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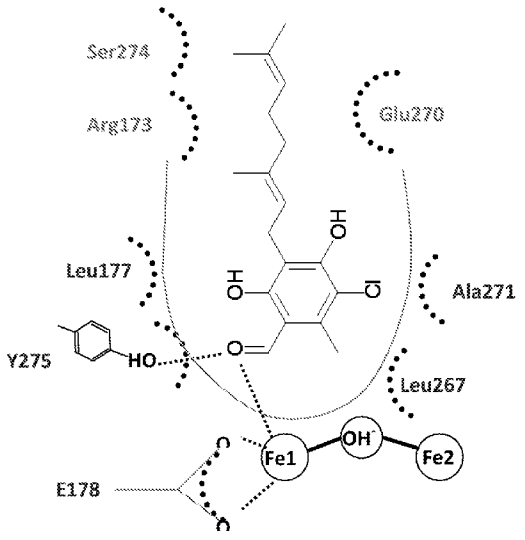
(54) Title: COMPOUNDS FOR USE AS INHIBITORS OF ALTERNATIVE OXIDASE OR CYTOCHROME BC1 COMPLEX

Figure: 3



(57) Abstract: The invention provides compounds for use in inhibiting a microbial alternative oxidase (AOX) and/or cytochrome bc_1 complex. The invention extends to the use of such inhibitors in agrochemicals and in pharmaceuticals, for treating microbial infections, including fungal infections.

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COMPOUNDS FOR USE AS INHIBITORS OF ALTERNATIVE OXIDASE OR CYTOCHROME BC1 COMPLEX

The present invention relates to alternative oxidases (AOXs) and, in particular, to inhibitors of alternative oxidases. The invention also relates to inhibitors of the cytochrome bc1 complex. The invention is especially concerned with dual inhibitors of both AOXs and the cytochrome bc1 complex. The invention extends to the use of such inhibitors in agrochemicals and in pharmaceuticals, for treating microbial infections, including fungal infections, as well as to agrochemical and pharmaceutical compositions *per se*.

Fungicides have long been used to control crop losses. The most successful class of agricultural fungicides are a set of specific inhibitors which specifically target the mitochondrial respiratory chain. Mitochondria are the power-house of the cell and, hence, inhibition of the processes that result in an organism's energy conservation have a major impact on their capability to survive. Over the last decade, inhibitors known as strobilurins, which target the oxidation of ubiquinol, a pivotal respiratory chain component, have improved the standards of disease control in plants (see Figure 1). Strobilurins inhibit fungal respiration and, hence, ATP synthesis by binding to the ubiquinol binding site of Complex III (the bc1 complex), which is essential for fungal respiration.

The strobilurins soon became one of the most important and successful agricultural fungicides accounting for over 20% of the global fungicide market. Since their introduction, this class of inhibitors has become essential to plant disease control programmes because of their wide-ranging efficacy against many agriculturally important fungal diseases. They have been registered in numerous countries for use on crops including cereals, turf grass, grapevine and numerous vegetables and ornamentals.

However, unfortunately, one of the apparent strengths of these systemic fungicides, namely their highly specific mode of action, is proving to be a significant weakness, since the rapid development of resistance to these fungicides and consequent control failure has become increasingly problematic.

In most cases, resistance was considered to be due to modification of the target site of the strobilurin, i.e. the apocytochrome b encoded by the mitochondrial genome. However, in recent years, an increasing amount of evidence suggests that resistance

may be caused by other mechanisms, such as the expression of an alternative oxidase (AOX). The AOX is a mitochondrial terminal oxidase, which, when engaged, by-passes the Qo (quinone-outside) site, and increases resistance of the plasma membrane efflux transporters to the fungicide. Efflux transporters enable fungi to survive exposure to toxic compounds by preventing their accumulation to toxic concentrations within fungal cells. Although there is some experimental evidence for cytochrome b mutations and increased resistance of efflux transporters, the possibility that the cause of fungicide resistance in phytopathogenic fungi is due to the expression of the alternative oxidase (AOX) is increasing.

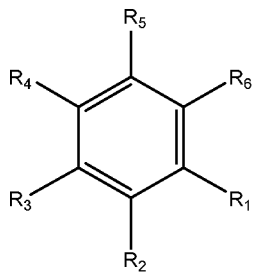
The AOX is a terminal mitochondrial respiratory chain complex that branches from the main respiratory chain at the level of ubiquinone and, hence, bypasses the bc1 complex and cytochrome c oxidase, as shown in Figure 1. AOX is not only relevant to plant fungal pathogens, because it is also present in human parasites including trypanosomes, which is the cause of African sleeping sickness, and also *Cryptosporidium parvum* and *Blastocystis hominis*, which are intestinal parasites.

In plants and fungi, expression of AOX is induced under conditions of oxidative stress, for instance when the main respiratory pathway is inhibited. Currently available inhibitors of the AOX include salicylhydroxamic acids and octylgallates. However, neither of these two classes of inhibitors is either a potent or specific target of AOX in cells, and they are therefore unsuitable for agrochemical (i.e. crop) or pharmaceutical applications.

There is therefore a need to design more specific and potent inhibitors of AOX in order to produce improved fungicidal agents, for use in either agrochemicals or anti-parasitic pharmaceuticals.

The inventors have elucidated the molecular structure and catalytic mechanism of the alternative oxidase enzyme (AOX) in the plant, *Sauromatum guttatum*, and demonstrated how this information relates to the protein's physiological role. Clearly, such fundamental knowledge is of considerable industrial relevance, and has enabled the rational design of AOX inhibitor compounds, which can be used in phytopathogenic fungicides and anti-parasitic pharmaceuticals that are targeted at mitochondrial respiration.

Thus, according to a first aspect of the invention, there is provided an alternative oxidase (AOX) inhibitor of formula I:-



[Formula I]

wherein R^1 is selected from a nitrile group, an alkyl, alkenyl, amine group with 1 to 4 C-atoms that is optionally mono- or polysubstituted by F, O, NH_2 or CN, and in which one or more non-adjacent CH_2 groups are optionally replaced, in each case independently from one another, by -O-, -NH-, -CO-, -COO-, or -OCO-;
R^2 is hydrogen or a hydroxy or alkoxy group with 1 to 3 C atoms;
R^3 is a straight chain or branched alkyl or alkylene with 4 to 20 C atoms, that is optionally mono- or polysubstituted by a C_1 to C_4 alkyl group;
R^4 is hydrogen or a hydroxy or alkoxy group with 1 to 3 C atoms;
R^5 is a halogen group; and
R^6 is H or a C_1 to C_4 alkyl group;
with the proviso that at least one of R^2 and R^4 is a hydroxy or alkoxy group with 1 to 3 C atoms.

Where any group is an alkyl group, it may be a C_1, C_2, C_3 or C_4 alkyl, for example a methyl, ethyl, propyl or butyl group. Optionally, the alkyl group may be substituted with one or more heteroatoms, for example nitrogen, oxygen, sulphur, phosphorous or a halogen.

R^1 may be a group selected from: CHO; CH_2OH; CN; CH_3; $C(O)NH_2$; $C(O)NHCH_3$; $C(O)CH_3$; CF_2CH_3; CH_2CH_3; CH_2OAc; COOH; and $COOCH_3$.

R^2 may be a short-chain alkyl, for example a methyl, ethyl or propyl group. However, preferably R^2 is a hydroxyl group.

R^3 may be a straight chain or branched alkyl or alkylene with 6 to 15 C atoms, 8 to 12 C atoms or 8 to 10 C atoms, that is optionally mono- or polysubstituted by a C_1 to C_4 alkyl group. For example, R^3 may be branched diene having 6 to 15 C atoms that is substituted with at least one, and preferably two, methyl groups.

R^4 may be a methyl, ethyl or propyl group. However, preferably R^4 is a hydroxyl group.

R^5 may be a chlorine, bromine, fluorine or iodine group. Preferably, R^5 is a chlorine group.

R^6 may be a methyl, ethyl or propyl group. However, preferably R^4 is a methyl group.

In one embodiment, the AOX inhibitor comprises a compound of formula I, wherein:-
R^1 is selected from CHO; CH_2OH; CN; CH_3; $C(O)NH_2$; $C(O)NHCH_3$; $C(O)CH_3$; CF_2CH_3; CH_2CH_3; CH_2OAc; COOH; and $COOCH_3$; and wherein
R^2 is a hydroxyl group;
R^3 is a straight chain or branched alkyl or alkylene with 4 to 20 C atoms, that is optionally mono- or polysubstituted by a C_1 to C_4 alkyl group;
R^4 is a hydroxyl group;
R^5 is a chlorine atom; and
R^6 is H or a C_1 to C_4 alkyl group.

In another embodiment, the AOX inhibitor comprises a compound of formula I, wherein:-
R^1 is selected from CHO; CH_2OH; CN; CH_3; $C(O)NH_2$; $C(O)NHCH_3$; $C(O)CH_3$; CF_2CH_3; CH_2CH_3; CH_2OAc; COOH; and $COOCH_3$; and wherein
R^2 is a hydroxyl group;
R^3 is a straight chain or branched alkyl or alkylene with 6 to 15 C atoms, that is optionally mono- or polysubstituted by a C_1 to C_2 alkyl group;
R^4 is a hydroxyl group;
R^5 is a chlorine atom; and
R^6 is H or a C_1 to C_4 alkyl group.

In one preferred embodiment, the AOX inhibitor comprises a compound of formula I, wherein:-

R^1 is selected from CHO; CH_2OH; CN; CH_3; $C(O)NH_2$; $C(O)NHCH_3$; $C(O)CH_3$; CF_2CH_3; CH_2CH_3; CH_2OAc; COOH; and $COOCH_3$; and wherein

R^2 is a hydroxyl group;

R^3 is an alkylene chain having 8 to 10 C atoms, and is substituted with at least one methyl group, preferably two methyl groups;

R^4 is a hydroxyl group;

R^5 is a chlorine atom; and

R^6 is a methyl group.

The inventors believe that the hydroxyl groups (R^2 and R^4) and the chlorine (R^5) and methyl (R^6) substituents on the benzene ring of the inhibitor compound may be important for high potency. In addition, the hydrophobic side chain, which may be a geranyl group, may also be important. However, the inventors believe that the aldehyde group present in a known compound, ascofuranone, may be problematic for anti-parasitic drug design for several reasons. Besides their ability to function as hydrogen bond acceptor and to undergo dipole-dipole interaction with AOX, aldehyde groups are chemically reactive enough to undergo reversible covalent modifications and would be generally unsuited to standard pharmaceutical formulations. Furthermore, aldehydes are prone to metabolic oxidation to the respective carboxylic acid with the concomitant non-specific binding to basic transport proteins. The inventors therefore believe that in some embodiments, it may be beneficial for there not to be an aldehyde group present.

Therefore, in some embodiments, R^1 is not a CHO group.

Preferably, the inhibitor of the first aspect is capable of inhibiting a microbial AOX. Preferably, the inhibitor is capable of inhibiting a fungal, bacterial or protist AOX. Examples of such micro-organisms which may be inhibited are provided herein. For example, the inhibitor may be capable of inhibiting an AOX from any of the organisms represented in Figure 4. In particular, the inhibitor is capable of binding with any of the amino acid residues shown in the boxes in Figure 4, as they represent conserved AOX residues which are believed to be involved in inhibitor binding.

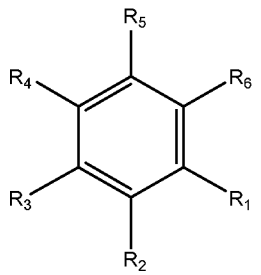
Advantageously, the inhibitor can be synthesised using only a two-step process. For example, one embodiment of such a process is shown in Figure 7.

