(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 3 653 617 A1

(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:
20.05.2020 Bulletin 2020/21

(51) Int Cl.:
C07D 401/04 *(2006.01)*

(21) Application number: **18206826.2**

(22) Date of filing: **16.11.2018**

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR
Designated Extension States:
BA ME
Designated Validation States:
KH MA MD TN

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(54) **PROCESS FOR THE PREPARATION OF (S)-NICOTIN FROM MYOSMINE**

(57) A process for synthetically producing (S)-nicotine ([(S)-3-(1-methylpyrrolidin-2-yl)pyridine]) is provided.

EP 3 653 617 A1

Description

Field of the invention

[0001] The present invention relates to a process for synthetically producing (S)-nicotine ([(*S*)-3-(1-methylpyrrolidin-2-yl)pyridine]).

Background of the invention

[0002] Nicotine (3-[1-methylpyrrolidin-2-yl]pyridine) is a natural product that may be obtained from the leaves of *Nicotiana,* i.e. the tobacco plant. There is considerable demand for nicotine products across the tobacco industry and also across the pharmaceutical field. For example, there remains a demand for traditional tobacco products e.g. traditional cigarettes, which is likely due to the addictive nature of nicotine. However, due to growing concern around the detrimental impact of traditional cigarette products on consumer health, there is an increasing demand for tobacco replacement products containing nicotine, such as electronic cigarette devices, patches, lozenges, nasal spray and chewing gum. Tobacco replacement products may be provided as a substitute for traditional tobacco products that would otherwise result in harmful carcinogenic effects; such as due to the presence of pyridine alkaloids, polycyclic aromatics, phenols and N-nitrosamines. Tobacco replacement products may be used specifically to treat nicotine dependence. Within the pharmaceutical field, there is also interest in the possible therapeutic applications of nicotine.

[0003] Challenges exist for obtaining nicotine with suitable levels of both enantiomeric purity and chemical purity. Nicotine is optically active, i.e. it may exist in one of two possible enantiomeric forms: (R)-nicotine or (S)-nicotine. Processes for obtaining racemic mixes of nicotine exist (e.g. WO2016065209). However, it is acknowledged that (S)-nicotine (i.e. [(S)-3-(1-methylpyrrolidin-2-yl)pyridine]) is significantly more active than (R)-nicotine. Therefore, the demand in the tobacco industry and in the pharmaceutical field is for nicotine with a high level of enantiomeric purity with respect to the (S) enantiomer. The pharmaceutical industry in particular imposes strict regulations on the required level of enantiomeric purity for new pharmaceutical products, and it is possible that the existing required level of enantiomeric purity for nicotine may increase. In addition to the demand for enantiomeric purity of nicotine, obtaining a high level of chemical purity is also of importance in both the pharmaceutical and tobacco industries - chemical purity referring to the amount of nicotine (i.e. both (R) and (S) enantiomeric forms) in comparison to non-nicotine impurities. The pharmaceutical industry already imposes very strict regulations on the required level of chemical purity of nicotine in comparison to non-nicotine impurities. In fact, the current U.S. Pharmacopeia reference standard for the chemical purity of nicotine is at least 99% with not more than 0.5% of any single impurity. A high chemical purity is also of significant importance to the tobacco industry, as the harmful carcinogenic effects mentioned above can be caused by impurities that are capable of exerting a carcinogenic effect.

[0004] (S)-Nicotine may be obtained by extraction from leaves of the tobacco plant. However, when nicotine is obtained this way, it typically has a chemical purity of less than 95% due of the presence of related alkaloid impurities. A typical composition of a nicotine sample obtained by extraction from tobacco leaves comprises 93% (S)-nicotine, 2.4% (S)-nornicotine, 3.9% (S)-anatabine and 0.5% (S)-anabasine (E. Leete and M. Mueller, J. Am. Chem., Soc., 1982, 104, 6440-44). The alkaloid impurities are of a similar chemical structure to nicotine and consequently are difficult to remove. The actual composition of nicotine is also dependent on such factors as the geographic source and the season of harvest.

[0005] (S)-Nicotine may also be obtained by a synthetic process. There are various examples in the prior art for synthetically producing (S)-nicotine. For example, in the prior art are processes where a racemic (i.e. equal) mix of (R)-nicotine and (S)-nicotine is made, where this racemic mix is subsequently resolved to obtain the (S) enantiomer (US 8,389,733, US 2014/0031554, and US 8,378,111). There is also an example in the prior art of a synthetic process for producing (S)-nicotine using an enzyme as a biocatalyst (WO 2014/174505); the use of biocatalysts in enantiomerically selective processes in general are known outside of the nicotine field (L.S. Bleicher et al, J. Org. Chem., 1998, 63, 1109-18, WO 2013/170050, WO2015/073555, P.N Scheller et al, Chembiochem, 2014, 15, 2201-4, Gand et al, J Mol. Cat. B, Enzymatic, 2014, 110, 126-32). Nevertheless, selectively synthesising (S)-nicotine in preference to the (R) enantiomer with high enantiomeric selectivity whilst also achieving high chemical purity remains a challenge.

Summary of the invention

[0006] In a first aspect, there is a process of making (S)-nicotine comprising the steps of:

(i) reducing myosmine with an enzyme with imine reductase activity to form (S)-nornicotine; and
(ii) methylating the (S)-nornicotine formed from step (i) to form (S)-nicotine.

