catalyzed conversion of the

alkaloid compound having chemical formula (I) to an alkaloid compound having chemical formula (II).

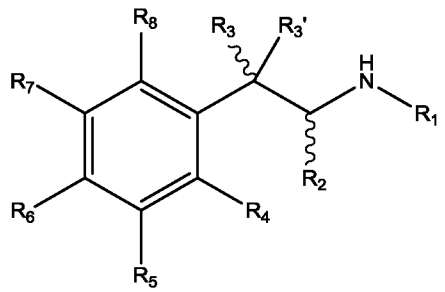
[00073] In some implementations, in the alkaloid compound having chemical formula (I) R_1 is a hydrogen atom, a methyl group or a halogen; R_2 is a hydrogen atom, a methyl group or a methoxy group; R_3 is a hydroxyl group or a halogen, R_3' is a hydrogen atom, or taken together, R_3 and R_3' form a carbonyl group (C=O); R_4 – R_8 are each independently or simultaneously a hydrogen atom, methyl group, hydroxyl group, halogen or methoxy group; and each R_1 to R_8 and R_3' in the alkaloid compound having chemical formula (I) are identical to R_1 to R_8 and R_3' in the alkaloid compound having chemical formula (II). R_3 halogen groups include chlorine, fluorine, bromine and iodine.

[00074] In one implementation, the present disclosure provides a method of making an alkaloid compound having a chemical formula (II):



(II),

the method comprising providing an alkaloid compound having a chemical formula (I):



(I),

contacting the alkaloid compound having a chemical formula (I) with sufficient quantities of a methyl-donor and a catalytic quantity of an N-methyl-transferase under reaction conditions permitting an enzyme catalyzed conversion of the

alkaloid compound having chemical formula (I) to an alkaloid compound having chemical formula (II), wherein R_1 is a hydrogen atom, a methyl group or a halogen; R_2 is a hydrogen atom, a methyl group or a methoxy group; R_3 is a hydroxyl group or a halogen, R_3' is a hydrogen atom, or taken together R_3 and $R_{3'}$ form a carbonyl group (C=O); R_4 – R_8 are each independently or simultaneously a hydrogen atom, methyl group, hydroxyl group, halogen or methoxy group; and each R_1 to R_8 and R_3' in the alkaloid compound having chemical formula (I) are identical to R_1 to R_8 R_3' in the alkaloid compound having chemical formula (II).

[00075] In some implementations, in both the alkaloid compound having chemical formula (I) and the alkaloid compound having chemical formula (II), R_1 is a hydrogen atom, R_2 is a methyl group, R_3 is a hydroxyl group, R_3' is a hydrogen atom, and R_4 – R_8 are hydrogen atoms. It is noted that in such implementations, the alkaloid compound having chemical formula (I) is also referred to as norephedrine and the alkaloid compound having chemical formula (II) is also referred to as ephedrine.

[00076] In some implementations, in both the alkaloid compound having chemical formula (I) and the alkaloid compound having chemical formula (II) R_1 is a methyl group, R_2 is a methyl group, R_3 is a hydroxyl group, R_3' is hydrogen atom and R_4 – R_8 are hydrogen atoms. It is noted that in such implementations, the alkaloid compound having chemical formula (I) is also referred to as ephedrine and the alkaloid compound having chemical formula (II) is also referred to as N-methylephedrine.

[00077] In some implementations, R_2 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 2R-enantiomer.

[00078] In some implementations, R_2 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 2S-enantiomer.

[00079] In some implementations, R_3 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 1R-enantiomer.

[00080] In some implementations, R_3 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 1S-enantiomer.

[00081] In some implementations, the alkaloid compound having chemical formula (I) is (1S, 2S)-pseudonorephedrine and the alkaloid compound having chemical formula (II) is (1S, 2S)-pseudoephedrine.

[00082] In some implementations, the alkaloid compound having chemical formula (I) is (1R, 2R)-norephedrine and the alkaloid compound having chemical formula (II) is (1R, 2R)-ephedrine.

[00083] In some implementations, the alkaloid compound having chemical formula (I) is (1R, 2S)-norephedrine and the alkaloid compound having chemical formula (II) is (1R, 2S)-ephedrine.

[00084] In some implementations, the alkaloid compound having chemical formula (I) is (1S, 2R)-norpseudoephedrine and the alkaloid compound having chemical formula (II) is (1S, 2R)-pseudoephedrine.

[00085] In some implementations, the alkaloid compound having chemical formula (I) is (1S, 2S)-pseudoephedrine and the alkaloid compound having chemical formula (II) is (1S, 2S)-N-methyl-pseudoephedrine.

[00086] In some implementations, the alkaloid compound having chemical formula (I) is (1R, 2R)-ephedrine and the alkaloid compound having chemical formula (II) is (1R, 2R)-N-methyl-ephedrine.

[00087] In some implementations, the alkaloid compound having chemical formula (I) is (1R, 2S)-ephedrine and the alkaloid compound having chemical formula (II) is (1R, 2S)-N-methyl-ephedrine.

[00088] In some implementations, the alkaloid compound having chemical formula (I) is (1S, 2R)-pseudoephedrine and the alkaloid compound having chemical formula (II) is (1S, 2R)-N-methyl-pseudoephedrine.

[00089] In some implementations, in both the alkaloid compound having chemical formula (I) and (II) R_1, and R_4-R_8 are each hydrogen atoms, R_2 is methyl and R_3 and R_3' taken together form a carbonyl group (C=O). It is noted that the compound having chemical formula (I) is known as cathinone, and the compound having chemical formula (II) is known as methcathinone. In some implementations, the alkaloid compound having chemical formula (I) is (S)-cathinone and the alkaloid compound having chemical formula (II) is (S)-methcathinone. In some implementations, the alkaloid compound having chemical formula (I) is (R)-cathinone and the alkaloid compound having chemical formula (II) is (R)-methcathinone.

[00090] In some implementations, in both the alkaloid compound having chemical formula (I) and (II), R_1, R_2, R_3' R_4, R_5, R_7 and R_8 are each hydrogen atoms,

and R_3 and R_6 are hydroxyl groups. It is noted that the compound having chemical formula (I) is known as p-octopamine, and the compound having chemical formula (II) is known as synephrine.

[00091] In some implementations, in both the alkaloid compound having chemical formula (I) and (II), R_1 is a methyl group, R_2, R_3', R_4, R_5, R_6 and R_8 are each hydrogen atoms, and R_3 and R_7 are hydroxyl groups. It is noted that the compound having chemical formula (I) is known as N-desmethylphenylephrine, and the compound having chemical formula (II) is known as phenylephrine.

