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(54) **Title**: COMPOUNDS AND USES THEREOF IN THE TREATMENT/PREVENTION OF GRAM-NEGATIVE BACTERIAL INFECTIONS

(57) **Abstract**: The present disclosure relates to compounds of formula I in the treatment and/or prevention of a Gram-negative bacterial infection in a multicellular organism. The disclosure also relates to a pharmaceutical composition comprising said compound, a method of treatment and/or prevention of said infection comprising the administration of said compound, and the use of said compound for the manufacture of a medicament for treating and/or preventing said infection. The disclosure also relates to the modulation of the activity of a type three secretion system in a Gram-negative bacterium.

Compounds and uses thereof in the treatment/prevention of Gram-negative bacterial infections

Field of the invention

The present disclosure relates to compounds for use in the treatment and/or prevention of an infection caused by a Gram-negative bacterium. The disclosure also relates to a pharmaceutical composition comprising said compounds, a method of treatment and/or prevention comprising administering said compound, and use of said compound in the preparation of a medicament.

Background

Antibiotic resistance

Antibiotics and similar drugs, together called antimicrobial agents, have been used for the last 70 years to treat patients with infectious diseases. Since the 1940s, these drugs have greatly reduced illness and death from infectious diseases. Antibiotic resistance is a form of drug resistance by which bacteria are able to survive exposure to one or more antibiotics.

In modern medicine, the emergence of resistant bacteria is a major problem, largely due to the misuse and overuse of antibiotics. For example, antibiotics are prescribed by doctors as treatment for viral infections, such as the common cold, and are even sold over the counter without a prescription in some countries. Furthermore, the addition of antibiotics to livestock feed contributes to increasing antibiotic resistance. The sheer volume of antibiotics used is the major factor in increasing rates of bacterial resistance.

Some clinically relevant pathogens have developed resistance to multiple antibiotics, and are referred to as multidrug resistant (MDR) pathogens, such as MRSA (methicillin-resistant *Staphylococcus aureus*), VISA (vancomycin-intermediate *S. aureus*), VRSA (vancomycin-resistant *S. aureus*), ESBL (extended spectrum beta-lactamase), VRE (vancomycin-resistant *Enterococcus*), MRAB (multidrug-resistant *Acinetobacter baumannii*) and multidrug-resistant *Pseudomonas aeruginosa*. Antibiotic resistance has

become an increasing problem in hospital environments, where a group of bacteria named "ESKAPE" pathogens (*Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumanii*, *P. aeruginosa*, and *Enterobacter* species) is causing the majority of these problems (Boucher *et al.* (2009) Clin Infect Dis 48:1-12).

Infections by organisms that are resistant to various antimicrobial agents pose a serious challenge to effective management of infections. Resistance to antimicrobial agents, which may be intrinsic or acquired, has been noted in a wide variety of microorganisms causing human infections. In both immunocompetent as well as immunocompromised individuals, with or without other associated co-morbidities, MDR pathogens may manifest as life threatening infections.

Additionally, "pan-resistant" strains (strains resistant to all available antibiotics) are still uncommon but are occurring.

As resistance towards antibiotics becomes more common, a greater need for alternative treatments arises. However, despite a push for new antibiotic therapies, there has been a continued decline in the number of newly approved drugs (Donadio *et al.* (2010) J Antibiot 63(8):423-430). Antibiotic resistance therefore poses a significant problem.

Spreading of antibiotic resistance

As previously mentioned, the increasing prevalence of antibiotic-resistant bacterial infections seen in clinical practice is considered to stem from antibiotic use both within modern medicine and agriculture. Any use of antibiotics can increase selective pressure in a population of bacteria, which allows the resistant bacteria to thrive and the susceptible bacteria to die off.

Antibiotic resistance may arise by spontaneous or induced genetic mutation, or by the acquisition of resistance genes from other bacterial species by horizontal gene transfer via conjugation, transduction or transformation. Many antibiotic resistance genes reside on transmissible plasmids which facilitate their transfer. Exposure to an antibiotic naturally selects for the survival of the organisms with the genes for resistance. In this

way, a gene for antibiotic resistance may readily spread through an ecosystem of bacteria. Antibiotic-resistance plasmids frequently contain genes conferring resistance to several different antibiotics. Some Gram-negative bacteria, for example *P. aeruginosa,* harbor an intrinsic resistance due to low penetration across their outer membrane and multi-drug efflux pumps that actively transport antibiotics out of the bacterial cells.

Gram-negative bacteria

Gram-negative bacteria, including *Escherichia coli* (*E. coli*), *Salmonella*, *Shigella*, *Pseudomonas*, *Helicobacter*, *Legionella* and numerous others, have evolved many mechanisms of attaching to, and invading, host epithelial and immune cells. In particular, many outer membrane proteins (OMPs) are involved in this initial interaction between the pathogen and its host.

The outer membrane (OM) of Gram-negative bacteria performs the crucial role of providing an extra layer of protection to the organism without compromising the exchange of material required for sustaining life. The pathogenic capability of Gram-negative bacteria is often associated with certain components of the OM, particularly the lipopolysaccharides (known as LPS or endotoxins) on its outside. The OM protects the bacteria from several antibiotics, dyes and detergents that would normally damage the inner membrane or peptidoglycan cell wall. The OM provides these bacteria with resistance to lysozyme and penicillin.

Many Gram-negative bacteria possess a type three secretion system, abbreviated TTSS or T3SS and also known as the injectisome or injectosome (Rosqvist *et al.* (1994) Embo J 13:964-72). Regulation of the T3SS machinery in *Yersinia pseudotuberculosis* is relatively well understood (Dewoody *et al.* (2013) Front Cell Infect Microbiol 3:4). Many of the genes required for the T3SS are carried on a 70-kbp plasmid. Most of the genes have one of three designations; Ysc (*Yersinia* secretion proteins), Yops (*Yersinia* outer proteins) or Sycs (specific yop chaperones). The machinery can be compared to a syringe that injects effector proteins from the bacteria directly into the

cytoplasm of the eukaryotic cell. The T3SS has been shown to be essential for the pathogenicity of many of these bacteria, and defects in the T3SS may render a bacterium non-pathogenic.

The T3SS is composed of approximately 30 different proteins, and its structure shows many similarities with bacterial flagella, which are long, rigid, extracellular structures used for motility. Some of the proteins participating in the T3SS share amino acid sequence homology to flagellar proteins. Some of the bacteria possessing T3SS also have flagella and are motile (for example *Salmonella and Pseudomonas*), while others do not (for example *Shigella*).

Alternative therapeutic approaches

Large efforts are currently being put into the development of new antimicrobial drugs, such as development of antibacterial vaccines, phage therapy, nanoparticles and new analogs of currently available drugs. Further, limiting the use of currently available drugs is also a priority.

For example, attempts to develop vaccines against bacterial infections have been made. While theoretically promising, anti-staphylococcal vaccines have shown limited efficacy due to immunological variation between *Staphylococcus* species and a limited duration of effectiveness of the antibodies produced (Verkaik *et al.* (2011) Immunotherapy 3:1063-73).

Phage therapy is the therapeutic use of lytic bacteriophages to treat pathogenic bacterial infections, and may prove to be an important alternative to antibiotics for treating multidrug resistant pathogens. Phage therapy is, however, still unavailable for clinical use (Burrowes *et al.* (2011) Expert Rev Anti Infect Ther 9:775-85).

A number of studies aimed at developing new analogs of known antibiotics, e.g. oxazolidinones, glycopeptides, quinolones, aminoglycosides, tetracyclines and ketolides have been reported (reviewed in Theuretzbacher (2011) Curr Opin Pharmacol 11:433-438). Furthermore, plant-derived compounds, metal nanoparticles and bacteriophage lysins may also be considered for use as new antimicrobial drugs due to their proven and substantial antibacterial effect, which, however, is significantly weaker than

that of common antibiotics in use today (Hemaiswarya *et al.* (2008) Phytomedicine 15:639-52).

In summary, rising antibiotic resistance is one of the greatest medical challenges of the twenty-first century. This creates an urgent need for innovative antimicrobial agents, where the efficacy would not be affected by current resistance mechanisms.

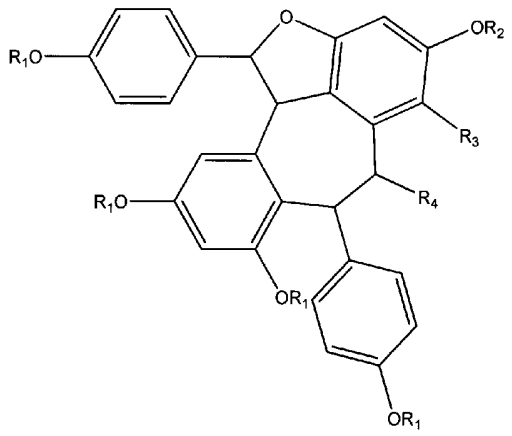
Summary of the Invention

It is an object of the present disclosure to provide compounds for use in treatment and/or prevention of an infection caused by Gram-negative bacteria while alleviating the above-mentioned and other drawbacks of current therapies.

It is another object of the present disclosure to provide a compound which may allow treatment and/or prevention of an infection caused by Gram-negative bacteria that are resistant to current antibiotic treatments.

These and other objects, which are evident to the skilled person from the present disclosure, are met by the different aspects of the invention as claimed in the appended claims and as generally disclosed herein.

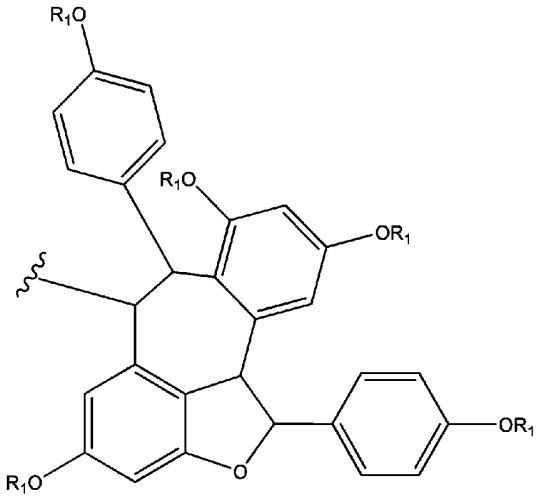
Thus, in the first aspect of the present disclosure, there is provided a method of treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism, comprising the step of administering a compound of Formula I, or a pharmaceutically acceptable salt thereof, to the multicellular organism:



Formula I

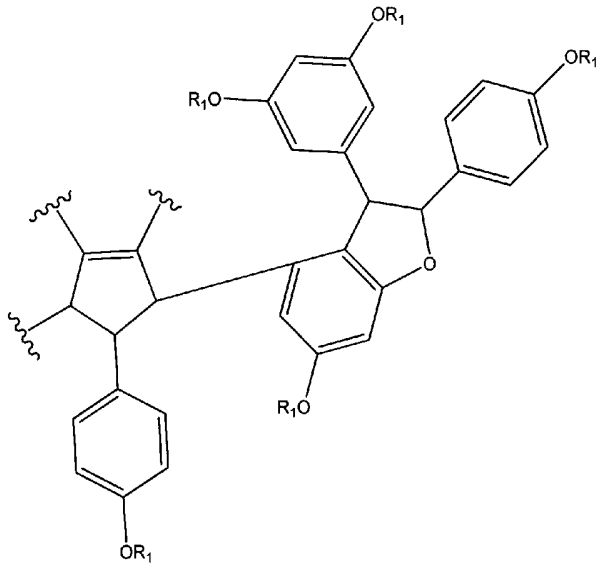
wherein, R_1 and R_2 are independently selected from the group consisting of hydrogen, C_1 to C_{12} alkyl, C_1 to C_{12} alkenyl, aryl, C_1 to C_{12} aldehyde, C_1 to C_{12} alkanone, C_1 to C_{12} carboxyl, C_1 to C_{12} carboxamide, C_1 to C_{12} alkanoyl and a sugar moiety, each of which groups may be substituted or unsubstituted;

R_3 and R_4 are independently selected from hydrogen or the structure shown below



wherein R_1 is as previously described,

or R_3 and R_4 may join to form a substituted five membered ring as shown below

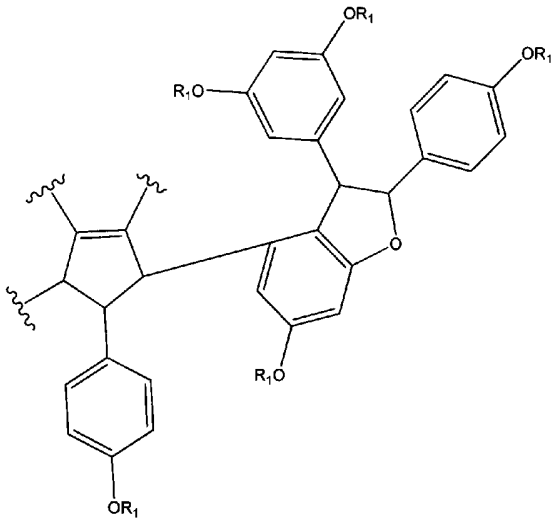


wherein R_1 is as previously described.

Preferably, R_1 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl, C_1 to C_6 alkenyl, aryl, C_1 to C_6 aldehyde, C_1 to C_6 alkanone, C_1 to C_6 carboxyl, C_1 to C_6 carboxamide and C_1 to C_6 alkanoyl, each of which groups may be substituted or unsubstituted;

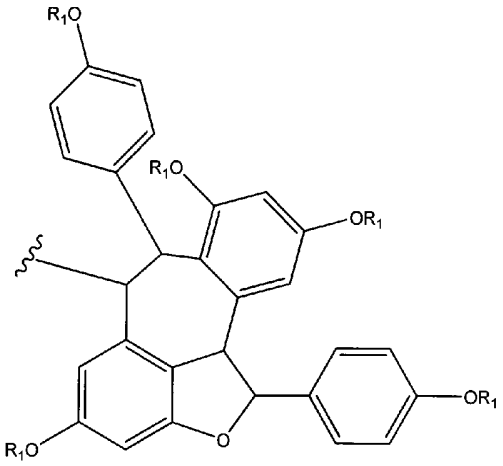
R_2 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl and a sugar moiety;

R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



wherein R_1 is as described,

or R_4 is

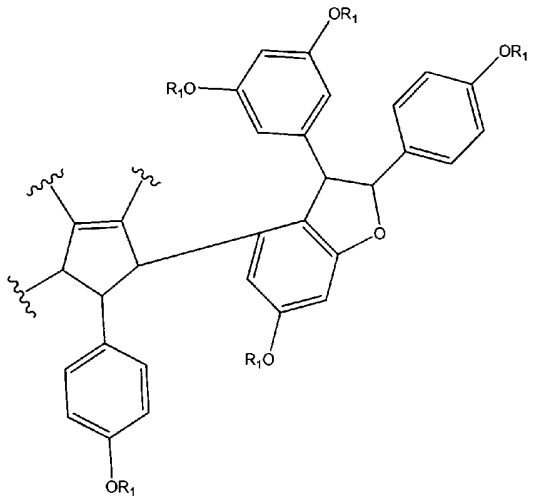


wherein R_1 is as described.

More preferably, R_1 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl, C_1 to C_6 alkenyl and aryl, each of which groups may be substituted or unsubstituted;

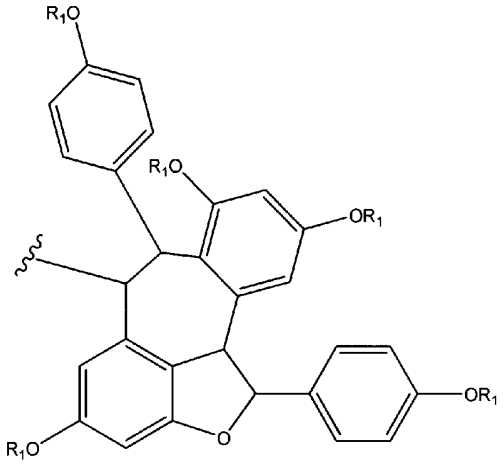
R_2 is selected from the group consisting of hydrogen, methyl, ethyl, propyl, butyl and a glucose moiety;

R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



wherein R_1 is as described,

or R_4 is

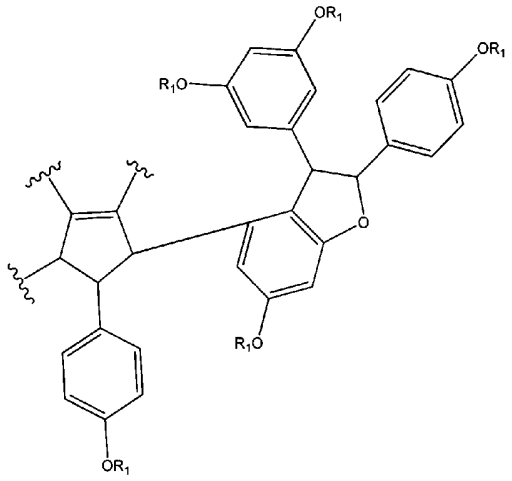


wherein R_1 is as described.

Even more preferably, R_1 is selected from the group consisting of hydrogen, methyl, ethyl, propyl and isopropyl, each of which groups may be substituted or unsubstituted;

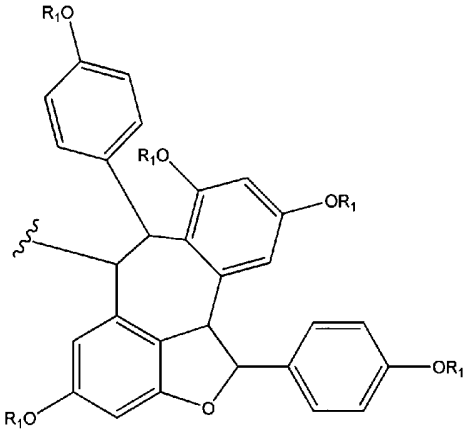
R_2 is selected from the group consisting of hydrogen, methyl, ethyl, propyl and a β-D-glucose moiety;

R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



wherein R_1 is as described,

or R_4 is



wherein R_1 is as described.

The structures given above for formula I and R_3 and R_4 take into account all stereochemistries which are covered by the structure and which would be known to the skilled addressee.

In one embodiment, the compound of formula I is selected from the group consisting of (-)-hopeaphenol, Vatalbinoside A, Vaticanol B, neohopeaphenol A and C_1 to C_6 O-alkyl derivatives thereof.

In one embodiment, the compound of formula I is(-)-hopeaphenol.

In one embodiment, the compound of formula I is Vatalbinoside A.

In one embodiment, the compound of formula I is Vaticanol B.

In one embodiment, the compound of formula I is neohopeaphenol A.

Suitably, the O-alkyl derivatives of the recited compounds are methyl, ethyl, propyl or butyl derivatives.

The term "O-alkyl derivatives thereof" in relation to the known compounds (-)-hopeaphenol, Vatalbinoside A, Vaticanol B and neohopeaphenol A means that each hydroxyl group of the compound has the recited alkyl group replace the hydrogen thereof.

In one embodiment of the disclosure, the compound of formula I is selected from the compounds represented by the structures shown in Figures 1 and 2.

It can be seen from a comparison of the structures of (-)-hopeaphenol and neohopeaphenol A that they are very closely related.

The term "*pharmaceutically acceptable salt*", as used herein, refers to salts which are toxicologically safe for systemic or localised administration such as salts prepared from pharmaceutically acceptable non-toxic bases or acids including inorganic or organic bases and inorganic or organic acids. The pharmaceutically acceptable salts may be selected from the group including alkali and alkali earth, ammonium, aluminium, iron, amine, glucosamine, chloride, sulphate, sulphonate, bisulphate, nitrate, citrate, tartrate, bitarate, phosphate, carbonate, bicarbonate, malate, maleate, napsylate, fumarate, succinate, acetate, benzoate, terephthalate, palmoate, piperazine, pectinate and S-methyl methionine salts and the like.

The term "alkyl" means a straight-chain or branched alkyl substituent containing from, for example, 1 to about 12 carbon atoms, preferably 1 to about 8 carbon atoms, more preferably 1 to about 6 carbon atoms, even more preferably from 1 to about 4 carbon atoms, still yet more preferably from 1 to 2 carbon atoms. Examples of such substituents include methyl, ethyl, propyl, isopropyl, *n*-butyl, *sec*-butyl, isobutyl, *tert*-butyl, pentyl, isoamyl, hexyl, and the like. The number of carbons referred to relates to the carbon backbone and carbon branching but does not include carbon atoms belonging to any substituents, for example the carbon atoms of an alkoxy substituent branching off the main carbon chain.

The term "alkenyl," as used herein, means a linear alkenyl substituent containing at least one carbon-carbon double bond and from, for example, 2 to 12 or 2 to 8 or 2 to 6 carbon atoms (branched alkenyls are 3 to 6 carbons atoms), preferably from 2 to 5 carbon atoms (branched alkenyls are preferably from 3 to 5 carbon atoms), more preferably from 3 to 4 carbon atoms. Examples of such substituents include vinyl, propenyl, isopropenyl, *n*-butenyl, *sec*-butenyl, isobutenyl, *tert*-butenyl, pentenyl, isopentenyl, hexenyl, and the like.