[0007] It was surprisingly found that by way of steps (i) and (ii) of this process, where myosmine is used as the starting

material, a very high enantiomeric and chemical purity was achieved for (S)-nicotine. This indicates that step (i) is a highly enantiomeric selective synthetic step with preference for the (S) isomer, and that step (ii) is such that this preference is retained in the final nicotine product, whilst also maintaining high chemical purity. This allows the production of (S)-nicotine without having to resort to resolution of a racemic mix. The high chemical purity is particularly advantageous; a reduced level of the undesirable impurities typically associated with nicotine results in a reduced risk of potential impurity-related negative effects. Furthermore, steps (i) and (ii) offer a convenient manufacturing process for making (S)-nicotine.

[0008] In a second aspect there is a process for producing a pharmaceutical composition, comprising forming (S)-nicotine using the process of the first aspect, and including the (S)-nicotine in the pharmaceutical composition together with one or more pharmaceutical excipients.

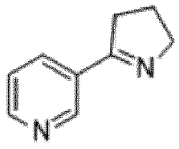
[0009] In a third aspect there is a process for producing a formulation for an electronic cigarette device, comprising forming (S)-nicotine using the process of the first aspect, and including the (S)-nicotine in a solvent with one or more additives.

[0010] In a fourth aspect there is the use of myosmine and an enzyme with imine reductase activity in a process of forming (S)-nicotine.

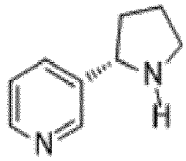
[0011] In a fifth aspect there is a kit comprising myosmine and an enzyme with imine reductase activity, for use in the above process of forming (S)-nicotine.

Description of the preferred embodiments

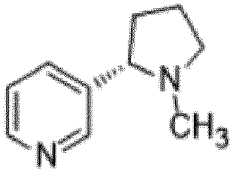
[0012] As the skilled person will appreciate, myosmine, (S)-nornicotine and (S)-nicotine, have the following structures:



myosmine



(S)-nornicotine



(S)-nicotine

[0013] The skilled person will be familiar with appropriate reaction schemes to make myosmine.

[0014] As used herein, an "enzyme with imine reductase activity" refers to an enzyme capable of asymmetrically reducing an imine group, in particular a secondary imine group, to the corresponding amine group, in particular a secondary amine group. In particular, the enzyme with imine reductase activity used in the process disclosed herein is an enzyme capable of catalysing the conversion of myosmine to (S)-nornicotine. The skilled person is familiar with such enzymes. The enzyme may be added to the reaction mixture in a variety of forms, such as in the form of spray dried cells.

[0015] Preferably, the process uses an enzyme capable of converting myosmine to (S)-nornicotine such that the

(S)-nornicotine is obtained with an enantiomeric excess of at least 90%, preferably at least 95%, more preferably at least 98%, most preferably at least 99%. Enantiomeric excess is measured in the manner given in the Examples. In the processes disclosed herein, this high enantiomeric excess is also achieved for the (S)-nicotine that is eventually achieved as the final product.

[0016] As the skilled person will appreciate, enzymes with imine reductase activity typically include NADH/NADPH dependent oxidoreductases, such as NADH/NADPH dependent dehydrogenases, and NADH/NADPH dependent imine reductases. NADH/NADPH dependent dehydrogenases include those referred to by enzyme classification number E.C.1.1.1, and include in particular 6-phosphogluconate dehydrogenases, referred to by enzyme classification number E.C.1.1.1.44. Imine reductases include those referred to with enzyme classification number E.C.1.5.1, in particular those referred to with enzyme classification number E.C 1.5.1.48.

[0017] Examples of different species of imine reductases include thiazolinyl imine reductase, dihydrofolate reductase, Δ^1 - pyrroline-2-carboxylate reductase, Δ^1 - piperideine-2-carboxylate reductase, sanguinarine reductase, and 1,2-dihydro reticuline reductase. Such enzymes can be isolated or derived from sources such as Streptomyces, Verrucosispora, Mesorhizobium, Yersinia, Pseudomonas, candida albicans, Eschscholzia, and Papaver.

[0018] Examples of possible enzymes also include those disclosed in WO2013170050 (the contents of which are incorporated by reference).

[0019] The enzyme may be IRED_A, IRED_B, IRED_C, IRED_D, IRED_E, IRED_F, IRED_P, IRED_X, IRED_AB, IRED-20, or a homologue thereof. IRED_A, IRED_B, IRED_C, IRED_D, IRED_E, IRED_F, IRED_P, IRED_X, and IRED_AB are available from Enzymicals; IRED-20 is available from Almac Group. For example, in one embodiment, the enzyme is IRED_A, IRED_B, IRED_C, IRED_D, IRED_E, IRED-20, or a homologue thereof.

[0020] Disclosed herein, the enzyme may comprise an amino acid sequence according to any one of SEQ I.D. NO: 1, SEQ I.D. NO: 2, SEQ I.D. NO: 3, SEQ I.D. NO: 4, or a homologue thereof. In another embodiment, the enzyme comprises an amino acid sequence according to any one of SEQ I.D. NO: 1, SEQ I.D. NO: 2, SEQ I.D. NO: 3, or SEQ I.D. NO: 4.

[0021] As used herein, "a homologue thereof" means an enzyme comprising an amino acid sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to any one of the enzymes disclosed herein. For example, "a homologue thereof" can mean an enzyme comprising an amino acid sequence having at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% sequence identity to the amino acid sequence according to any one of SEQ I.D. NO: 1, SEQ I.D. NO: 2, SEQ I.D. NO: 3, or SEQ I.D. NO: 4..