As described in Example 6, the inventors have also surprisingly demonstrated that the compound of formula I is a specific inhibitor of the cytochrome bc_1 complex as well as being a potent inhibitor of AOX. Accordingly, the compound of formula I may be capable of inhibiting the cytochrome bc_1 complex. Advantageously, compounds of formula I can act as a specific and potent dual function fungicide, as not only do they inhibit the alternative oxidase (AOX), but also the cytochrome bc_1 complex. This makes the compound a very potent inhibitor of respiration even in the absence of an alternative oxidase.

Surprisingly, the data also suggest that the compound inhibits the cytochrome bc_1 complex at both the Qo (Quninone outside) and Qi (Quinone inside) binding sites of the Cytochrome bc_1 complex. Thus, the compound of formula I may inhibit the Qo and/or Qi binding site of the Cytochrome bc_1 complex. Preferably, however, the compound of formula I inhibits the Qo and Qi binding sites of cytochrome bc_1 complex. It will be appreciated that commercially available fungicides, such as azoxystrobin, inhibit only one site (Qo) within the bc1 complex. Accordingly, since compounds of the invention inhibit the cytochrome bc_1 complex at both the Qo and Qi binding sites, and also the AOX, such compounds act as highly potent and robust inhibitors.

In a second aspect, the invention provides a use of the compound of the first aspect, for inhibiting an alternative oxidase (AOX).

In a third aspect, there is provided use of a compound, for inhibiting a microbial alternative oxidase (AOX) and/or cytochrome bc_1 complex, wherein the compound is represented by formula I:-



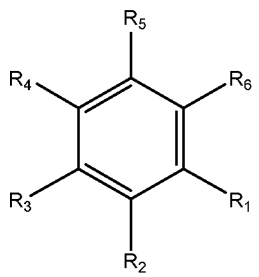
[Formula I]

wherein R^1 is selected from a nitrile group, an alkyl, alkenyl, amine group with 1 to 4 C-atoms that is optionally mono- or polysubstituted by F, O, NH_2 or CN, and in which one

or more non-adjacent CH_2 groups are optionally replaced, in each case independently from one another, by -O-, -NH-, -CO-, -COO-, or -OCO-;
R^2 is hydrogen or a hydroxy or alkoxy group with 1 to 3 C atoms;
R^3 is a straight chain or branched alkyl or alkylene with 4 to 20 C atoms, that is optionally mono- or polysubstituted by a C_1 to C_4 alkyl group;
R^4 is hydrogen or a hydroxy or alkoxy group with 1 to 3 C atoms;
R^5 is a halogen group; and
R^6 is H or a C_1 to C_4 alkyl group;
with the proviso that at least one of R^2 and R^4 is a hydroxy or alkoxy group with 1 to 3 C atoms.

Accordingly, the compound that is used may be the inhibitor as defined herein. The use may be carried out *in vitro* or *in vivo*. The use may comprise inhibiting a microbial AOX, which may be a fungal, bacterial or protist AOX, as defined herein. The use may comprise inhibiting the cytochrome bc_1 complex, preferably the Qo and/or Qi binding site of the cytochrome bc_1 complex. The inventors believe that this is an important feature of the invention.

Hence, in a fourth aspect, the invention provides a use of a compound of formula I, for inhibiting the cytochrome bc_1 complex, wherein the compound of formula I is represented as:-



[Formula I]

wherein R^1 is selected from a nitrile group, an alkyl, alkenyl, amine group with 1 to 4 C-atoms that is optionally mono- or polysubstituted by F, O, NH_2 or CN, and in which one or more non-adjacent CH_2 groups are optionally replaced, in each case independently from one another, by -O-, -NH-, -CO-, -COO-, or -OCO-;
R^2 is hydrogen or a hydroxy or alkoxy group with 1 to 3 C atoms;

R^3 is a straight chain or branched alkyl or alkylene with 4 to 20 C atoms, that is optionally mono- or polysubstituted by a C_1 to C_4 alkyl group;
R^4 is hydrogen or a hydroxy or alkoxy group with 1 to 3 C atoms;
R^5 is a halogen group; and
R^6 is H or a C_1 to C_4 alkyl group;
with the proviso that at least one of R^2 and R^4 is a hydroxy or alkoxy group with 1 to 3 C atoms.

R^1 to R^6 may be defined as above. The use may comprise inhibiting the Qo and/or Qi binding site of the cytochrome bc_1 complex, and preferably both the Qo and Qi binding sites.

The inventors have found that the AOX inhibitors of the invention can be effectively used to treat infections of plant pathogens which comprise an AOX enzyme.

Thus, in a fifth aspect, there is provided use of the compound of formula I, which may be the alternative oxidase (AOX) inhibitor of the first aspect, as an agrochemical.

In a sixth aspect, there is provided use of the compound of formula I, which may be the alternative oxidase (AOX) inhibitor of the first aspect, for use as an agrochemical.

In a seventh aspect, there is provided an agrochemical composition comprising the compound of formula I, which may be alternative oxidase (AOX) inhibitor of the first aspect.

In an eighth aspect, there is provided use of the agrochemical composition of the seventh aspect, for treating an agrochemical disease or infection.

An agrochemical disease, which may be treated, may be caused by an organism selected from a group of organisms consisting of: *Chalara fraxinea*; *Septoria tritici*; *Gaeumannomyces gramminis var titici*; *Magnaporthe grisea*; *Magnaporthe oryzae*; *Rhizoctonia solani*; *Botrytis cinerea*; *Fusicladium effusum* syn. *Cladosporium caryigenum and Fusicladosporium effusum*; *Carya illinoinensis*; *Podosphaera fusca*; *Microdochium nivale*; *Microdochium majus*; *Septoria nodoum*; *Tapesia acuformis*; and *Metarhizium anisopliae*.

It will be appreciated that *Septoria tritici* and *Gaeumannomyces gramminis var titici* are wheat pathogens (take-all); *Magnaporthe grisea* and *Magnaporthe oryzae* may cause rice blast and grey leaf spot of rye grass; *Rhizoctonia solani* may cause black scurf of potato, *Botrytis cinerea* may cause grey mold - a necrotrophic fungus that affects many plant species, although its most notable hosts are wine grapes; *Fusicladium effusum* (syn. *Cladosporium caryigenum and Fusicladosporium effusum* is known as the Pecan scab and is the most devastating disease of the commercial pecan; *Carya illinoinensis* is involved in the production in South Eastern United States; *Podosphaera fusca* is the main causal agent of cucurbit powdery mildew in Spain and one of the most important limiting factors for cucurbit production worldwide; *Microdochium nivale & majus* may attack barley, durum wheat and soft wheat and is present in all areas of France; *Septoria nodoum* may cause leaf and glume blotch; and *Tapesia acuformis* may cause eyespot. In addition, there is growing agrochemical interest in *Metarhizium anisopliae* which is an entomopathogenic fungus as an alternative for the management of pest insects.

It will be appreciated that *Chalara fraxinea* causes Chalara ash dieback disease. The first DNA sequence for *Chalara fraxinea* has been published, as follows:-

TTTATATATCCGATGTTTGGTGACAGCATTCTTGGTGCATGAAAAGTTACTCCCGGCGGCGAG
GAACCGGTGACTTGGGAGAAGGGAGCGTTTGTTAATTGAAGGACTGGTACATGGATACCTGCGT
ACTTCATGCGGTCCTTTCTCGTGGTGGTAGGCCTGCATTTAGGTTTCTTTGATGTTTGACGCCG
CAGTTACACAGGCTGAGGCTGACTTGGATTTCGTGTGTCCCGATAAGACATCGTGAACGAGTTA
TTTCCATCCCTTCTTTTCTTGTTCTCTTTTAGTTTGCTTAGAAGACGTTGCCGGCTTTTTTTCT
TGACTGAGGTTGCTCGGGCTTTTATTCATTCACCTCTTCTTAGAGTTACCCGTTGCTTGCCGTG
TTTCTTTGCTAGTGGCAATTGAAATACAACACCTACCTACTGTACCTTCACACACCTACAAACC
TTTTTCTTATTTCGTCAAAATAACAGGATTCTGATTGAGACCACTATGTATGTGGCAAGAGTAT
CCACGAGGGTACAGTTCTCCAAACAGACTGCTTCGCATCTTTCCAAGGTCGTAGCAGCCAACTT
TTCACAATCATGTTCTGGCTCCCTCCATCGCGTTGGTCTTGGTGCGAGTCCAGTACTTCATACT
TCACAATCTCATCGTGAGTTCTCTACGACGCCCCGAGCAGCGTTGAGAGATTTCTTCCCTCAGA
AGGAAACGGAACTGATCCGGAAAACCAAACCAGCATGGGAACATCCCGACTTCAGCTACGAAGA
CATGAAAACCAAGGTCTTCTATGCCCACCGCGAACCAGCCGATTTCTCAGATCGTGTCGCATTA
TGGATGGTTCGCCTTTTAAGATGGGGAACCGACCTAGCAACGGGCTACAAACACGATGTAGAAG
TGCCAAAGAAAATCGGTGATGCCAATGCCGTTGCAGAGACGAAGCCATATGGTATGAGTGAGCG
AAAATGGTTGATTCGAATTATATTTTTGGAATCTGTTGCGGGTGTGCCAGGGATGGTTGCGGCT
ATGTTGAGGCATTTGCATTCGATGAGGAGGTTGAAGAGGGATAATGGATGGATTGAGACGCTTT
TGGAGGAGAGTCAGAATGAGAGGATGCATCTCCTCACCTTCCTCAAAATGGCCGAACCAGGCTG
GTTCATGAAATTCATGCTCCTGGGCGCCCAAGGCGTCTTCTTCAACAGCATGTTCATCTCCTAC
CTCATCTCCCCACGAACCTGCCACCGCTTCGTCGGCTACCTCGAAGAAGAAGCCGTCTTCACGT
ACACGCTCGCCATCCAAGACCTGGAAGCGGGCAAGCTGCCCCAATGGACGCACCCGGACTTCCG
CGTCCCAGACATCGCCGTCGATTACTGGAAGATGCCCGAGGACAAACGCACCATGAGGGATCTC
ATGCTCTATGTGAGAGCGGATGAGGCGAAACATCGTGAGGTTAATCATACCCTGGGGAATCTGG
ATCAGGATGAGGATCCGAATCCGTTTGTTTCCGAGTATAAGGATGTGGGGAGGCCGCATCCTGG
GAAGGGGATTGAGCATGTGCAGCCGATTGGGTGGGAGAGGAAGGATGTTATTTGAGAGTTGGAG

CGAAGTCTTTTGCTCTTTTCTTGATCGCGATCGATGGCTCTCGACGACTAGATGAGGGACTTGA
AGTCTTAAACTGCGACCAGGACTGCATAGAGATTACTACAGAGAGGCGTTTTGAGGTTTTTGGC
GTTGGTTTATAGGTGTGCAAGATGGGTTCGGGCGTTTGTTCTGCTTTT

[SEQ ID No:1]

This sequence can be found online at https://github.com/ash-dieback-crowdsource/data. The sequence of DNA can be found at this position within the open source file: CHAFR746836.1.1_0053300.1 gene=CHAFR746836.1.1_0053300. loc:Cf746836_TGAC_s1v1_scaffold_1027()115146-117337 segs:1-260,261-620,621-1098 CDS=493-1590 loc:Cf746836_TGAC_s1v1_scaffold_1027|115146-117337| exons:115146-115897,115987-116346,116416-117337 segs:1-752,753-1112,1113-2034.