Whenever a range of the number of atoms in a structure is indicated (e.g., a C_1-C_{12}, C_1-C_8, C_1-C_6, C_1-C_4, or C_2-C_{12}, C_2-C_8, C_2-C_6, C_2-C_4 alkyl, alkenyl, alkynyl, etc.), it is specifically contemplated that any sub-range or individual number of carbon atoms falling within the indicated range also can be used. Thus, for instance, the recitation of a range of 1-12 carbon atoms (e.g., C_1-C_{12}), 1-6 carbon atoms (e.g., C_1-C_6), 1-4 carbon atoms (e.g., C_1-C_4), 1-3 carbon atoms (e.g., C_1-C_3), or 2-8 carbon atoms (e.g., C_2-C_8) as used with respect to any chemical group (e.g., alkyl, alkenyl, etc.) referenced herein encompasses and specifically describes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, and/or 12 carbon atoms, as appropriate, as well as any sub-range thereof (e.g., 1-2 carbon atoms, 1-3 carbon atoms, 1-4 carbon atoms, 1-5 carbon atoms, 1-6 carbon atoms, 1-7 carbon atoms, 1-8 carbon atoms, 1-9 carbon atoms, 1-10 carbon atoms, 1-11 carbon atoms, 1-12 carbon atoms, 2-3 carbon atoms, 2-4 carbon atoms, 2-5 carbon atoms, 2-6 carbon atoms, 2-7 carbon atoms, 2-8

carbon atoms, 2-9 carbon atoms, 2-10 carbon atoms, 2-11 carbon atoms, 2-12 carbon atoms, 3-4 carbon atoms, 3-5 carbon atoms, 3-6 carbon atoms, 3-7 carbon atoms, 3-8 carbon atoms, 3-9 carbon atoms, 3-10 carbon atoms, 3-11 carbon atoms, 3-12 carbon atoms, 4-5 carbon atoms, 4-6 carbon atoms, 4-7 carbon atoms, 4-8 carbon atoms, 4-9 carbon atoms, 4-10 carbon atoms, 4-11 carbon atoms, and/or 4-12 carbon atoms, etc., as appropriate).

"*Aryl*" means a C_6-C_{14} membered monocyclic, bicyclic or tricyclic carbocyclic ring system having up to 7 atoms in each ring, wherein at least one ring is aromatic. Examples of aryl groups include, but are not limited to, phenyl, naphthyl, tetrahydronaphthyl, indanyl and biphenyl. The aryl may comprise 1-3 benzene rings. If two or more aromatic rings are present, then the rings may be fused together, so that adjacent rings share a common bond.

"*Alkanoyl*" means an acyl moiety of a straight or branched configuration having 1-12, 1-8, 1-6 or 1-4 carbon atoms. Examples of alkanoyl groups include, but are not limited to, acetyl, propionoyl, butyryl, isobutyryl, pentanoyl and hexanoyl.

"*Alkanone*" refers to a ketone substituent with 2-12, 2-8, 2-6 or 2-4 carbon atoms in a linear, branched or cyclic arrangement, optionally substituted with 1 to 5 substituents independently selected at each occurrence from halogens, cyano or nitro.

In a second aspect of the present disclosure, there is provided a pharmaceutical composition comprising a compound as described for the first aspect, or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable carrier, diluent and/or excipient.

Suitably, the pharmaceutical composition is for the treatment or prophylaxis of an infection caused by a Gram-negative bacterium.

In a third aspect of the present disclosure, there is provided a compound, or a pharmaceutically acceptable salt thereof, for use in the treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism, wherein said compound is as described for the first aspect.

In a fourth aspect of the present disclosure, there is provided a use of a compound, or a pharmaceutically acceptable salt thereof, as described for the first aspect in the manufacture of a medicament for use in the treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism.

In a fifth aspect of the present disclosure, there is provided a method of modulating the activity of a type three secretion system in a Gram-negative bacterium comprising the step of exposing the Gram-negative bacterium to a compound as described for the first aspect, or a pharmaceutically acceptable salt thereof.

Suitably, the step of modulating the activity is an inhibition of the activity of the type three secretion system in the Gram-negative bacterium.

The person skilled in the art knows that treatment or prevention of bacterial infections may include ways of decreasing bacterial virulence. Bacterial virulence includes events that enable bacteria to enter the host, disarm the defenses of the host, multiply and spread within the host or to a new host. The person skilled in the art will appreciate that the compounds and compositions disclosed herein are not antibacterial agents in the classical sense but are agents that modify the virulence of bacteria and thereby exhibit antibacterial effect.

In one embodiment of the second aspect of the present disclosure, the composition further comprises at least one additional active agent, such as at least two additional active agents, such as at least three additional active agents. Non-limiting examples of additional active agents that may prove useful in such a combination are other compounds with anti-virulence properties, compounds with antibiotic properties, current antibiotic therapies, current antimicrobial therapies and phage therapies.

In one embodiment, said composition is for use in the treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism.

The listing of embodiments that follows is equally relevant for all four aspects of the present invention, unless otherwise stated, and would be understood as such by the person skilled in the art.

In one embodiment of any aspect of the present disclosure, said Gram-negative bacterium possesses a type three secretion system. In one embodiment, said compound acts to inhibit the activity of the T3SS, wherein said inhibition may be partial or complete. Thus, in one embodiment, said compound blocks the activity of the T3SS. Non-limiting examples of blocking T3SS activity are preventing the T3SS from docking on the target cell and preventing the delivery of target effector molecules to the target cell.

In another embodiment of any aspect of the present disclosure, said Gram-negative bacterium possesses at least one flagellum. In one embodiment, said compound acts to inhibit, partially or completely, the activity of at least one flagellum. In an alternative embodiment, said compound blocks the activity of at least one flagellum. Non-limiting examples of blocking or inhibiting the activity of a flagellum are preventing assembly of the flagellum or preventing changes in flagellum structure, thereby inhibiting transition between swarming and swimming flagellum motility phases. In this way, the motility of the Gram-negative bacterium as a whole may be decreased or inhibited.

In yet another embodiment of any aspect of the present disclosure, said Gram-negative bacterium possesses both a type three secretion system and at least one flagellum.

In one embodiment of any aspect of the present disclosure, said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter, Chlamydia, Klebsiella, Cronobacter, Serratia, Acinetobacter, Morganella, Lawsonia, Actinobacillus, Francisella, Erwinia, Ralstonia, Rhizobium* and *Xanthomonas*. In another embodiment, said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella,*

Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter and *Chlamydia*. In yet another embodiment, said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella* and *Shigella*. In a specific embodiment, said bacterium belongs to the genus *Pseudomonas*.

Bacterial infections, especially infections caused by bacteria that are resistant to current antibiotic treatment, are a large problem in modern healthcare leading to a large degree of suffering and even to death. Bacterial infections also pose large problems in veterinary medicine as well as in agriculture, leading to large financial losses and decreased food production due to infected crops. Hence, in one embodiment of any aspect of the present disclosure, said multicellular organism is selected from the group consisting of a plant, a fungus and an animal. Preferably, as used herein, the term "multicellular organism" may refer to any subject, particularly a vertebrate subject, and even more particularly a mammalian subject, for whom therapy or prophylaxis is desired. Suitable vertebrate animals include, but are not restricted to, primates, avians, livestock animals (e.g., sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g., rabbits, mice, rats, guinea pigs, hamsters), companion animals (e.g., cats, dogs) and captive wild animals (e.g., foxes, deer, dingoes). A preferred subject is a human in need of treatment or prophylaxis for a condition related to an infection caused by a Gram-negative bacterium. However, it will be understood that this do not imply that symptoms are necessarily present.

Many Gram-negative bacteria have been found to act as pathogens in mammals, such as humans and are known to cause a wide variety of diseases. For example, *Escherichia coli* is a common cause of food-borne disease, and *Vibrio cholerae* is a water-borne pathogen which causes cholera. Some Gram-negative bacteria cause respiratory problems (for example *Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Chlamydia pneumoniae*), urinary problems (for example *Proteus mirabilis*, *Enterobacter cloacae*, *Serratia marcescens*, *Pseudomonas*

aeruginosa), and/or gastrointestinal problems (for example *Helicobacter pylori*, *Salmonella enterica*, *Yersinia* spp.). Gram-negative bacteria are also the cause of some sexually transmitted diseases, for example *Chlamydia trachomatis*. Furthermore, there are Gram-negative bacteria that are associated with nosocomial infections, including *Acinetobacter baumannii*, which cause bacteremia, secondary meningitis and ventilator-associated pneumonia in intensive-care units of hospitals.

The following is a non-limiting listing of clinically relevant Gram-negative bacteria contemplated as targets in connection with the various aspects of the present disclosure: *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Escherichia coli*, *Salmonella enterica*, *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Chlamydia trachomatis*, *Chlamydia pneumoniae (Chlamydophila pneumoniae)*, *Salmonella bongori*, *Legionella pneumophila*, *Proteus vulgaris*, *Proteus penneri*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter cancerogenus*, *Enterobacter gergoviae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*, *Enterobacter nimipressuralis*, *Helicobacter pylori*, *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas veronii*, *Citrobacter freundii*, *Citrobacter rodentium*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Burkholderia cepacia*, *Burkholderia cenocepacia*, *Burkholderia multivorans*, *Vibrio cholerae*, *Vibrio anguillarum*, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Bordetella parapertussis*, *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter jejuni*, *Cronobacter sakazakii*, *Serratia marcescens*, *Lawsonia intracellularis*, *Pseudomonas oryzihabitans*, *Pseudomonas plecoglossicida*, *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Escherichia albertii*, *Escherichia fergusonii*, *Escherichia vulneris*, *Escherichia hermannii*, *Legionella longbeachae*, *Legionella bozemanii*, *Legionella micdadei*, *Legionella feeleii*, *Legionella dumoffii*, *Legionella wadsworthii*, *Legionella anisa*, *Pantoea agglomerans*, *Helicobacter cinaedi*, *Helicobacter fennelliae*, *Helicobacter felis*, *Helicobacter pullorum*, *Helicobacter canadensis*, *Helicobacter winghamensis*, *Helicobacter canis*,

Aeromonas aquariorum, *Aeromonas salmonicida*, *Aeromonas popoffii*, *Aeromonas jandaei*, *Aeromonas schubertii*, *Citrobacter amalonaticus*, *Citrobacter koseri*, *Citrobacter braakii*, *Citrobacter farmeri*, *Citrobacter sedlakii*, *Burkholderia gladioli*, *Burkholderia fungorum*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio mimicus*, *Vibrio fluvialis*, *Vibrio campbellii*, *Vibrio mimicus*, *Vibrio harveyi*, *Bordetella petrii*, *Bordetella trematum*, *Bordetella holmesii*, *Bordetella hinzii*, *Campylobacter concisus*, *Campylobacter curvus*, *Campylobacter gracilis*, *Campylobacter hyointestinalis*, *Campylobacter insulaenigrae*, *Campylobacter lari*, *Campylobacter mucosalis*, *Campylobacter rectus*, *Campylobacter showae*, *Campylobacter sputorum*, *Campylobacter upsaliensis*, *Chlamydia suis*, *Chlamydia muridarum*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella granulomatis*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Serratia plymuthica*, *Serratia liquefaciens*, *Serratia rubidaea*, *Serratia odorifera*, *Serratia fonticola*, *Serratia proteamaculans*, *Acinetobacter baumannii*, *Acinetobacter pittii*, *Acinetobacter nosocomialis*, *Acinetobacter lwoffii*, *Acinetobacter junii*, *Acinetobacter haemolyticus*, *Acinetobacter calcoaceticus*, *Morganella morganii*, *Actinobacillus ureae*, *Actinobacillus hominis*, *Actinobacillus suis*, *Actinobacillus lignieresii*, *Actinobacillus pleuropneumoniae*, *Actinobacillus equuli*, *Francisella tularensis*, *Francisella novicida*, *Francisella noatunensis*, *Francisella philomiragia* and *Francisella piscicida*.

Particularly relevant in connection with the various aspects of the present disclosure is the following subset of the above listing: *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Escherichia coli*, *Salmonella enterica*, *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Chlamydia trachomatis*, *Chlamydia pneumoniae (Chlamydophila pneumoniae)*, *Salmonella bongori*, *Legionella pneumophila*, *Proteus vulgaris*, *Proteus penneri*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter cancerogenus*, *Enterobacter gergoviae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*,

Enterobacter nimipressuralis, *Helicobacter pylori*, *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas veronii*, *Citrobacter freundii*, *Citrobacter rodentium*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Burkholderia cepacia*, *Burkholderia cenocepacia*, *Burkholderia multivorans*, *Vibrio cholerae*, *Vibrio anguillarum*, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Bordetella parapertussis*, *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter jejuni*, *Cronobacter sakazakii*, *Serratia marcescens* and *Lawsonia intracellularis*.

Highly relevant in connection with the various aspects of the present disclosure is the following subset: *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Escherichia coli*, *Salmonella enterica*, *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Chlamydia trachomatis* and *Chlamydia pneumoniae (Chlamydophila pneumoniae)*.

In a specific embodiment, said Gram-negative bacterium is *Pseudomonas aeruginosa*.

In one embodiment, said multicellular organism is suffering, or is at risk of suffering, from a disease associated with infection by a Gram-negative bacterium, such as a disease associated with T3SS- and/or flagella-expressing bacteria. Such a disease may for example be selected from the group consisting of pneumonia, diarrhea, severe urinary tract infections, eye infections, skin and soft tissue infections (in particular infections in deep burn wounds), dermatitis, postoperative infections and infections in individuals suffering from cystic fibrosis or chronic obstructive lung disease (COLD). Individuals suffering from cystic fibrosis or chronic obstructive lung disease (COLD) are particularly sensitive to infection, and such individuals may in particular benefit from the invention disclosed herein as a preventive measure.

In one embodiment, said compound is administered systemically. In another embodiment, the compound may be administered locally. The administration may for example be oral or parenteral.

As used herein, the term "systemic administration" refers to a route of administration which is such that the substance of interest enters into the circulatory system, affecting the entire body. The skilled person is aware that systemic administration can take place via enteral administration (absorption of the drug through the gastrointestinal tract) or parenteral administration (generally injection, infusion, or implantation).

In certain embodiments, systemic administration of said compound may be used. In one embodiment, said compound is administered orally. In another embodiment, said administration may be parenteral.

In another embodiment, said compound is administered by a local route. For example, local administration may be topical, such as in the form of an ointment, paste, foam, cream or powder, but may also be oral.

In yet other embodiments, the administration route may be selected from the group consisting of upper respiratory, lower respiratory, intranasal, intraurethreal, intradermal, intramuscular, intravenous, subcutaneous, gastrointestinal, vaginal and rectal administration.

The compounds of the invention can, for example, be incorporated in pharmaceutical compositions suitable for any administration route as described above, e.g. in tablets and capsules for oral administration, solutions for intravenous and intramuscular administration, and ointments for topical administration.

Because a number of Gram-negative bacteria have developed resistance to one or more currently available antibiotic treatments and hence do not respond, or respond insufficiently, to said antibiotic treatments, it may be beneficial to treat or prevent infections caused by these bacteria by compounds disclosed herein. Such treatment or prevention may be used as a complement to other antimicrobial treatments, or may be the only antimicrobial treatment or prevention administered to the multicellular organism.

Thus, in one embodiment according to any one of the aspects of this disclosure, said multicellular organism is one that does not respond, or responds insufficiently, to antibiotic treatment.

In one embodiment, said compound is administered at a dose sufficient to significantly reduce, eliminate or prevent the virulence of the Gram-negative bacterium causing the infection to be treated and/or prevented. In one embodiment, said compound is administered at a dose corresponding to a dose of 0.1-50 mg/kg body weight, such as a dose of 2-20 mg/kg, such as a dose of 4-10 mg/kg. In one particular embodiment, said compound is administered at a dose of 4-7 mg/kg body weight, such as a dose of 4-6 mg/kg, such as a dose of 5 mg/kg.

A person skilled in the art will be aware that the dose may need to be adjusted based for example on whether the compound disclosed herein is a complement to other antimicrobial treatment or the only antimicrobial treatment administered to the multicellular organism. Furthermore, it is within the skill of the treating physician or veterinarian to adjust the dose with regard to any parameter, including the subject's age, weight, sex and other such known factors.

In one preferred embodiment, the compound referred to in any of the aspects is selected from the group consisting of (-)-hopeaphenol, Vatalbinoside A, Vaticanol B, neohopeaphenol A and C_1 to C_6 O-alkyl derivatives thereof.

While the invention has been described with reference to various exemplary aspects and embodiments, it will be understood by those skilled in the art that various changes may be made, and that elements may be substituted for equivalents thereof, without departing from the scope of the invention. In addition, modifications may be made to adapt a particular situation to the teachings of the invention without departing from the essential scope thereof. Therefore, it is intended that the invention not be limited to any particular embodiment contemplated, but that the invention will include all embodiments falling within the scope of the appended claims.

Brief Description of the Figures

Figure 1 shows the structural formulas of (-)-hopeaphenol, Vatalbinoside A, Vaticanol B, (-)-hopeaphenol permethyl ether and Vaticanol B permethyl ether;

Figure 2 shows the structural formula of neohopeaphenol A;

Figure 3 is an ORTEP view of the compact tetrameric resveratrol rotamer (–)-hopeaphenol;

Figure 4 is an ORTEP view of the extended tetrameric resveratrol rotamer vaticanol B permethyl ether;

Figure 5A and 5B are bar graphs representing YopH secretion and yopE expression measured by phosphatase and luciferase activity, respectively, after incubation of *Y. pseudotuberculosis* strain YPIII(pIB102EL) with 10 µM, 20 µM, 50 µM and 100 µM of compound 1 as indicated. Values are given as percentage of control (-Ca);

Figure 6A and 6B show optical density at 600 nm of *Y. pseudotuberculosis* strain YPIII(pIB102) cultures, incubated at 37 °C for 7 hours with varying concentrations of compound 1 as indicated. OD600 was measured every hour;

Figure 7A and 7B show optical density at 600 nm of *P. aeruginosa* strain PAK cultures, incubated at 37 °C for 7 hours with varying concentrations of compound 1 as indicated. OD600 was measured every hour;

Figure 8 shows inverted phase contrast microscope images of HeLa cells infected with *P. aeruginosa* strain PAK incubated for 5 hours at 37 °C. Figure 8A and 8B show images of uninfected cells, untreated infected cells, and infected cells treated with the indicated concentrations of (-)-hopeaphenol;

Figure 9 is a bar graph representing the percentage of J774 cell metabolism. Cells were infected with *P. aeruginosa* strain PAK and incubated for 4 hours at 37 °C, together with (-)-hopeaphenol at a concentration of 20 µM, 50 µM, 100 µM and 150 µM. Uninfected J774 cells, J774 cells infected with PAK*exsA*::Ω (a T3SS defective mutant strain) and J774 cells infected

with PAK were used as controls. Values are given as percentage of uninfected control;

Figure 10 is a bar graph representing the percentage of sheep red blood cells (RBCs) that undergo hemolysis after infection with *P. aeruginosa* strain PAK. RBCs were incubated for 4 hours at 37 °C, after which absorbance at 560 nm was measured. Controls included RBC alone, RBC + DMSO, PAK and PAK*popB* (a T3SS defective mutant strain that does not cause hemolysis);

Figure 11 is a bar graph representing the level of secreted ExoS (media) and expressed ExoS (media and cells) after treatment of *P. aeruginosa* strain PAK with (-)-hopeaphenol for 3 hours at 37 °C. PAK*exsA*::Ω was included as a control. ExoS content was examined by Western blot analysis and band intensities were analyzed. Values are given as percentage of PAK control;

Figure 12 is a bar graph representing swimming zone diameters of *P. aeruginosa* strain PAK on agar plates containing various amounts of (-)-hopeaphenol. PAK*fliaA*::Gm, a mutant strain unable to swim, and PAK were included as controls. Values are given as percentages of untreated PAK;

Figure 13 is a bar graph representing swarming zone areas of *P. aeruginosa* strain PAO1 on agar plates containing various amounts of (-)-hopeaphenol. Values are given as percentage of control (PAO1 without compound);

Figure 14A and 14B are bar graphs representing level of LDH release 5 hours post infection of J774 cells by enteropathogenic *E. coli* (**A**, EPEC) and enterohemorrhagic *E. coli* (**B**, EHEC), respectively. Level of LDH release is given as percentage of LDH release measured from EPEC (A) or EHEC (B) infected J774 cells;

Figure 15 shows the percent survival of animals infected by *P. aeruginosa* strain Xen5 by intranasal inhalation and treated with (-)-hopeaphenol or vehicle as indicated at 2 and 12 hours post infection (n = 25). Survival was monitored for 24 hours. ($P < 0.01$, Chi-square);

Figure 16A-C show (-)-hopeaphenol inhibits the effector protein secretion in *Y. pseudotuberculosis* but does not affect growth; (A) Circles show inhibition of YopE and the luciferase light signal with an IC50 of 6.6 μM and triangles show inhibition of the enzymatic signal from YopH in *Y. pseudotuberculosis*. (B) Growth inhibition curve of *Y. pseudotuberculosis* treated with three different concentrations of (-)-hopeaphenol (C) Time study of the YopE and the luciferase light signal in *Y. pseudotuberculosis*.;

Figure 17 shows a Western analysis on *Y. pseudotuberculosis* treated with (-)-hopeaphenol;

Figures 18 A (total culture) and B (supernatant) show a Western blot analysis of the reversibility of (-)-hopeaphenol treatment of *Y. pseudotuberculosis*;

Figure 19 is a graph showing (-)-hopeaphenol affects translocation by *Y. pseudotuberculosis* in a dose dependent manner;

Figure 20 shows (-)-hopeaphenol inhibits secretion and expression of the *P. aeruginosa* T3SS effector protein ExoS;

Figure 21 A-G is a series of images showing (-)-hopeaphenol inhibits the infection of *P. aeruginosa* in HeLa cells; and

Figure 22 shows the affect of (-)-hopeaphenol pretreatment on *C. trachomatis* infection and growth.