[0022] As used herein, the term "sequence identity" refers to a relationship between two or more amino acid sequences. When a position in one sequence is occupied by the same amino acid residue in the corresponding position of the comparator sequence, the sequences are said to be "identical" at that position. The percentage "sequence identity" is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of "identical" positions. The number of "identical" positions is then divided by the total number of positions in the comparison window and multiplied by 100 to yield the percentage of "sequence identity." Percentage of "sequence identity" is determined by comparing two optimally aligned sequences over a comparison window. In order to optimally align sequences for comparison, the portion of a polypeptide sequence in the comparison window may comprise additions or deletions termed gaps while the reference sequence is kept constant. An optimal alignment is that alignment which, even with gaps, produces the greatest possible number of "identical" positions between the reference and comparator sequences. Levels of sequence identity between coding sequences may be calculated using known methods.

[0023] The sequence identity can be calculated using publicly available computer-based methods for determining sequence identity including the BLASTP, BLASTN and FASTA (Atschul et al., J. Molec. Biol., 215: 403-410, (1990)), the BLASTX programme available from NCBI, and the Gap programme from Genetics Computer Group (Madison WI). Levels of sequence identity are obtained using the Gap programme, with a Gap penalty of 50 and a Gap length penalty of 3 for the amino acid sequence comparisons.

[0024] Generally, step (i) comprises reducing myosmine with the enzyme in the presence of a suitable cofactor, in particular NADH or NADPH. As the skilled person will appreciate, the enzyme and the cofactor may be introduced to the reaction mixture as separate components, or they may be introduced to the reaction mixture as part of the same component for example in the form of whole microbial cells which contain both the enzyme and the appropriate cofactor. A suitable cofactor recycling system may be present to convert the cofactor from its oxidised form (NAD+ or NADP+) to its reduced form (NADH or NADPH). The skilled person will be familiar with appropriate cofactor recycling systems, such cofactor recycling systems including glucose(monohydrate) /glucose dehydrogenase, formate/formate dehydrogenase and isopropanol/ alcohol dehydrogenase. When a cofactor recycling system is present, the cofactor may be added to the reaction mixture in its oxidised form i.e. as NAD+ or NADP+.

[0025] The cofactor itself may be present in the range of 0.02 parts to 10 parts by weight per 100 parts of myosmine. Preferably, the cofactor may be present in the range of 0.05 part to 5 parts by weight per 100 parts of myosmine. More preferably, the cofactor may be present in the range of 0.5 part to 2 parts by weight per 100 parts of myosmine..

[0026] The amount of enzyme present in step (i) can be present in an amount of 0.1 parts to 30 parts by weight per 100 parts of myosmine. Preferably, the amount of enzyme present in step (i) can be present in an amount of 0.5 parts to 10 parts by weight of myosmine. The skilled person will appreciate that the amount of enzyme present in step (i) can be tailored depending on the desired time period for the reaction of step (i), where more enzyme can be used for a shorter reaction time, and vice versa.

[0027] Step (i) may be carried out in the presence of an ion exchange resin, however preferably step (i) is carried out in absence of an ion exchange resin. The ion exchange resin, when present, is an Amberlite resin, an Amberlyst resin, an Amberjet resin, such as Amberlite IR-120, or a Dowex resin, where each of these ion exchange resins is available from Aldrich.

[0028] The possible pH for step (i) can be in the range of pH 5-9.

[0029] The (S)-nornicotine is converted to (S)-nicotine by a further step of:

(ii) methylating the (S)-nornicotine formed from step (i) to form (S)-nicotine.

[0030] It was surprisingly found that following step (ii) the (S)-nicotine was achieved with particularly high chemical purity and particularly high enantiomeric excess.

[0031] The methylation step, i.e., step (ii), may be carried out by way of a muti-step process. For example, step (ii) may comprise forming a compound (e.g. N-formyl-(S)-nornicotine), and then subsequently reducing this compound to arrive at the methylated product i.e. (S)-nicotine. Preferably however, step (ii) is carried out by way of a single step process such as reductive methylation. As the skilled person will appreciate, the term "reductive methylation" refers to a process whereby a species is formed and reduced to arrive at the methylated product (i.e. (S)-nicotine) by way of a single step.

[0032] Preferably, the (S)-nornicotine is reductively methylated using formaldehyde or a formaldehyde-based compound. Step (ii) is particularly effective when using such reagents.

[0033] As used herein, a formaldehyde-based compound is used to refer to a compound that is capable of generating formaldehyde in-situ during a chemical reaction. The skilled person will appreciate that this means the formaldehyde-based compound is added to the reaction mixture, and then subsequently breaks down to release formaldehyde (and other related compounds) which may then react with the (S)-nornicotine to form (S)-nicotine. In the case of the addition of a formaldehyde-based compound, the skilled person will be familiar with how to tailor the appropriate amount of the formaldehyde-based compound added in order to achieve the release of a particular amount of formaldehyde in situ.

[0034] Formaldehyde itself has the formula HC(O)H and is generally introduced as a liquid or a gas. The formaldehyde may be introduced to the reaction mixture as part of an aqueous solution of formaldehyde (such aqueous solutions may be referred to as formalin).

[0035] The formaldehyde-based compound is generally introduced as a solid or a liquid. The formaldehyde-based compound may be a dimer of formaldehyde, a polymer of formaldehyde, or an acetal of formaldehyde. Preferably, the formaldehyde-based compound is a polymer of formaldehyde.