As represented by the underlined bases shown in SEQ ID No:1, the DNA sequence for *C. fraxinea* encodes an AOX. Accordingly, the AOX inhibitors of the invention, as defined herein, will be effective against this fungus, especially its AOX encoded by SEQ ID No:1.

The agrochemical composition may comprise one or more solvents in which the AOX inhibitor is mixed. The amount of solvents in the composition may range from 1% to 99%, or from 30% to 80%. Suitable solvents include, for example, a non-polar water-immiscible solvent, or a polar aprotic water miscible organic solvent. Non-polar solvents include, for example substituted or unsubstituted aliphatic or aromatic hydrocarbons and esters of plant oils or mixtures thereof. Non-limiting examples of aromatic hydrocarbons include benzene or substituted benzene derivatives such as toluene, xylene, 1,2,4-trimethylbenzene, naphthalene or mixtures thereof. In one embodiment, a solvent includes a mixture of napthalen and 1,2,4-trimethylbenzene. In another embodiment, a solvent is Aromatic 150, a heavy aromatic naptha solvent containing <10% naphthalene and <1.7% 1,2,4-trimethylbenzene.

Alkyl esters can also be used as non-polar, water immiscible solvents. Plant oils may be esterified with various alcohols to form alkyl esters of plant oils. Fatty acids of these plant oils have 5 to 20, or 6 to 15 carbon atoms. Alkyl esters of plant oils include, without limitation, methyl, ethyl and butyl esters of canola (B. napus), linseed, safflower (Carthamus tinctorius L), soybean and sunflower oils. In one embodiment, the solvent is a mixture of methyl esters. A specific non-limiting example of methyl esters is Agent 2416-21 manufactured by Stepan Company (22 W. Frontage Road,

Northfield, Illinois).

Water-miscible polar aprotic solvents can include, for example, alkyl lactates, isopropyl lactate, alkyl carbonates, polyethylene glycols, polyethylene glycol alkyl ethers, polypropylene glycols, and polypropylene glycol alkyl ethers, or mixtures thereof.

The composition may comprise one or more adjuvants. An adjuvant may enhance or improve herbicidal performance, for example. Adjuvants may be added to the composition at the time of formulation, or by the applicator to a mix prior to treatment. Adjuvants include, for example surfactants (emulsifier), crop oil, fertilizers, dispersing agents, compatibility agents, foaming activators, foam suppressants, correctives, and spray colorants (dyes). An adjuvant may be present in any desired amount. For example, a formulation may contain 1% to 3% adjuvant, 3% to 8% of adjuvant, 8% to 16% adjuvant, 17% to 30% adjuvant, or 30% or (e.g. 40% or more) more adjuvant.

The composition may comprise one or more surfactant. A surfactant may increase solubility of the AOX inhibitor in a solution. A surfactant may also affect spray retention, droplet spreading, and dry rates. A surfactant may be anionic or non-ionic. Examples of anionic surfactants include phosphoric mono- and di- esters of long-chain alcohols having 14 to 22 carbon atoms and the salts thereof; phosphoric mono-and di-esters of alkylene oxide addition products of long-chain alcohols having 14 to 22 carbon atoms and the salts thereof; alkylsulphates having 14 to 22 carbon atoms; polyoxyethylene alkyl ether sulphates of alcohols having 14 to 22 carbon atoms; alkane sulphonates having 14 to 22 carbon atoms; and olefin sulphonates having 14 to 22 carbon atoms.

Suitable non-ionic surfactants include, for example, ethoxylated fatty acids, alcohol ethoxylates, tristyrylphenol ethoxylates, ethoxylated sorbitan fatty acid esters or mixtures thereof. Ethoxylated fatty acids include castor or canola oil ethoxylates having at least 25, preferably 27 to 37 ethoxy units, such as Sunaptol RTM CA350 (castor oil ethoxylate with 35 ethoxy units) of Uniqema (formerly ICI Surfactants), Mergital RTM EL33 (castor oil ethoxylate with 33 ethoxy units) of Henkel KGaA, Eumulgin RTM C03373 (canola oil ethoxylate with 30 ethoxy units) of Henkel KGaA and Ukanil RTM 2507 (castor oil ethoxylate) of Uniqema.

Surfactants may be present in any desired amount. For example, a surfactant may be

present in an amount of about 0.1 to about 30% by weight in the formulation. In a particular embodiment, a surfactant is present in an amount of about 1 to about 9 % by weight in the formulation. In another embodiment, a surfactant is present in an amount of about 10 to about 20 % by weight in the formulation.

The composition may comprise one or more emulsifier. An emulsifier is a type of surfactant typically used to keep emulsion well-dispersed. Non-limiting examples of the emulsifier include Agent 2201-76, Agent 2416-20, Emulpon CO-360, T-Det C-40(R), and Agnique(TM) SBO-IO. Agent 2201-76 is manufactured by Stepan Company (22 W. Frontage Road, Northfield, Illinois), which is a blend of nonionic and anionic surfactants (82%). The ingredients in Agent 2201-76 are alkylbenzene sulfonate and fatty acid ethoxylate, aromatic petroleum hydrocarbon, 1-hexanol and naphthalene. Agent 2416-20 is also manufactured by Stepan Company (22 W. Frontage Road, Northfield, Illinois), which is a blend of nonionic and anionic surfactants (35-37%). Agent 2416-20 also includes aromatic petroleum hydrocarbon (57-58%), and naphthalene (6-7%). Emulpon CO- 360 is manufactured by Akzo Nobel Chemicals Ltd. (525 West Van Buren, Chicago, Illinois), which contains ethoxylated castor oil (100% by weight) and oxirane (<0.001% by weight). T-Det C-40(R) may be purchased from Harcros Organics (5200 Speaker Road., P.O. Box 2930, Kansas City, Kansas), or from Akzo Nobel Chemicals Ltd. (525 West Van Buren, Chicago, Illinois), which is a non-ionic emulsifier, and a brand of ethoxylated (polyethoxylated) castor oil. Agnique(TM) SBO-IO is manufactured by Cognix GmbH headquartered in Monheim, Germany, which contains alkoxylated triglycerides as an ethoxylated soybean oil.

A crop oil, or a crop oil concentrate, may be used to increase the efficacy of a herbicide formulation. Although not wishing to be bound by any particular theory, a crop oil is believed to keep the leaf surface moist longer than water, which in turn allows more time for the herbicide to penetrate, thereby increasing the amount of herbicide that will enter the plant (e.g. weed). A crop oil can improve uptake of herbicide by plant (e.g. weed). A crop oil can therefore improve, enhance, increase or promote herbicidal efficacy or activity. Crop oils may contained from 1% to 40% by weight, or 1% to 20% by weight in the formulation. A crop oil can be derived from either petroleum oil or vegetable oil. Non-limiting examples of crop oil include soybean oils and petroleum based oils.

The agrochemical composition of the invention may be in customary formulations.

Non-limiting examples include solutions, emulsions, suspensions, wettable powders, powders, dusts, pastes, soluble powders, granules, pellets, emulsifiable concentrate, oil spray, aerosol, natural and synthetic materials impregnated with active compound, and very fine capsules (e.g. in polymeric substances). In certain embodiments, the composition is in a form of an emulsifiable concentrate, wettable powder, granule, dust, oil spray or aerosol.

The composition may optionally include adherent coatings. Such coatings include those that aid the AOX/bc1 inhibitor to adhere to the intended environment, for example, a plant being treated. Adherent coatings include carboxymethylcellulose, natural and synthetic polymers in various forms, such as powders, granules or latexes. Other adherent coatings include gum arabic, polyvinyl alcohol and polyvinyl acetate. Phospholipids, such as cephalins and lecithins, and synthetic phospholipids are also examples of adherent coatings. Further additives may be mineral and vegetable oils.

Colourants can also be included in the compositions. Non-limiting examples are inorganic pigments, such as iron oxide, titanium oxide and Prussian Blue, and organic dyestuffs, such as alizarin dyestuffs, azo dye-stuffs and metal phthalocyanine dyestuffs, and trace nutrients such as salts of iron, manganese, boron, copper, cobalt, molybdenum and zinc.

The agrochemical compositions according to the invention can be applied in the form of ready mixes. Herbicidal compositions can also be formulated individually and mixed upon use, i.e. applied in the form of tank mixes. The compositions of the invention can be used as such or in the form of their formulations, and furthermore also as mixtures with herbicides, ready mixes or tank mixes. The compositions may also be mixed with other active compounds, such as other fungicides, insecticides, acaricides, nematicides, bird repellents, growth substances, plant nutrients and agents which improve soil structure. For particular application purposes, in particular when applied post-emergence, formulations such as mineral or vegetable oils which are tolerated by plants (for example the commercial product "Oleo DuPont 1 IE") or ammonium salts such as, for example, ammonium sulphate or ammonium thiocyanate, as further additives can be included.

The compositions can be used as such, in the form of their formulations or in the forms prepared therefrom by dilution of a concentrated form, such as ready-to-use or

concentrated liquids, solutions, suspensions, emulsions, or solids, such as, powders, pastes, granules and pellets. They are dispersed in the customary manner, for example by watering, spraying, atomizing, dusting or scattering.

The compositions of the invention can be produced by mixing or suspending one or more stabilizers, an active ingredient, and optionally an adjuvant, a diluent or a solvent. In certain embodiments, compositions of the invention can be produced, for example by first mixing or suspending one or more AOX/bc1 inhibitor with a diluent or solvent. Next, the appropriate amount of adjuvant is combined to the resulting mixture containing the AOX/bc1 inhibitor. The AOX/bc1 inhibitor can be added at the end and blended until the formulation becomes mostly or entirely homogeneous.

Plants that may be treated with the agrochemical composition are generally referred to herein as "crop plants". The term "crop plants" as used herein, includes any edible or non-edible plant, including decorative, plant species with commercial value, which is planted and cultivated for commercial use. Thus, crop plants include floral and non-floral plants, trees, vegetable plants, turf, and ground cover. Non-limiting specific examples of crop plants include canola, flax, peas, lentils, beans, linola, mustard, chickpeas, sunflowers, potatoes, seedling alfalfa, onions, soybeans and turf grass. The term "plants" is meant to include germinant seeds, emerging seedlings, and established vegetation, including roots and above-ground portions (for example, leaves, stalks, flowers, fruits, branches, limbs, root, etc.). The term "turf" used herein refers to grass which grow in areas in which they are desired, or purposely planned for and maintained, for example, a lawn. Turf also refers to a sod, where the surface layer of ground consisting of a mat of grass and grass roots.

The application rate of AOX/bc1 inhibitor varies depending, for example, on the crop being treated with the agrochemical composition. In general, the application rate may be from 0.01 kg/ha to 5.00kg/ha or from 0.03 kg/ha to 3.00kg/ha of the AOX/bc1 inhibitor.

The inventors have also found that the inhibitors of the invention can be effectively used to treat infections of animal or human pathogens which comprise an AOX enzyme.

In a ninth aspect, there is provided the compound of formula I, which may be alternative oxidase (AOX) inhibitor of the first aspect, for use in therapy or diagnosis.

In a tenth aspect, there is provided the compound of formula I, which may be alternative oxidase (AOX) inhibitor of the first aspect, for use in treating a microbial infection.

In an eleventh aspect, there is provided a method of treating, ameliorating or preventing a microbial infection in a subject, the method comprising, administering to a subject in need of such treatment, a therapeutically effective amount of the compound of formula I, which may be alternative oxidase (AOX) inhibitor of the first aspect.