Examples

The following Examples disclose data showing that compounds of formula I can act as virulence blockers to inhibit bacterial infections by targeting the type three secretion system and/or the bacterial flagellum.

Unless otherwise indicated, cell lines, bacteria and culturing conditions as follows and as listed in Table 1 were used herein.

HeLa cells (ATCC CCL-2) and J774 cells (ATCC TIB-67) were grown in Dulbecco's modified Eagle medium (DMEM) with GlutaMAX (l-alanyl-l-glutamine) and phenol red (Life Technologies, Carlsbad, CA, USA), supplemented with 10 % fetal bovine serum (FBS; Life Technologies) and 3 μg/ml gentamicin (Life Technologies) at 37 °C in humidified air with 5 % CO_2.

Table 1: Bacterial strains

Bacterial strain	Characteristics	Reference or source
Pseudomonas aeruginosa		
PAK	Wild type	D. Bradley
180	Wild type	ATCC 19660
PAO1	Wild type	Holloway *et al.* (1979)[1]
Xen5	Strain 180 with *luxCDABE* operon	Caliper Life Sciences, Hopkinton, MA
PAK *exsA*::Ω	T3SS defective	Frank *et al.* (1994)[2]
PAK*popB*	T3SS translocation mutant	Sundin *et al.* (2002)[3]
PAK *fliA*::Gm	Swimming motility defective	Starnbach and Lory (1992)[4]
Yersinia pseudotuberculosis		
YPIII(pIB102)	Wild type	Bölin and Wolf-Watz (1984)[5]
YPIII(pIB102EL)	*yopE* promoter controlled *luxAB* fusion	Forsberg and Rosqvist (1994)[6]
Escherichia coli		
Enteropathogenic *E. coli*	Serotype O111:H2	CCUG 38068
Enterohemorrhagic *E. coli*	Serotype O111:NM	CCUG 58078

1. Holloway *et al.* (1979) Microbiol Rev 43:73-102

2. Frank *et al.* (1994) Infect Immun 62:554-63

3. Sundin *et al.* (2002) Microb Pathog 33:265-277

4. Starnbach and Lory (1992) Mol Microbiol 6:459-69

5. Bölin and Wolf-Watz (1984) Infect Immun 43:72-8

6. Forsberg and Rosqvist (1994) Infect Agents Dis 2:275-278

Extraction and isolation of plant material

The following discloses the extraction and isolation of plant material resulting in the identification of the natural products (-)-hopeaphenol, Vatalbinoside A and Vaticanol B (labeled compounds **1**, **2** and **3**, respectively, in Figure 1 and the numbering is used herein interchangeably with the associated compound name).

General experimental for plant material extraction

NMR spectra were recorded at 30 °C on either a Varian 500 MHz or 600 MHz Unity INOVA spectrometer (Varian, Walnut Creek, CA, USA). The latter spectrometer was equipped with a triple resonance cold probe. The ^{1}H and ^{13}C NMR chemical shifts were referenced to the solvent peak for acetone-d_6 (δ_H 2.05 and δ_C 29.9) or CD_3OD (δ_H 3.31 and δ_C 49.15). LRESIMS were recorded on a Waters ZQ mass spectrometer (Waters, Milford, MA, USA). HRESIMS were recorded on a Bruker Daltronics Apex III 4.7e Fourier-transform mass spectrometer (Bruker, Karlsruhe, Germany). A BIOLINE orbital shaker (Edwards Instrument Company, Narellan, NSW, Australia) was used for the large-scale extraction of plant material. Machery Nagel Polyamide CC6 (0.05–0.016 mm) was used for tannin/polyphenolic removal (Machery Nagel, Düren, Germany). Alltech Davisil 40–60 µm 60 Å C_{18} bonded silica was used for pre-adsorption work (Alltech, Deerfield, IL, USA). Merck 40–63 µm silica (Kiesselgel 60) was used for flash chromatography. A Waters 600 pump equipped with a Waters 996 PDA detector and a Waters 717 autosampler (Waters, Milford, MA, USA) were used for HPLC. A ThermoElectron C_{18} Betasil 5 µm 143 Å column (21.2 mm × 150 mm) (Thermo Scientific, Los Angeles, CA, USA) and a Phenomenex Luna C_{18} column 5 µm 143 Å column (Phenomenex, Torrance, CA, USA) were used for semi-preparative (21.2 mm × 250 mm) and preparative (50 × 150 mm) HPLC separations. All solvents used for chromatography, UV, and MS were Lab-Scan HPLC grade (RCI Lab-Scan, Bangkok, Thailand), and the H_2O was Millipore Milli-Q PF filtered (Millipore, Billerica, MA, USA). All synthetic

reagents were purchased from Sigma Aldrich and used without further purification.

Plant material

The leaves of *Anisoptera thurifera* (Blanco) Blume and *A. thurifera* subsp. *polyandra* (Blume) P.S.Ashton (syn. *Anisoptera polyandra*) were collected in Papua New Guinea. Voucher samples (QID016405; *A. thurifera*) and (QID018040; *A. polyandra*) have been lodged with the Biodiversity Research Pty Ltd, Port Moresby, Papua New Guinea.

Extraction and isolation of plant material

Bioassay-directed Isolation

In separate extraction processes, the air-dried and ground leaves of *A. thurifera* (10 g) and *A. polyandra* (10 g) were defatted (250 mL *n*-hexane, 2 h) and then sequentially extracted with CH_2Cl_2 (250 mL, 2 h) and CH_3OH (2 × 250 mL, 2 h then 24 h) with stirring (200 rpm). The filtered CH_2Cl_2 and CH_3OH extractions were combined and dried under reduced pressure to yield a crude extracts (1.9 g, *A. thurifera*, and 2.0 g *A. polyandra*).

The organic extracts were resuspended in CH_3OH (150 mL) and loaded onto a polyamide gel column (30 g bed volume, pre-equilibrated with CH_3OH) to remove pigments. The column was eluted with CH_3OH (300 mL), and the eluent evaporated to obtain a crude extract (1.0 g, *A. thurifera*, and 1.1 g, *A. polyandra*). A portion of this crude material (0.8 g) was pre-adsorbed to C_{18}-bonded silica (1 g) then packed into a stainless steel guard cartridge (10 × 30 mm) that was subsequently attached to a C_{18} semi-preparative HPLC column. Isocratic HPLC conditions of H_2O-CH_3OH-CF_3COOH (90:10:0.1) were initially employed for the first 10 min, then a linear gradient to CH_3OH (0.1% CF_3COOH) was run over 40 min, followed by isocratic conditions of CH_3OH (0.1% CF_3COOH) for a further 10 min, all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected from the start of the run then submitted to bioassay.

In the case of *A. thurifera*: Bioassay data indicated that fractions 25–30 were the active fractions. ^{1}H NMR analysis of the fractions identified that fractions 25–26 contained vatalbinoside A (**2**, 44.7 mg, 1.0% dry wt). Fractions 27–30 were a mixture of related oligostilbenes and were combined (73.4 mg) and further fractionated using identical C_{18} HPLC conditions to those detailed above. ^{1}H NMR analyis of all UV-active peaks resulted in the identification of the previously reported natural products, vatalbinoside A (**2**, 4.2 mg, 0.10% dry wt), vaticanol B (**3**, 10.8 mg, 0.25% dry wt) and (–)-hopeaphenol (**1**, 3.4 mg, 0.08% dry wt).

In the case of *A. polyandra*: Bioassay data indicated that fractions 29–31 were active. Fractions 29–31 were combined (32.7 mg), the mixtures were fractionated using identical C_{18} HPLC conditions to those detailed above. ^{1}H NMR analysis of all UV-active peaks resulted in the identification of the previously reported natural product, the vaticanol B (**3**, 19.2 mg, 0.26% dry wt). Compounds **1** and **2** were identified in extracts of *A polyandra* but were not isolated in sufficient quantities during the bioassay-directed fractionation step.

*Alternative enrichment of **1** and optimized large-scale purification of **1–3***

Several strategies, with broader application, to the optimal enrichment or semi-purification of the major stilbenoid tetramers prior to HPLC purification were developed.

Enzymatic hydrolysis of stilbenoid glycosides using endogenous leaf enzymes

The air-dried and ground leaf material of *A. polyandra* (100 g) was suspended in tap water (400 mL) in a glass container. The leaf material/water was maintained at 35 °C in a water bath for 7 days. The water level was maintained at 400 mL with the addition of fresh tap water. Prior to filtration, methanol (400 mL) was added to the plant material to solubilize the aglycone product. The sample was filtered through a glass frit (Pore 1) then

sequentially extracted with acetone (2 × 400 mL) at ambient temperatures for 24 h with shaking. The resulting H_2O/CH_3OH and acetone extracts were filtered then combined and dried under reduced pressure to yield a crude extract (14.5 g).

Enrichment of stilbene glycosides and stilbene aglycones by normal phase column chromatography

The aglycone-enriched extract of the leaf material was resuspended in acetone and pre-adsorbed to silica gel (100 g). The dry pre-adsorbed sample was loaded onto a silica flash column (200 g, 12.5 cm diam. × 4 cm high; pre-equilibrated with 1:1 hexane:EtOAc. The extract was fractionated using a step-wise gradient consisting of: 1:1 hexane:EtOAc; 1:9 hexane:EtOAc; 19:1 acetone:CH_3OH. Fraction 1, which contained plant pigments, was discarded. Fraction 2 contained a crude mixture of stilbenoid aglycones and fraction 3 contained a crude mixture of stilbenoid glycosides.

*Purification of compounds **1** – **3** by reversed phase preparative HPLC*

Fraction 2 (10.3 g) was re-suspended in CH_3OH and pre-adsorbed onto C_{18} bonded silica (40 g). The dried pre-adsorbed extract (sufficient for three guard cartridges) was then loaded into a stainless steel guard cartridge (50 × 25 mm diam.) A C_{18} Betasil preparative column (Thermo Electron Company Betasil C18; 150 × 50 mm; 5 µm) was pre-equilibrated with the starting mobile phase H_2O-CH_3OH-CF_3COOH (85:15:0.1) Isocratic HPLC conditions of H_2O-CH_3OH-CF_3COOH (85:15:0.1) were employed for the first 2 min, then a linear gradient to H_2O-CH_3OH-CF_3COOH (63:37:0.1) was run over 32 min, followed by isocratic conditions of H_2O-CH_3OH-CF_3COOH (63:37:0.1) for a further 51 min, and lastly, a linear gradient to H_2O-CH_3OH-CF_3COOH (20:80:0.1) over 68 min, all at a flow rate of 18 mL/min. 118 Fractions (84 sec.) were collected over 150 min from the start of the run. Pure (–)-hopeaphenol (**1**) eluted between fractions 26 – 40 (1.02 g, 99% purity; 0.5 g 60% purity; 1.3% dry wt., cumulative yield). Vaticanol B (**3**) eluted between fractions 41–72 (4.15 g, ca. 70–99% purity; ca. 2.9% yield dry wt.).

Fraction 3 contained a moderate amount of the unhydrolyzed β-glycoside **2** (1.7 g, 30% purity; 0.5% yield dry wt.). Crystals of **2** were obtained after further purification by reversed-phase HPLC. A portion of this crude material (0.1 g) was pre-adsorbed to C_{18}-bonded silica (1 g) then packed into a stainless steel cartridge (10 × 30 mm) that was subsequently attached to a C_{18} Betasil semi-preparative HPLC column (21.2 mm × 150 mm). A linear gradient from H_2O-CH_3OH-CF_3COOH (80:20:0.1) to H_2O-CH_3OH-CF_3COOH (72:28:0.1) was run over 30 min, followed by isocratic conditions of H_2O-CH_3OH-CF_3COOH (72:28:0.1) for a further 30 min, all at a flow rate of 9 mL/min. Compound **2** eluted between 36–42 min. Crystalline **2** was obtained by slow evaporation of the mobile phase.

Characterisation of Isolated Compounds

*(–)-Hopeaphenol (**1**)*

Colourless plates (CH_3OH /H_2O/0.1% CF_3COOH); decomp. ~280°C; Lit: 351°C (evac. cap); $[a]_D^{25}$= -454° (*c* 0.1, CH_3CH_2OH); Lit: $[a]_D^{20}$= -407° (*c* not specified, CH_3CH_2OH) .)(the above data was consistent with Coggon et al 1965, 61. J. Chem. Soc. 0, 406-409); ^1H and ^{13}C NMR data were consistent with literature values (Kawabata et al, 1992. Magn. Reson. Chem. 30, 6-10); (+)-LRESIMS (rel. int.) *m/z* 907 (100) $[M+H]^+$. Crystals of the monohydrate of **1** suitable for X-ray diffraction studies were obtained by slow evaporation of the CH_3OH/H_2O.0.1% CF_3COOH HPLC eluent of **1**.

*Vatalbinoside A (**2**)*

Colourless needles decomp. ~280 °C; $[a]_D^{25}$= -341° (*c* 0.1, CH_3OH); Lit: $[a]_D^{25}$= -320° (*c* 0.1, CH_3OH) (Abe et al 2010. J. Nat. Prod. 73, 1499-1506); ^1H and ^{13}C NMR data were consistent with literature values (Abe et al 2010 as above); IR ν_{max} (KBr) 3265, 1612, 1513, 1345, 1240, 1022, 839 cm^{-1}; (+)-LRESIMS (rel. int.) *m/z* 1091.4 (100) $[M+Na]^+$.

*Acid hydrolysis of **2***

A solution of **2** (10 mg in 1 mL of 5% H_2SO_4 in CH_3CH_2OH) was refluxed for 4 h at 65 °C. The reaction mixture was allowed to cool, then evaporated to dryness. The crude reaction mass was partitioned between EtOAc (2 × 10 mL) and sat. $NaHCO_3$ (10 mL). The organic layer was washed with brine solution, and subsequently dried over Na_2SO_4. The organic layer was evaporated and the reaction mixture was further purified by C_{18} preparative HPLC using the conditions described for fraction 3 in §4.4.3. to yield (–)-hopeaphenol (**1**, 8 mg, 93% yield, 98% purity).

*Vaticanol B (**3**)*

White amorphous powder; m.p. decomp. ~280 °C; $[a]_D^{25}$= -46° (*c* 0.06, CH_3OH); Lit: $[a]_D^{20}$= -14 (*c* 0.1, CH_3OH)(Tanaka et al, 2000. Phytochemistry, 54, 63-69); ^1H and ^{13}C NMR data were consistent with literature values (Tanaka et al as above); (+)-LRESIMS (rel. int.) *m/z* 907 (100) $[M+H]^+$, (+)-HRESIMS *m/z* 907.2740 ($C_{62}H_{52}O_{17}$ $[M+H]^+$ requires 907.2749).

Synthesis of *O*-Alkylated Compounds 4 and 5

*Methylation of (–)-hopeaphenol (**1**)*

(–)-Hopeaphenol (**1**, 20 mg, 0.02 mmol) was dissolved in CH_3OH–CH_2Cl_2 (1:1, 1.5 mL) at room temperature before TMS-diazomethane (2.0 M in Et_2O, 650 µL, 1.30 mmol) was added dropwise. The reaction was stirred overnight at room temperature, then evaporated to dryness. The crude reaction mixture was adsorbed to C_{18}-bonded silica then purified by C_{18} semi-preparative HPLC (Betasil column, 21.2 mm × 150 mm). A linear gradient from H_2O-CH_3OH-CF_3COOH (50:50:0.1) to H_2O-CH_3OH-CF_3COOH (5:95:0.1) was run over 30 min, followed by a linear gradient to CH_3OH (0.1% CF_3COOH) over 10 min followed by isocratic conditions of CH_3OH (0.1% CF_3COOH) for a further 20 min, all at a flow rate of 9 mL/min. (–)-Hopeaphenol permethyl ether (**4**) (15.6 mg, 68% yield, ≥95% purity) eluted between 33–37 minutes. m.p. 169-172 °C (CH_3OH-H_2O); Lit: 162–164°C (benzene-CH_3OH) (Coggon et al 1965, 61. J. Chem. Soc. 0, 406-409); [*a*]

$_D^{25}$= -387° (*c* 0.1, CH_3OH); Lit.: [*a*] $_D^{RT}$ -378° (*c* not specified, $CHCl_3$); ^1H NMR (500 MHz, CD_3OD): δ 3.29 (s, H-13a-OMe/13d-OMe), 3.65 (s, H-13b-OMe/13c-OMe), 3.72 (s, H-4b-OMe/4c-OMe), 3.77 (s, H-11a-OMe/11d-OMe), 3.80 (s, H-4a-OMe/4d-OMe), 3.87 (brs, H-8b/8c), 4.15 (d, 11.8, H-8a/8d), 4.92 (d, 2.4, H-14b/14c), 5.73 (brs, H-7b/7c), 5.77 (d, 11.8, H-7a/7d), 5.84 (d, 2.4, H-12b/12c), 6.41 (d, 2.4, H-14a/14d), 6.66 (d, 2.4, H-12a/12d), 6.71 (d, 8.6, H-3b,5b/3c,5c), 6.85 (d, 8.7, H-3a,5a/3d,5d), 6.89 (d, 8.4, H-2b,6b/2c,6c), 7.14 (d, 8.7, H-2a,6a/2d,6d); ^{13}C NMR (126 MHz, CD_3OD); 41.5 (C-7b/7c),48.3 (C-8b/8c), 50.8 (C-8a/8d), 55.9 (C-11a-OMe/11d-OMe), 55.9 (C-4b-OMe/4c-OMe), 56.0 (C-4a-OMe/4d-OMe), 56.1 (C-13a-OMe/13d-OMe), 56.6 (C-13b-OMe/13c-OMe), 89.0 (C-7a/7d), 95.8 (C-12b/12c), 96.9 (C-12a/12d), 105.0 (C-14a/14d),108.8 (C-14b/14c), 114.5 (C-3b,5b/3c,5c), 115.1 (C-3a,5a/3d,5d), 120.7 (C-10b/10c), 124.3 (C-10a/10d), 129.5 (C-2b,6b/2c,6c), 130.3 (C-2a,6a/2d,6d), 132.2 (C-1a/1d), 136.2 (C-1b/1c), 140.3 (C-9b/9c), 142.6 (C-9a/9d), 159.2 (C-4b/4c), 159.8 (C-11b/11c), 160.9 (C-13a/13d), 161.2 (C-4a/4d), 161.7 (C-11a/11d), 161.8 (C-13b/13c); (+)-LRESIMS (rel. int.) *m/z* 1048 (100) $[M+H]^+$; HRESIMS *m/z* 1047.4307 ($C_{66}H_{63}O_{12}$ $[M+H]^+$ requires 1047.4319).

*Methylation of vaticanol B (**3**)*

Vaticanol B (**3**, 22.1 mg, 0.024 mmol) and K_2CO_3 (320 mg, 2.32 mmol) were dissolved in dry acetone (5 mL) at room temperature before MeI (200 µL, 3.2 mmol) was added dropwise. The reaction was refluxed for 2 h at 40°C then evaporated to dryness. The crude reaction mixture was adsorbed to C_{18} bonded silica then purified using the same conditions described above for compound **4**. Evaporation of fractions 35–38 yielded the crystalline vaticanol B permethyl ether (**5**) as colourless prisms suitable for X-ray diffraction studies (21.0 mg, 90% yield, ≥95% purity); m.p. 240–242°C (CH_3OH-H_2O); [*a*] $_D^{25}$= +60° (*c* 0.01, CH_3CH_2OH) tentative value only, this compound has solubility issues in a range of solvents; ^1H and ^{13}C NMR data were consistent with literature values (Tanaka et al as above); (+)-LRESIMS (rel. int.) *m/z* 1048 (100) $[M+H]^+$.