[00092] In some implementations, in both the alkaloid compound having chemical formula (I) and (II), R_1 is a methyl group, R_2, R_3', R_4, R_5, R_7 and R_8 are each hydrogen atoms, and R_3 and R_6 are hydroxyl groups. It is noted that the compound having chemical formula (I) is known as synephrine, and the compound having chemical formula (II) is known as N-methylsynephrine.

[00093] In some implementations, the alkaloid compound having chemical formula (I) is selected from the group of compounds consisting of 3'-chloro-2-aminopropiophenone; normephedrone; normethedrone; oxidopamine; m-tyramine; amphetamine; noradrenaline; normetraminol; gepefrine; dopamine, p-tyramine; p-octopamine; phenylethanolamine (β-hydroxy phenylethanolamine); 2-phenethylamine; ortetamine; β-methyl-phenethylamine; 3-methoxy-tyramine; normethoxyphenamine; mescaline, 3,4dimethoxyphenethylamine; normacromerine; and S-methcathinone.

***In vitro* synthesis**

[00094] In accordance with certain aspects of the present disclosure, an alkaloid compound having a chemical formula (I) is brought in contact with sufficient quantities of a methyl-donor and catalytic quantities of an N-methyltransferase under reaction conditions permitting an enzyme catalyzed chemical conversion of an alkaloid compound having chemical formula (I) to an alkaloid compound having chemical formula (II) under *in vitro* reaction conditions. Under such *in vitro* reaction conditions the initial reaction constituents are provided in more or less pure form and are contacted with each other and mixed under conditions that permit the requisite chemical reactions, upon enzyme catalysis, to substantially proceed. Substantially pure forms of the initial alkaloid compound having a chemical formula (I) may be chemically synthesized or

isolated from natural sources, including from plant species belonging to the genus *Khat* and plant species belonging to the genus *Ephedra*. Suitable plant species include, without limitation, *Catha edulis*, *Ephedra sinica* and *Ephedra distachya*

[00095] In accordance herewith, more or less pure forms of the enzymes may be isolated from natural sources, microbial species, and the hereinbefore mentioned plant species, or they may be prepared recombinantly. Thus, provided herein is further a method for preparing an N-methyltransferase comprising:

(a) providing a chimeric nucleic acid sequence comprising as operably linked components:

(i) one or more nucleic acid sequences encoding an N-methyltransferase; and

(ii) one or more nucleic acid sequences capable of controlling expression in a host cell;

(b) introducing the chimeric nucleic acid sequence into a host cell and growing the host cell to produce an N-methyltransferase; and

(c) recovering the N-methyltransferase from the host cell.

[00096] In preferred implementations, the N-methyltransferase is a polypeptide obtainable or obtained from a plant belonging to a plant genus selected from the group consisting of *Ephedra*, *Catha*, *Sesamum*, *Brassica* and *Erythranthe*. In particularly preferred implementations, the N-methyltransferase is a polypeptide obtainable or obtained from a plant species selected from the group consisting of *Ephedra sinica*, *Catha edulis*, *Sesamum indicum*, *Brassica rapus* and *Erythranthe guttata*.

[00097] In preferred implementations, the N-methyltransferase is a polypeptide having a polypeptide sequence represented by SEQ.ID NO: 3, SEQ.ID NO: 5; SEQ.ID NO: 7 or SEQ.ID NO: 9.

[00098] Growth of the host cells leads to production of the N-methyltransferase. The polypeptides subsequently may be recovered, isolated and separated from other host cell components by a variety of different protein purification techniques including, e.g. ion-exchange chromatography, size exclusion chromatography, affinity chromatography, hydrophobic interaction chromatography, reverse phase chromatography, gel filtration, etc. Further general guidance with respect to protein purification may for example be found in:

Cutler, P. Protein Purification Protocols, Humana Press, 2004, Second Ed. Thus substantially pure preparations of the N-methyltranferase polypeptides may be obtained.

[00099] In accordance herewith, an alkaloid compound having chemical formula (I) is brought in contact with sufficient quantities of a methyl-donor and catalytic quantities of N-methyl transferase under reaction conditions permitting an enzyme catalyzed chemical conversion of the alkaloid compound having chemical formula (I) to an alkaloid compound having chemical formula (II). A variety of methyl- donors may be used. In preferred embodiments, S-adenosyl methionine (SAM) is used as a methyl-donor. In other embodiments other methyl donors are used including, natural or synthetic methyl-donors, including, without limitation, L-methionine; L-methionine ethyl ester (MEE); methyl ester of methionine (MME); N-derivatized methionine analogues, such as N-acetyl-L-methionine (NAM), and N,N-dimethyl-L-methionine (DMM); aziridinium-based SAM analogues; SAM analogues comprising a substituted L-methyl-group, for example, a terminal alkynyl, keto or amino group; or S/Se-Met analogues. Further reference to these and other methyl donors that may be used in accordance herewith can be found in Biochemistry (2014) 53:1521-1526; Microbiology (2015) 161 (Pt 3):674-682; Agnew. Chem. Int. Ed. (2014) 53:3965-3969; Nature Chemical Biology (2006) 2:31-32; Org. Biomol. Chem. (2013) 11:7606-7610; and Anal. Biochem. (2014) 450:11-19. The quantities of methyl-donor that are used may vary. In some implementations, equimolar, or approximately equimolar amounts of a methyl-donor and an alkaloid compound having chemical formula (I) are provided. In some implementations, the methyl-donor is provided in excess of an equimolar quantity of and an alkaloid compound having chemical formula (I).

[000100] In some implementations, the agents are brought in contact with each other and mixed to form a mixture. In some implementations, the mixture is an aqueous mixture comprising water and further optionally additional agents to facilitate enzyme catalysis, including buffering agents, salts, pH modifying agents, or other enzymes. The reaction may be performed at a range of different temperatures. In preferred implementations, the reaction is performed at a temperature between about 18 °C and 37 °C. Upon completion of the *in vitro*

reaction and an alkaloid compound having chemical formula (II) may be obtained in more or less pure form.

***In vivo* synthesis**

[000101] In accordance with certain aspects of the present disclosure, an alkaloid compound having a chemical formula (I) is brought in contact with sufficient quantities of a methyl-donor and catalytic quantities of an N-methyltransferase under reaction conditions permitting an enzyme catalyzed chemical conversion of an alkaloid compound having chemical formula (I) to an alkaloid compound having chemical formula (II) under *in vivo* reaction conditions. Under such *in vivo* reaction conditions living cells are modified in such a manner that they produce an alkaloid compound having chemical formula (II). In certain implementations, the living cells are microorganisms, including bacterial cells and fungal cells. In other embodiments, the living cells are multicellular organisms, including plants.