In a twelfth aspect, there is provided a method of inhibiting activity of a microbial alternative oxidase (AOX) and/or cytochrome bc_1 complex, the method comprising contacting a microbial alternative oxidase (AOX) and/or cytochrome bc_1 complex with an effective amount of the compound of formula I, which may be alternative oxidase (AOX) inhibitor of the first aspect.

The AOX/bc1 inhibitor may be used to treat a bacterial infection, for example a Gram-positive or a Gram-negative bacterial infection.

Preferably, the AOX/bc1 inhibitor is used to treat a fungal infection. For example, fungi against which the inhibitor is effective may include a filamentous fungus, such as an *Ascomycete*. Examples of fungi against which the inhibitor is effective may be selected from a group of genera consisting of *Aspergillus*; *Blumeria*; *Candida*; *Cryptococcus*; *Encephalitozoon; Fusarium; Leptosphaeria; Magnaporthe; Phytophthora; Plasmopara; Pneumocystis; Pyricularia; Pythium; Puccinia; Rhizoctonia; richophyton*; and *Ustilago*.

Further examples of fungi may be selected from a group of genera consisting of *Aspergillus* and *Candida*. The fungus may be selected from a group of species consisting of *Aspergillus flavus*; *Aspergillus fumigatus; Aspergillus nidulans; Aspergillus niger; Aspergillus parasiticus; Aspergillus terreus; Blumeria graminis; Candida albicans*; *Candida cruzei ; Candida glabrata; Candida parapsilosis; Candida tropicalis; Cryptococcus neoformans; Encephalitozoon cuniculi; Fusarium solani; Leptosphaerianodorum; Magnaporthe grisea; Phytophthora capsici; Phytophthora infestans; Plasmopara viticola; Pneumocystis jiroveci; Puccinia coronata; Puccinia graminis; Pyricularia oryzae; Pythium ultimum; Rhizoctonia solani;

Trichophytoninterdigitale; Trichophyton rubrum; and *Ustilago maydis*. Further examples of fungi include yeast, such as *Saccharomyces spp*, eg *S. cerevisiae*, or *Candida spp*, and *C.albicans*, which is know to infect humans.

The AOX/bc1 inhibitor may be used to treat a disease associated with human pathogens, such as intestinal disease; Leishmaniasis; Candidiasis; and diseases associated with contact lens usage.

It will be appreciated that *Trypanosoma*, *Cryptosporidium parvum* and *Blastocystis hominis* can cause intestinal diseases; Leishmaniasis is caused by the *Leishmani* parasite; Candidiasis is caused by *Candida albicans* (commonly known as thrush); and diseases associated with contact lens usage may be caused by the free-living protozoan *Acanthamoeba*.

It will be appreciated that AOX/bc1 inhibitors according to the invention may be used in a medicament, which may be used in a monotherapy, i.e. use of only the AOX/bc1inhibitor for treating, ameliorating, or preventing a microbial infection. Alternatively, AOX/bc1 inhibitors may be used as an adjunct to, or in combination with, known therapies for treating, ameliorating, or preventing microbial infections, for example known antibacterial agents or antifungal agents.

The AOX/bc1 inhibitors according to the invention may be combined in compositions having a number of different forms depending, in particular, on the manner in which the composition is to be used. Thus, for example, the composition may be in the form of a powder, tablet, capsule, liquid, ointment, cream, gel, hydrogel, aerosol, spray, micellar solution, transdermal patch, liposome suspension or any other suitable form that may be administered to a person or animal in need of treatment. It will be appreciated that the vehicle of medicaments according to the invention should be one which is well-tolerated by the subject to whom it is given.

Medicaments comprising AOX/bc1 inhibitors according to the invention may be used in a number of ways. For instance, oral administration may be required, in which case the inhibitors may be contained within a composition that may, for example, be ingested orally in the form of a tablet, capsule or liquid. Compositions comprising inhibitors of the invention may be administered by inhalation (e.g. intranasally).

Compositions may also be formulated for topical use. For instance, gels, creams or ointments may be applied to the skin, for example, adjacent the treatment site.

Inhibitors according to the invention may also be incorporated within a slow- or delayed-release device. Such devices may, for example, be inserted on or under the skin, and the medicament may be released over weeks or even months. The device may be located at least adjacent the treatment site. Such devices may be particularly advantageous when long-term treatment with modulators used according to the invention is required and which would normally require frequent administration (e.g. at least daily injection).

In a preferred embodiment, inhibitors and compositions according to the invention may be administered to a subject by injection into the blood stream or directly into a site requiring treatment. Injections may be intravenous (bolus or infusion) or subcutaneous (bolus or infusion), or intradermal (bolus or infusion).

It will be appreciated that the amount of the AOX/bc1 inhibitor that is required is determined by its biological activity and bioavailability, which in turn depends on the mode of administration, the physiochemical properties of the inhibitor and whether it is being used as a monotherapy or in a combined therapy. The frequency of administration will also be influenced by the half-life of the inhibitors within the subject being treated. Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the particular inhibitors in use, the strength of the pharmaceutical composition, the mode of administration, and the advancement of the disease being treated. Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of administration.

Generally, a daily dose of between 0.01µg/kg of body weight and 0.5g/kg of body weight of the inhibitors according to the invention may be used for treating, ameliorating, or preventing the microbial infection, depending upon which inhibitor is used. More preferably, the daily dose of inhibitor is between 0.01mg/kg of body weight and 500mg/kg of body weight, more preferably between 0.1mg/kg and 200mg/kg body weight, and most preferably between approximately 1mg/kg and 100mg/kg body weight.

The inhibitors may be administered before, during or after onset of the microbial infection. Daily doses may be given as a single administration (e.g. a single daily injection). Alternatively, the inhibitors may require administration twice or more times during a day. As an example, inhibitors may be administered as two (or more depending upon the severity of the disease being treated) daily doses of between 25mg and 7000 mg (i.e. assuming a body weight of 70 kg). A patient receiving treatment may take a first dose upon waking and then a second dose in the evening (if on a two dose regime) or at 3- or 4-hourly intervals thereafter. Alternatively, a slow release device may be used to provide optimal doses of inhibitors according to the invention to a patient without the need to administer repeated doses.

Known procedures, such as those conventionally employed by the pharmaceutical industry (e.g. *in vivo* experimentation, clinical trials, etc.), may be used to form specific formulations comprising the inhibitors according to the invention and precise therapeutic regimes (such as daily doses of the inhibitors and the frequency of administration). The inventors believe that they are the first to describe a composition for treating microbial infections, based on the use of the inhibitors of the invention.

Hence, in a thirteenth aspect of the invention, there is provided an antimicrobial composition comprising the compound as represented by formula I, which may be an AOX inhibitor according to the first aspect, and a pharmaceutically acceptable vehicle.

The term "antimicrobial composition" can mean a pharmaceutical formulation used in the therapeutic amelioration, prevention or treatment of any microbial infection, for example a fungal, bacterial or pathogenic infection.

The invention also provides in an fourteenth aspect, a process for making the antimicrobial composition according to the thirteenth aspect, the process comprising contacting a therapeutically effective amount of the compound of formula I, which may be an AOX inhibitor according to the first aspect, and a pharmaceutically acceptable vehicle.

A "subject" may be a vertebrate, mammal, or domestic animal. Hence, inhibitors, compositions and medicaments according to the invention may be used to treat any mammal, for example livestock (e.g. a horse), pets, or may be used in other veterinary applications. Most preferably, however, the subject is a human being.

A "therapeutically effective amount" of the AOX/bc1 inhibitor is any amount which, when administered to a subject, is the amount of medicament or drug that is needed to treat the microbial infection, or produce the desired effect.

For example, the therapeutically effective amount of AOX/bc1 inhibitor used may be from about 0.01 mg to about 800 mg, and preferably from about 0.01 mg to about 500 mg. It is preferred that the amount of inhibitor is an amount from about 0.1 mg to about 250 mg, and most preferably from about 0.1 mg to about 20 mg.

A "pharmaceutically acceptable vehicle" as referred to herein, is any known compound or combination of known compounds that are known to those skilled in the art to be useful in formulating pharmaceutical compositions.

In one embodiment, the pharmaceutically acceptable vehicle may be a solid, and the composition may be in the form of a powder or tablet. A solid pharmaceutically acceptable vehicle may include one or more substances which may also act as flavouring agents, lubricants, solubilisers, suspending agents, dyes, fillers, glidants, compression aids, inert binders, sweeteners, preservatives, dyes, coatings, or tablet-disintegrating agents. The vehicle may also be an encapsulating material. In powders, the vehicle is a finely divided solid that is in admixture with the finely divided active agents according to the invention. In tablets, the active agent (e.g. the modulator) may be mixed with a vehicle having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active agents. Suitable solid vehicles include, for example calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, polyvinylpyrrolidine, low melting waxes and ion exchange resins. In another embodiment, the pharmaceutical vehicle may be a gel and the composition may be in the form of a cream or the like.

However, the pharmaceutical vehicle may be a liquid, and the pharmaceutical composition is in the form of a solution. Liquid vehicles are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The inhibitor according to the invention may be dissolved or suspended in a pharmaceutically acceptable liquid vehicle such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid vehicle can contain other suitable

pharmaceutical additives such as solubilisers, emulsifiers, buffers, preservatives, sweeteners, flavouring agents, suspending agents, thickening agents, colours, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid vehicles for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the vehicle can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid vehicles are useful in sterile liquid form compositions for parenteral administration. The liquid vehicle for pressurized compositions can be a halogenated hydrocarbon or other pharmaceutically acceptable propellant.

Liquid pharmaceutical compositions, which are sterile solutions or suspensions, can be utilized by, for example, intramuscular, intrathecal, epidural, intraperitoneal, intravenous and particularly subcutaneous injection. The inhibitor may be prepared as a sterile solid composition that may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium.

The inhibitor and pharmaceutical compositions of the invention may be administered orally in the form of a sterile solution or suspension containing other solutes or suspending agents (for example, enough saline or glucose to make the solution isotonic), bile salts, acacia, gelatin, sorbitan monoleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like. The inhibitors according to the invention can also be administered orally either in liquid or solid composition form. Compositions suitable for oral administration include solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

All of the features described herein (including any accompanying claims, abstract and drawings), and/or all of the steps of any method or process so disclosed, may be combined with any of the above aspects in any combination, except combinations where at least some of such features and/or steps are mutually exclusive.