X-ray Crystallography

Unique data sets for compounds **1** as the monohydrate and **5** were measured at 200 K on an Oxford-Diffraction GEMINI S Ultra CCD diffractometer (Mo-K_α radiation, graphite monochromation) utilizing CrysAlis software. The structures were solved by direct methods and refined by full matrix least squares refinement on F^2. Anisotropic thermal parameters were refined for non-hydrogen atoms; $(x, y, z, U_{iso})_H$ were included and constrained at estimated values. Conventional residuals at convergence are quoted; statistical weights were employed. Computation used, SIR-97, SHELX97, ORTEP-3 and PLATON programs and software systems. In the absence of significant anomalous scatterers in compounds **1** and **5**, Friedel equivalents were merged with the absolute configuration established on the basis of optical activity.

*Crystal data for (–)-hopeaphenol (**1**)*

Following large-scale purification of the natural product **1**, crystals of the monohydrate were obtained and the structure and relative stereochemistry were determined by single crystal X-ray crystallographic studies. The crystal structure is indicated in Figure 3 in an ORTEP view. $C_{56}H_{42}O_{12}.H_2O$, M_r = 924.9. Orthorhombic, space group $P2_12_12_1$, a = 18.7861(6), b = 22.2885(7), c = 11.0953(3) Å, V = 4645.8(2) Å^3. D_c (Z = 4) = 1.32 g cm^{-3}. μ_{Mo} = 0.09 mm; specimen: 0.20 × 0.18 × 0.07 mm; $T'_{min/max}$ = 0.99/0.98. $2\theta_{max}$ = 53.0°; N_t = 11468, N = 5288 (R_{int} = 0.046), N_o = 2697; $R1$ = 0.042, $wR2$ = 0.099; S = 0.75.

*Crystal data for vaticanol B permethyl ether (**5**)*

Purification of the reaction mixture using C_{18} HPLC afforded **5**, (91% yield) which readily formed colourless prisms upon evaporation of the mobile phase. X-ray crystallography confirmed the enantiomeric purity of **5**, and indirectly **3**. An ORTEP representation of the extended-rotamer of vaticanol B

(**5**) is shown in Figure 4. The C1–C7–C8–C9 torsion angles for each ring system in **5** are: A -98.9(5)°; B -59.8(6)°; C 156.8(4)°; and D -138.9(5)°. Further data: $C_{66}H_{62}O_{12}$, M_r = 1047.2. Monoclinic, space group $P2_1$, a = 15.5983(6), b = 12.4215(3), c = 16.8136(7) Å, β = 117.230(5), V = 2896.7(2) Å^3. D_c (Z = 2) = 1.20 g cm^{-3}. μ_{Mo} = 0.08 mm; specimen: 0.54 × 0.35 × 0.10 mm; $'T'_{min/max}$ = 0.99/0.96. $2\theta_{max}$ = 55.0°; N_t = 22650, N = 6728 (R_{int} = 0.044), N_o = 3205; $R1$ = 0.057, $wR2$ = 0.117; S = 0.84.

Full .cif depositions reside with the Cambridge Crystallographic Data Centre, CCDC Nos. 953124 [(–)-hopeaphenol, **1**] and 953125 (vaticanol B permethyl ether, **5**).

The straightforward O-alkylation of compounds **1** and **3** indicates that further functionalisation can be undertaken at these positions in a straightforward manner using known chemistry.

Pharmacology

Determination of YopE expression using a reporter-gene assay

YPIII(pIB102-Elux) from a culture grown on LB agar containing 25 μg/mL chloramphenicol (Sigma) for 48 h at 26 °C was used to inoculate a liquid culture, then grown for 12–16 h in Brain Heart Infusion Broth (BHI; Difco) containing 25 μg/mL chloramphenicol (Sigma) on an orbital shaker at 26 °C. Liquid cultures were diluted to an OD_{620} of 0.2, then further diluted 1 in 4 in Ca^{2+} depleted media (BHI media with 5 mM EGTA and 20 mM $MgCl_2$) before addition of 30 μL of bacteria to a 384-well white solid lidded Optiplate™ (Perkin Elmer). Before bacterial addition, 5 μL of fraction or compound/controls were added to the plate, by diluting plates of library fractions or compounds/controls in DMSO with a Minitrak™ (PerkinElmer) liquid handler, by addition of 1 μL of fraction to 4 μL of H_2O.

Plates were incubated at 26 °C for 1 h followed by incubation at 37 °C for 3 h and then transferred back to 26 °C for 15 min. 15 μL of 0.1% decanal (Sigma) emulsified in H_2O was added to each well. Plates were read on a

Trilux (PerkinElmer) counter using a luminescence protocol. A dose response of streptomycin was used as an antibacterial control. Bacteria in BHI with 2.5 mM Ca^{2+} was used as an in plate negative control for the assay since no Yop protein is expressed during these conditions. Ca^{2+} depletion at 37 °C stimulates Yop production and the Yop production will eventually suppresses growth.

Fractions were tested at single point concentrations of 7.14 µge/µL Active fractions and controls were retested at 7.14, 1.42, 0.71, 0.14 and 0.071 µge/µL. Pure compounds were screened at 10, 20, 50 and 100 µM. YopE suppression was calculated using linear regression on the basis of the reduction in luminescent signal versus sample concentration (µg/mL). IC_{50} values were calculated from interpolation of the dose response curves.

Combined reporter-gene and phosphatase assay for the determination of YopH secretion

Before addition of decanal, and as according to the YopH protocol, 5 µL of the final assay volume was added to a clear 384-well plate (Becton Dickinson) containing 45 µL of YopH substrate *p*-nitrophenyl phosphate (pNPP; Acros Organics; 12.5 mM in 20 mM MES pH 5.0 and 0.8 mM DTT), with a Biomek FX liquid handler (Beckman Coulter). Plates were incubated for 15 min at 37 °C before addition of 10 µL of sodium hydroxide to stop the reaction. Plates were then read at 405 nm on a VictorII Wallac plate reader (PerkinElmer). To the remainder of the assay volume decanal was added and luminescence was measured as described above. Controls were as described for the reporter-gene assay. Fractions were tested at single point concentrations of 7.14 µge/µL Active fractions and controls were retested at 7.14, 1.42, 0.71, 0.14 and 0.071 µge/µL. Pure compounds were screened at 10, 20, 50 and 100 µM. IC_{50} values were calculated from interpolation of the dose response curves.

Antibacterial optical density (OD_{620}) assay.

YPIII(pIB102-Elux) cultures were grown overnight, then diluted to an OD_{620} of 0.2 in BHI medium, with 2.5 mM Ca^{2+}. A further 1:4 dilution in BHI with 2.5 mM Ca^{2+} was prepared before addition to the assay. 50 µL of the diluted bacteria was added to clear, lidded 384 well plates (Becton Dickinson), containing 5 µL of fraction/compound or controls. Active fractions and controls were screened at a single point concentration of 7.14 µge/µL then retested at 4.45, 0.91, 0.45, 0.091 and 0.045 µge/µL to determine a dose response. Plates were incubated for 3 h at 37 °C and then transferred to room temperature for 15 min before reading at 620 nm on a VictorII Wallac plate reader. Streptomycin was used as an in-plate negative growth control and an external plate contained a dose response of streptomycin for the estimation of the antibacterial IC_{50} values. Pure compounds were screened at 10, 20, 50 and 100 µM.

Results of Pharmacology Tests

The results of the above tests are indicated in Table 2.

Table 2. The YopE, YopH anti-infective and antibacterial activities of compounds **1–3**

Compound	IC_{50} (µM)		
	YopE	YopH	Antibacterial assay [a]
1	8.8	2.9	Not active [b]
2	12.5	4.5	Not active [b]
3	9.9	3.3	Not active [b]
Streptomycin [c]	38.0	31.0	36.0

[a] *Y. pseudotuberculosis* optical density assay; [b] at 364 µM; [c] commercially available antibiotic used as positive control.

Vatalbinoside A (**2**), vaticanol B (**3**) and (–)-hopeaphenol (**1**) exhibited IC_{50} values of 12.4, 9.9 and 8.8 µM (reporter-gene assay) and 4.5, 3.3 and 2.9 µM (YopH phosphatase assay) without detectable inhibition of bacterial growth (Table 2). These results strongly suggest that this class of molecules

targets the T3SS and may be developed as novel antibacterial agents against Gram-negative bacteria.

It has been shown above, that acid hydrolysis could produce **1** in high yield from the more abundant glycosylated compound **2**. Compound **1** is slightly more active than **2** and **3** and consequently, enrichment of **1** by enzymatic hydrolysis of **2** prior to isolation was successfully explored. In a procedure inspired by the enzymatic hydrolysis of ruberythric acid to alizarin in madder root (Derksen et al, 2003. Phytochemical Analysis 14, 137-144), the biotransformation of **2** to **1**was achieved by utilizing the endogenous enzymes present in the leaf. The dried ground leaf material of *A. polyandra* was suspended in tap water for 7 days at 35°C. Following chromatographic purification, a sixteen-fold enrichment of **1** (0.08–1.3% dry wt.) was achieved over this time-frame.

Further Pharmacology on (-)-hopeaphenol

Combined YopH/*yopE*-lux assay

(-)-Hopeaphenol was assayed for inhibition of YopH secretion and *yopE* expression, using the YPIII(pIB102EL) strain of *Y. pseudotuberculosis*. YopH secretion and *yopE* expression was measured through phosphatase and luciferase activity, respectively, and a dose dependent inhibitory effect was observed in both assays. Commercially purchased (-)-hopeaphenol was used in certain of the assays as a control and so for the following results and associated figures references to compound 1 and compound 2 refer to isolated (-)-hopeaphenol and purchased (-)-hopeaphenol, respectively.

Materials and methods

Combined YopH/*yopE*-lux assay

Y. pseudotuberculosis strain YPIII(pIB102EL) was grown overnight in BHI medium (Becton Dickinson, Franklin Lakes, NJ, USA), containing 25 µg/ml chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA), at 26 °C with

shaking (250 rpm). Optical density at 600 nm (OD_{600}) was measured on a Beckman Coulter DU 530 spectrophotometer (Beckman Coulter, Brea, CA, USA) and the culture was diluted to an OD_{600} of 0.04 in BHI medium containing 5 mM EGTA and 20 mM $MgCl_2$ for calcium depletion. 100 µl of bacterial solution was added to the wells of a white 96-well plate followed by addition of 1 µl of serially diluted test compounds in DMSO. To negative control wells, 100 µl of the same bacterial solution was added but with 1 µl DMSO instead. Bacteria were added to positive control wells diluted to an OD_{600} of 0.04 in BHI medium containing 2.5 mM $CaCl_2$, along with 1 µl DMSO. The plate was incubated for 1 h at 26 °C with shaking (250 rpm), followed by an additional 2 h at 37 °C. For the YopH assay, 10 µl of the induced bacteria were mixed with 90 µl freshly prepared substrate mixture, consisting of 25 mM para-nitrophenyl phosphate (pNPP; Acros Organics, Geel, Belgium), 40 mM 2-(N-morpholino)ethanesulfonic acid (MES) pH 5.0 (Acros Organics, Geel, Belgium) and 1.6 mM dithiothreitol (DTT; Acros Organics, Geel, Belgium) in ultrapure water, in a transparent 96-well plate. The new plate was incubated for 15 min at 37 °C with shaking (250 rpm), and 20 µl 1 M NaOH was added to all wells. Absorbance was measured at 405 nm on a Tecan GENios microplate reader (Tecan Group, Männedorf, Switzerland). In parallel with the above, the white 96-well plate was incubated for 30 min at room temperature, after which 50 µl 0.01 % aqueous solution of decanal (Sigma-Aldrich, St. Louis, MO, USA) was added and luciferase activity was measured on the microplate reader. Percent of control values were calculated for both assays relative to the negative (calcium depleted) control, which was set to 100 %.

Growth inhibition

Bacteria were grown overnight in LB medium (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C with shaking (250 rpm). When *Y. pseudotuberculosis* strain YPIII(pIB102) was used, 50 µg/ml kanamycin (Duchefa, Haarlem, The Netherlands) was added to the medium. OD_{600} was then measured on a Beckman Coulter DU 530 spectrophotometer and the bacteria were diluted to

an OD_{600} of 0.1 in LB medium. For *Y. pseudotuberculosis*, 2.5 mM $CaCl_2$ was added to the medium. 100 µl of bacterial solution was added to the wells of a transparent 96-well plate followed by addition of 1 µl of serially diluted test compounds in DMSO. To control wells were added an equal amount of DMSO. The plate was incubated at 37 °C with shaking (250 rpm), and absorbance at 600 nm was periodically determined during 24 h (once every hour for the first seven hours, then once after 24 h) using a Tecan GENios microplate reader.

Results

For confirmation of screening results, (-)-hopeaphenol was re-tested in a combined assay for inhibition of YopH secretion and *yopE* expression, using the YPIII(pIB102EL) strain of *Y. pseudotuberculosis*. YopH secretion and *yopE* expression was measured through phosphatase and luciferase activity, respectively. Dose dependent inhibitory effect was seen in both assays, with full inhibition at 50 µM (Figures 5A, for phosphatase, and 5B, for luciferase, wherein compound 1 is isolated (-)-hopeaphenol and compound 2 is purchased (-)-hopeaphenol).

Furthermore, (-)-hopeaphenol did not affected the growth of *Y. pseudotuberculosis* (Figures 6A and 6B), which indicates that the compounds act on targets connected to the T3SS. It also did not affect the growth of *P. aeruginosa* either (Figures 7A and 7B). This was a pre-requisite for further testing, as the compounds for use in the present methods should act as virulence blockers and not as traditional antibiotics. Due to the color of the compounds themselves, the absorbance values are higher than for the DMSO control at several concentrations.

HeLa cell based infection assay

Next, the inhibitory potential of (-)-hopeaphenol against *P. aeruginosa* infection was investigated in an infection assay based on HeLa cell morphology.

Materials and methods

HeLa cells were washed with PBS (pH 7.2) and harvested in growth medium using a cell scraper. Cells were seeded into flat-bottomed 96-well plates (0.2 x 10^5 cells/well) and incubated overnight at 37 °C in humidified air with 5 % CO_2. Different strains of *P. aeruginosa* (PAK, strain 180 and Xen5) were grown overnight in LB medium at 37 °C with shaking (250 rpm). On infection day, the bacteria were diluted 2x in DMEM without phenol red (Life Technologies), supplemented with 3.97 mM l-alanyl-l-glutamine, and incubated for 1 h at 37 °C with shaking (250 rpm). OD_{600} was measured on a Beckman Coulter DU 530 spectrophotometer and the bacteria were diluted to an OD_{600} of 0.0004. HeLa cells were washed with PBS (pH 7.2) and 50 µl of serially diluted test compounds (in DMEM without phenol red, supplemented with 10 % FBS and 3.97 mM l-alanyl-l-glutamine) were added to the plate, followed by addition of 50 µl bacterial solution to a final OD_{600} of 0.0002. To control wells, 50 µl of the same medium was added containing an amount of DMSO corresponding to the amount in the test compound wells (to a final maximum of 1 %). 50 µl bacterial solution or medium was added to the infected and the uninfected control wells, respectively. PAK *exsA*::Ω, a T3SS defective mutant strain, was used as a control for T3SS specificity. A plate with the same layout but with no addition of bacteria was run in parallel, to assess the cytotoxicity of the test compounds. The plates were incubated at 37 °C in humidified air with 5 % CO_2, typically for 5 h. Cell morphology was investigated in an inverted phase contrast microscope (Nikon Eclipse TE2000-S; Nikon, Tokyo, Japan) to evaluate the ability of the test compounds to protect against infection by *P. aeruginosa*. Infected cells round up and detach from the bottom of the well. Photographs of the cells were taken using a Nikon Digital Sight DS-U2 (Nikon, Tokyo, Japan) and imaging software NIS-Elements (Nikon, Tokyo, Japan).

Results

The inhibitory potential of (-)-hopeaphenol against *P. aeruginosa* infection was investigated in a HeLa cell based infection assay, in which cell morphology was observed in an inverted phase contrast microscope after infection by strains of *P. aeruginosa* (PAK, strain 180 and Xen5) for 5 h.

Infected cells display changes in morphology; they round up and detach from the bottom of the well, due to depolarization of actin microfilaments by the T3SS-toxins, Exotoxin S and T (Frithz-Lindsten *et al.* (1997) Mol Microbiol 25:1125-39; Sundin *et al.* (2001) Cell Microbiol 3:237-46). PAK *exsA*::Ω, a T3SS defective mutant strain, was used as a control for T3SS specificity. With the PAK strain, the compounds inhibited infection in a dose dependent manner with complete inhibition at 100 µM, where cell morphology in infected wells was indistinguishable from that in uninfected control wells (Figures 8A and 8B). A similar inhibitory effect was observed using either *Pseudomonas* strain 180 or Xen5.

These results indicate that (-)-hopeaphenol inhibits infection of HeLa cells by *P. aeruginosa* in a dose-dependent manner.

J774-based infection assay (UptiBlue)

Next, the ability of (-)-hopeaphenol to inhibit infection by *P. aeruginosa* of J774 cells, in a mouse macrophage cell line, was tested. Cell viability was scored by using UptiBlue, an oxidation-reduction fluorescent indicator of cell viability.

Materials and methods

J774 cells were washed with PBS (pH 7.2) and harvested in growth medium using a cell scraper. Cells were seeded into flat-bottomed 96-well plates (0.3×10^5 cells/well) and incubated overnight at 37 °C in humidified air with 5 % CO_2. Strains of *P. aeruginosa* (PAK, strain 180 and Xen5) were grown overnight in BHI medium at 37 °C with shaking (250 rpm). On infection day, OD_{600} was measured on a Beckman Coulter DU 530 spectrophotometer and the bacteria were diluted to an OD_{600} of 0.001. J774 cells were washed

with PBS (pH 7.2) and 40 µl of serially diluted test compounds (in DMEM without phenol red, supplemented with 6.25 % FBS and 3.97 mM l-alanyl-l-glutamine) were added to the plate, followed by addition of 10 µl bacterial solution to a final OD_{600} of 0.0002. To control wells, 40 µl of the same medium was added containing an amount of DMSO corresponding to the amount in the test compound wells (to a final maximum of 1 %). 10 µl bacterial solution or medium was added to the infected and the uninfected control wells, respectively. PAK *exsA*::Ω, a T3SS defective mutant strain, was included as a control for T3SS specificity. A plate with the same layout but with no addition of bacteria was run in parallel, to assess the cytotoxicity of the test compounds. The plates were incubated for 4 h at 37 °C in humidified air with 5 % CO_2. 10 µl UptiBlue (Interchim, Montluçon, France), a fluorescent reagent used to assess cell viability, was added to all wells and the plates were incubated at 37 °C in humidified air with 5 % CO_2 for an additional 40 min. Fluorescence was measured on a Tecan GENios microplate reader using excitation and emission wavelengths of 535 nm and 595 nm, respectively, with large values indicating healthy cells. Percent of control values were calculated relative to the uninfected control, which was set to 100 %. Also, cell morphology was observed in an inverted phase contrast microscope, to check for conformity with fluorescence readings.

Results

(-)-Hopeaphenol was tested in a J774 cell based infection assay with strains of *P. aeruginosa* (PAK, strain 180 and Xen5), using UptiBlue, a fluorescent reagent used to assess cell viability via cellular metabolism. Fluorescence was measured after 4.67 h of infection, with excitation and emission wavelengths of 535 nm and 595 nm, respectively. Infected cells give rise to lower fluorescence readings and vice versa. PAK *exsA*::Ω, a T3SS defective mutant strain, was included as a control for T3SS specificity.

The data disclosed show that (-)-hopeaphenol inhibited infection of J774 cells by the PAK strain in a dose dependent manner (Figure 9). These results are similar to, but slightly better than, the results obtained using the

HeLa cell assay as described above, and show almost complete inhibition of infection by (-)-hopeaphenol already at 50 µM, at least when using the PAK strain. The inhibitory effect was marginally lower when cells were infected with strain 180 or Xen5 instead.

Contact hemolysis assay

Next, (-)-hopeaphenol was tested in a hemolysis assay, where the release of hemoglobin from red blood cells was measured. Sheep blood was infected with *P. aeruginosa* strain PAK and the T3SS defective mutant strain PAK*popB,* that does not give rise to hemolysis, was used as a control for T3SS specificity.