[000102] In one implementation, the living cells are selected to be host cells capable of producing an alkaloid compound having chemical formula (I), but not an alkaloid compound having chemical formula (II). Such cells include, without limitation, bacteria, yeast, other fungal cells, plant cells, or animal cells. Thus, by way of example only, a host cell may be a yeast host cell capable of producing cells capable of producing an alkaloid compound having chemical formula (I), but not an alkaloid compound having chemical formula (II).

[000103] In some implementations, such a host cell, a yeast cell, for example, is used which is capable of producing one or more of the following compounds serving as a precursor to the alkaloid compound having chemical formula (I): (1R)-phenylacetylcarbinol (R-PAC), (1S)-phenylacetylcarbinol (S-PAC), (S)-cathione, 1 phenylpropane-1,2-dione, benzoyl-CoA, benzaldehyde or benzoic acid, such a cell not being capable of producing an alkaloid compound having chemical formula (II). In some implementations, one or more of the compounds (1R)-phenylacetylcarbinol (R-PAC), (1S)- phenylacetylcarbinol (S-PAC), (S)-cathione, 1 phenylpropane-1,2-dione, benzoyl-CoA, benzaldehyde or benzoic acid serving as a precursor compound to the alkaloid compound having chemical formula (I) is synthesized by a cell exogenously supplemented with benzaldehyde. In order to modulate such host cells in such a manner that they produce an alkaloid

compound having chemical formula (II), an N-methyltransferase in accordance herewith may be heterologously introduced and expressed in the host cells.

[000104] In some implementations, the host cells comprise one or more enzymes capable of producing an alkaloid compound having chemical formula (I) and/or a precursor thereof.

[000105] In some implementations, the host cells comprises a reductase capable of catalyzing a reaction resulting in the production of norephedrine from (S)-cathinone.

[000106] In some implementations, the host cell comprises a transaminase capable of catalyzing a reaction resulting in the production of norephedrine from (1R)-phenylacetylcarbinol (R-PAC), (1S)- phenylacetylcarbinol (S-PAC).

[000107] In some implementations, the host cell comprises transaminase capable of catalyzing a reaction resulting in the production of (S)-cathione from 1-phenyl-propane-1,2-dione.

[000108] In some implementations, the host cell comprises a carboligase capable of catalyzing a reaction resulting in the production of (1R)-phenylacetylcarbinol (R-PAC), (1S)- phenylacetylcarbinol (S-PAC) from benzaldehyde.

[000109] In some implementations, the host cell comprises a carboligase capable of catalyzing a reaction resulting the production of 1-phenyl-propane-1,2-dione from benzoyl-CoA.

[000110] In some implementations, the host cell comprises a CoA-ligase capable of catalyzing a reaction resulting in the production of benzoyl-CoA from benzoic acid.

[000111] In some implementations, the host cell comprises a dehydrogenase capable of catalyzing a reaction resulting in the production of benzoic acid from benzaldehyde.

[000112] In other embodiments, the living cells naturally produce an alkaloid compound having chemical formula (II), however the living cells are modulated in such a manner that the level of an alkaloid compound having chemical formula (II) produced is modulated, relative to the level produced by the cell without heterologous introduction of any of the aforementioned enzymes in such living cells.

[000113] In order to produce alkaloid compound having chemical formula (II), provided herein is further a method for preparing an alkaloid compound having chemical formula (II) comprising:

(a) providing a chimeric nucleic acid sequence comprising as operably linked components:

(i) a nucleic acid sequence encoding an N-methyltransferase polypeptide; and

(ii) one or more nucleic acid sequences capable of controlling expression in a host cell;

(b) introducing the chimeric nucleic acid sequence into a host cell and growing the host cell to produce the N-methyltransferase and to produce the alkaloid compound having chemical formula (II); and

(c) recovering the alkaloid compound having chemical formula (II).

[000114] In some implementations, the nucleic acid sequences may be isolated from the hereinbefore mentioned plant species. In some implementations, the nucleic acid sequences are selected from the nucleic acid sequences set forth herein as one SEQ.ID NO: 1, SEQ.ID NO: 2, SEQ.ID NO: 4, SEQ.ID NO: 6 or SEQ.ID NO: 8.

[000115] In accordance herewith, the nucleic acid sequence encoding N-methyltransferase is linked to a nucleic acid sequence capable of controlling expression of N-methyltransferase in a host cell. Accordingly, the present disclosure also provides a nucleic acid sequence encoding N-methyltransferase linked to a promoter capable of controlling expression in a host cell. Nucleic acid sequences capable of controlling expression in host cells that may be used herein include any transcriptional promoter capable of controlling expression of polypeptides in host cells. Generally, promoters obtained from bacterial cells are used when a bacterial host is selected in accordance herewith, while a fungal promoter will be used when a fungal host is selected, a plant promoter will be used when a plant cell is selected, and so on. Further nucleic acid elements capable elements of controlling expression in a host cell include transcriptional terminators, enhancers and the like, all of which may be included in the chimeric nucleic acid sequences of the present disclosure.

[000116] In accordance with the present disclosure, the chimeric nucleic acid sequences comprising a promoter capable of controlling expression in host cell linked to a nucleic acid sequence encoding an N-methyltransferase, can be integrated into a recombinant expression vector which ensures good expression in the host cell. Accordingly, the present disclosure includes a recombinant expression vector comprising as operably linked components:

(i) a nucleic acid sequence capable of controlling expression in a host cell; and

(ii) a nucleic acid sequence encoding an N-methyltransferase,

wherein the expression vector is suitable for expression in a host cell. The term "suitable for expression in a host cell" means that the recombinant expression vector comprises the chimeric nucleic acid sequence of the present disclosure linked to genetic elements required to achieve expression in a host cell. Genetic elements that may be included in the expression vector in this regard include a transcriptional termination region, one or more nucleic acid sequences encoding marker genes, one or more origins of replication and the like. In preferred embodiments, the expression vector further comprises genetic elements required for the integration of the vector or a portion thereof in the host cell's genome, for example if a plant host cell is used the T-DNA left and right border sequences which facilitate the integration into the plant's nuclear genome.

[000117] Pursuant to the present disclosure, the expression vector may further contain a marker gene. Marker genes that may be used in accordance with the present disclosure include all genes that allow the distinction of transformed cells from non-transformed cells, including all selectable and screenable marker genes. A marker gene may be a resistance marker such as an antibiotic resistance marker against, for example, kanamycin or ampicillin. Screenable markers that may be employed to identify transformants through visual inspection include β-glucuronidase (GUS) (U.S. Pat. Nos. 5,268,463 and 5,599,670) and green fluorescent protein (GFP) (Niedz *et al.*, 1995, Plant Cell Rep., 14: 403).