[0036] As the skilled person will appreciate, the term "polymer of formaldehyde" refers to a compound with three or more polymerised formaldehyde repeat units. Preferably, the polymer of formaldehyde is paraformaldehyde. As used herein, the term "paraformaldehyde" refers to polymer of formaldehyde with a degree of polymerization of 8-100 units.

[0037] When the (S)-nornicotine is reductively methylated using formaldehyde or a formaldehyde-based compound, the formaldehyde or formaldehyde-based compound may be added in an amount of 50 parts to 110 parts by weight, preferably 60 parts to 90 parts by weight, per 100 parts of (S)-nornicotine. Such amounts refer to the actual amounts of formaldehyde, formaldehyde-based compound and (S)-nornicotine present. Therefore, where for example the (S)-nornicotine is formed as part of a solution (e.g. an aqueous solution) and/or when the formaldehyde or formaldehyde-based compound is introduced to the reaction mixture as part of a solution (e.g. an aqueous solution) the parts by weight disclosed herein refer to the actual amounts of the formaldehyde, formaldehyde-based compound and (S)-nornicotine contained in the respective solutions.

[0038] Where the methylation step is a reductive methylation step, the reductant may be formic acid, sodium cyanoborohydride, or palladium/hydrogen, preferably formic acid. As the skilled person will appreciate, the appropriate amount of reductant will depend on the specific reductant used. For example, when the reductant is formic acid, the reductant may be present in an amount of 40-110 parts, preferably 40-100 parts, more preferably 50 parts to 70 parts by weight per 100 parts of (S)-nornicotine. Such amounts refer to the actual amounts of reductant and (S)-nornicotine present.

[0039] Preferably, steps (i) and (ii) may be carried out without isolating the (S)-nornicotine formed from step (i). This allows the formation of (S)-nicotine with both a high enantiomeric excess and a high chemical purity whilst using a particularly convenient synthetic route. Avoiding the need for isolation of the (S)-nornicotine from the reaction mixture formed from step (i) before converting this to (S)-nicotine has the benefit of offering a particularly convenient synthetic route, as isolation of the (S)-nornicotine can be process intensive as a result of costly plant time and energy (for example

due to the need for large quantities of solvent for extraction and/or the boiling down of the solution). For example, in step (i) the (S)-nornicotine may be formed as part of an aqueous solution, where the aqueous solution containing the (S)-nornicotine is then carried through for direct use in step (ii). Consequently, the methylation step (step (ii)) is performed on the aqueous solution of (S)-nornicotine formed from step (i). When the process is carried out in this manner, it is preferable for the (S)-nornicotine to be reductively methylated either by using paraformaldehyde, or, by using formaldehyde that is introduced to the reaction mixture as part of an aqueous solution. When the process is carried out in this manner, it is more preferable for the (S)-nornicotine to be reductively methylated by using formaldehyde that is introduced to the reaction mixture as part of an aqueous solution, as it has been found that this reduces undesirable frothing of the reaction mixture as the process proceeds.

[0040] The (S)-nicotine produced using the processes disclosed herein has an enantiomeric excess of at least 90%, preferably of at least 95%, more preferably of at least 98%, most preferably of at least 99%. The skilled person will be familiar with how to measure the enantiomeric excess. Enantiomeric excess may for instance be measured in the manner given in the Examples.

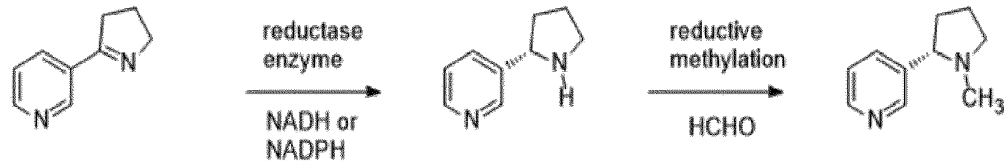
[0041] The (S)-nicotine produced using the processes disclosed herein has a chemical purity of at least 98%, preferably of at least 99%. The skilled person will be familiar with how to measure the chemical purity. Chemical purity may for instance be measured in the manner given in the Examples. The level of chemical purity achieved by the examples is particularly high.

[0042] The (S)-nicotine produced using the method steps above may be included in a pharmaceutical composition together with one or more pharmaceutical excipients. Preferably, the pharmaceutical composition is a transdermal patch, a lozenge, or an inhalation formulation.

[0043] The (S)-nicotine produced using the method steps above may also be included in a formulation for inclusion in an electronic cigarette device. The formulation includes (S)-nicotine in a solvent with one or more additives. The solvent may comprise glycerol, propylene glycol, water, or mixtures thereof. Preferably, the solvent comprises glycerol and propylene glycol, wherein the proportion of glycerol to propylene glycol is in the range of 80:20 to 20:80 by volume. The one or more additives may include one or more flavouring agents.

[0044] Also provided herein is a kit comprising myosmine and an enzyme with imine reductase activity for use a process of forming (S)-nicotine.

[0045] A particularly preferred reaction scheme is displayed below as scheme 1:



myosmine (S)-nornicotine (S)-nicotine

[0046] The invention will be demonstrated with the following non-limiting examples.

EXAMPLES

[0047] The following examples demonstrate results associated with the process disclosed herein. Various reagents have been used to exemplify the process.

[0048] The enzymes used include the following:

IRED_A from Verrucosispora maris (strain AB-18-032, Uniprot: F4F8G5_VERMA) with the amino acid sequence (a) or (b) given below - sequence (a) corresponds with SEQ I.D. NO: 1, and sequence (b) corresponds with SEQ I.D. NO: 2.