Materials and methods

P. aeruginosa strains PAK and PAK*popB*, a T3SS defective translocation mutant used as a control for T3SS specificity, were grown overnight in LB medium at 37 °C with shaking (250 rpm). On infection day, the overnight cultures were centrifuged at 18000 x g for 10 min and supernatants were removed. The resulting pellets were resuspended in an equal amount of LB medium containing 5 mM EGTA and 20 mM $MgCl_2$ (for calcium depletion), followed by 1.5x dilution in the same medium. The bacteria were grown for 2 h at 37 °C with shaking (250 rpm). OD_{600} was measured on a Beckman Coulter DU 530 spectrophotometer and the cultures were diluted to an OD_{600} of 0.84. Blood cells were washed by centrifugation: 12.5 ml sheep blood (Håtunalab, Håtunaholm, Sweden) was added to two 50 ml tubes and the tubes were filled with PBS (pH 7.2) up to 50 ml, followed by centrifugation at 858 x g for 10 min. Supernatants were removed and PBS (pH 7.2) was added up to the same volume, and the pellet was resuspended. The cells were washed at least two more times (to obtain a clear supernatant) and then resuspended in 8 ml PBS (pH 7.2), after which the tubes were pooled. OD_{600} was measured on a Beckman Coulter DU 530

spectrophotometer and the blood was diluted to an OD_{600} of 100. 100 µl of blood was seeded to two 96-well plates (one for infection and one for assessment of cytotoxicity) and 75 µl PBS (pH 7.2) was added to all wells. 75 µl bacterial solution (PAK in calcium depleted LB medium) or 75 µl calcium depleted LB medium was added to the infection plate and the cytotoxicity plate, respectively. 2.5 µl of serially diluted test compounds (in DMSO) were added to both plates. Four different controls were included: PAK, PAK*popB* and blood only, all with the addition of 2.5 µl DMSO, and finally blood without DMSO. The plates were centrifuged at 500 x g for 20 min at 20 °C and incubated for 4 h at 37 °C. After incubation, the blood was resuspended by careful pipetting, and the plates were centrifuged at 500 x g for 20 min at 20 °C. 100 µl of the supernatants were transferred to new 96-well plates and absorbance at 560 nm was measured on a Tecan GENios microplate reader. Percent of control values were calculated relative to the infected control, which was set to 100 %.

Results

(-)-Hopeaphenol was tested in a hemolysis assay, where the release of hemoglobin from red blood cells was measured. Sheep blood was infected with P. aeruginosa strains PAK or PAK*popB*, a T3SS defective mutant that does not give rise to hemolysis (used as a control for T3SS specificity), and absorbance at 560 nm was measured after 4 h. The data shows that (-)-hopeaphenol completely inhibited infection at 50 µM, where hemoglobin release was at control levels (Figure 10).

Western blot for expression and secretion of ExoS

Next, the ability of (-)-hopeaphenol to affect expression and secretion of ExoS, a toxin secreted by *P. aeruginosa* via the T3SS, was investigated by Western blot analysis of i) bacteria and surrounding medium and ii) surrounding medium only, respectively.

Materials and methods

P. aeruginosa strains PAK and PAK exsA::Ω were grown overnight in BHI medium at 37 °C with shaking (250 rpm). The overnight cultures were diluted 100x in BHI medium containing either 2.5 mM $CaCl_2$ or 5 mM EGTA and 20 mM $MgCl_2$ (for calcium depletion). 2 ml of the diluted cultures were transferred to culture tubes and 20 µl test compound (in DMSO) was added. Three different controls were included: PAK without calcium, PAK *exsA*::Ω without calcium and PAK with calcium, all with the addition of DMSO instead of test compound. The tubes were incubated for 3 h at 37 °C with shaking (250 rpm). After incubation, OD_{600} was measured on a Beckman Coulter DU 530 spectrophotometer for later adjustment of gel loading volumes. 75 µl of the cultures were mixed with 25 µl 4x NuPAGE LDS sample buffer (Life Technologies, Carlsbad, CA, USA) forming the total protein samples, which were stored in the fridge until loading on gel. Supernatant samples were prepared by transferring 1 ml of the cultures to micro centrifuge tubes followed by centrifugation at 18000 x g for 10 min at 4 °C. 75 µl of the supernatants were mixed with 25 µl sample buffer and stored in the fridge until loading on gel. DTT was added to each sample to a final concentration of 50 mM and the tubes were placed in a heating block at 70 °C for 10 min. Samples were loaded on a NuPAGE 10% Bis-Tris gel (Life Technologies) and electrophoresis was performed in MES running buffer at 200 V for approximately 30 min, with NuPAGE antioxidant (Life Technologies) added to the inner buffer chamber. Loading volumes were adjusted based on the OD_{600} of the original cultures; the sample with the lowest value was set to 10 µl for the total protein samples and 15 µl for the supernatant, and the volumes for samples with higher values were lowered accordingly. After electrophoresis, the gel was placed in NuPAGE transfer buffer (Life Technologies). An Immobilon-P membrane (Millipore, Billerica, MA, USA) was activated in methanol for 15 s and placed on top of four filter papers, pre-wet in transfer buffer. The gel was placed on the membrane, followed by another four pre-wet filter papers. Blotting was performed at 20 V for 40 min, after which the

membrane was blocked in 5 % milk in TBS-T for at least 1 h. The membrane was then placed in 1 % milk in TBS-T containing primary rabbit anti-ExoS antiserum for 1 h and washed with TBS-T for 3 x 10 min, followed by 1 h incubation with secondary donkey anti-rabbit horseradish peroxidase linked antibody (GE Healthcare, Little Chalfont, UK) diluted in 1 % milk in TBS-T. The membrane was again washed with TBS-T for 3 x 10 min and developed using ECL Plus Western blotting detection reagents (GE Healthcare). Band intensities were analyzed in a FluorChem Q MultiImage III (Alpha Innotech, San Leandro, CA, USA) and percent of control values were calculated relative to the total protein and supernatant samples, respectively, which were set to 100 %.

Results

The testing of (-)-hopeaphenol was continued by Western blot analysis for secretion and expression of ExoS, a toxin secreted by *P. aeruginosa* via the T3SS. *P. aeruginosa* strain PAK was incubated with the compound for 3 h at 37 °C, after which expressed ExoS (ExoS present in both bacteria and the surrounding medium) and secreted ExoS (ExoS present in medium only) samples were collected. The PAK *exsA*::Ω strain was included as a negative control. ExoS content in the samples was examined by Western blot analysis using an anti-ExoS antiserum and by analyzing band intensities.

As seen in Figure 11, (-)-hopeaphenol efficiently inhibited the secretion of ExoS at 50 µM as well as the expression of ExoS. ExoS expression was, however, more markedly inhibited at 100 µM. The ability to inhibit ExoS expression and secretion supports the notion that (-)-hopeaphenol acts on the T3SS.

Swimming and swarming motility assay

As many proteins involved in the bacterial flagella are homologous to proteins of the T3SS, it was interesting to test if (-)-hopeaphenol could act on

the bacterial flagellum as well. To investigate this, bacterial swimming and swarming motility assays were used.

Materials and methods

Swimming motility assay

P. aeruginosa strains PAK and PAK *fliA*::Gm, a mutant strain unable to swim, were grown overnight in LB medium at 37 °C with shaking (250 rpm). PAK *fliA*::Gm was grown with 20 µg/ml gentamicin. On the next day, 4 ml 0.3 % LB agar containing different concentrations of test compound in DMSO was added to 6-well plates and left to solidify. Experiments were performed in triplicate. For control wells, the same amount of DMSO was added instead. The overnight cultures were diluted 10x in LB medium and incubated for 3 h at 37 °C with shaking (250 rpm). OD_{600} was measured on a Beckman Coulter DU 530 spectrophotometer and the cultures were diluted to an OD_{600} of 0.3. 1 µl of PAK solution was placed at the center of each test compound well. To control wells, either 1 µl of PAK solution or 1 µl of PAK *fliA*::Gm solution was added, and the plates were incubated at 30 °C for 16 h. Swimming zone diameters were measured using a Bio-Rad GS-800 densitometer (Bio-Rad Laboratories, Hercules, CA, USA) and Quantity One 4.6.6 software (Bio-Rad Laboratories, Hercules, CA, USA) and percent of control values were calculated relative to the PAK control, which was set to 100 %.

Swarming motility assay

P. aeruginosa strain PAO1 was grown overnight in LB medium at 37 °C with shaking (250 rpm). On the next day, 4 ml 0.5 % agar in M8 medium containing different concentrations of test compound in DMSO was added to 6-well plates and left to solidify. Experiments were performed in triplicate. For control wells, the same amount of DMSO was added instead. OD_{600} of the overnight culture was measured on a Beckman Coulter DU 530 spectrophotometer and the culture was diluted to an OD_{600} of 0.3. 1 µl of bacterial solution was placed at the center of each test compound well, and the plates were incubated at 37 °C for 20 h. Swarming zone areas were

measured using a Bio-Rad GS-800 densitometer and Quantity One 4.6.6 software and percent of control values were calculated relative to the control, which was set to 100 %.

Results

With the amassed evidence for the T3SS specificity of (-)-hopeaphenol in mind, it was deemed interesting to test whether it could act on bacterial flagella as well, as many proteins involved in secretion and assembly are homologous to proteins in the T3SS (Aldridge and Hughes (2002) Curr Opin Microbiol 5:160-5). This was tested in motility agar assays, where the ability of the compound to inhibit swimming and swarming motility was assessed. *P. aeruginosa* strains PAK and PAO1 were used to study swimming and swarming, respectively. Bacterial solution was placed on agar containing the compound, and swimming zone diameters and swarming zone areas were measured after incubation.

(-)-Hopeaphenol showed inhibition of both swimming (Figure 12) and swarming (Figure 13) motility in a dose dependent manner.

Inhibition of *E.coli* infection

Next, the inventors proceeded to test the ability of (-)-hopeaphenol to inhibit infection of J774 cells by enteropathogenic and enterohemorrhagic *E. coli* (EPEC and EHEC, respectively) using a cell viability assay based on the release of lactate dehydrogenase (LDH) from infected cells.

Materials and methods

J774 cells were washed with PBS (pH 7.2) and harvested in growth medium using a cell scraper. Cells were seeded into flat-bottomed 96-well plates (0.3×10^5 cells/well) and incubated overnight at 37 °C in humidified air with 5 % CO_2. EPEC or EHEC (enteropathogenic *E. coli* and enterohemorrhagic *E. coli*) bacteria were grown overnight in LB medium at 37 °C with shaking (250 rpm). On infection day, the bacteria were diluted 10x

in DMEM without phenol red, supplemented with 3.97 mM l-alanyl-l-glutamine, and incubated for 1 h at 37 °C with shaking (250 rpm). OD_{600} was measured on a Beckman Coulter DU 530 spectrophotometer and the bacteria were diluted to an OD_{600} of 0.004. J774 cells were washed with PBS (pH 7.2) and 50 µl of serially diluted test compounds (in DMEM without phenol red, supplemented with 10 % FBS and 3.97 mM l-alanyl-l-glutamine) were added to the plate, followed by addition of 50 µl bacterial solution to a final OD_{600} of 0.002. To control wells, 50 µl of the same medium was added containing an amount of DMSO corresponding to the amount in the test compound wells (to a final maximum of 1 %). 50 µl bacterial solution or medium was added to the infected and the uninfected control wells, respectively. For technical reasons, wells with medium only (to be used as background) and wells for subsequent addition of lysis buffer (method validation) were included. A plate with the same layout but with no addition of bacteria was run in parallel, to assess the cytotoxicity of the test compounds. The plates were incubated for 5 h at 37 °C in humidified air with 5 % CO_2. An LDH release assay was performed, using the Cytotoxicity Detection KitPLUS (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, but with some modifications. 5 µl lysis buffer was added to the lysis control wells and the plates were incubated for 15 min at 37 °C in humidified air with 5 % CO_2. The plates were centrifuged at 250 x g for 5 min at 4 °C. 50 µl of the supernatants were transferred to new 96-well plates and mixed with an equal amount of reaction mixture. The new plates were incubated for 25 min in the dark at room temperature. Finally, 25 µl stop solution was added and absorbance was measured at 492 nm and 650 nm (reference wavelength) on a SpectraMax Plus microplate reader (Molecular Devices, Sunnyvale, CA, USA). Percent of control values were calculated relative to the infected control, which was set to 100 %.

Results

(-)-Hopeaphenol was tested for inhibition of infection by enteropathogenic and enterohemorrhagic *E. coli* (EPEC and EHEC,

respectively), as these bacteria possess a T3SS as well, using a J774 cell based LDH release assay. LDH release was measured after 5 h of infection, with infected cells giving rise to higher release. The compound completely inhibited EPEC and EHEC infection at 50 μM and 100 μM, respectively, where LDH release was at or slightly lower than uninfected control levels (Figures 14A and 14B). (-)-Hopeaphenol has also been shown to inhibit infection of *Citrobacter rodentium* and *Y. pseudotuberculosis* in *ex vivo* assays.

Taken together, (-)-hopeaphenol seems to affect bacteria harboring T3SS, meaning that it should be able to block infection with other pathogenic bacteria, for example *Shigella* spp. and *Salmonella* spp.

In vivo efficacy assay

The inventors further investigated the ability of (-)-hopeaphenol to inhibit *P. aeruginosa* strain Xen5 infections *in vivo* by employing an acute murine lung infection model with (-)-hopeaphenol treatment at 2 and 12 hours post-infection and following survival of treated and non-treated mice was for 24 hours post-infection.

Materials and methods

Immune competent female BALB/c mice obtained from Harlan Laboratories were allowed to acclimatize, and were randomized into groups based on bodyweight. The mice were 6-8 weeks of age at the start of the study. Prior to infection, the P. aeruginosa strain Xen 5 was prepared from multiple sweeps of single colonies from agar plates incubated at 37 °C overnight in ambient air. Multiple sweeps of CFU (Colony Forming Units) were taken into 20 ml DPBS (with Ca^{2+} and Mg^{2+}) with an inoculation loop.

The suspension was then allowed to settle for 10 min, permitting any clumps to sediment. The resulting stock was adjusted to an OD_{600} of 0.5. Based on a relationship of a suspension in DPBS at an OD_{600} of 0.5 yielding 3.75×10^8 CFU/ml, an appropriate amount of stock was sampled for further

dilution or concentration into DPBS (with Ca^{2+} and Mg^{2+}). All prepared stocks remained on ice until needed (inoculation took place within 1 h of preparation). Aliquots were taken from the stock inoculum for dilution and plating to verify the actual inoculum used in the infection. The mice were anesthetized with isofluorane in oxygen at 1 l/min. A stock solution of bacteria at 2.9 x 10^7 CFU/ml was made. Infection was established by intranasal inhalation of 35 µl of bacteria, containing 1 x 10^6 CFU bacteria as small droplets. Immediately after infection, verification cultures were made of both stock solutions to ensure correct infection doses for all groups. Filter tops were used on cages to prevent bacterial aerosol formation. Mice were treated by intranasal inhalation of a 35 µl droplet of compound 1 (1 mM compound 1 in PBS; 1 % DMSO) 2 and 12 hours after infection. The control mice were given 35 µl PBS (containing 1 % DMSO). The progression of infection was monitored using IVIS imaging at 0, 8, 16 and 24 h post-inoculation. At termination (24 h), lungs were assessed for signs of gross pathology. Termination of animals was made when animals were judged to have reached a humane endpoint.

Results

In order to investigate the effect of (-)-hopeaphenol *in vivo*, an acute murine lung model was used. The mice were infected with *P. aeruginosa* strain Xen5 and treated with (-)-hopeaphenol at 2 and 12 h after infection. The treatment showed a statistically significant increased survival of mice treated with (-)-hopeaphenol (Figure 15).

Further Biological Testing: (-)-hopeaphenol 'alone' series of tests

A series of tests in part overlapping with those already described were carried out on (-)-hopeaphenol alone to confirm results and allow for different analyses. The experimental protocol is given below followed by a discussion of the results obtained with reference to the appropriate figures.

Experimental YopE reporter-gene and YopH phosphatase assays

Luciferase analysis was performed essentially as described before (Kauppi et al 2003, Chem Biol 10, 241-249). The *Yersinia pseudotubeculosis* serotype III strain YPIII(pIB102) *yopE-luxAB* was grown over night in LB supplemented with Km, diluted to OD600 0.1 in Ca2+ depleted LB and dispensed in 96-well plates (NuncTM, flat bottom, white) with 100 µL / well. Compound was added to a final concentration between 1 and 100 µM. Final DMSO concentration did not exceed 1 %. The plates were incubated on a rotary shaker for 1 h at 26 °C followed by 2 h at 37 °C. The plates were allowed to adjust to room temperature on the bench for 2 h before 50 µL of decanal in water (10 µL / 100 mL) was added. After 4 minutes chemiluminescence was detected by a microplate reader (TECAN Infinite M200, gain 150, integration time 20 ms). An enzymatic YopH phosphatise assay was performed in parallel to the luciferase analysis. After 1 h incubation at 26 °C and 2 h at 37 °C the bacterial suspension (10 µL per well) was transferred to new 96-well plates (NuncTM, flat bottom, transparent) containing the YopH substrate mixture (90 µL, 25 mM, p-nitro phenyl phosphate, 40 mM 2-(N-morpholino)ethanesulfonic acid, pH 5.0, and 1.6 mM dithiothreitol in water). The plates were incubated at 37 °C for 15 min. To quench the reaction NaOH (20 µL, 1 M) was added to each well. The absorbance was measured at 405 nm in a microplate reader (TECAN Infinite M200).

Western blot analysis of total culture and supernatant samples from *Y. pseudotuberculosis*

Dose-response experiment: An overnight culture of *Yersinia pseudotuberculosis* YPIII(pIB102) was diluted 1:20 in Ca2+ depleted LB. The test compound was added to final concentration between 1.6 - 100 µM. The cultures were incubated for 1 h at 26 °C followed by 3 h at 37 °C. The cultures were centrifuged and the supernatant was mixed with sample buffer and loaded on to a 12 % SDS polyacrylamide gel. The gel was blotted onto a PVDF membrane. Blots were probed with polyclonal rabbit anti Yop-

antiserum and polyclonal goat anti-rabbit immunoglobulins/HRP (Dako Denmark). Chemiluminiscense was generated using Millipore ImmobilonTM Western chemiluminescent HRP substrate and detected with CCD camera.

Reversibility experiment: An overnight culture of YPIII(pIB102) was diluted 1:20 in LB supplemented with 2.5 mM CaCl2. Compound was added to a final concentration of 40 µM, control cultures received only DMSO. Cultures were first incubated at 26 °C for 30 min followed by 2 h at 37 °C for induction of Yop expression. To study the reversibility of the compound the samples was centrifuged and the pellet washed with LB once. Then LB complemented with 20 mM MgCl2 and 5 mM EGTA for Ca2+ depletion or 2.5 mM CaCl2, with 40 µM compound or DMSO was added followed by incubation at 37 °C for 45 min. The supernatant was mixed with sample buffer and the rest of the experiment was the same as described for the dose-response experiment.

Time study: Analyses of the reporter-gene signal with compound added at different time points of the T3SS induction. Overnight culture of YPIII(pIB102) *yopE-luxAB* was diluted to OD600 0.1 and dispensed in 96-well plate with 100 µL in each well. Compound (1 µL, 5 mM) was added at different time points t = 0, 30, 60, 90, 120, 150, and 180 minutes, where t = 60 is at the temperature shift. Incubation and measurements was the same as for luciferase experiments.

Inhibition of effector protein translocation in *Y. pseudotuberculosis*

HeLa 229 cells (ATCC® CCL-2.1TM) were seeded into 96-well plates (NuncTM, flat bottom, transparent), 100 µL 1x105 cells / mL one day before translocation experiment. Overnight cultures of YPIII(pIB102) *yopE-bla* and YPIII(pIB604) *yopE-bla* was diluted 1:10 in Ca2+ depleted LB and grown 1 h at 26 °C followed by 2 h at 37 °C. (-)-Hopeaphenol was added to the HeLa 229 cells at the same time as infected with MOI 50. The cells were infected for 1 h at 37 °C 5 % CO_2 followed by 15 min at room temperature. The cells were loaded with LiveBLAzerTM FRET - B/G loading kit with CCF4-AM protocol from Invitrogen according to the manufacture's instructions.

Fluorescence was measured in a microplate reader (Synergy H4 Hybrid Reader, BioTek®). For microscope pictures HeLa 229 cells were seeded into 35 mm glass-bottom microwell dishes (MatTek) 2 mL, 1.5x105 cells/mL the day before translocation experiments. Translocation experiments and labeling was undertaken as written above.

Western blot analysis of total culture and supernatant samples from *P. aeruginosa*

Overnight cultures of *P. aeruginosa* (strains PAK and PAK *exsA*::Ω, a T3SS mutant, Table 1) in brain heart infusion medium (BHI; BD) were diluted 1:100 in either Ca2+ enriched (2.5 mM CaCl2) or Ca2+ depleted (5 mM EGTA and 10 mM MgCl2) BHI medium and (-)-hopeaphenol or 1 % DMSO (as a control) were added. The tubes were incubated for 3 h at 37 °C with shaking (250 rpm). The total and supernatant samples were prepared and run on NuPAGE 10 % Bis-Tris gel (Life Technologies) according to the manufacturer's protocol. In brief, by mixing the samples with 4x NuPAGE LDS sample buffer (Life Technologies) and DTT, heating to 70 °C and loading onto the gel. ExoS protein was visualized using rabbit anti-ExoS serum and donkey anti-rabbit HRP-linked Ig (Amersham Bioscience) by ECL Western blot (GE Healthcare), which was performed according to standard procedures. Band intensities were analyzed in a FluorChem Q MultiImage III image system (Alpha Innotech).