Embodiments of the invention will now be further described, by way of example only, with reference to the following Examples, and to the accompanying diagrammatic drawings, in which:-

Figure 1 is a schematic drawing showing the mitochondrial respiratory chain, in which I-IV: Respiratory chain complexes; V: ATP synthase; Q: Ubiquinone; $N_{ext/int}$: NADH dehydrogenases; Stb: Strobilurin site of action; SHAM: salicylhydroxamic acid site of action; CN/CO: Cyanide/Carbon monoxide inhibition site; AO: Alternative oxidase; IMS/IM/M: Inter-membrane space/inner membrane/matrix of mitochondria;

Figure 2 shows a crystal structure of an alternative oxidase (AOX) protein from *Trypanosoma brucei* in the presence of a stoichiometric inhibitor;

Figure 3 is a schematic figure showing the hydrophobic pocket and hydrogen-bonding of the inhibitor to the di-iron site of the alternative oxidase (AOX) using *Sauromatum* numbering;

Figure 4 is a sequence alignment of various alternative oxidases (AOX) of various species showing a consensus sequence. AXIB-ARATH is the AOX enzyme from *Arabidposis thaliana* (SEQ ID No:2), AOX1_SOYBN is the AOX enzyme from soybean (SEQ ID No:3), AOX1_TOBAC is the AOX enzyme from tobacco (SEQ ID No:4), AOX1A_ORYS is the AOX enzyme from *Oryza sativa*-rice (SEQ ID No:5), AOX1_SAUGU is the AOX enzyme from *Sauromatum guttatum* (SEQ ID No:6), AOX_CATRO is the AOX enzyme from *Catharanthus roseus* (SEQ ID No:7), AOX1_MANIN is the AOX enzyme from *Mangifera indica* (SEQ ID No:8), AOX_ZEAMA is the AOX enzyme from Maize (SEQ ID No:9), AOX1_CHLRE is the AOX enzyme from *Chlamydomonas reinhardtii* (SEQ ID No:10), AOX_NEUCR is the AOX enzyme from *Neuropsora crassa* (SEQ ID No:11), AOX_HANAN is the AOX enzyme from *Hansenula anomola* (SEQ ID No:12), AOX_TRYBB is the AOX enzyme from *Trypanosoma brucei* (SEQ ID No:13), and AOX_CHLSP is the AOX enzyme from *Chlamydomonas* species (SEQ ID No:14);

Figure 5 represents the chemical formula of Ascofuranone (left-hand side) and various embodiments of the AOX inhibitor according to the invention;

Figure 6 represents the chemical formula of Colletochlorin B;

Figure 7 shows the chemical synthesis of Colletochlorin B;

Figure 8 is a graph showing IC_{50} values for Colletochlorin B; and

Figure 9 is a graph showing the effects of Colletochlorin B on cytochrome bc_1 activity.

Examples

Materials & Methods

The alternative oxidase (AOX) protein was purified and crystallized according to the techniques outlined in Kido, Y. et al (2010) Biochim. Biophys. Acta 1797, 443-450, and in Kido, Y. et al (2010) Acta. Crystallogr. Sect. F Struct. Biol. Cryst. Commun., 66, 275-278. The crystal structure of the protein was obtained both in the presence and absence of an inhibitor using the vapour hanging-drop technique, as outlined in the papers given above. The inhibitor-binding site was identified from the crystal structure. Analysis of the residues surrounding the pocket revealed that L177, E178, L267, A271 and Y275 shown in Figure 3 are 100% conserved across all fungal, plant and trypanosomatid species.

Example 1 – Characterisation of the alternative oxidase (AOX) binding pocket

A major breakthrough in this study was the determination of the first ever crystal structure of an alternative oxidase (AOX) protein both in the presence and absence of a stoichiometric inhibitor, as illustrated in Figure 2. The inventors found that the pocket does not change, i.e. it is substantially the same in the presence or absence of the inhibitor. Knowledge of the crystal structure of AOX in the presence of an inhibitor put the inventors in a very powerful position to undertake some rational fungicidal molecular design, which, as discussed below, has resulted in the production of a library of AOX inhibitor compounds that have the capacity to act as phytopathogenic fungicides specifically targeted at the AOX.

Accordingly, once the inventors had generated the crystal structure of AOX shown in Figure 2, they went on to characterise the AOX quinone-binding pocket in detail using site-directed mutagenesis. Referring to Figure 3, there is shown the fully characterised quinone-binding site or pocket of the alternative oxidase enzyme (AOX) of the plant, *Sauromatum guttatum* (Voodoo Lily). The Figure also shows a representative inhibitor (Colletochlorin B) positioned inside the pocket. The six-membered ring of the inhibitor tightly interacts with the hydrophobic residues of the pocket, and the isoprenyl tail of the inhibitor interacts with Arg173, Glu270 and Ser274 residues of the pocket. Figure 3 also shows that the inhibitor binding pocket (R173, L177, E178, L267, E270, A271, S274 & Y275) is located near the membrane surface, and is within 4 Å of the active-site of the protein. It should be noted that the numbering on Figure 3 refers to the plant (i.e. *Sauromatum guttatum*) AOX protein, as all AOXs tend to be compared with this

protein. The head group aldehyde oxygen is hydrogen-bonded by Glu178 and Tyr275. Although not wishing to be bound by theory, these hydrogen bonds are believed to be important for the potent inhibitory activity of these compounds.

As discussed in the Examples below, the inventors have confirmed that the quinone-binding site of the AOX shown in Figure 4 is a promising target in the treatment of fungal pathogens. The inventors have prepared a sequence alignment of a number of AOX enzymes, which is shown in Figure 4, and it can be seen that the architecture of the AOX binding-site is highly conserved across all AOXs, irrespective of the species from which they are derived. The boxed residues in Figure 4 represent AOX residues which are involved in inhibitor binding. The Arg173, Glu270 and Ser274 residues shown in Figure 3 are less conserved. However, as these residues are only involved in the binding of the tail, variation is believed to be less significant with respect to inhibitor sensitivity.

Thus, the detailed knowledge of the nature of the binding site of the AOX of *S. guttatum* is important, as it has revealed that there is a common architecture that can be applied to quinol-binding sites in general, and hence provides further insight into the mechanism of binding. More importantly, this information has assisted in the rational design of phytopathogenic fungicides and human parasites that are specifically targeted to the alternative oxidase.

Based on this information, the inventors set out to design and synthesize a new library of AOX inhibitors, and also to gain further detailed structural knowledge of the nature of the protein-ligand interaction and kinetics. They also tested the extent to which structurally modified inhibitors targeted at the AOX could also inhibit the fungal Qo site, thereby providing a new generation of dual-mode fungicides.

Example 2 – Design and synthesis of AOX inhibitors

The inventors have designed and synthesised a number of AOX inhibitors based on the compound, ascofuranone, the chemical structures of which are illustrated in Figure 5. Ascofuranone has a complex synthetic route, and has a reactive aldehyde group (-CHO). Several ascofuranone derivatives were synthesised, namely Colletochlorin B (labelled structure "2" in Figure 5, where R is CHO), compound "3" shown in Figure 5, where R is CH_2OH, and 4a-4h, where R is as shown in Figure 5.

The inhibitory effects of some of these compounds were assessed, and the results are summarised in Table 1.

Table 1 – Inhibitory effects on recombinant AOX protein

Inhibitor	IC50
Ascofuranone	58pM
Colletochlorin B	165pM
Octyl Gallate	105nM
Salicylhydroxamic acid (SHAM)	7μM

Table 1 summarises the concentration of inhibitor required to reduce the respiration of purified recombinant AOX protein by 50%. Respiration was measured as the rate of oxygen consumption in the presence of 1mM NADH as substrate and the numbers represent the final I_{50} concentration of the inhibitor.

Of the derivatives that were synthesized, Colletochlorin B was one of the most promising candidates, because it has an IC_{50} value of approx 165pM (IC_{50} for Ascofuranone 58pM) when tested upon recombinant AOX proteins. This inhibitor was specific for membrane-bound and purified AOX, and did not appear to inhibit other quinol oxidases. Furthermore, Colletochlorin B can also be synthesized by a simple two-step process, which is a significant advantage over ascofuranone.

Example 3 – Synthesis of Colletochlorin B

The chemical structure of Colletochlorin B is shown in Figure 6, and the method used for its synthesis is shown in Figure 7.

Step 1: Compound (1) to compound (2)

Orcinol (5g, 40mmol) and $Zn(CN)_2$ (7.1g, 60mmol) were placed into a 3 necked flask with mechanical stirrer under N_2. 50ml of Ether was added, and the reaction was saturated with HCl gas. After 2 hours, the Ether was decanted off and 50mls of water added to the reaction mixture. This was heated to 100°C where the product crashed out of solution. The crude product was collected via buchner filtration, and recrystallised from water to yield the aldehyde (4.6g) in 76% yield.

Step 2: Compound (2) to compound (3)

Orcinol Aldehyde (527mg, 3.5 mmol) was put under N_2 and dissolved in anhydrous ether (60ml) on an ice bath. SO_2Cl_2 (1.35ml, 4.7mmol) was diluted in ether (15ml) and

then added dropwise over 15minutes. The reaction was left to stir overnight, and quenched with the addition of water. The Ether layer was washed with 0.1M $NaHCO_3$ and water, then dried over $MgSO_4$ and concentrated under vacuum. The crude solid was then purified via flash chromatography (Toluene: Ethyl acetate 2:1 -> 1:1) to obtain the product (459mg) in 75% yield.

Step 3: Compound (3) to compound (4)

3-chloro-4,6-dihydroxy-2-methyl-benzaldehyde (150mg, 0.8mmol) was dissolved in 10% KOH (0.9ml, 0.8mmol) yielding a deep red solution. The reaction was placed on an ice bath, and geranyl bromide (0.39ml, 1.6mmol) was added. The reaction was stirred vigorously overnight, and extracted with ether. The organic layer was washed with $NaHCO_3$ and brine, before being concentrated under vacuum. The resultant oil was purified via flash chromatography (petrol ether 40-60 : ether 10:1 -> 3:1) to obtain pure Colletochlorin B (52mg) in 20% yield.

Example 4 – Characterisation of Colletochlorin B

The inventors have confirmed that whilst the hydroxyl groups and the chlorine and methyl substituents on the benzene ring of the inhibitor are believed to be important for high potency, the furanone moiety is redundant as long as a hydrophobic side chain, such as the geranyl group, is retained, as in Colletochlorin B (2).

However, the aldehyde group present in ascofuranone (1) and Colletochlorin (2) is believed to represent a problem for anti-parasitic design for several reasons. Besides their ability to function as hydrogen bond acceptor and to undergo dipole-dipole interaction with AOX, aldehyde groups are chemically reactive enough to undergo reversible covalent modifications and would be generally unsuited to standard pharmaceutical formulations. Furthermore, aldehydes are prone to metabolic oxidation to the respective carboxylic acid with the concomitant non-specific binding to basic transport proteins. Therefore, the inventors set out to remove this aldehyde group using the reducing agent $NaBH_4$, as shown in Figure 5. Synthesis and analysis of an alcohol-derivative (3) revealed that its inhibitory properties are retained, which is why the various aldehyde bioisosteres, which are represented as compounds 4a-4h in Figure 5, were produced.

Example 5 - Site-directed mutagenesis studies

The inventors have generated mutants of E123 and Y220, and have demonstrated that they are important for enzyme activity and inhibitor-binding.

Site-directed mutagenesis and plasmid construction

Construction of pREP1-AOX, pREP1-E123A and pREP1-Y220F (used to express wild type AOX and the E123A and Y220F mutants in *S. pombe*) has been described previously [M.S. Albury, C. Affourtit, P.G. Crichton, A.L. Moore, Structure of the plant alternative oxidase - Site-directed mutagenesis provides new information on the active site and membrane topology, J. Biol. Chem. 277 (2002) 1190-1194: M.S. Albury, P. Dudley, F.Z. Watts, A.L. Moore, Targeting the plant alternative oxidase protein to *Schizosaccharomyces pombe* mitochondria confers cyanide-insensitive respiration, J. Biol. Chem. 271 (1996) 17062-17066.]. Mutagenesis of AOX was performed using the Quick Change mutagenesis kit (Stratagene) according to manufacturer's instructions, with plasmid pSLM-AOR [M.S. Albury, C. Affourtit, P.G. Crichton, A.L. Moore, Structure of the plant alternative oxidase - Site-directed mutagenesis provides new information on the active site and membrane topology, J. Biol. Chem. 277 (2002) 1190-1194]. Each full length mutant AOX was excised on a *Bsp*HI-*Bam*HI fragment and ligated to the yeast expression vector pREP1/N (a modified version of pREP1 [K. Maundrell, *Nmt1* of fission yeast - a highly transcribed gene completely repressed by thiamine, J. Biol. Chem. 265 (1990) 10857-10864.] in which the *Nde*I site was replaced with *Nco*I) which had been digested with *Nco*I and *Bam*HI, yielding pREP1-E123A and pREP1-Y220F.