(a) As used with hexahistidine tag, total 302 amino acid residues:

MHHHHHHAADSRAPVTVIGLGAMGSALARAFLAAGHPTTVWNRSPDKADDLV
GQGAVRAATVADAMSAGNLIVICVLDYRAMREIIDSTGHSPADRVIVNLTSGTP
GDARATAAWAQEQGMEYIDGAIMATPSMIGSEETLIFYGGPQEVYDAHADTLR
SIAGAGTYLGEEPGLPSLYDVALLGLMWTTWAGFMHSAALLASEKVPAAAFLP
YAQAWFEYVISPEVPNLATQVDTGAYPDNDSTLGMQTVAIEHLVEASRTQGVD
PTLPEFLHARAEQAIRRGHAGDGFGAVFEVLRAPAAQ

(b) Original enzyme, total of 296 amino acid residues:

MAADSRAPVTVIGLGAMGSALARAFLAAGHPTTVWNRSPDKADDLVGQGAVR
AATVADAMSAGNLIVICVLDYRAMREIIDSTGHSPADRVIVNLTSGTPGDARATA
AWAQEQGMEYIDGAIMATPSMIGSEETLIFYGGPQEVYDAHADTLRSIAGAGTY
LGEEPGLPSLYDVALLGLMWTTWAGFMHSAALLASEKVPAAAFLPYAQAWFE
YVISPEVPNLATQVDTGAYPDNDSTLGMQTVAIEHLVEASRTQGVDPTLPEFLH
ARAEQAIRRGHAGDGFGAVFEVLRAPAAQ

[0049] IRED_B from Mesorhizobium sp. L48C026A00 aka a 6-phosphogluconate dehydrogenase, with the amino acid sequence (a) or (b) given below - sequence (a) corresponds with SEQ I.D. NO: 3, and sequence (b) corresponds with SEQ I.D. NO: 4.

(a) As used with hexahistidine tag, total 310 amino acid residues:

MHHHHHHASNVCVLGAGRMGSSIARTLLDRGYPTWVWNRTAAKCEPLAALG
AKVASSVQEGIQAAEVVIINVLDYAASDALLKRDGIASALAGKAVVQLTSGSPRL
AREEARWVEAHGAGYLDGAIMATPDFIGKPETAMLYSGSRDVYEKHKPLLFAL
GGGTNYVGELPGQASALDTALLTQMWGGLFGALQGMAVAEAEGLDLETFRN
HLSAFKPVVDASLFDLVDRTNARRFAGDDATLASLGAHYSAFQHLLEACEERG
LDAAMPRAMDMIFRQALSLGSMEDDLASLALLFRNGSPRQSREPANA

(b) Original enzyme, total of 304 amino acid residues

MASNVCVLGAGRMGSSIARTLLDRGYPTWVWNRTAAKCEPLAALGAKVASSV
QEGIQAAEVVIINVLDYAASDALLKRDGIASALAGKAVVQLTSGSPRLAREEAR
WVEAHGAGYLDGAIMATPDFIGKPETAMLYSGSRDVYEKHKPLLFALGGGTNY
VGELPGQASALDTALLTQMWGGLFGALQGMAVAEAEGLDLETFRNHLSAFKP
VVDASLFDLVDRTNARRFAGDDATLASLGAHYSAFQHLLEACEERGLDAAMPR
AMDMIFRQALSLGSMEDDLASLALLFRNGSPRQSREPANA

Example 1

[0050] Biotransformations were undertaken at 0.5 mL scale with a solution of 10mM myosmine and NADP+ (0.5mM), glucose (25 mM), glucose dehydrogenase (10U/ml), and the enzyme with imine reductase activity. The enzymes used

are detailed in table 1, available from Enzymicals. For each enzyme, the amount of enzyme was 9mg/ml of cell free extract (estimated approx. 0.9mg/ml contained enzyme). For IRED_B and IRED_C specifically, additional tests were run which used 0.9 mg/ml cell free extract.

[0051] The enantiomeric excess of the (S) nornicotine obtained from the biotransformation was determined using a Chiralpak AD-H column (250 x 4.6mm id) eluting with a mixture of hexane:ethanol:diethylamine 74.9 : 25.0: 0.1 (v/v/v) at 1 ml/min over 18 min at 30 °C. This method was also used to measure the conversion of myosmine into nornicotine, a relative response factor of 2.18 : 1 having been determined for uv absorption detection at 254 nm.

[0052] The results are displayed in table 1 below.

Table 1

	Enzyme	Amount	Conversion [%]	Enantiomeric Excess [%S]
i	IRED_A	9 mg/ml	99.3	99.8
ii	IRED_B	9 mg/ml	99.9	98.4
iii	IRED_B	0.9 mg/ml	99.3	98.4
iv	IRED_C	9 mg/ml	99.3	92.9
v	IRED C	0.9 mg/ml	100.0	99.1
vi	IRED_D	9 mg/ml	99.6	99.8
vii	IRED_E	9 mg/ml	99.4	99.8
viii	IRED_F	9 mg/ml	99.1	86.5
ix	IRED_P	9 mg/ml	97.7	86.6
x	IRED_X	9 mg/ml	99.8	95.7
xi	IRED_AB	9 mg/ml	99.6	96.8

[0053] The % enantiomeric excess for (S)-nornicotine was identified according to the equation [(S)-(R)]/((S)+(R)] × 100 where (S) and (R) are the amounts of (S) and (R) enantiomers present respectively. The % conversion was identified according to the amount of myosmine consumed i.e. according to the equation 100-(final amount of myosmine)/(starting amount of myosmine) × 100.