[000118] One host cell that particularly conveniently may be used is *Escherichia coli*. The preparation of the *E. coli* vectors may be accomplished using commonly known techniques such as restriction digestion, ligation, gelectrophoresis, DNA sequencing, the Polymerase Chain Reaction (PCR) and

other methodologies. A wide variety of cloning vectors is available to perform the necessary steps required to prepare a recombinant expression vector. Among the vectors with a replication system functional in E. coli, are vectors such as pBR322, the pUC series of vectors, the M13 mp series of vectors, pBluescript etc. Typically, these cloning vectors contain a marker allowing selection of transformed cells. Nucleic acid sequences may be introduced in these vectors, and the vectors may be introduced in *E. coli* by preparing competent cells, electroporation or using other well known methodologies to a person of skill in the art. *E. coli* may be grown in an appropriate medium, such as Luria-Broth medium and harvested. Recombinant expression vectors may readily be recovered from cells upon harvesting and lysing of the cells. Further, general guidance with respect to the preparation of recombinant vectors and growth of recombinant organisms may be found in, for example: Sambrook *et al.*, Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press, 2001, Third Ed.

[000119] Further included in the present disclosure are a host cell wherein the host cell comprises a chimeric nucleic acid sequence comprising in the 5' to 3' direction of transcription as operably linked components one or more nucleic acid sequences encoding an N-methyltransferase. As hereinbefore mentioned the host cell is preferably a host cell capable of producing an alkaloid compound having chemical formula (I), but is unable to produce an alkaloid compound having chemical formula (II), but for the introduction of the chimeric nucleic acid sequences of the present disclosure.

[000120] As hereinbefore mentioned, in other embodiments, the host cells naturally produce an alkaloid having chemical formula (II), however the host cells are modulated in such a manner that the levels of an alkaloid having chemical formula (II) produced in the cells is modulated, relative to levels of such alkaloid produced by the cell without heterologous introduction of any of the aforementioned enzymes in such host cells. Such modulations may be achieved by a variety of modification techniques, including, but not limited to, the modulation of the enzymatic activity of an N-methyltransferase, for example by modulating the native nucleic acid sequences encoding the N-methyltransferase, for example by gene silencing methodologies, such as antisense methodologies; or by the use of modification techniques resulting in modulation of activity of the enzymes using

for example site directed mutagenesis, targeted mutagenesis, random mutagenesis, virus-induced gene silencing, the addition of organic solvents, gene shuffling or a combination of these and other techniques known to those of skill in the art, each methodology designed to alter the activity of the enzymes of the N-methyltransferase, in such a manner that level of alkaloid compound having chemical formula (II) in the host cells increases.

EXAMPLES

[000121] Hereinafter are provided examples of specific implementations for performing the methods of the present disclosure, as well as implementations representing the compositions of the present disclosure. The examples are provided for illustrative purposes only, and are not intended to limit the scope of the present disclosure in any way.

Example 1 – Ephedrine, norephedrine, pseudoephedrine, cathinone, synephrine and octopamine as substrates for *Escherichia coli* and *Ephedra sinica* produced N-methyltransferase

[000122] This example describes the use ephedrine and five other related chemical compounds as substrates for N-methyltransferase. The N-methyltransferase is obtained from a plant belonging to the genus *Ephedra*, notably *Ephedra sinica,* and expressed in microbial host cells, notably *Escherichia coli*. This example further describes the use of an N-methyltransferase obtained from *Ephedra sinica* using norephedrin as a substrate.

Construct assembly and protein expression in E. coli.

[000123] Open reading frames (ORFs) of *N*-methyltransferase (NMT) enzyme sequences (i.e. SEQ.ID NO: 1 and SEQ.ID NO: 2) were subcloned into *Escherichia coli* expression vector pET47b (Merck Millipore) in-frame with vector-encoded His-tag for downstream purification purposes. Constructs were transformed into *E. coli* strains ArcticExpress (Agilent Technologies) or Rosetta (Novagen). Recombinant His-tagged protein production was performed according to standard procedures (Farrow *et al*. 2013. J. Biol. Chem. 288: 28977) and manufacturer's instructions. Briefly, cultures of *E. coli* harboring pET47b were grown on an orbital shaker (200 rpm) to log phase in 1 L of LB medium containing gentamicin, streptomycin and kanamycin (Arctic Express) or chloramphenicol and kanamycin (Rosetta). Recombinant His-tagged protein production was initiated by the

addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). To produce NMT, cultures were grown for 24 h at 15°C.

Enzyme purification and activity assays.

[000124] For purification, bacterial pellets obtained following induction / incubation were resuspened in 10 mL of resuspension buffer (50 mM Tris pH 8.5, 10% v/v glycerol, 1% w/v polyvinylpyrrolidone, 5 mM dithiothreitol) and lysed at 4°C using a French pressure cell (1,000 psi). After centrifugation (10,000 g) to remove insoluble debris, the supernatant was incubated on ice with buffer-equilibrated Talon resin (Clontech) on an orbital shaker (60 rpm) for 60 minutes. The protein-charged resin was washed twice with 10 mL of cold resuspension buffer containing 2.5 mM imidazole, and proteins were eluted stepwise with increasing concentrations (10 to 200 mM) of imidazole in resuspension buffer. Total proteins from a 40 mM imidazole elution were desalted using a PD-10 column (EMD Millipore) and resuspension buffer free of imidazole. Recombinant proteins were analyzed by SDS-PAGE to assess yield and purity, and immunoblot analyses were conducted using α-His primary antibodies and goat-anti-mouse, horseradish peroxidase-conjugated secondary antibodies. Total protein concentration was determined using BCA Protein Assay kit (Thermo Scientific). Calculated protein concentrations were adjusted on gel densitometry. Standard enzyme assays included approximately 100 μg/mL purified NMT protein, which was determined to be in the linear range of catalyst performance. Specifically, standard enzyme reactions consisted of 50 mM Tris pH 8.5, 10 v/v glycerol, 1% w/v polyvinylpyrrolidone, 5 mM dithiothreitol, 1 mM alkaloid substrate, 1mM SAM (S-adenosylmethionine) and 100 μg/mL purified NMT protein in a total of 100 μl reaction volume. Reactions were incubated for 4 h at 37°C, and stopped via 1) basification of the reaction mixture to pH 10-11, and 2) immediate addition of 1 mL ethyl acetate. Quenched reactions were vortexed and centrifuged (10,000 rpm) to separate organic and aqueous phases, and organic phase was carefully drawn off, placed in a new tube, reduced to dryness under vacuum, and resuspended in 500 μL of Solvent A (10 mM ammonium acetate pH 5.5, 5% v/v methanol). The resuspensions were centrifuged (10,000 rpm) prior to analysis by LC-MS to remove insoluble matter.

Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

[000125] Ten μL sample was injected for LC-MS analysis using an Agilent 6410B triple quadrupole mass analyzer equipped with a 1200 Agilent HPCL system. Samples were fractionated using a previously described LC-ESI-MS/MS method [J. Chromatography B. 879:727, 2011] employing a Prodigy Phenyl-3 column (Phenomenex) which separated enantiomeric pairs (e.g. pseudoephedrine / ephedrine). Briefly, the HPLC gradient began as 95% Solvent A (Solvent B = 100% methanol) for 1 minute at a flow rate of 0.2 ml/min, and decreased to 30% Solvent A / 70% Solvent B over 19 minutes, then graded to 10% Solvent A / 90% Solvent A over 0.5 minutes, then immediately returned to 100% Solvent A and equilibrated for 5 minutes. Retention times (minutes) were as follows: norephedrine = 4.3; ephedrine = 6.9; pseudoephedrine = 7.9; cathinone = 7.1; methylephedrine = 10.6; methylpseudoephedrine = 11.9; methcathinone = 10.1. ESI and MS conditions were essentially the same as described [J. Chromatography B. 879:727, 2011]. Briefly, the MS was operated in positive ion mode with probe voltage of 4000 V and an extractor potential of 3 V, dwell time of 200 ms for all transitions, and CID spectra were acquired for product identification. Nitrogen was used for CID analyses. CID spectra and retention times of enzyme products were compared with those of authentic standards. Additional comparisons were made with published values [J. Chromatography B. 879:727, 2011; http://www.massbank.jp]. Enzyme activity was measured by integrating total area under product peaks of TIC (total ion count) spectra. Control samples consisting of boiled enzyme yielded negligible product. MS data analysis was performed using MassHunter Workstation software (Agilent), and subsequent enzyme activity calculations were performed manually with the aid of Microsoft Excel (Microsoft Corporation).

Analysis of Ephedra sinica plant tissue.

[000126] For comparative purposes, 2.0 grams of *Ephedra sinica* plant tissue was analyzed for NMT activity according to the protocol of Krizevski *et al.* [Phytochemistry 71:895, 2010]. Briefly, the tips of the young shoots (5 cm from the tip inward) were harvested from 2-year old *E. sinica* plants and ground to fine powder in a mortar and pestle under liquid nitrogen. The powder was added to cold extraction buffer [50 mM Tris pH 8.5, 10 v/v glycerol, 1% w/v

polyvinylpyrrolidone, 5 mM dithiothreitol] and vortexed. Centrifugation (10,000 g) was performed to remove insolubles, and the supernatant was concentrated to 1 mL using an Amicon Ultra Centrifugal filters (10 kD cutoff; Millipore) according to manufacturer's instructions. Several washes were performed using three fresh columns to ensure that all traces of alkaloid present in the plant-derived extraction mixture were removed prior to NMT assay. Assays were performed as described above for enzymes raised in *E. coli* host.

Results

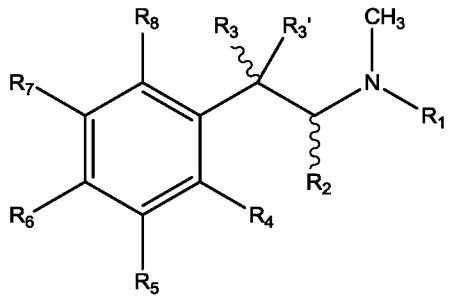
[000127] Expression of native *Ephedra sincia* NMT (SEQ.ID NO: 1) in *E. coli* yielded suboptimal results. Improved expression was observed when a codon-optimized synthetic gene (SEQ.ID NO: 2) was used. Western blotting and SDS-PAGE analysis revealed that expression of this synthetically-encoded NMT was superior using ArcticExpress *E. coli* compared to protein production in Rosetta cells, and therefore all experiments were conducted using the ArticExpress cell line and protocol. Enzyme assays were conducted using a variety of time points and enzyme amounts to determine the linear range of activity. Preliminary results showed that the enzyme activity continued linearly for over 4 hours, and therefore 4 hours was chosen as the time frame of standard assays. Tandem assays conducted with *Ephedra sinica*-derived protein revealed comparable rates of product turnover. As such, 4 hour assays were also conducted for plant-derived enzyme. Norephedrine was used as a substrate for plant-derived extract, yielding ephedrine at comparable levels to *E. coli*-produced NMT under standard assay conditions. Plant-derived NMT assays were conducted as a positive control to ensure that standard assay conditions were adequate for activity. The following subtrates were examined in the case of purified, *E. coli*-derived NMT: ephedrine, norephedrine, pseudoephedrine, cathinone, synephrine and octopamine. Results, as shown in **FIG. 3.** It is noted that control samples using identical conditions with the exception that enzyme was boiled for 20 minutes prior to assay initiation did not yield detectable product. The results illustrate that the NMT accepts a broad range of molecules for *N*-methylation. Thus NMT accepts primary amines (norephedrine) and secondary amines (ephedrine, pseudoephedrine) with amphetamine analogue-type molecular structure (e.g. with a methyl group attached to the alpha carbon). Another amphetamine-type alkaloid acting as

substrate included cathinone, which NMT converts to methcathinone. These results indicated that the NMT was able to turnover an alkaloid with a β-keto group in place of a β-hydroxyl. Synephrine and octopamine do not have an amphetamine-type structure, but were accepted as substrates.

[000128] In order to enable quantitative comparisons between alkaloids in terms of their effectiveness as substrates, standard curves were prepared for three alkaloids and their *N*-methylated products. This enabled absolute product quantification in terms of ng of alkaloid. Results shown in **FIG. 4** revealed norephedrine to be the preferred NMT substrate, followed closely by pseudoephedrine. The conversion efficiency of ephedrine to *N*-methylephedrine was somewhat lower. Control samples using identical conditions with the exception that enzyme was boiled for 20 minutes prior to assay initiation did not yield detectable product.