Results

Table 2

Condition	Activity (nmol oxygen/min/mg protein	% Inhibition (compared to wt)
pREP1-AOX - wt	55	0
pREP1-E123A – E123 mutant	2	96
pREP1-Y220F – Y220 mutant	0	100

Activity was measured as oxygen consumed/min/mg protein using NADH as substrate when isolated yeast mitochondria (*Schizosaccharomyces pombe)* containing the wild-

type and mutant form of the AOX. Note that the inhibitor does not bind to the mutant forms of the oxidase.

Example 6 - Effect of Colletochlorin B on cytochrome bc_1 respiratory activity

Colletochlorin B and its derivatives have been demonstrated in Examples 1-5 to be a specific inhibitor of the alternative oxidase (AOX) in plants and fungi. Following on from this work, the inventors set out to test whether or not these compounds also have any effect on the respiratory activity of cytochrome bc_1 complex. Mitochondria from two sources (rat liver and potato) were titrated with Colletochlorin B (CB), as indicated in typical data summarised in Figure 9. Both mitochondrial sources did not contain any alternative oxidase (AOX), and so the respiratory activity measured must have been from the cytochrome bc_1 complex.

Respiratory activity was measured in a medium containing 0.3M mannitol, 10mM KCl, 5mM $MgCl_2$, 1mM potassium phosphate and 10mM MOPS (3-(N-morpholino)propanesulfonic acid) buffer pH7.4. Either 0.9mg of rat liver mitochondria respiring on 5mM succinate or 0.3mg potato mitochondria respiring on 1mM NADH were used. Respiration was measured using a Rank oxygen electrode of 0.4ml volume at 25°C in the presence of 1µM CCCP (Carbonyl cyanide m-chloromethoxy phenylhydrazone). The results were compared with ascochlorin (a known bc_1 inhibitor) and azoxystrobin (a commercial fungicide targeted at the bc_1 complex).

Results

Table 3 - IC_{50} (Rat liver mitochondria)

Compound	**IC_{50}/nM**
Ascochlorin	187
Colletochlorin B	515
Azoxystrobin	525

Table 4 - IC_{50} (Potato mitochondria)

Compound	**IC_{50}/µM**
Ascochlorin	0.5
Colletochlorin B	0.75
Azoxystrobin	1.4
Ascofuranone	30

Tables 3 and 4 summarise the concentration of inhibitor that was required to reduce the respiration of cytochrome bc_1 complex (in the absence of AOX) by 50%. Respiration was measured as the rate of oxygen consumption in the presence of 1mM NADH or 5mM succinate as substrate, and the numbers represent the final IC_{50} concentration of the inhibitor tested. The lower the IC_{50} value the better, since it means that a lower amount of the compound is needed to halve the respiratory activity.

Of the inhibitors that were synthesized, Colletochlorin B was a promising candidate, because it has a lower IC_{50} value (approx 515nM) than Azoxystrobin (approx 525nM) when tested on rat liver mitochondria. When tested on potato mitochondria, the IC_{50} value of Colletochlorin B was only 0.75μM, which was half of the IC_{50} of Azoxystrobin (approx 1.4μM), and significantly less than the IC_{50} of Ascofuranone (approx 30μM).

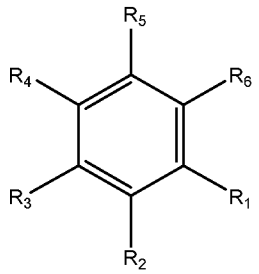
Conclusions

Careful titration using mitochondria in which the alternative oxidase is absent has surprisingly revealed that the compound is a specific inhibitor of the cytochrome bc_1 complex in addition to inhibiting AOX. The data suggest that the compound inhibits the cytochrome bc_1 complex at both the Qo and Qi binding-sites of this complex thereby making it a very potent inhibitor of respiration even in the absence of the alternative oxidase. The implications of such a finding suggest that derivatives of this compound would be very specific and potent dual function fungicide, as not only do they inhibit the alternative oxidase (AOX), but also the cytochrome bc_1 complex.

It will be appreciated that commercially available fungicides, such as azoxystrobin, against which Colletochlorin B has been tested herein, inhibit only one site (qo) within the bc1 complex. Accordingly, since Colletochlorin B inhibits the cytochrome bc_1 complex at both the Qo and Qi binding-sites, and also the AOX, this compound and its derivatives can act as a highly potent and robust inhibitor.

CLAIMS

1. Use of a compound, for inhibiting a microbial alternative oxidase (AOX) and/or cytochrome bc_1 complex, wherein the compound is represented by formula I:-



[Formula I]

wherein R^1 is selected from a nitrile group, an alkyl, alkenyl, amine group with 1 to 4 C-atoms that is optionally mono- or polysubstituted by F, O, NH_2 or CN, and in which one or more non-adjacent CH_2 groups are optionally replaced, in each case independently from one another, by -O-, -NH-, -CO-, -COO-, or -OCO-;

R^2 is hydrogen or a hydroxy or alkoxy group with 1 to 3 C atoms;

R^3 is a straight chain or branched alkyl or alkylene with 4 to 20 C atoms, that is optionally mono- or polysubstituted by a C_1 to C_4 alkyl group;

R^4 is hydrogen or a hydroxy or alkoxy group with 1 to 3 C atoms;

R^5 is a halogen group; and

R^6 is H or a C_1 to C_4 alkyl group;

with the proviso that at least one of R^2 and R^4 is a hydroxy or alkoxy group with 1 to 3 C atoms.

2. Use according to claim 1, wherein R^1 is a group selected from: CHO; CH_2OH; CN; CH_3; $C(O)NH_2$; $C(O)NHCH_3$; $C(O)CH_3$; CF_2CH_3; CH_2CH_3; CH_2OAc; COOH; and $COOCH_3$.

3. Use according to either claim 1 or claim 2, wherein R^2 is a short-chain alkyl, for example a methyl, ethyl or propyl group,

4. Use according to either claim 1 or claim 2, wherein R^2 is a hydroxyl group.

5. Use according to any preceding claim, wherein R^3 is a straight chain or branched alkyl or alkylene with 6 to 15 C atoms, 8 to 12 C atoms or 8 to 10 C atoms, that is optionally mono- or polysubstituted by a C_1 to C_4 alkyl group.

6. Use according to any preceding claim, wherein R^3 is a branched diene having 6 to 15 C atoms that is substituted with at least one, and preferably two, methyl groups.

7. Use according to any preceding claim, wherein R^4 is a methyl, ethyl or propyl group.

8. Use according to any preceding claim, wherein R^4 is a hydroxyl group.

9. Use according to any preceding claim, wherein R^5 is a chlorine, bromine, fluorine or iodine group.

10. Use according to any preceding claim, wherein R^5 is a chlorine group.

11. Use according to any preceding claim, wherein R^6 is a methyl, ethyl or propyl group.

12. Use according to any preceding claim, wherein R^4 is a methyl group.

13. Use according to any preceding claim, wherein:-
R^1 is selected from CHO; CH_2OH; CN; CH_3; $C(O)NH_2$; $C(O)NHCH_3$; $C(O)CH_3$; CF_2CH_3; CH_2CH_3; CH_2OAc; COOH; and $COOCH_3$; and wherein
R^2 is a hydroxyl group;
R^3 is a straight chain or branched alkyl or alkylene with 4 to 20 C atoms, that is optionally mono- or polysubstituted by a C_1 to C_4 alkyl group;
R^4 is a hydroxyl group;
R^5 is a chlorine atom; and
R^6 is H or a C_1 to C_4 alkyl group.

14. Use according to any preceding claim, wherein:-
R^1 is selected from CHO; CH_2OH; CN; CH_3; $C(O)NH_2$; $C(O)NHCH_3$; $C(O)CH_3$; CF_2CH_3; CH_2CH_3; CH_2OAc; COOH; and $COOCH_3$; and wherein
R^2 is a hydroxyl group;

R^3 is a straight chain or branched alkyl or alkylene with 6 to 15 C atoms, that is optionally mono- or polysubstituted by a C_1 to C_2 alkyl group;
R^4 is a hydroxyl group;
R^5 is a chlorine atom; and
R^6 is H or a C_1 to C_4 alkyl group.

15. Use according to any preceding claim, wherein:-
R^1 is selected from CHO; CH_2OH; CN; CH_3; $C(O)NH_2$; $C(O)NHCH_3$; $C(O)CH_3$; CF_2CH_3; CH_2CH_3; CH_2OAc; COOH; and $COOCH_3$; and wherein
R^2 is a hydroxyl group;
R^3 is an alkylene chain having 8 to 10 C atoms, and is substituted with at least one methyl group, preferably two methyl groups;
R^4 is a hydroxyl group;
R^5 is a chlorine atom; and
R^6 is a methyl group.

16. Use according to any preceding claim, wherein R^1 is not a CHO group.

17. Use according to any preceding claim, wherein the compound inhibits a fungal, bacterial or protist AOX and/or cytochrome bc_1 complex.

18. Use according to any preceding claim, wherein the compound inhibits a fungal AOX and/or cytochrome bc_1 complex.

19. Use according to any preceding claim, wherein the compound inhibits the Qo or Qi binding site of the cytochrome bc_1 complex.

20. Use according to any preceding claim, wherein the compound inhibits the Qo and Qi binding sites of cytochrome bc_1 complex.

21. A compound as defined in any one of claims 1-20, for use in inhibiting a microbial alternative oxidase (AOX) and/or cytochrome bc_1 complex.

22. Use of a compound as defined in any one of claims 1-20, as an agrochemical.

23. An agrochemical composition comprising the compound as defined in any one of claims 1-20.

24. Use of the agrochemical composition according to claim 23, for treating an agrochemical disease or infection caused by an organism selected from a group of organisms consisting of: *Chalara fraxinea*; *Septoria tritici*; *Gaeumannomyces gramminis var titici*; *Magnaporthe grisea*; *Magnaporthe oryzae*; *Rhizoctonia solani*; *Botrytis cinerea*; *Fusicladium effusum* syn. *Cladosporium caryigenum and Fusicladosporium effusum*; *Carya illinoinensis*; *Podosphaera fusca*; *Microdochium nivale*; *Microdochium majus*; *Septoria nodoum*; *Tapesia acuformis*; and *Metarhizium anisopliae*.

25. The compound as defined in any one of claims 1-20, for use in therapy or diagnosis.

26. The compound as defined in any one of claims 1-20, for use in treating a microbial infection.

27. The compound according to either claim 25 or claim 26, wherein the compound is for use in treating a bacterial infection, for example a Gram-positive or a Gram-negative bacterial infection.

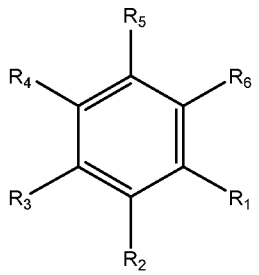
28. The compound according to either claim 25 or claim 26, wherein the compound is for use in treating a fungal infection.

29. The compound according to claim 28, wherein the compound is for use in treating a disease associated with human pathogens, such as intestinal disease; Leishmaniasis; Candidiasis; or diseases associated with contact lens usage.