Example 2

[0054] Reactions were carried out in a similar manner to that of example 1, except that 1.5 equivs glucose and 1 mol% NADP+ were used relative to the myosmine substrate, and a 24hr reaction time was employed. The enzymes used are detailed in each of tables 2, 3 and 4 (available from Enzymicals).

[0055] At 100mM myosmine concentration, using 0.9mg/mL enzyme cell free extract, the results were as displayed in the table below:

Table 2

	Enzyme	Conversion [%]	Enantiomeric Excess [%S]
i	IRED_A	63.6	99.8
ii	IRED_B	99.9	98.7
iii	IRED_C	99.9	99.8
iv	IRED_D	99.0	99.9
v	IRED_E	99.9	99.9

[0056] At 100mM myosmine concentration, using 9mg/mL enzyme cell free extract, the results were as displayed in the table below:

Table 3

	Enzyme	Conversion [%]	Enantiomeric Excess [%S]
i	IRED_A	99.9	99.8
ii	IRED_B	99.8	98.8
iii	IRED_C	99.8	99.9
iv	IRED_D	99.9	100.0
v	IRED_E	99.9	99.9

[0057] At 250mM myosmine concentration, using 9mg/mL enzyme cell free extract, the results were as displayed in the table below:

Table 4

	Enzyme	Conversion [%]	Enantiomeric Excess [%S]
i	IRED_A	100.0	99.7
ii	IRED_B	99.9	98.6
iii	IRED_C	100.0	99.9
iv	IRED_D	100.0	99.9
v	IRED_E	99.9	99.9

Example 3

[0058] A solution of myosmine (20 mmol, 2.924 g), D-Glucose (30 mmol, 5.405 g) nicotinamide adenine dinucleotide phosphate sodium salt (0.2 mmol, 157 mg), enzyme IRED_A (available from Enzymicals) cell free extract lyophilizate (1.0 g), glucose dehydrogenase (2000 U, 40 mg) in pH7.5 100 mM sodium phosphate buffer (200 mL) was mixed by an overhead stirrer at 200 rpm at 30°C for 24 hours. The solution was analysed for nornicotine during the course of the reaction with HPLC showing 77% conversion after 8 hours, and over 99% conversion after 24 h with 98.7% e.e. (S)-Nornicotine. This solution was then treated with 37% formaldehyde solution (8.1g) and formic acid (2.8g) at 80 °C for 4h, with the reaction being complete after 2h. After cooling, 6g solid sodium hydroxide was added (pH 12.7) and the mixture extracted with 2 x 75ml MTBE. After drying over sodium sulphate, the solvent was removed to afford 2.25 g crude (S)-nicotine which was >99% pure by HPLC (area% at 260nm) and had 98.7% enantiomeric excess.

Example 4

[0059] A solution of myosmine (20 mmol, 2.924 g), D-Glucose (30 mmol, 5.405 g) nicotinamide adenine dinucleotide phosphate sodium salt (0.2 mmol, 157 mg), enzyme IRED_B (available from Enzymicals) cell free extract lyophilizate (0.5 g), glucose dehydrogenase (2000 U, 40 mg) in pH7.5 100 mM sodium phosphate buffer (200 mL) was mixed by overhead stirrer at 200 rpm at 30°C for 24 hours. The solution was analysed for nornicotine during the course of the reaction with HPLC showing 91% conversion after 4 hours, and over 99% conversion after 6 hours. After 24 hours the (S)-Nornicotine was 98.2% e.e. This solution was then treated with paraformaldehyde (3g) and formic acid (2.8g) at 80 °C for 6h, with the reaction being complete after 4h. After cooling, 6g solid sodium hydroxide was added (pH 12.7) and the mixture extracted with 2 x 75ml MTBE. After drying over sodium sulphate, the solvent was removed to afford 2.31g crude (S)-nicotine which was >99% pure by HPLC (area% at 260nm) and had 98.3% enantiomeric excess.

Example 5

[0060] This example demonstrates the enantioselectivity and conversion rate at high substrate concentrations. This example was carried out in a similar manner to example 1 except that all reactions used glucose 1.5 equivs, NADP (1% relative to the myosmine), imine reductase, specifically IRED_C available from Enzymicals (4.5 mg/ml cell free extract, GDH (10U/ml per 250mM of myosmine concentration), sodium phosphate buffer pH7.5 100mM over a 24 hour time period. The results are shown below.

Table 5

	Concentration of myosmine starting material	Conversion [%]	Enantiomeric Excess [%S]
i	250mM	99.9	99.7
ii	400mM	99.6	99.8
iii	600mM	68.8	99.8
iv	800mM	56.5	99.7
v	1000mM	52.4	99.6

Example 6

[0061] This example demonstrates the enantioselectivity and conversion rate on a larger scale.

[0062] A solution of myosmine (400 mmol, 58.5 g), D-Glucose (600 mmol, 118.9 g) nicotinamide adenine dinucleotide phosphate sodium salt (4 mmol, 3.15 g), enzyme IRED_C (available from enzymicals) cell free extract lyophilisate (10.0 g), glucose dehydrogenase CFE (0.32 g) in pH7.5 100 mM sodium phosphate buffer (1000 mL) was mixed with an overhead stirrer at 200 rpm at 30°C for 24 hours. The solution was analysed for nornicotine after 24 hours with HPLC and showed over 98% conversion.