CLAIMS

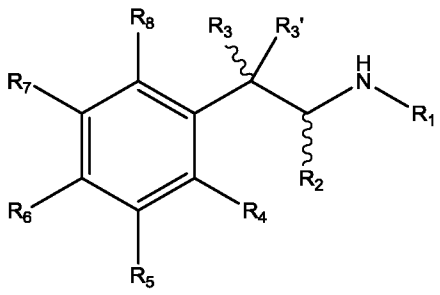
1. A method of making an alkaloid compound having a chemical formula (II):



(II),

the method comprising

providing an alkaloid compound having a chemical formula (I):



(I),

contacting the alkaloid compound having a chemical formula (I) with sufficient quantities of a methyl-donor and a catalytic quantity of an N-methyl-transferase under reaction conditions permitting an enzyme catalyzed conversion of the alkaloid compound having chemical formula (I) to an alkaloid compound having chemical formula (II), wherein R_1 is a hydrogen atom, a methyl group or a halogen; R_2 is a hydrogen atom, a methyl group or a methoxy group; R_3 is a hydroxyl group or a halogen, R_3' is a hydrogen atom, or taken together R_3 and $R_{3'}$ form a carbonyl group (C=O); R_4 – R_8 are each independently or simultaneously a hydrogen atom, methyl group, hydroxyl group, halogen or methoxy group; and each R_1 to R_8 and R_3' in the alkaloid compound having chemical formula (I) are identical to R_1 to R_8 R_3' in the alkaloid compound having chemical formula (II).

2. The method according to claim **1** wherein in both the alkaloid compound having chemical formula (I) and the alkaloid compound having chemical formula (II), R_1 is a hydrogen atom, R_2 is a methyl group, R_3 is a hydroxyl group, R_3' is a hydrogen atom and R_4 – R_8 are hydrogen atoms.

3. The method according to claim **1** wherein in both the alkaloid compound having chemical formula (I) and the alkaloid compound having chemical formula (II) R_1 is a methyl group, R_2 is a methyl group, R_3 is a hydroxyl group, R_3' is a hydrogen atom and R_4 – R_8 are hydrogen atoms.

4. The method according to claim **1** wherein R_2 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 2R-enantiomer.

5. The method according to claim **1** wherein R_2 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 2S-enantiomer.

6. The method according to claim **1** wherein R_3 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 1R-enantiomer.

7. The method according to claim **1** wherein R_3 is spatially oriented so that the alkaloid compound having a chemical formula (I) or (II) is a 1S-enantiomer.

8. The method according to claim **1** wherein the alkaloid compound having chemical formula (I) is (1S, 2S)-pseudonorephedrine and the alkaloid compound having chemical formula (II) is (1S, 2S)-pseudoephedrine.

9. The method according to claim **1** wherein the alkaloid compound having chemical formula (I) is (1R, 2R)-norephedrine and the alkaloid compound having chemical formula (II) is (1R, 2R)-ephedrine.

10. The method according to claim 1 wherein the alkaloid compound having chemical formula (I) is (1R, 2S)-norephedrine and the alkaloid compound having chemical formula (II) is (1R, 2S)-ephedrine.

11. The method according to claim 1 wherein the alkaloid compound having chemical formula (I) is (1S, 2R)-norpseudoephedrine and the alkaloid compound having chemical formula (II) is (1S, 2R)-pseudoephedrine.

12. The method according to claim **1** wherein the alkaloid compound having chemical formula (I) is (1S, 2S)-pseudoephedrine and the alkaloid compound having chemical formula (II) is (1S, 2S)-N-methyl-pseudoephedrine.

13. The method according to claim **1** wherein the alkaloid compound having chemical formula (I) is (1R, 2R)-ephedrine and the alkaloid compound having chemical formula (II) is (1R, 2R)-N-methyl-ephedrine.

14. The method according to claim **1** wherein the alkaloid compound having chemical formula (I) is (1R, 2S)-ephedrine and the alkaloid compound having chemical formula (II) is (1R, 2S)-N-methyl-ephedrine.

15. The method according to claim 1 wherein the alkaloid compound having chemical formula (I) is (1S, 2R)-pseudoephedrine and the alkaloid compound having chemical formula (II) is (1S, 2R)-N-methyl-pseudoephedrine.

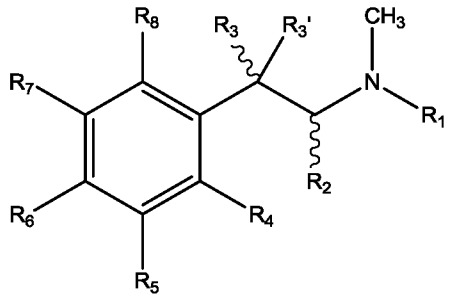
16. The method according to claim **1** wherein the N-methyl-transferase is obtainable from a plant species belonging to the genus *Catha* or from a plant belonging to the genus *Ephedra.*

17. The method according to claim **1 – 15** wherein the N-methyltransferase is selected from the group consisting of SEQ.ID NO: 3. SEQ. ID NO: 5, SEQ.ID NO: 7 and SEQ.ID NO: 9.

18. The method according to claim **1** wherein, the method is conducted *in vivo*.

19. The method according to claim **1** wherein the method is conducted *in vitro.*

20. A method for preparing an alkaloid compound having chemical formula (II)



(II)

the method comprising:

(a) providing a chimeric nucleic acid sequence comprising as operably linked components:

(i) a nucleic acid sequence encoding an N-methyltransferase polypeptide; and

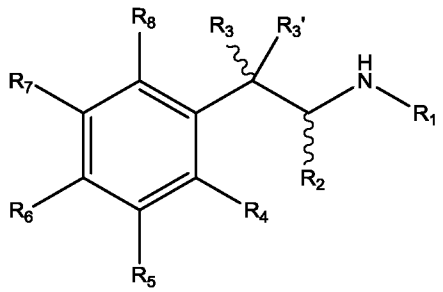
(ii) one or more nucleic acid sequences capable of controlling expression in a host cell;

(b) introducing the chimeric nucleic acid sequence into a host cell and growing the host cell to produce the N-methyltransferase and to produce the alkaloid compound having chemical formula (II); and

(c) recovering the alkaloid compound having chemical formula (II);

wherein R_1 is a hydrogen atom, a methyl group or a halogen; R_2 is a hydrogen atom, a methyl group or a methoxy group; R_3 is a hydroxyl group or a halogen, R_3' is a hydrogen atom, or taken together R_3 and $R_{3'}$ form a carbonyl group (C=O); and R_4 – R_8 are each independently or simultaneously a hydrogen atom, methyl group, hydroxyl group, halogen or methoxy group.

21. The method according to claim 20 wherein the cells are capable or producing compound (I)



(I);

and wherein in (I) R_1 is a hydrogen atom, a methyl group or a halogen; R_2 is a hydrogen atom, a methyl group or a methoxy group; R_3 is a hydroxyl group, or a halogen , R_3' is a hydrogen atom, or taken together R_3 and R_3' form a carbonyl group (C=O); R_4 – R_8 are each independently or simultaneously a hydrogen atom, methyl group, hydroxyl group, halogen or methoxy group; and each R_1 to R_8 and R_3' in the alkaloid compound having chemical formula (I) are identical to R_1 to R_8 and R_3' in the alkaloid compound having chemical formula (II).