30. An antimicrobial composition comprising a compound as defined in any one of claims 1-20, and a pharmaceutically acceptable vehicle.

31. A process for making the antimicrobial composition according to claim 30, the process comprising contacting a therapeutically effective amount of a compound according to any one of claims 1-20, and a pharmaceutically acceptable vehicle.

32. A microbial alternative oxidase (AOX) and/or cytochrome bc_1 complex inhibitor, of formula I:-



[Formula I]

wherein R^1 is selected from a nitrile group, an alkyl, alkenyl, amine group with 1 to 4 C-atoms that is optionally mono- or polysubstituted by F, O, NH_2 or CN, and in which one or more non-adjacent CH_2 groups are optionally replaced, in each case independently from one another, by -O-, -NH-, -CO-, -COO-, or -OCO-;
R^2 is hydrogen or a hydroxy or alkoxy group with 1 to 3 C atoms;
R^3 is a straight chain or branched alkyl or alkylene with 4 to 20 C atoms, that is optionally mono- or polysubstituted by a C_1 to C_4 alkyl group;
R^4 is hydrogen or a hydroxy or alkoxy group with 1 to 3 C atoms;
R^5 is a halogen group; and
R^6 is H or a C_1 to C_4 alkyl group;
with the proviso that at least one of R^2 and R^4 is a hydroxy or alkoxy group with 1 to 3 C atoms.