[0063] Details of the workup are as follows: the biocatalytic reaction mixture was acidified with concentrated sulphuric acid to pH 1-2, then heated to 90°C for 20 minutes to precipitate all the proteins. Proteins were filtered out of the mixture over Celite. The resulting clear solution was basified with 40% NaOH solution to pH > 11 and extracted four times with 500 mL methyl tert-butyl ether (MTBE). The combined MTBE phases were dried over anhydrous magnesium sulfate and the solvent evaporated. The isolated yield of nornicotine was 41.1 g (70 %) as a brown-yellow liquid.

[0064] A separate sample of the nornicotine reaction mixture prior to work up and isolation was taken through to the methylation step. Specifically, without isolation of the nornicotine, to the biocatalytic reaction mixture, paraformaldehyde (60 g) and formic acid (49.2 g) were added. The reaction was heated to 85°C and stirred vigorously to form (S)-nicotine.

Example 7

[0065] The general experimental method to form (S)-nicotine was as follows. Biocatalysis of myosmine into (S)-nor-nicotine using IRED_C (available from Enzymicals) was conducted at a concentration of 400mM myosmine. Either the (S)-nornicotine was isolated by way of extraction with methyl-tert butyl ether and removal of the solvent, or the aqueous solution from the biocatalysis was heated at 90 °C for 15 min to precipitate proteins, then after cooling the mixture was acidified to pH 1-2 with sulfuric acid, the precipitated protein removed by filtration through Celite, and the solution then neutralised with aqueous sodium hydroxide to about pH7.

Example 7a

[0066] Crude isolated nornicotine from the enzyme reduction of myosmine (92g) was added to 800ml water. Paraformaldehyde (74g, 4eq) and formic acid (58g, 2 eq) were added. The mixture was gradually warmed to 80-85 degrees C. HPLC analysis after 2h indicated completion of the reaction. The mixture was kept at the same temperature for a further 2h and then cooled to room temperature. 50% Sodium hydroxide solution was added to obtain a pH of approximately 13. The mixture was extracted with 2 x 500ml MTBE and dried over sodium sulphate. The solvent was removed and the crude (S)-nicotine distilled under vacuum. After a forerun of about 4g, 87g of purified nicotine was obtained (>99% by HPLC and 99.6%ee by chiral HPLC).

Example 7b

[0067] To 2.5 litres of aqueous nor-nicotine solution from the same biocatalysis as used in Example 1 (5.63g/100ml) was added paraformaldehyde (112.5g, 4eq) and formic acid (88g, 2eq). The mixture was gradually heated to 80-85 degrees C, with reaction beginning at about 70 degrees C with some foaming due to gas evolution. After 1h at 80-85 degrees C, HPLC indicated the reaction to be complete. The reaction was heated for 4h in total and then cooled. The mixture was basified with 50% sodium hydroxide solution and extracted with MTBE (800ml then 500ml). After drying, the crude mixture was distilled to give 118.7g (S)-nicotine (>99% by HPLC and 99.5%ee by HPLC).

Example 7c

[0068] To 2.5 litres of aqueous nor-nicotine solution from the same biocatalysis as used in Example 1 (5.63g/100ml) was added 37% formaldehyde solution (290ml, ~4eq) and formic acid (88g, 2eq). The mixture was gradually heated to 80-85 degrees C, with reaction beginning at about 60 degrees C with some foaming due to gas evolution. After 1h at 80-85 degrees C, HPLC indicated the reaction to be complete. The reaction was heated for 4h in total and then cooled. The mixture was basified with 50% sodium hydroxide solution and extracted with MTBE (800ml then 500ml). After drying, the crude mixture was distilled to give 119.1g (S)-nicotine (>99% by HPLC and 99.5%ee by HPLC).

Example 8

[0069] A solution of myosmine (298 g) and glucose monohydrate (505 g) was made in 0.1M dipotassium hydrogen phosphate buffer (6L). Amberlite IR-120 resin (2kg, wet) was added as the ion exchange resin and the solution adjusted to pH7 with 12M sodium hydroxide (about 0.3L), then stirred overnight at 25°C to ensure a stable pH. Glucose dehydrogenase GDH-102 (6 g), beta-NADP+ (6 g), and enzyme IRED-20 available from the Almac Group (30 g) were added, then the mixture stirred at 150 rpm while held at 25°C with the pH maintained in the range 6.8-7.0 through additions of 4M postassium hydroxide. After 72 h the solution was decanted and the Amberlite resin was washed with deionized water (3 x 3 L). Then Amberlite resin was transferred to a column and further washed with deionized water (4 L), then shaken for 3 hours with 2M ammonia solution (4 L) and further washed with 2M ammonia (10 L). The combined solutions were concentrated under reduced pressure to dryness to give (S)-Nornicotine (131.2 g) as a yellow liquid. In order to recover further nornicotine out of the reaction mixture, reactivated Amberlite resin (2 kg) was added to it and the mixture stirred overnight at room temperature. The same treatment was repeated as above to recover further (S)-Nornicotine (59.8 g) bringing the total yield to 191.0g. The above two batches were converted into (S)-nicotine separately. For the larger batch the (S)-nornicotine (126.2 g of) was combined with paraformaldehyde (154.5 g) and formic acid (118 g) in water (1 L) and the resulting stirred mixture heated to 85°C overnight. The mixture was then cooled to 0°C, and adjusted to pH 14 with 12M sodium hydroxide. The mixture was extracted with methyl tert-butyl ether (3 x 8 vols). The organic phase was dried with anhydrous magnesium sulfate and concentrated to dryness to give crude (S)-nicotine as a yellow liquid (131.2 g). The second batch of (S)-nornicotine (59.8 g) had likewise been transformed into crude (S)-nicotine (60.7 g) in the same manner making a total yield of crude nicotine of 191.9 g. These were combined and distilled under reduced pressure (b.p. 70-77°C at 0.53-0.67 mbar) to provide (S)-nicotine (174.5 g) as a colourless liquid with an enantiomeric excess of 99.38% as determined by HPLC, and a chemical purity of 99.96 %, as determined by HPLC. Further of the process used to measure enantiomeric excess and chemical purity are given below.