22. The method according to claim **20** wherein the nucleic acid sequence encoding an N-methyltransferase polypeptide is selected from the group SEQ.ID NO:1, SEQ.ID NO: 2, SEQ.ID NO: 4, SEQ.ID NO: 6 and SEQ.ID NO: 8.

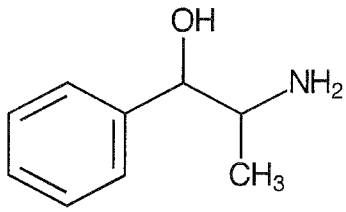


FIG. 1A

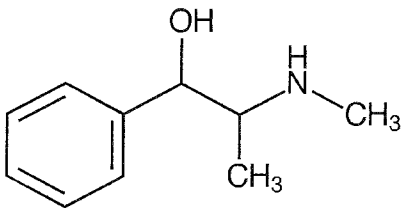


FIG. 1B

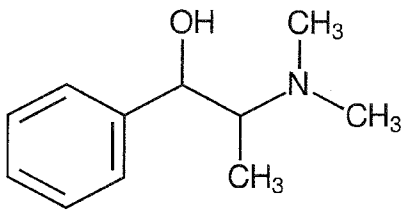
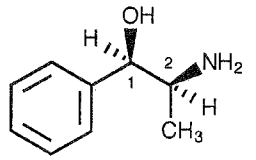


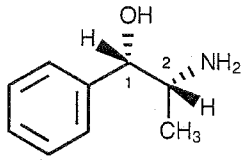
FIG. 1C

FIGURE 1



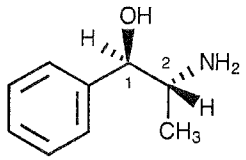
(1R, 2S)-norephedrine

FIG. 2A



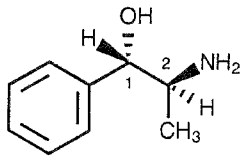
(1S, 2R)-pseudonorephedrine

FIG. 2B



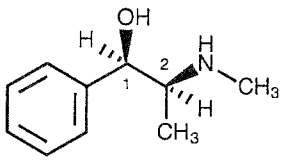
(1R, 2R)-norephedrine

FIG. 2C



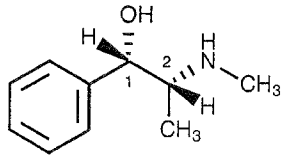
(1S, 2S)-pseudonorephedrine

FIG. 2D



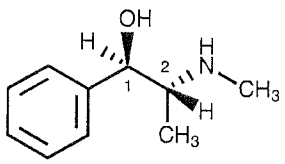
(1R, 2S)-ephedrine

FIG. 2E



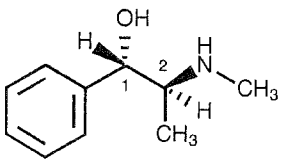
(1S, 2R)-pseudoephedrine

FIG. 2F



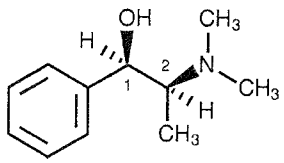
(1R, 2R)-ephedrine

FIG. 2G



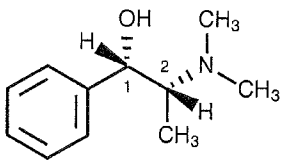
(1S, 2S)-pseudoephedrine

FIG. 2H



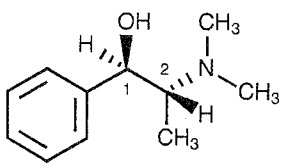
(1R, 2S)-N-methylephedrine

FIG. 2I



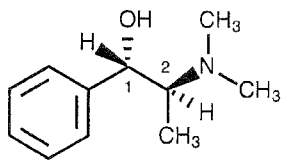
(1S, 2R)-N-methylpseudoephedrine

FIG. 2J



(1R, 2R)-N-methylephedrine

FIG. 2K



(1S, 2S)-N-methylpseudoephedrine

FIG. 2L

FIGURE 2

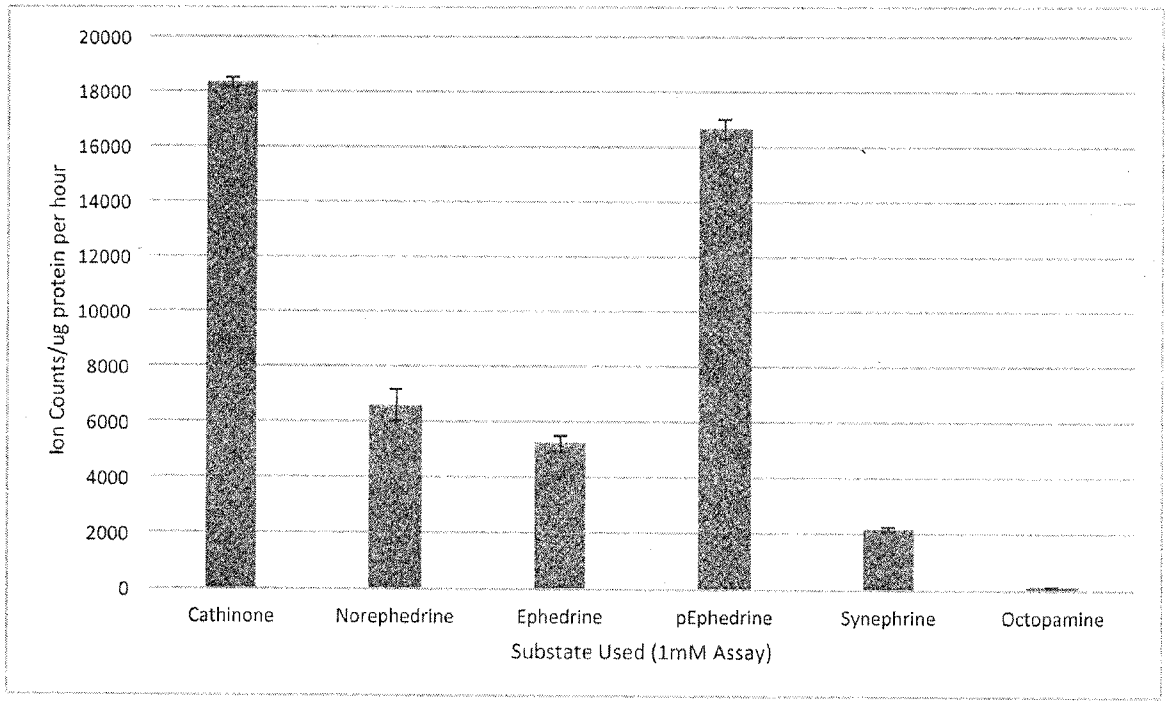

20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Ion Counts/ug protein per hour
Cathinone
Norephedrine
Ephedrine
pEphedrine
Synephrine
Octopamine
Substate Used (1mM Assay)