Infection of HeLa cells with *P. aeruginosa*

HeLa cells were seeded into flat-bottom 96-well plates (0.2 × 105 cells/well) in DMEM (GlutaMAX –I, Gentamicin, FBS:Life Technologies) and incubated overnight at 37 °C 5 % CO2. Overnight cultures of *P. aeruginosa* in Luria-Broth (LB; Sigma- Aldrich) were diluted 1:2 in DMEM without phenol red (Life Technologies), supplemented with 3.97 mM l-alanyl-lglutamine, and incubated for 1 h at 37 °C with shaking (250 rpm). The bacteria were then diluted to an OD600 of 0.0004. HeLa cells were washed (in DMEM without phenol red, supplemented with 10 % FBS and 3.97 mM l-alanyl-lglutamine)

and different concentrations of (-)-hopeaphenol were added to the plate, followed by addition of bacterial solution to a final OD600 of 0.0002. The cytotoxicity of (-)- hopeaphenol was tested on a plate with the same layout but with no addition of bacteria. The plates were incubated at 37 °C 5 % CO2 for 5 h and cell morphology was investigated using an inverted phase contrast microscope (Nikon Eclipse TE2000-S; Nikon) and photographs were taken using a Nikon Digital Sight DS-U2 camera (Nikon) and imaging software NIS-Elements (Nikon).

Infection experiments with *C. trachomatis*

For *Chlamydia* experiments, HeLa 229 cells were seeded into 96-well plates and 24-well plates with coverslips. Cells were infected the next day with 0.1 multiplicities of infection (MOI) of *C. trachomatis* serovar L2 (VR-902B, ATCC®) in Hank's Balanced Salt Solution (HBSS). Infection was done in triplicates in 96-well plates and in duplicate in 24-well plates. To determine the minimum inhibitory concentration (-)- hopeaphenol was added 1 h after infection at concentrations ranging from 100 to 6 µM. For the pretreatment experiment either the cells or the *C. trachomatis* was treated for 1 h with indicated concentrations of (-)-hopeaphenol in room temperature. The (-)-hopeaphenol was removed from pretreated: host cells by changing the medium to HBSS containing DMSO treated *C. trachomatis*, and from pretreated *C. trachomatis* by centrifuging the bacteria down at 21,000 × *g* removing the supernatant and suspending the bacteria in fresh HBSS. Controls were treated similarly with equal amount of DMSO added. The 96-well plates were fixed with methanol 19 h post infection and stained with DAPI and in-house antichlamydial rabbit antibody labeled with secondary FITC anti-rabbit antibody. The infection was analyzed using ArrayScan VTI HCA Reader (Thermo Fisher Scientific Inc. Waltham. MA), which automatically generated photomicrographs using 20 × objective. The *Chlamydia* inclusions were calculated with spot detection method included in the ArrayScan software. The 24-well plates were fixed with methanol 48 h post infection and labeled similarly to 96-well plates. The micrographs were taken with Confocal

Nikon 90i Eclipse microscope equipped with C1 Plus confocal (Nikon Instruments Europe B.V., Amsterdam, Netherlands) and EZ-C1 3.91 software. Exaction wavelengths were 405 and 488 nm and emissions were detected at 430/435 and 515/530 nm. The picture content was clarified by adjusting the curves of individual micrographs in Adobe Photoshop C5 Extended 12.0.4 (Adobe Systems Inc., San Jose, CA). Adjustments were done in similar manner for each micrograph.

Treatment of five gram-negative and five gram-positive bacteria

Overnight cultures of the gram-positive bacteria *Micrococcus luteus, Staphylococcus epidermis, Staphylococcus aeureus, Bacillus subtilis*, and *Enterococcus faecalis* and the gramnegative bacteria *Y. pseudotuberculosis P. aeruginosa, Proteus mirabilis, Klebsiella pneumonia*, and *E. coli K12* were diluted to OD600 0.1 in 96-well plates 25, 50, and 100 µM (-)- hopeaphenol final concentration was added to the wells in triplicate. Plates were incubated at 37 °C and samples were mixed before OD600 was checked at start, after 8 and 24 h.

Results and Discussion for (-)-hopeaphenol 'alone' series of tests

(-)-Hopeaphenol inhibits YopE expression and YopH secretion

As a first step the activity of (-)-hopeaphenol was again confirmed in the primary screening YopE reporter-gene assay and the YopH phosphatase assay. Figure 16A shows the dose-response curves from the reporter-gene and phosphatase assays with IC50 values of 6.6 µM and 3.3 µM, respectively. (-)- Hopeaphenol had no or limited effect on bacterial growth at concentrations up to 100 µM (Figure 16B). To establish if addition of (-)-hopeaphenol at different stages of the induction and secretion phases of *Y. pseudotuberculosis* T3SS affect the efficacy the compound was added at seven time points during infectious conditions. The compound (50 µM) was added every 30th min for 3 h and the temperature shift from 26 to 37 °C was made at t = 60 min, mimicking eukaryote cell contact that trigger effector

protein secretion and translocation. Addition of (-)-hopeaphenol up to t = 90 min strongly attenuated the reporter-gene signal while addition at later time points resulted in a diminished effect (Figure 16C). This indicated that the effect of (-)-hopeaphenol was rapid.

Dose-dependent inhibition of expression and secretion of the translocator protein YopD

Western blot analysis of effector proteins in total culture and supernatant, of YPIII(pIB102) wild-type bacteria incubated together with 7 different concentrations of (-)-hopeaphenol for 1 h at 26 °C followed by 3 h at 37 °C showed a clear dosedependent response for both secretion and expression of translocator protein YopD (Figure 17). The expression of YopD was reduced but could be detected at all concentrations, whereas concentrations above 13 μM completely blocked the secretion of YopD. These results are in line with the data obtained from the YopE reporter-gene and YopH phosphatise assays (Figure 16A), suggesting that (-)-hopeaphenol targets the secretion machinery directly rather than a general attenuation of T3SS genes transcription.

(-)-Hopeaphenol is an irreversible T3SS inhibitor

To investigate if the effect of (-)-hopeaphenol on the T3SS is reversible, overnight cultures of YPIII(pIB102) were diluted and divided into two cultures that were treated with either (-)- hopeaphenol (40 μM) or DMSO alone. To fully induce the T3SS without triggering effector protein secretion, the cultures were grown in calcium containing medium for 30 min at 26 °C followed by 2 h at 37 °C. The cultures were then divided into eight tubes, with or without 40 μM (-)-hopeaphenol. The cultures were incubated at 37 °C for an additional 45 min to trigger effector protein secretion. Western blot analysis was performed on the total culture and the supernatant. The total culture samples all showed Yop expression indicating that (-)- hopeaphenol inhibited secretion but not expression (Figure 18A, lane 1-8). None of the cultures grown in the presence of calcium secreted any Yops irrespective of

the pretreatment conditions (Figure 18B, lane 1, 3, 5, and 7). The culture pretreated with DMSO grown in calcium depletion secretes Yops into the media (Figure 18B, lane 4). When (-)-hopeaphenol was added to the culture pretreated with DMSO in presence of calcium secretion was completely blocked (Figure 18B, lane 2).

This result shows that the effect of (-)-hopeaphenol on the T3SS is rapid. The cultures pretreated with (-)-hopeaphenol were unable to secrete effector proteins both in the DMSO control and when treated with (-)-hopeaphenol independent of the presence or absence of calcium (Figure 18B, lane 5-8). Thus, the effect of (-)-hopeaphenol is not reversible and the results suggest that the compound might bind covalently to its target(s) and that the targets possibly are located on the bacterial cell surface e.g. the extracellular structure of the T3SS.

(-)-Hopeaphenol inhibits YopE translocation

To investigate if (-)-hopeaphenol also inhibits *Y. pseudotuberculosis* effector protein translocation and thereby virulence, a β-lactamase reporter system was used. The bacterial strains used have the effector protein YopE translationally fused to β-lactamase. HeLa cells were infected with YPIII(pIB102) *yopE-bla* with a multiplicity of infection (MOI) of 50 times and (-)-hopeaphenol was added at different concentrations.

As a control experiment HeLa cells were also infected with a translocation deficient mutant YPIII(pIB604) Δ*yopB yopE-bla*. After infection the HeLa cells were loaded with CCF4-AM, which consists of a cephalosporin core linking 7-hydroxycoumarine to a fluorescein. Inside the cells the ester of the substrate will be hydrolyzed to its negatively charged form CCF4, and retained in the cytosol. In the absence of β- lactamase, excitation of the coumarine at 409 nm will result in a Förster resonance energy transfer (FRET) and the emitted light at 520 nm can be detected as a green fluorescent signal. This was the case for the HeLa cells where no translocation of YopE-Bla has occurred. When translocation has occurred the β-lactamase will act on cephalosporin and cleave the substrate resulting in

disruption of the FRET, which after excitation at 409 nm result in light emitted at 450 nm, a blue light signal. The light signal was measured in a microplate reader and percentage of translocation, the blue light signal, was calculated and normalized to DMSO treated YPIII(pIB102) wildtype set to 100 % translocation. The infected HeLa cells were analyzed with fluorescent microscopy as a supplement to the microplate reader data.

Treatment with (-)-hopeaphenol resulted in a dose-dependent response (Figure 19). A series of images (not shown) were also obtained indicating the level of the blue light signal and hence translocation. (-)-Hopeaphenol inhibits translocation of YopE completely at 50, 25, and 13 µM (Figure 19) and at lower concentrations translocation could be observed (Figure 19). Green fluroescence was seen entirely dominating the images at these concentrations with some blue light appearing at 6.5 µM. Infected cells treated with or without DMSO as control have the same amount of translocated YopE-Bla. As additional controls cells were infected with YPIII(pIB604) Δ*yopB* (green fluorescence dominated the image), a translocation deficient mutant, YPIII(pIB102) wild-type (blue light and green light were visible in the image) and left uninfected, with or without 50 µM (-)-hopeaphenol (both images showed only green fluorescence).

(-)-Hopeaphenol blocks ExoS expression and secretion by *P. aeruginosa*

It was decided to examine if (-)-hopeaphenol was able to inhibit the T3SS in *Pseudomonas aeruginosa* a gram-negative pathogen with a T3SS system that is closely related to the T3SS of *Y. pseudotuberculosis. P. aeruginosa* is an opportunistic human pathogen infecting burn wounds, as well as immunocompromised, and leukemia patients. It belongs to the six ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter species, P. aeruginosa, and Entero-bacter species*). Together with *Mycobacterium tuberculosis* they are recognized as the most significant emerging threats of this century. *P. aeruginosa* is a "superbug" with a unique capacity to develop resistance due

to a combination of intrinsic, acquired and adaptive mechanisms. As a consequence, today we have multi-drug resistant *P. aeruginosa* strains for which there are no effective antibiotic treatments available and the trend is that these strains are becoming more common. The need for new antimicrobial drugs for treatment of these multi-resistant strains is extremely urgent and should not be underestimated.

The effector protein ExoS from *P. aeruginosa* is highly similar to the *Y. pseudotuberculosis* effector protein YopE and as with *Yersinia* the T3SS can be triggered by growth at 37 °C and removal of calcium. The wild-type *P. aeruginosa*, strain PAK was grown in calcium-depleted media with or without (-)- hopeaphenol. Western blot analysis of the effector protein ExoS showed that the amount expressed and secreted ExoS was reduced in bacteria treated with (-)-hopeaphenol compared to the DMSO control. Secretion of ExoS is essentially completely blocked by (-)-hopeaphenol at 50 and 100 µM (Figure 20), while treatment with 10 and 20 µM (-)- hopeaphenol reduced the secretion. Inhibition of ExoS expression is dose-dependent as ExoS levels are higher in the bacteria treated with 10 µM (-)-hopeaphenol compared to bacteria treated with 100 µM (Figure 20).

(-)-Hopeaphenol inhibits *P. aeruginosa* virulence

To investigate if (-)-hopeaphenol could inhibit the virulence of *P. aeruginosa*, HeLa cells were infected for 5 h with the wildtype *P. aeruginosa*, strain PAK in the presence of (-)- hopeaphenol at four different concentrations ranging from 20-150 µM. Infected cells treated with 100 and 150 µM (-)-hopeaphenol were completely protected from infection (Figure 21A and 21B), whereas treatment with 50 µM cells were partly protected (Figure 21C). When cells were treated with 20 µM (-)- hopeaphenol, inhibition of virulence could not be observed (Figure 21D). Uninfected cells treated with 150 µM (-)-hopeaphenol for 5 h did not show any signs of toxicity. The results from the experiments with *Y. pseudotuberculosis* and *P. aeruginosa* corroborate each other and support the notion that (-)-hopeaphenol is a T3SS inhibitor.

(-)-Hopeaphenol prevents cell entry and intracellular growth of *C. trachomatis*

Chlamydia is an obligate intracellular pathogen that can only grow inside a eukaryotic cell. *Chlamydiae* have a very different life cycle compared to both *Yersinia* and *Pseudomonas* and the role of the T3SS in *Chlamydia* is still enigmatic. It has a biphasic life cycle consisting of two distinct forms, the infectious elementary body (EB) and the replicative reticulate body (RB). *C. trachomatis* is mostly known to causes the sexually transmitted disease but it can also cause eye infections. It has been stated that T3SS is most likely essential for the survival of *Chlamydia*, and we therefore we tested the effect of (-)-hopeaphenol on *C. trachomatis* growth. HeLa cells were infected with *C. trachomatis* EBs and treated with five different concentrations, ranging between 6 and 100 µM of (-)-hopeaphenol 1 h after infection as per the standard procedure. After 19 h infected cells and *C. trrachomatis* inclusions were stained and analysed with an automated microscope. However we could not observe any effect on *C. trachomatis* growth when treated with natural product at concentrations up to 100 µM (Figure 21).

Since (-)- hopeaphenol is large with many polar hydroxyl groups it probably has low permeability both in eukaryotic and prokaryotic cells. The *Chlamydia* inclusion have low permeability for compounds larger than 520 Da, and we therefore decided to pretreat the bacteria with (-)-hopeaphenol before infection of host cells in order to address if the compound can prevent cell entry. *C. trachomatis* EBs were pretreated with different concentrations of (-)-hopeaphenol for 1 h followed by washing and infection of HeLa cells. Analyses of this experimental setup show that (-)-hopeaphenol inhibit infection and growth of *C. trachomatis* with a clear doseresponse pattern (Figure 21). Pretreatment of the HeLa cells with (-)-hopeaphenol resulted in little or no effect on the *Chlamydia* infection, indicating that the compound directly targets *Chlamydia* (Figure 21). This data corresponds with the data showing an irreversible effect in *Y. pseudotuberculosis*.

(-)-Hopeaphenol does not affect growth of a panel of gram-positive and gram-negative pathogens

Finally a panel of five gram-positive were tested for growth inhibition by (-)-hopeaphenol. The gram-positive bacteria tested were: *Micrococcus luteus, Staphylococcus epidermis, Staphylococcus aeureus, Bacillus subtilis*, and *Enterococcus faecalis*. None of the strains were significantly affected when grown together with 25, 50, or 100 µM (-)-hopeaphenol for 24 h (data not shown). The data suggest that (-)- hopeaphenol is a selective inhibitor of the T3SS in gram-positive bacteria, particularly in *Y. pseudotuberculosis* and *P. aeruginosa*.

Conclusion on (-)-hopeaphenol results

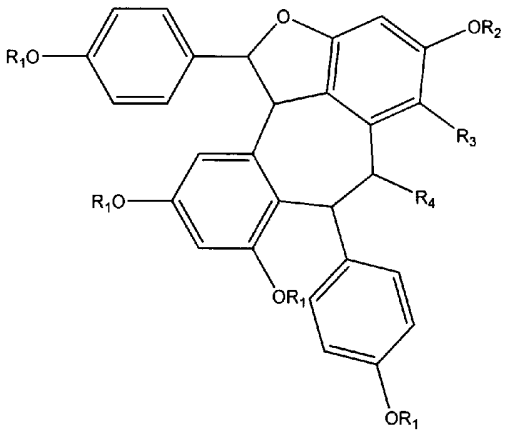
In summary, (-)-hopeaphenol was found to act as a T3SS inhibitor with an IC50 value of 6.6 µM in the YopE reporter-gene assay. Western blot analysis indicated dose-dependent inhibition of secretion with less reduction of Yop expression. Complete inhibition of Yop secretion occurred at concentrations that do not cause any detrimental effect on bacterial growth, indicating a selective inhibition of the T3SS. This is further corroborated by lack of growth inhibition in a panel of grampositive and gram-negative bacteria. Interestingly pretreatment and washout experiments with (-)-hopeaphenol showed that the compound is an irreversible inhibitor of Yop secretion and suggest that the compound possibly binds covalently to its target(s). Considering the size and molecular weight of (-)- hopeaphenol cell permeability might be low which in turn suggest that inhibition occurs by interaction of T3SS components on the bacterial surface. However, no predictive models for permeability of bacteria exist and many antibiotics are typically larger and more hydrophilic than other types of drugs.

It was also identified that (-)-hopeaphenol inhibits the T3SS in the clinically challenging pathogen *P. auerginosa*. In *ex vivo* infection models using HeLa cells the compound blocks translocation of effector proteins by *Y. pseudotuberculosis* and prevents cytotoxicity caused by *P. aeruginosa*. In addition, it has been demonstrated that (-)-hopeaphenol inhibits growth of the

intracellular pathogen *C. trachomatis* when pretreated with the natural product. These results are in line with the hypothesis that (-)-hopeaphenol has low cell permeability. As for *Y. pseudotuberculosis* the data indicates that (-)-hopeaphenol binds covalently to *C. trachomatis*, inhibits successful infection and possibly reduces their growth inside the eukaryotic cell.

ITEMIZED LISTING OF EMBODIMENTS

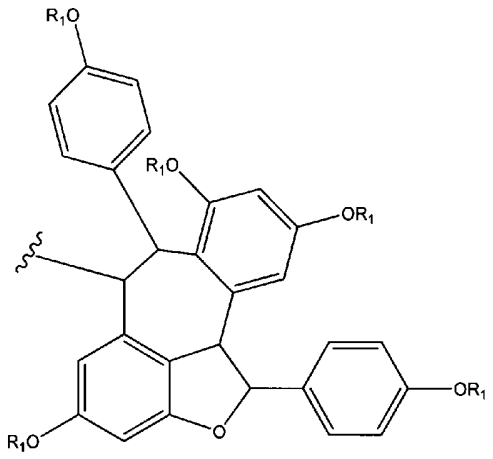
1. Compound for use in the treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism, wherein said compound is a compound of Formula I, or a pharmaceutically acceptable salt thereof:



Formula I

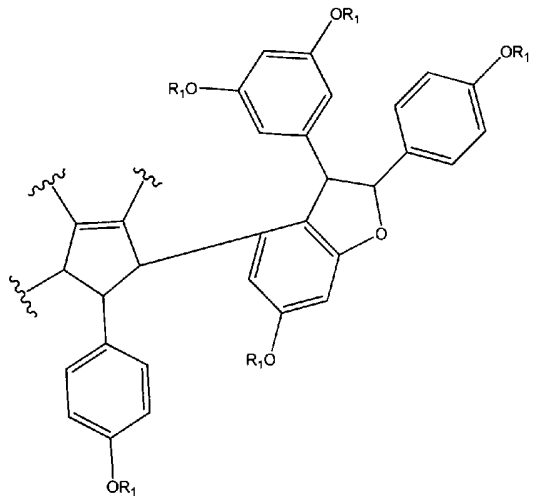
wherein, R_1 and R_2 are independently selected from the group consisting of hydrogen, C_1 to C_{12} alkyl, C_1 to C_{12} alkenyl, aryl, C_1 to C_{12} aldehyde, C_1 to C_{12} alkanone, C_1 to C_{12} carboxyl, C_1 to C_{12} carboxamide, C_1 to C_{12} alkanoyl and a sugar moiety, each of which groups may be substituted or unsubstituted;

R_3 and R_4 are independently selected from hydrogen or the structure shown below



wherein R_1 is as previously described,

or R_3 and R_4 may join to form a substituted five membered ring as shown below



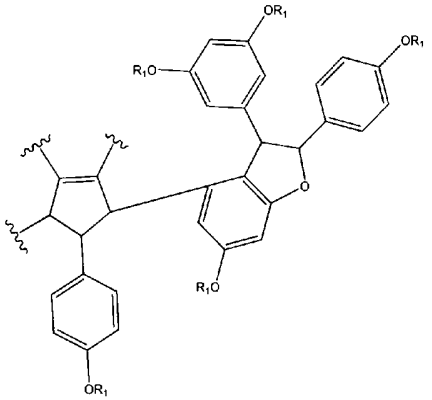
wherein R_1 is as previously described.