[0070] Enantiomeric purity by HPLC: using a Chiracel OD-H column eluting with n-hexane and 1-butanol in a ratio of 95:5 and containing 0.1% diethylamine. The (R)-enantiomer eluted at 6.1 min and the (S)-enantiomer at 5.6 min. The enantiomeric excess is determined from the area of the peaks identified according to the equation [(S)-(R)]/((S)+(R)]. The enantiomeric excess was thus determined as 99.38%.

[0071] Chemical purity by HPLC: Using an X-Bridge C18 column with an eluant comprising a mixture of (i) 20mM ammonium bicarbonate in water (pH=8.7) and (ii) acetonitrile in a gradient programme of 0-10 mins at 95:5, 10-13 mins at 70:30; 13-16 mins at 10:90; and subsequently 95:5. Temperature was 35 degrees C. The conditions of the detector were of UV absorption at a wavelength of 260nm. A single impurity at 12.132 minutes at 0.04% area was found versus nicotine at 9.925 mins. With a single impurity at 0.04% area the purity was deemed as 99.96%. In comparison, prior to distillation the weighted average of the two batches used was 99.70%.

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Claims

1. A process of making (S)-nicotine comprising the steps of:

   (i) reducing myosmine with an enzyme with imine reductase activity to form (S)-nornicotine; and
   (ii) methylating the (S)-nornicotine formed from step (i) to form (S)-nicotine.

2. The process of claim 1, wherein step (ii) is carried out by way of reductive methylation.

3. The process of claim 2, wherein in step (ii) the (S)-nornicotine is reductively methylated using formaldehyde or a formaldehyde-based compound in the presence of a reductant.

4. The process of claim 3, wherein the formaldehyde is introduced as part of an aqueous solution.

5. The process of claim 3, wherein the formaldehyde-based compound is a dimer of formaldehyde, a polymer of formaldehyde, or an acetal of formaldehyde.

6. The process of any of claims 2-4, wherein the reductant is formic acid, sodium cyanoborohydride, or palladium/hydrogen.

7. The process of any of claims 2-4, wherein the reductant is formic acid.

8. The process of any preceding claim, wherein the process is carried out without isolation of the (S)-nornicotine formed

from step (i).

9. The process of any preceding claim, wherein in step (i) the (S)-nornicotine is formed as part of an aqueous solution, and wherein step (ii) comprises methylating the (S)-nornicotine contained within the aqueous solution.

10. The process according to claim 9, wherein in step (ii) the (S)-nornicotine is reductively methylated using formaldehyde introduced as part of an aqueous solution.

11. The process of any preceding claim, wherein the (S)-nicotine is obtained with an enantiomeric excess of at least 90%, preferably at least 95%, more preferably at least 98%, most preferably at least 99%.

12. A process for producing a pharmaceutical composition, comprising forming (S)-nicotine according to the process of any preceding claim, and including the (S)-nicotine in the pharmaceutical composition together with one or more pharmaceutical excipients.

13. The process of claim 12, wherein the pharmaceutical composition is a transdermal patch, a lozenge, or an inhalation formulation.

14. A process for producing a formulation for an electronic cigarette device, comprising forming (S)-nicotine according to the process of any of claims 1-11, and including the (S)-nicotine in a solvent with one or more additives.

15. The use of myosmine and an enzyme with imine reductase activity in a process of forming (S)-nicotine, preferably wherein the process comprises the features of any one of claims 1-11.

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EUROPEAN SEARCH REPORT

Application Number
EP 18 20 6826

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A,D	WO 2014/174505 A2 (PERRIGO API LTD [IL]) 30 October 2014 (2014-10-30) * Claims 1, 32, 34; examples 1, 4, 6. *	1-15	INV. C07D401/04
A,D	WO 2016/065209 A2 (NEXT GENERATION LABS LLC [US]) 28 April 2016 (2016-04-28) * Abstract; claims 1-2; page 3, scheme 1; pages 11-12, example 6. *	1-15	
A	MITSUKURA ET AL.: "A NADPH-dependent (S)-imine reductase (SIR) from Streptomyces sp. GF3546 for asymmetric synthesis of optically active amines: purification, characterization, gene cloning, and expression", APPL. MICROBIOL. BIOTECHNOL., vol. 97, no. 18, 21 December 2012 (2012-12-21), pages 8079-8086, XP055538015, DE ISSN: 0175-7598, DOI: 10.1007/s00253-012-4629-4 * Abstract; page 8084, table 2. *	1-15	TECHNICAL FIELDS SEARCHED (IPC) C07D

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Munich	3 January 2019	Weisbrod, Thomas

CATEGORY OF CITED DOCUMENTS

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L : document cited for other reasons

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03-01-2019

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WO 2016065209 A2	28-04-2016	AU 2015335770 A1	08-06-2017
		CN 107406411 A	28-11-2017
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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- WO 2013170050 A **[0005] [0018]**
- WO 2015073555 A **[0005]**

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- **ATSCHUL et al.** *J. Molec. Biol.,* 1990, vol. 215, 403-410 **[0023]**