FIGURE 3

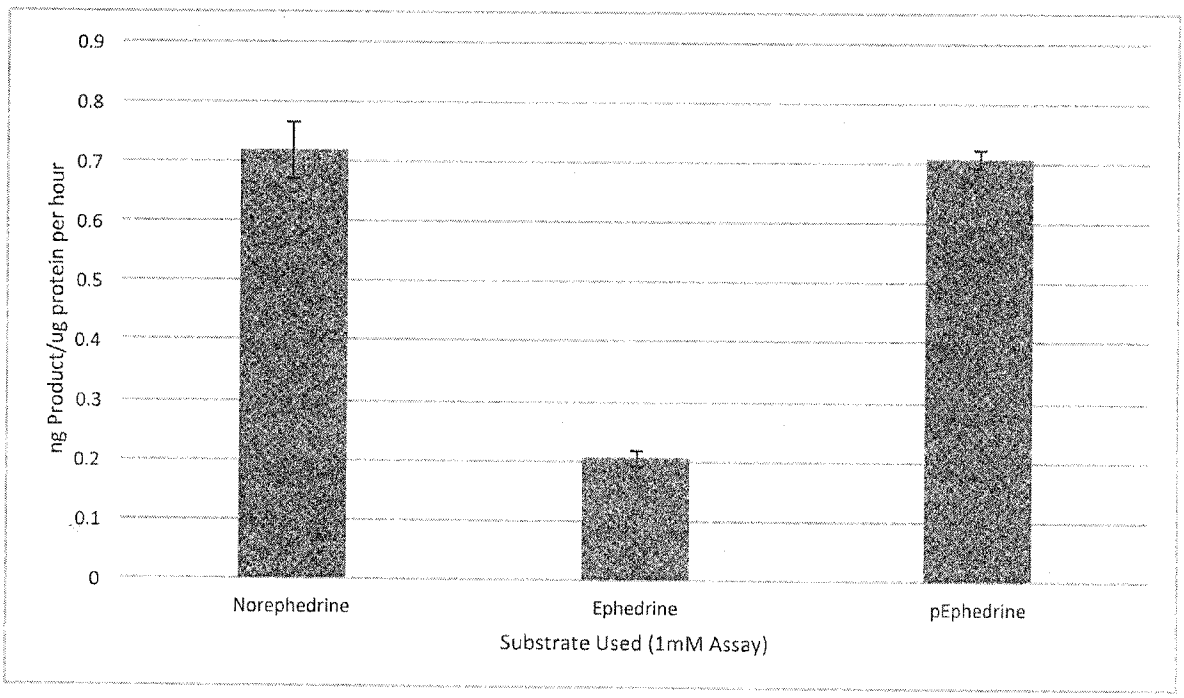

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ng Product/ug protein per hour
Norephedrine
Ephedrine
pEphedrine
Substrate Used (1mM Assay)

FIGURE 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2016/050730

A. CLASSIFICATION OF SUBJECT MATTER
IPC: ***C12P 13/00*** (2006.01), ***C07C 213/08*** (2006.01) , ***A61K 31/137*** (2006.01) , ***C12N 9/10*** (2006.01)

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)
C12P 13/00 (2006.01), ***C07C 213/08*** (2006.01) , ***A61K 31/137*** (2006.01) , ***C12N 9/10*** (2006.01)

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

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

Databases: Canadian Patent Database, Scopus, Google, Genbank, Questel Orbit; Keywords: N-methyltransferase, ephedrine, phenylethylamine, phenylethanolamine, alkaloid, methylation
SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	AXELROD, JULIUS :"Purification and Properties of Phenylethanolamine-N-methyl Transferase" THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 237, No. 5, Pages 1657-1660 (1962), ISSN 1096-0384. Methods and Materials, Table III	1-3, 5-7, 10, 19 4, 8, 9, 11-16, 18
X Y	KRIZEVSKI ET AL. :"Composition and Stereochemistry of Ephedrine Alkaloids Accumulation in *Ephedra sinica* Stapf" PHYTOCHEMISTRY, Vol. 71, Pages 895-903 (2010), ISSN 0031-9422. The whole document	1-3, 5-8, 10, 12, 14, 16, 19 4, 9, 11, 13, 15, 18
X	US 7 371 523 B1 (THOMAE ET AL.) 13 May 2008 (13-05-2008) Example 1	1, 19, 20, 21

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

*	Special categories of cited documents:	"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
"A"	document defining the general state of the art which is not considered to be of particular relevance	"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
"E"	earlier application or patent but published on or after the international filing date	"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
"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)	"&"	document member of the same patent family
"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		

Date of the actual completion of the international search 23 August 2016 (23-08-2016)	Date of mailing of the international search report 13 September 2016 (13-09-2016)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 819-953-2476	Authorized officer May Ling Nung (819) 639-9347

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GRUNEWALD, GARY L. ET AL.:"Stereochemical Aspects of Phenylethanolamine Analogues as Substrates of Phenylethanolamine N-Methyltransferase" JOURNAL OF MEDICINAL CHEMISTRY, Vol. 31, Pages 1984-1986 (1988), ISSN 0022-2623.	1, 19, 20, 21
Y	The whole document	1-15
A	GROVES, RYAN A. ET AL. :"Transcriptome Profiling of Khat (*Catha edulis*) and *Ephedra sinica* Reveals Gene Candidates Potentially Involved in Amphetamine-Type Alkaloid Biosynthesis" PLoS ONE, 25 March 2015, 10(3) Retrieved from the Internet: <https://eds.b.ebscohost.com/eds/pdfviewer/pdfviewer?sid=31797aea-71a8-4b90-9373-8bee8ffc1c6a%40sessionmgr105&vid=9&hid=127>	1-22
P,A	WO 2015/181823 (DANGOOR ET AL.) 03 December 2015 (03-12-2015) SEQ ID NOs. 6721, 6731, 1637, 1647	17, 22
A	HAGEL ET AL. "Biosynthesis of Amphetamine Analogs in Plants" TRENDS IN PLANT SCIENCE, Vol. 17, No. 7, Pages 404-412 (July 2012)	1-22

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
US7371523B1	13 May 2008 (13-05-2008)	US7371523B1	13 May 2008 (13-05-2008)
		US7482146B1	27 January 2009 (27-01-2009)
WO2015181823A1	03 December 2015 (03-12-2015)	None	