2. Compound for use according to item 1, wherein R_1 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl, C_1 to C_6 alkenyl, aryl, C_1 to C_6 aldehyde, C_1 to C_6 alkanone, C_1 to C_6 carboxyl, C_1 to C_6

carboxamide and C_1 to C_6 alkanoyl, each of which groups may be substituted or unsubstituted;

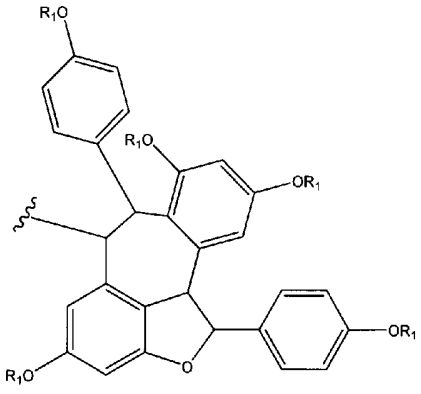
R_2 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl and a sugar moiety;

R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



wherein R_1 is as described,

or R_4 is



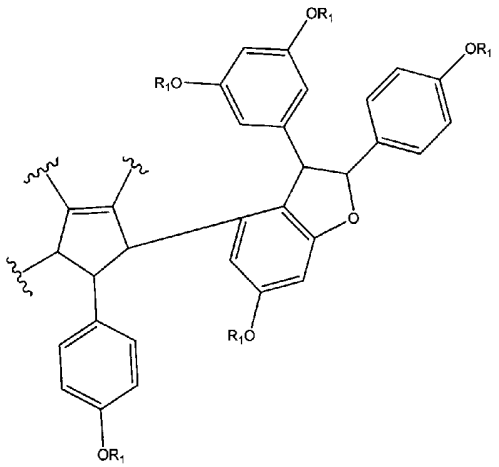
wherein R_1 is as described.

3. Compound for use according to item 1 or item 2, wherein R_1 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl, C_1 to C_6

alkenyl and aryl, each of which groups may be substituted or unsubstituted;

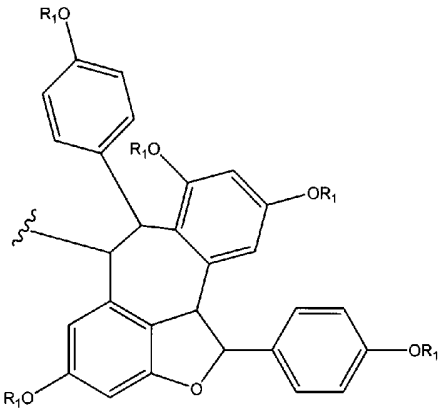
R_2 is selected from the group consisting of hydrogen, methyl, ethyl, propyl, butyl and a glucose moiety;

R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



wherein R_1 is as described,

or R_4 is

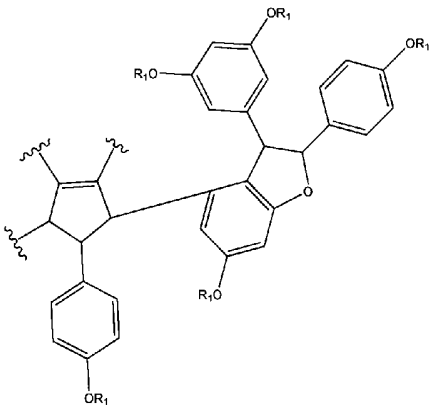


wherein R_1 is as described.

4. Compound for use according to any one of the preceding items, wherein R_1 is selected from the group consisting of hydrogen, methyl, ethyl, propyl and isopropyl, each of which groups may be substituted or unsubstituted;

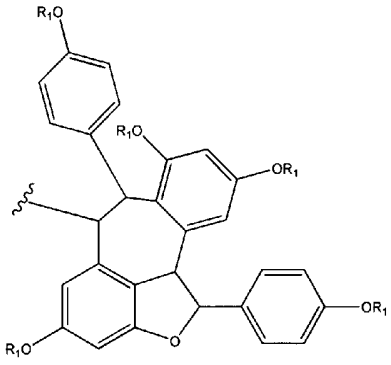
R_2 is selected from the group consisting of hydrogen, methyl, ethyl, propyl and a β-D-glucose moiety;

R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



wherein R_1 is as described,

or R_4 is



wherein R_1 is as described.

5. Compound for use according to any one of the preceding items, wherein the compound of formula I is selected from the group consisting of (-)-hopeaphenol, Vatalbinoside A, Vaticanol B, neohopeaphenol A and C_1 to C_6 O-alkyl derivatives thereof.

6. Compound for use according to item 5, wherein the O-alkyl derivatives of the recited compounds are methyl, ethyl, propyl or butyl derivatives.

7. Compound for use according to any one of the preceding items, wherein said Gram-negative bacterium possesses a type three secretion system.

8. Compound for use according to any one of the preceding items, wherein said Gram-negative bacterium possesses at least one flagellum.

9. Compound for use according to any one of the preceding items, wherein said Gram-negative bacterium possesses a type three secretion system and at least one flagellum.

10. Compound for use according to any one of the preceding items, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter, Chlamydia, Klebsiella, Cronobacter, Serratia, Acinetobacter, Morganella, Lawsonia, Actinobacillus, Francisella, Erwinia, Ralstonia, Rhizobium* and *Xanthomonas.*

11. Compound for use according to item 10, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella,*

Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter and *Chlamydia.*

12. Compound for use according to item 11, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella* and *Shigella.*

13. Compound for use according to item 12, wherein said bacterium belongs to the genus *Pseudomonas.*

14. Compound for use according to any one of the preceding items, wherein said multicellular organism is selected from the group consisting of a plant, a fungus and an animal.

15. Compound for use according to item 14, wherein said multicellular organism is an animal selected from the group consisting of birds, fish, reptiles and mammals.

16. Compound for use according to item 14, wherein said multicellular organism is an animal selected from the group consisting of vertebrate animals including primates, avians, livestock animals (e.g., sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g., rabbits, mice, rats, guinea pigs, hamsters), companion animals (e.g., cats, dogs) and captive wild animals (e.g., foxes, deer, dingoes).

17. Compound for use according to item 15, wherein said mammal is a human.

18. Compound for use according to any one of items 14-17, wherein said Gram-negative bacterium is selected from the group consisting of *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Yersinia pestis*,

Yersinia pseudotuberculosis, *Escherichia coli*, *Salmonella enterica*, *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Chlamydia trachomatis*, *Chlamydia pneumoniae* (*Chlamydophila pneumoniae*), *Salmonella bongori*, *Legionella pneumophila*, *Proteus vulgaris*, *Proteus penneri*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter cancerogenus*, *Enterobacter gergoviae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*, *Enterobacter nimipressuralis*, *Helicobacter pylori*, *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas veronii*, *Citrobacter freundii*, *Citrobacter rodentium*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Burkholderia cepacia*, *Burkholderia cenocepacia*, *Burkholderia multivorans*, *Vibrio cholerae*, *Vibrio anguillarum*, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Bordetella parapertussis*, *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter jejuni*, *Cronobacter sakazakii*, *Serratia marcescens*, *Lawsonia intracellularis*, *Pseudomonas oryzihabitans*, *Pseudomonas plecoglossicida*, *Pseudomonas stutzeri*, *Pseudomonas mendocina*, *Escherichia albertii*, *Escherichia fergusonii*, *Escherichia vulneris*, *Escherichia hermannii*, *Legionella longbeachae*, *Legionella bozemanii*, *Legionella micdadei*, *Legionella feeleii*, *Legionella dumoffii*, *Legionella wadsworthii*, *Legionella anisa*, *Pantoea agglomerans*, *Helicobacter cinaedi*, *Helicobacter fennelliae*, *Helicobacter felis*, *Helicobacter pullorum*, *Helicobacter canadensis*, *Helicobacter winghamensis*, *Helicobacter canis*, *Aeromonas aquariorum*, *Aeromonas salmonicida*, *Aeromonas popoffii*, *Aeromonas jandaei*, *Aeromonas schubertii*, *Citrobacter amalonaticus*, *Citrobacter koseri*, *Citrobacter braakii*, *Citrobacter farmeri*, *Citrobacter sedlakii*, *Burkholderia gladioli*, *Burkholderia fungorum*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio alginolyticus*, *Vibrio mimicus*, *Vibrio fluvialis*, *Vibrio campbellii*, *Vibrio mimicus*, *Vibrio harveyi*, *Bordetella petrii*, *Bordetella trematum*, *Bordetella holmesii*, *Bordetella hinzii*, *Campylobacter concisus*,

Campylobacter curvus, *Campylobacter gracilis*, *Campylobacter hyointestinalis*, *Campylobacter insulaenigrae*, *Campylobacter lari*, *Campylobacter mucosalis*, *Campylobacter rectus*, *Campylobacter showae*, *Campylobacter sputorum*, *Campylobacter upsaliensis*, *Chlamydia suis*, *Chlamydia muridarum*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella granulomatis*, *Cronobacter malonaticus*, *Cronobacter turicensis*, *Serratia plymuthica*, *Serratia liquefaciens*, *Serratia rubidaea*, *Serratia odorifera*, *Serratia fonticola*, *Serratia proteamaculans*, *Acinetobacter baumannii*, *Acinetobacter pittii*, *Acinetobacter nosocomialis*, *Acinetobacter lwoffii*, *Acinetobacter junii*, *Acinetobacter haemolyticus*, *Acinetobacter calcoaceticus*, *Morganella morganii*, *Actinobacillus ureae*, *Actinobacillus hominis*, *Actinobacillus suis*, *Actinobacillus lignieresii*, *Actinobacillus pleuropneumoniae*, *Actinobacillus equuli*, *Francisella tularensis*, *Francisella novicida*, *Francisella noatunensis*, *Francisella philomiragia* and *Francisella piscicida*.

19. Compound for use according to item 18, wherein said Gram-negative bacterium is selected from the group consisting of *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Escherichia coli*, *Salmonella enterica*, *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Chlamydia trachomatis*, *Chlamydia pneumoniae (Chlamydophila pneumoniae)*, *Salmonella bongori*, *Legionella pneumophila*, *Proteus vulgaris*, *Proteus penneri*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Enterobacter asburiae*, *Enterobacter cancerogenus*, *Enterobacter gergoviae*, *Enterobacter hormaechei*, *Enterobacter kobei*, *Enterobacter ludwigii*, *Enterobacter nimipressuralis*, *Helicobacter pylori*, *Aeromonas hydrophila*, *Aeromonas caviae*, *Aeromonas veronii*, *Citrobacter freundii*, *Citrobacter rodentium*, *Burkholderia mallei*, *Burkholderia pseudomallei*, *Burkholderia cepacia*, *Burkholderia cenocepacia*, *Burkholderia*

multivorans, *Vibrio cholerae*, *Vibrio anguillarum*, *Bordetella bronchiseptica*, *Bordetella pertussis*, *Bordetella parapertussis*, *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter jejuni*, *Cronobacter sakazakii*, *Serratia marcescens* and *Lawsonia intracellularis*.

20. Compound for use according to item 19, wherein said Gram-negative bacterium is selected from the group consisting of *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Escherichia coli*, *Salmonella enterica*, *Shigella boydii*, *Shigella dysenteriae*, *Shigella flexneri*, *Shigella sonnei*, *Chlamydia trachomatis* and *Chlamydia pneumoniae (Chlamydophila pneumoniae)*.

21. Compound for use according to item 20, wherein said Gram-negative bacterium is *Pseudomonas aeruginosa*.

22. Compound for use according to any one of the preceding items, wherein said multicellular organism is suffering from, or is at risk of suffering from, a disease associated with T3SS- and/or flagella-expressing bacteria, such as for example selected from the group consisting of pneumonia, diarrhea, severe urinary tract infections, eye infections, skin and soft tissue infections, dermatitis, postoperative infections and infections in individuals suffering from cystic fibrosis or chronic obstructive lung disease (COLD).

23. Compound for use according to any one of the preceding items, wherein said compound is administered by a route selected from a systemic route and a local route.

24. Compound for use according to any one of the preceding items, wherein said multicellular organism does not respond, or responds insufficiently, to antibiotic treatment.

25. Compound for use according to any one of the preceding items, wherein said compound is administered at a dose corresponding to a dose of 0.1-50 mg/kg body weight, for example 2-20 mg/kg, for example 4-10 mg/kg, for example 4-7 mg/kg, for example 4-6 mg/kg, for example 5 mg/kg.

26. Compound for use according to any one of the preceding items, wherein said compound is selected from the group consisting of (-)-hopeaphenol, Vatalbinoside A, Vaticanol B, neohopeaphenol A and C_1 to C_6 O-alkyl derivatives thereof.

27. Pharmaceutical composition comprising a compound as described in any one of items 1 to 5, and at least one pharmaceutically acceptable carrier, diluent or excipient.

28. Pharmaceutical composition according to item 27, further comprising at least one additional active ingredient.

29. Pharmaceutical composition according to any one of items 27-28, wherein said compound is selected from the group consisting of (-)-hopeaphenol, Vatalbinoside A, Vaticanol B, neohopeaphenol A and C_1 to C_6 O-alkyl derivatives thereof.

30. Pharmaceutical composition according to any one of items 27-29, for use in the treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism.

31. Pharmaceutical composition for use according to item 30, wherein said Gram-negative bacterium possesses a type three secretion system.

32. Pharmaceutical composition for use according to item 30, wherein said Gram-negative bacterium possesses at least one flagellum.

33. Pharmaceutical composition for use according to any one of items 31 and 32, wherein said Gram-negative bacterium possesses a type three secretion system and at least one flagellum.

34. Pharmaceutical composition for use according to any one of items 30-33, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter, Chlamydia, Klebsiella, Cronobacter, Serratia, Acinetobacter, Morganella, Lawsonia, Actinobacillus, Francisella, Erwinia, Ralstonia, Rhizobium* and *Xanthomonas.*

35. Pharmaceutical composition for use according to item 34, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter* and *Chlamydia.*

36. Pharmaceutical composition for use according to item 35, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella* and *Shigella.*

37. Pharmaceutical composition for use according to item 36, wherein said bacterium belongs to the genus *Pseudomonas.*

38. Pharmaceutical composition for use according to any one of items 30-37, wherein said multicellular organism is selected from the group consisting of a plant, a fungus and an animal.

39. Pharmaceutical composition for use according to item 38, wherein said multicellular organism is an animal selected from the group consisting of birds, fish, reptiles and mammals.

40. Pharmaceutical composition for use according to item 39, wherein said animal is a mammal.

41. Pharmaceutical composition for use according to item 40, wherein said mammal is human.

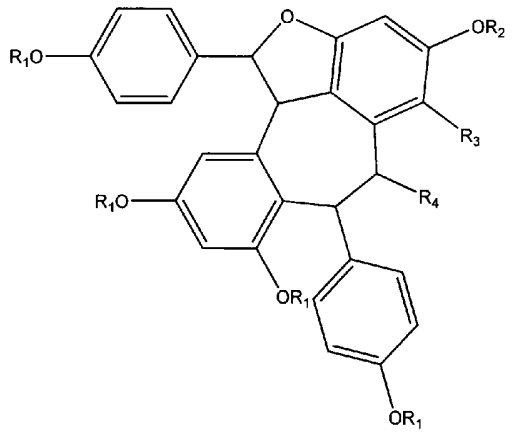
42. Pharmaceutical composition for use according to any one of items 30-41, wherein said multicellular organism is suffering from, or is at risk of suffering from, a disease associated with T3SS- and/or flagella-expressing bacteria, such as for example selected from the group consisting of pneumonia, diarrhea, severe urinary tract infections, eye infections, skin and soft tissue infections, dermatitis, postoperative infections and infections in individuals suffering from cystic fibrosis or chronic obstructive lung disease (COLD).

43. Pharmaceutical composition for use according to any one of items 30-42, wherein said compound is administered by a route selected from a systemic route and a local route.

44. Pharmaceutical composition for use according to any one of items 30-43, wherein said multicellular organism does not respond, or responds insufficiently, to antibiotic treatment.

45. Pharmaceutical composition for use according to any one of items 30-44, wherein said composition is administered at a dose corresponding to a dose of 0.1-50 mg/kg body weight, for example 2-20 mg/kg, for example 4-10 mg/kg, for example 4-7 mg/kg, for example 4-6 mg/kg, for example 5 mg/kg.

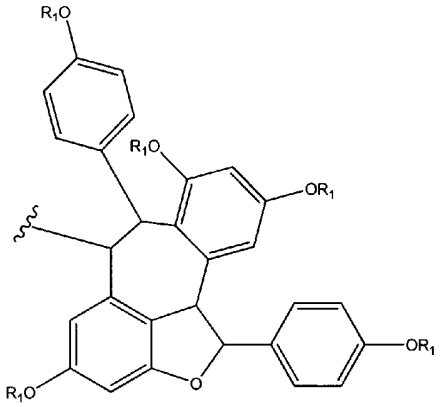
46. Method of treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism, comprising administering a compound, or a pharmaceutical composition comprising said compound, to a multicellular organism in need thereof, wherein said compound is a compound of Formula I, or a pharmaceutically acceptable salt thereof:



Formula I

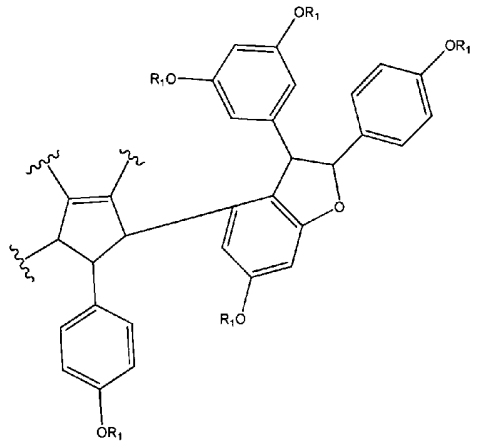
wherein, R_1 and R_2 are independently selected from the group consisting of hydrogen, C_1 to C_{12} alkyl, C_1 to C_{12} alkenyl, aryl, C_1 to C_{12} aldehyde, C_1 to C_{12} alkanone, C_1 to C_{12} carboxyl, C_1 to C_{12} carboxamide, C_1 to C_{12} alkanoyl and a sugar moiety, each of which groups may be substituted or unsubstituted;

R_3 and R_4 are independently selected from hydrogen or the structure shown below



wherein R_1 is as previously described,

or R_3 and R_4 may join to form a substituted five membered ring as shown below

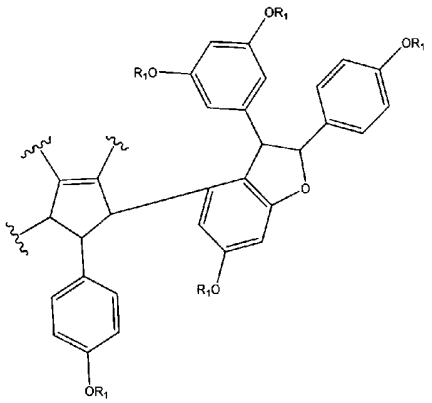


wherein R_1 is as previously described.

47. Method according to item 46, wherein R_1 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl, C_1 to C_6 alkenyl, aryl, C_1 to C_6 aldehyde, C_1 to C_6 alkanone, C_1 to C_6 carboxyl, C_1 to C_6 carboxamide and C_1 to C_6 alkanoyl, each of which groups may be substituted or unsubstituted;

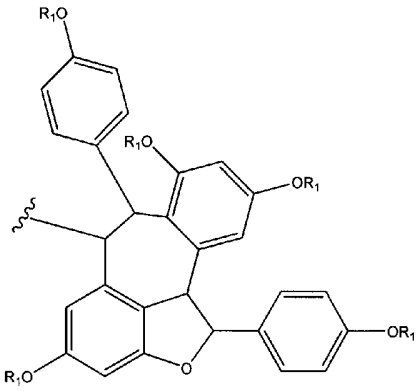
R_2 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl and a sugar moiety;

R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



wherein R_1 is as described,

or R_4 is

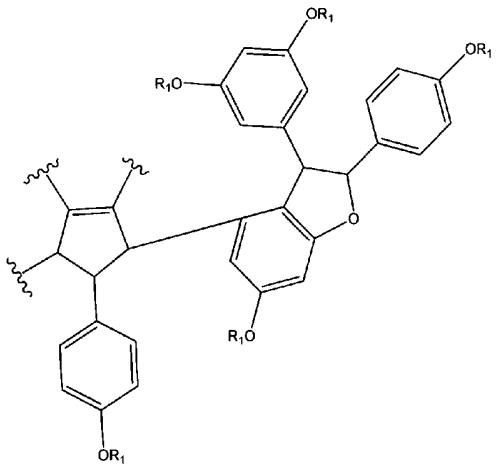


wherein R_1 is as described.