33. An inhibitor according to claim 32, wherein the inhibitor is as defined in any one of claims 1-20.

Figure: 1

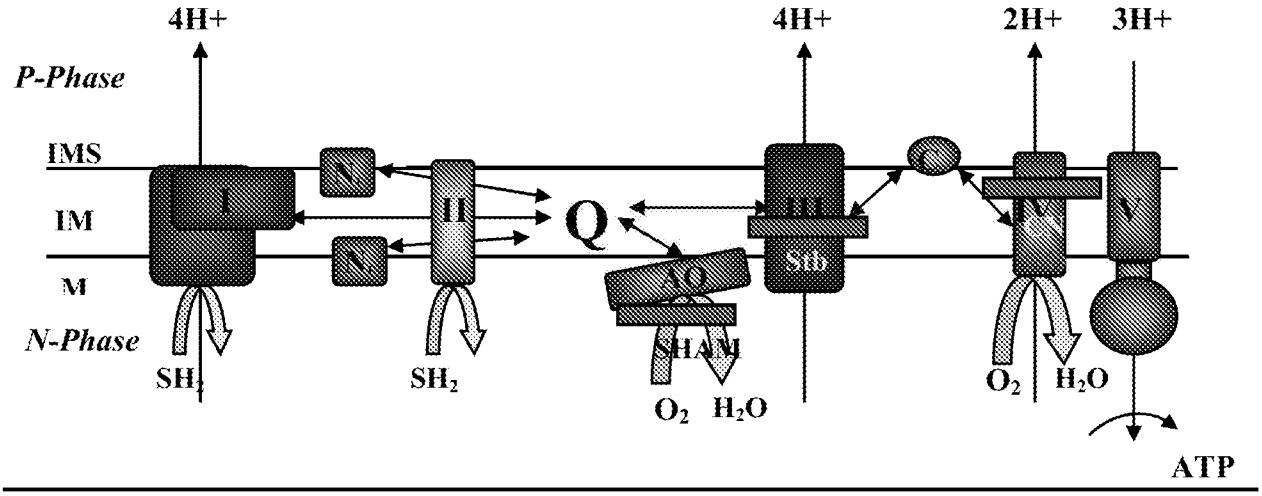


Figure: 2

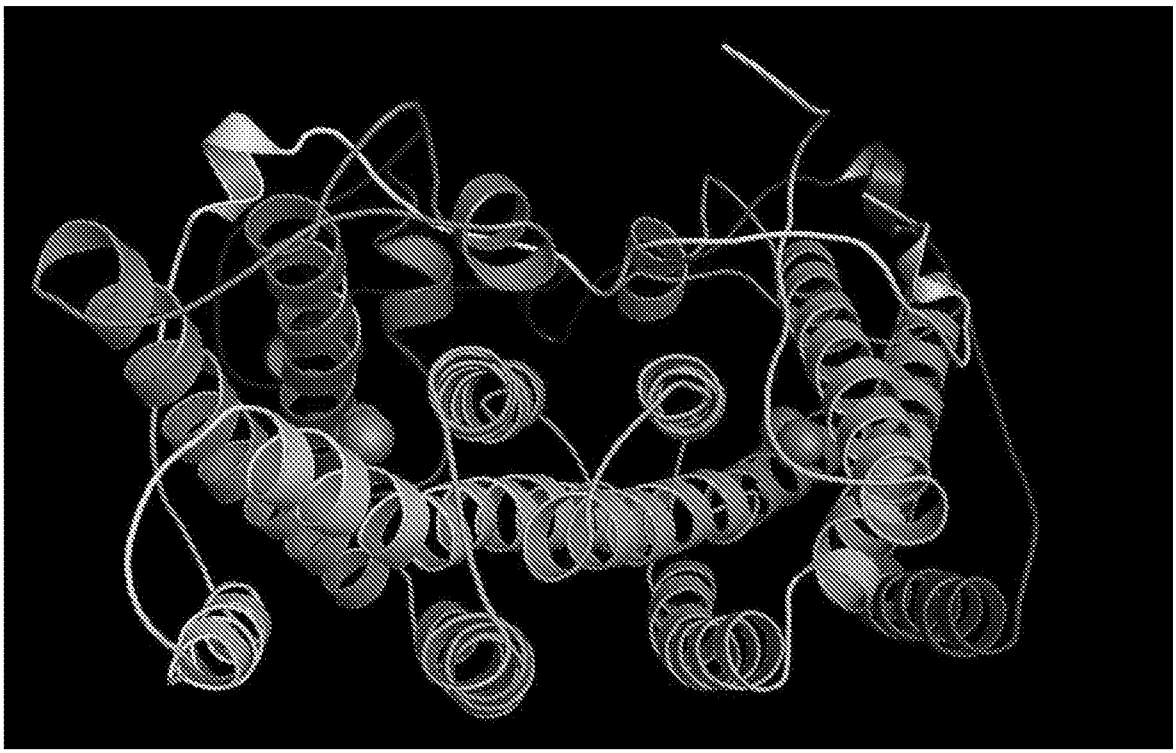


Figure: 3

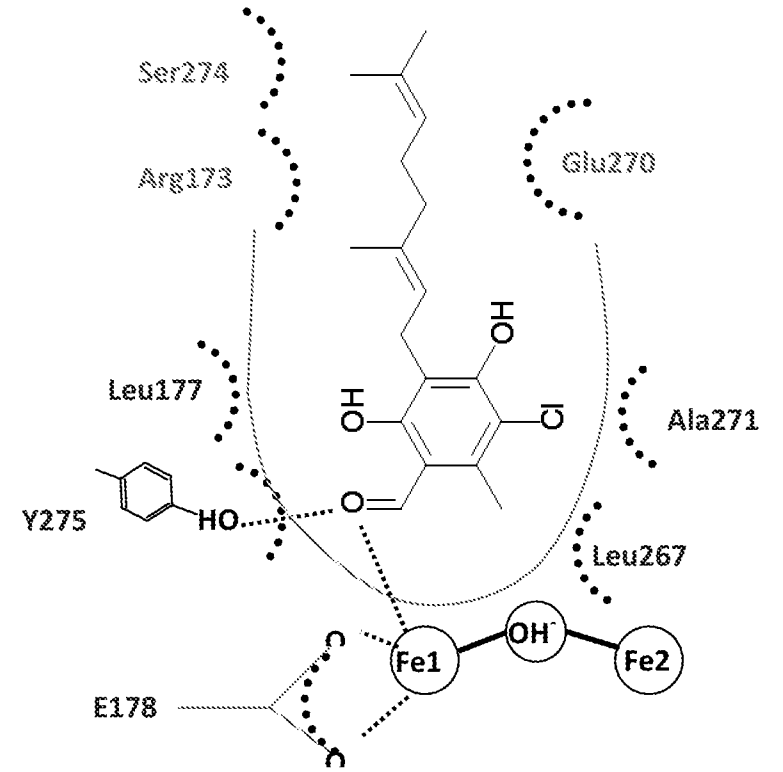

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Figure: 4

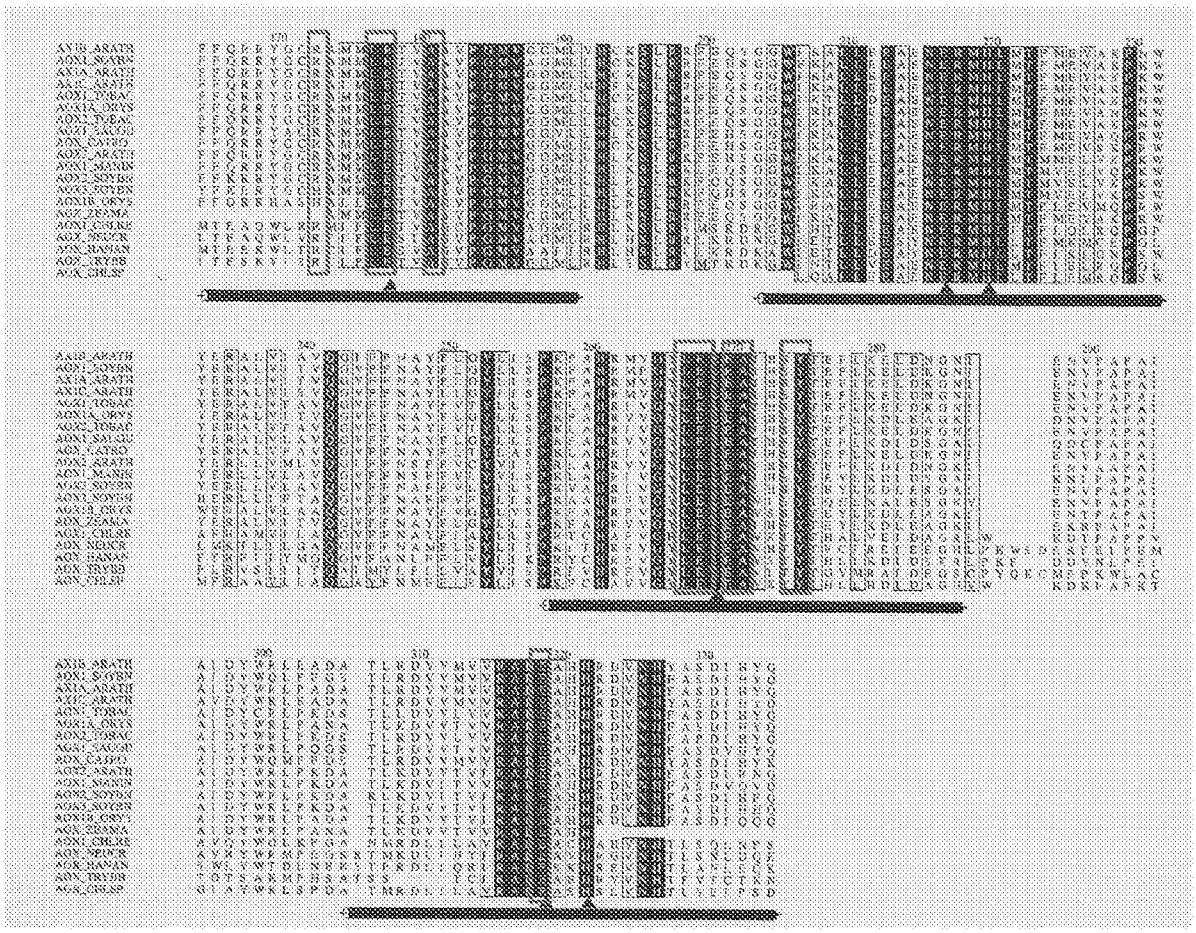


Figure: 5

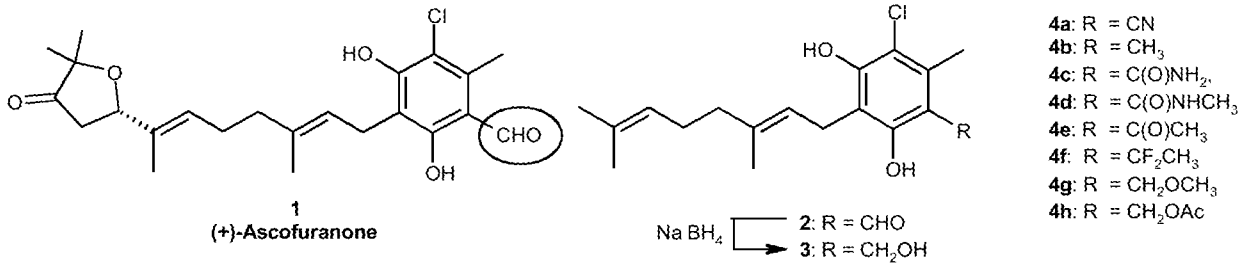


Figure: 6

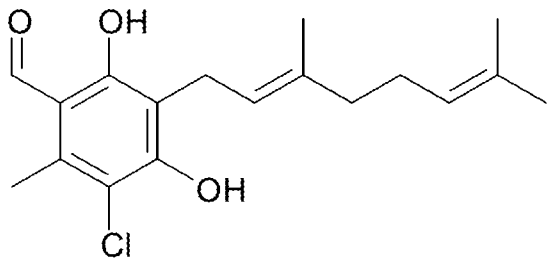


Figure: 7

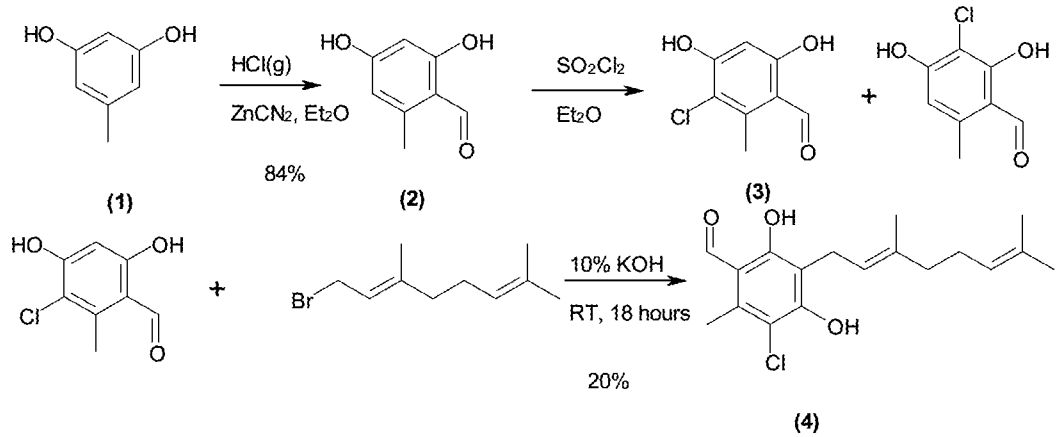


Figure: 8

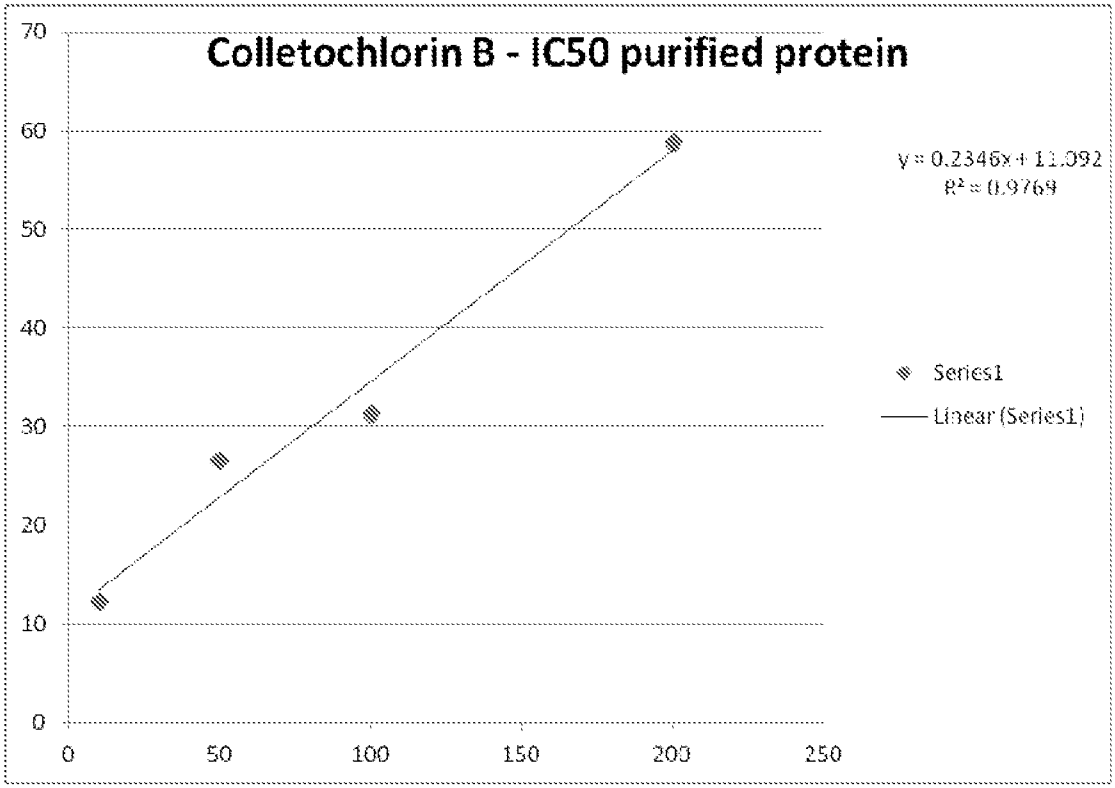
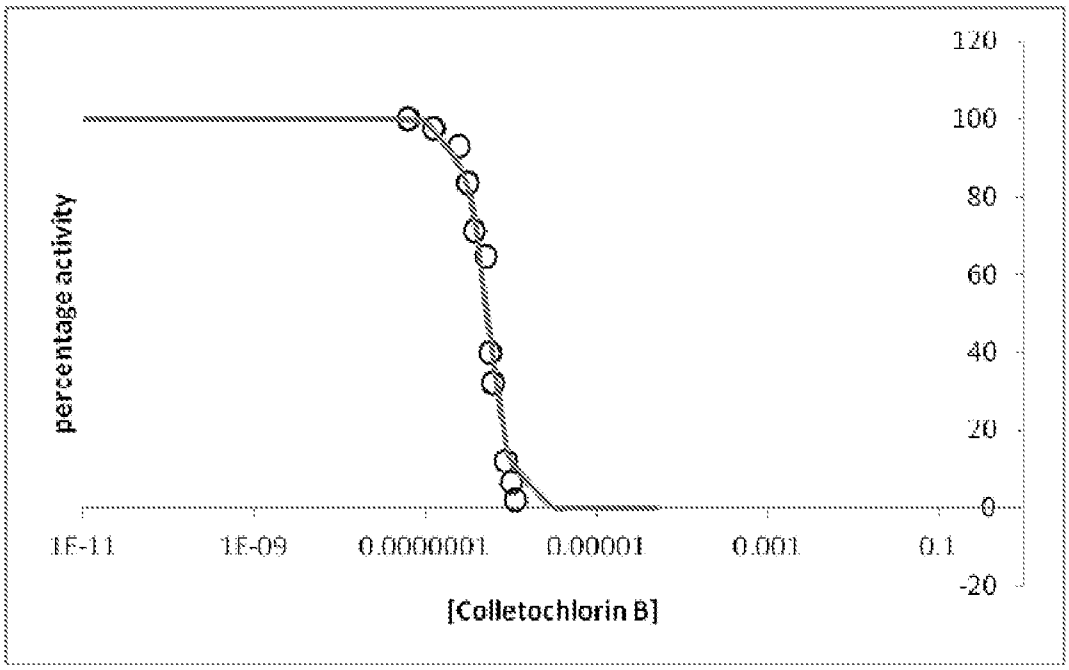


Figure: 9



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/051030

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07C39/24 A01N35/04 A61K31/11 C07C69/63 C07C39/373
C07C43/178 C07C47/565 C07C49/835 A61P31/04 A61P31/10
C07C255/53 C07C235/60 A01N49/00

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)
C07C A01N A61K

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NORIKO TAKAHASHI ET AL: "Differentiation induction of human promyelocytic leukemia cells with colletochlorin B and its analogues.", CHEMICAL & PHARMACEUTICAL BULLETIN, vol. 36, no. 1, 1 January 1988 (1988-01-01), pages 452-455, XP055073479, ISSN: 0009-2363, DOI: 10.1248/cpb.36.452 abstract table 1; compounds 1,2,5,7 page 454, line 8 - line 14 ----- -/--	32,33

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 August 2013	08/08/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Lacombe, Céline

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/051030

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SHOHEI HAYAKAWA ET AL: "THE ILICICOLINS, ANTIBIOTICS FROM CYLINDROCLADIUM ILICICOLA", THE JOURNAL OF ANTIBIOTICS, vol. 24, no. 9, 1 January 1971 (1971-01-01), pages 653-654, XP055073497, ISSN: 0021-8820, DOI: 10.7164/antibiotics.24.653 the whole document -----	1-21,23, 25-27, 30-33
X	MOGI T ET AL: "Antibiotics LL-Z1272 identified as novel inhibitors discriminating bacterial and mitochondrial quinol oxidases", BIOCHIMICA ET BIOPHYSICA ACTA. BIOENERGETICS, AMSTERDAM, NL, vol. 1787, no. 2, 1 February 2009 (2009-02-01), pages 129-133, XP025942205, ISSN: 0005-2728, DOI: 10.1016/J.BBABIO.2008.11.016 [retrieved on 2009-02-01] abstract paragraph [0001]; figure 1; table 1 -----	1-23, 25-33
X	BERRY E A ET AL: "Ascochlorin is a novel, specific inhibitor of the mitochondrial cytochrome bc1 complex", BIOCHIMICA ET BIOPHYSICA ACTA. BIOENERGETICS, AMSTERDAM, NL, vol. 1797, no. 3, 1 March 2010 (2010-03-01), pages 360-370, XP026879265, ISSN: 0005-2728 [retrieved on 2009-12-16] abstract paragraph [0004]; figures 1,8; table 1 -----	1-31
X,P	H. SAIMOTO ET AL: "Pharmacophore identification of ascofuranone, potent inhibitor of cyanide-insensitive alternative oxidase of Trypanosoma brucei", JOURNAL OF BIOCHEMISTRY, vol. 153, no. 3, 23 December 2012 (2012-12-23), pages 267-273, XP055073541, ISSN: 0021-924X, DOI: 10.1093/jb/mvs135 paragraph [0002]; figure 2; tables 2,4,8,9,10 -----	1-33