48. Method according to item 46 or item 47, wherein R_1 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl, C_1 to C_6 alkenyl and aryl, each of which groups may be substituted or unsubstituted;

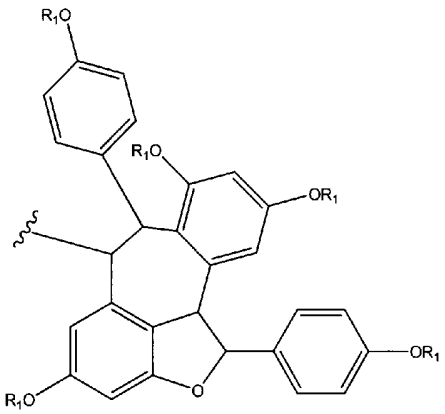
R_2 is selected from the group consisting of hydrogen, methyl, ethyl, propyl, butyl and a glucose moiety;

R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



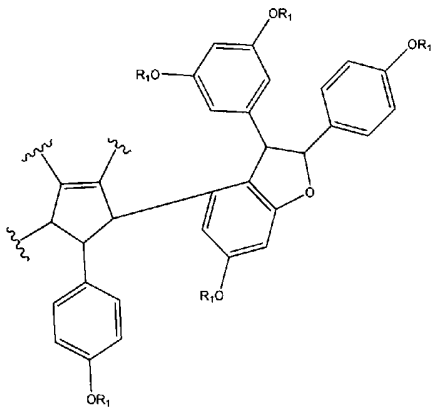
wherein R_1 is as described,

or R_4 is

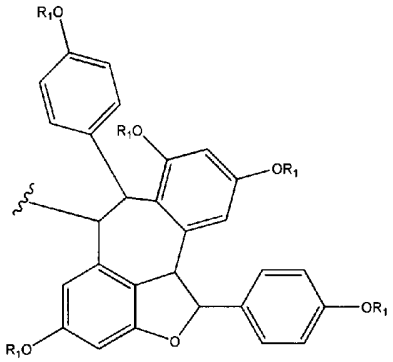


wherein R_1 is as described.

49. Method according to any one of items 46 to 48, wherein R_1 is selected from the group consisting of hydrogen, methyl, ethyl, propyl and isopropyl, each of which groups may be substituted or unsubstituted;
R_2 is selected from the group consisting of hydrogen, methyl, ethyl, propyl and a β-D-glucose moiety;
R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



wherein R_1 is as described,
or R_4 is



wherein R_1 is as described.

50. Method according to any one of items 46 to 49, wherein the compound of formula I is selected from the group consisting of (-)-hopeaphenol,

Vatalbinoside A, Vaticanol B, neohopeaphenol A and C_1 to C_6 O-alkyl derivatives thereof.

51. Method according to item 50, wherein the O-alkyl derivatives of the recited compounds are methyl, ethyl, propyl or butyl derivatives.

52. Method according to any one of items 46 to 51, wherein said Gram-negative bacterium possesses a type three secretion system.

53. Method according to item 52, wherein said Gram-negative bacterium possesses at least one flagellum.

54. Method according to any one of items 52 and 53, wherein said Gram-negative bacterium possesses a type three secretion system and at least one flagellum.

55. Method according to any one of items 46-54, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter, Chlamydia, Klebsiella, Cronobacter, Serratia, Acinetobacter, Morganella, Lawsonia, Actinobacillus, Francisella, Erwinia, Ralstonia, Rhizobium* and *Xanthomonas.*

56. Method according to item 55, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter* and *Chlamydia.*

57. Method according to item 56, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella* and *Shigella.*

58. Method according to item 57, wherein said bacterium belongs to the genus *Pseudomonas.*

59. Method according to any one of items 46-58, wherein said multicellular organism is a selected from the group consisting of a plant, a fungus and an animal.

60. Method to according to item 59, wherein said multicellular organism is an animal selected from the group consisting of birds, fish, reptiles and mammals.

61. Method according to item 59, wherein said animal is a mammal.

62. Method according to item 61, wherein said mammal is human.

63. Method according to any one of items 55-58, wherein said Gram-negative bacterium is as defined in any one of claims 18-21.

64. Method according to any one of items 46-63, wherein said multicellular organism is suffering from, or is at risk of suffering from, a disease associated with T3SS- and/or flagella-expressing bacteria, such as for example selected from the group consisting of pneumonia, diarrhea, severe urinary tract infections, eye infections, skin and soft tissue infections, dermatitis, postoperative infections and infections in individuals suffering from cystic fibrosis or chronic obstructive lung disease (COLD).

65. Method according to any one of items 46-64, wherein said compound is administered by a route selected from a systemic route and a local route.

66. Method according to any one of items 46-65, wherein said multicellular organism does not respond, or responds insufficiently, to antibiotic treatment.

67. Method according to any one of items 46-66, wherein said compound or said pharmaceutical composition is administered at a dose corresponding to a dose of 0.1-50 mg/kg body weight, for example 2-20 mg/kg, for example 4-10 mg/kg, for example 4-7 mg/kg, for example 4-6 mg/kg, for example 5 mg/kg.

68. Method according to any one of items 46-67, wherein said compound is selected from the group consisting of (-)-hopeaphenol, Vatalbinoside A, Vaticanol B, neohopeaphenol A and C_1 to C_6 O-alkyl derivatives thereof.

69. Method according to any one of items 46-67, wherein said compound is (-)-hopeaphenol.

70. Use of a compound as described in any one of items 1 to 5, or of a composition comprising a compound as described in any one of items 1 to 5, for the manufacture of a medicament for use in the treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism.

71. Use according to item 70, wherein said Gram-negative bacterium possesses a type three secretion system.

72. Use according to item 70, wherein said Gram-negative bacterium possesses at least one flagellum.

73. Use according to any one of items 71 and 72, wherein said Gram-negative bacterium possesses a type three secretion system and at least one flagellum.

74. Use according to any one of items 70-73, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter, Chlamydia, Klebsiella, Cronobacter, Serratia, Acinetobacter, Morganella, Lawsonia, Actinobacillus, Francisella, Erwinia, Ralstonia, Rhizobium* and *Xanthomonas.*

75. Use according to item 74, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter* and *Chlamydia.*

76. Use according to item 75, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella* and *Shigella.*

77. Use according to item 76, wherein said bacterium belongs to the genus *Pseudomonas.*

78. Use according to any one of items 70-77, wherein said multicellular organism is selected from the group consisting of a plant, a fungus and an animal.

79. Use according to item 78, wherein said multicellular organism is an animal selected from the group consisting of birds, fish, reptiles and mammals.

80. Use according to item 78, wherein said animal is a mammal.

81. Use according to item 80, wherein said mammal is human.

82. Use according to any one of items 70-81, wherein said multicellular organism is suffering from, or is at risk of suffering from, a disease associated with T3SS- and/or flagella-expressing bacteria, such as for example selected from the group consisting of pneumonia, diarrhea, severe urinary tract infections, eye infections, skin and soft tissue infections, dermatitis, postoperative infections and infections in individuals suffering from cystic fibrosis or chronic obstructive lung disease (COLD).

83. Use according to any one of items 70-82, wherein said medicament is intended for administration by a route selected from a systemic route and a local route.

84. Use according to any one of items 70-83, wherein said multicellular organism does not respond, or responds insufficiently, to antibiotic treatment.

85. Use according to any one of items 70-84, wherein said medicament is intended for administration at a dose corresponding to a dose of 0.1-50 mg/kg body weight, for example 2-20 mg/kg, for example 4-10 mg/kg, for example 4-7 mg/kg, for example 4-6 mg/kg, for example 5 mg/kg.

86. Use according to any one of items 70-85, wherein said compound is selected from the group consisting of (-)-hopeaphenol, Vatalbinoside A, Vaticanol B, neohopeaphenol A and C_1 to C_6 O-alkyl derivatives thereof.

87. Method of modulating the activity of a type three secretion system in a Gram-negative bacterium comprising the step of exposing the Gram-negative bacterium to a compound according to any one of item 1 to 5, or a pharmaceutically acceptable salt thereof.

88. Method according to item 87, wherein the step of modulating the activity is an inhibition of the activity of the type three secretion system in the Gram-negative bacterium.

89. Method according to item 87 or item 88, wherein said Gram-negative bacterium possesses a type three secretion system.

90. Method according to item 87 or 88, wherein said Gram-negative bacterium possesses at least one flagellum.

91. Method according to any one of items 89 and 90, wherein said Gram-negative bacterium possesses a type three secretion system and at least one flagellum.

92. Method according to any one of items 87-91, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter, Chlamydia, Klebsiella, Cronobacter, Serratia, Acinetobacter, Morganella, Lawsonia, Actinobacillus, Francisella, Erwinia, Ralstonia, Rhizobium* and *Xanthomonas*.

93. Method according to item 92, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter* and *Chlamydia.*

94. Method according to item 93, wherein said bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella* and *Shigella.*

95. Method according to item 94, wherein said bacterium belongs to the genus *Pseudomonas.*

96. Method according to any one of items 87-95, wherein said multicellular organism is selected from the group consisting of a plant, a fungus and an animal.

97. Method according to item 96, wherein said multicellular organism is an animal selected from the group consisting of birds, fish, reptiles and mammals.

98. Method according to item 96, wherein said animal is a mammal.

99. Method according to item 98, wherein said mammal is human.

100. Method according to any one of items 87-99, wherein said multicellular organism is suffering from, or is at risk of suffering from, a disease associated with T3SS- and/or flagella-expressing bacteria, such as for example selected from the group consisting of pneumonia, diarrhea, severe urinary tract infections, eye infections, skin and soft tissue infections, dermatitis, postoperative infections and infections in

individuals suffering from cystic fibrosis or chronic obstructive lung disease (COLD).

101. Method according to any one of items 87-100, wherein said medicament is intended for administration by a route selected from a systemic route and a local route.

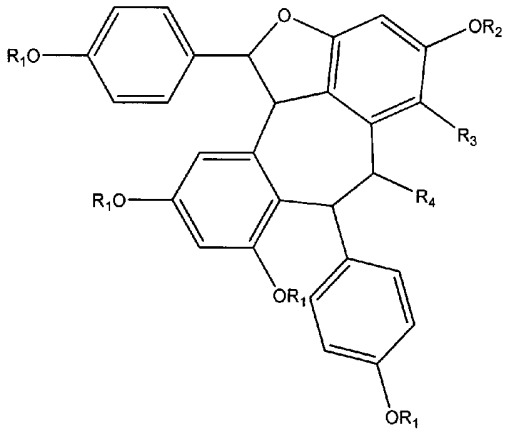
102. Method according to any one of items 87-101, wherein said multicellular organism does not respond, or responds insufficiently, to antibiotic treatment.

103. Method according to any one of items 87-102, wherein said medicament is intended for administration at a dose corresponding to a dose of 0.1-50 mg/kg body weight, for example 2-20 mg/kg, for example 4-10 mg/kg, for example 4-7 mg/kg, for example 4-6 mg/kg, for example 5 mg/kg.

104. Method according to any one of items 87-104, wherein said compound is selected from the group consisting of (-)-hopeaphenol, Vatalbinoside A, Vaticanol B, neohopeaphenol A and C_1 to C_6 O-alkyl derivatives thereof.

CLAIMS

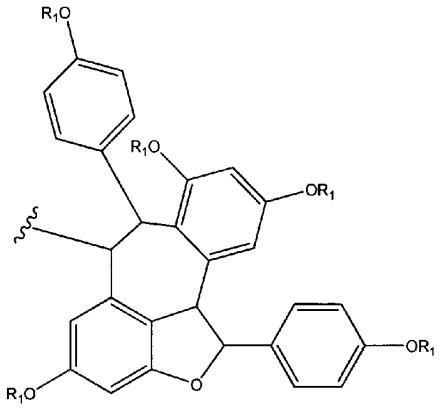
1. A method of treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism, comprising the step of administering a compound of Formula I, or a pharmaceutically acceptable salt thereof, to the multicellular organism:



Formula I

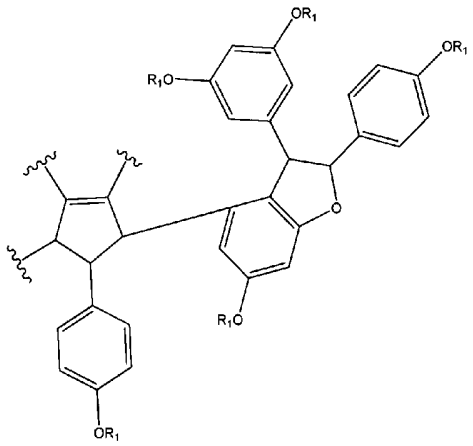
wherein, R_1 and R_2 are independently selected from the group consisting of hydrogen, C_1 to C_{12} alkyl, C_1 to C_{12} alkenyl, aryl, C_1 to C_{12} aldehyde, C_1 to C_{12} alkanone, C_1 to C_{12} carboxyl, C_1 to C_{12} carboxamide, C_1 to C_{12} alkanoyl and a sugar moiety, each of which groups may be substituted or unsubstituted;

R_3 and R_4 are independently selected from hydrogen or the structure shown below



wherein R_1 is as previously described,

or R_3 and R_4 may join to form a substituted five membered ring as shown below

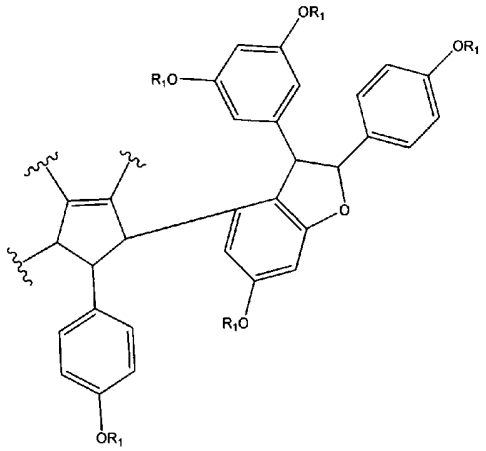


wherein R_1 is as previously described.

2. The method of claim 1 wherein R_1 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl, C_1 to C_6 alkenyl, aryl, C_1 to C_6 aldehyde, C_1 to C_6 alkanone, C_1 to C_6 carboxyl, C_1 to C_6 carboxamide and C_1 to C_6 alkanoyl, each of which groups may be substituted or unsubstituted;

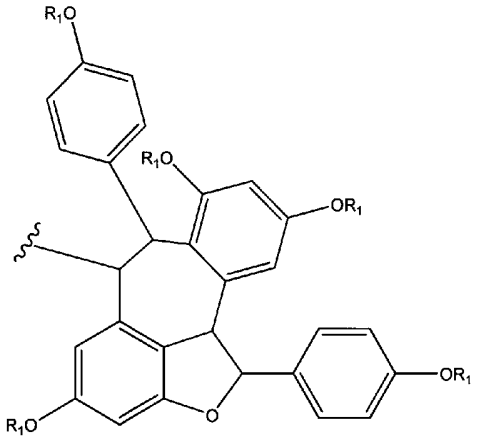
R_2 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl and a sugar moiety;

R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



wherein R_1 is as described,

or R_4 is

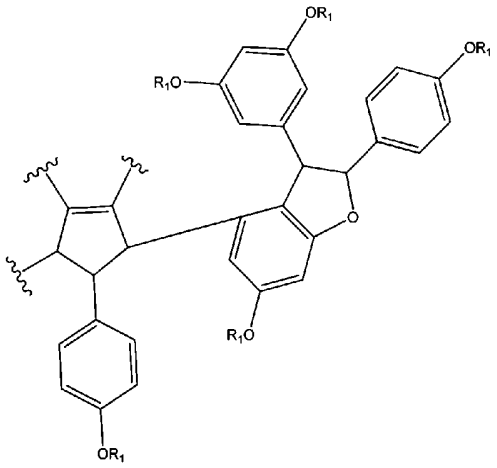


wherein R_1 is as described.

3. The method of claim 1 or claim 2 wherein R_1 is selected from the group consisting of hydrogen, C_1 to C_6 alkyl, C_1 to C_6 alkenyl and aryl, each of which groups may be substituted or unsubstituted;

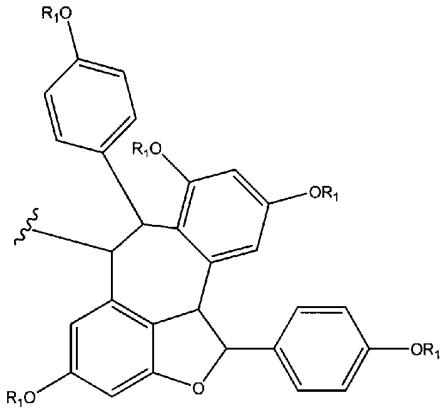
R_2 is selected from the group consisting of hydrogen, methyl, ethyl, propyl, butyl and a glucose moiety;

R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



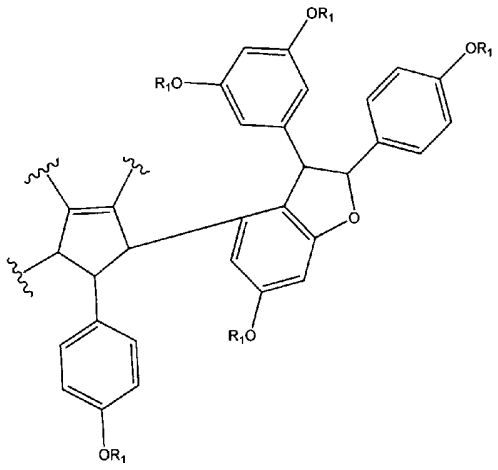
wherein R_1 is as described,

or R_4 is

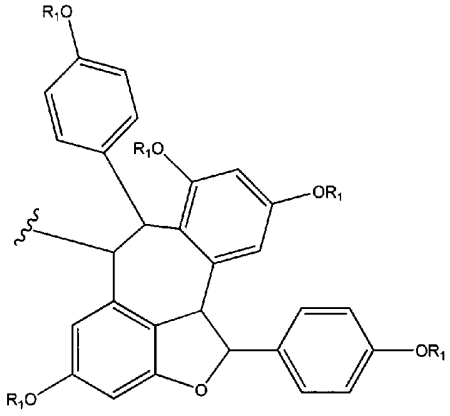


wherein R_1 is as described.

4. The method of any one of the preceding claims wherein R_1 is selected from the group consisting of hydrogen, methyl, ethyl, propyl and isopropyl, each of which groups may be substituted or unsubstituted;
R_2 is selected from the group consisting of hydrogen, methyl, ethyl, propyl and a β-D-glucose moiety;
R_3 may be hydrogen or R_3 and R_4 may join to form a substituted five membered ring as shown below



wherein R_1 is as described,
or R_4 is



wherein R_1 is as described.

5. The method of any one of the preceding claims wherein the compound of formula I is selected from the group consisting of (-)-hopeaphenol, Vatalbinoside A, Vaticanol B, neohopeaphenol A and C_1 to C_6 O-alkyl derivatives thereof.

6. The method of claim 5 wherein the O-alkyl derivatives of the recited compounds are methyl, ethyl, propyl or butyl derivatives.

7. The method of any one of the preceding claims wherein the Gram-negative bacterium possesses a type three secretion system and/or at least one flagellum.

8. The method of any one of the preceding claims wherein the bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter, Chlamydia, Klebsiella, Cronobacter, Serratia, Acinetobacter, Morganella, Lawsonia, Actinobacillus, Francisella, Erwinia, Ralstonia, Rhizobium* and *Xanthomonas*.

9. The method of claim 8 wherein the bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter* and *Chlamydia*.

10. The method of claim 9 wherein the bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella* and *Shigella.*

11. The method of any one of the preceding claims wherein the multicellular organism is a selected from the group consisting of a plant, a fungus and an animal.

12. The method of claim 11 wherein the multicellular organism is an animal selected from the group consisting of birds, fish, reptiles and mammals.

13. The method of any one of the preceding claims wherein the multicellular organism is suffering from, or is at risk of suffering from, a disease associated with T3SS- and/or flagella-expressing bacteria, including those selected from the group consisting of pneumonia, diarrhea, severe urinary tract infections, eye infections, skin and soft tissue infections, dermatitis, postoperative infections and infections in individuals suffering from cystic fibrosis or chronic obstructive lung disease (COLD).

14. The method of any one of the preceding claims wherein the compound is administered by a route selected from a systemic route and a local route.

15. The method of any one of the preceding claims wherein the multicellular organism does not respond, or responds insufficiently, to antibiotic treatment.

16. The method of any one of the preceding claims wherein the compound or said pharmaceutical composition is administered at a dose corresponding to a dose of 0.1-50 mg/kg body weight.

17. A pharmaceutical composition comprising a compound of Formula I, or a pharmaceutically acceptable salt thereof, and at least one pharmaceutically acceptable carrier, diluent and/or excipient.

18. The pharmaceutical composition of claim 17 further comprising at least one additional active ingredient.

19. The pharmaceutical composition according to claim 17 or claim 18 for use in the treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism.

20. The pharmaceutical composition according to claim 19 wherein the Gram-negative bacterium possesses a type three secretion system and/or at least one flagellum.

21. A compound, or a pharmaceutically acceptable salt thereof, for use in the treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism, wherein the compound is a compound of Formula I.

22. The compound for use of claim 21 wherein the Gram-negative bacterium possesses a type three secretion system and/or at least one flagellum.

23. The compound for use of claim 21 or claim 22 wherein the bacterium belongs to a genus selected from the group consisting of *Pseudomonas, Yersinia, Escherichia, Salmonella, Shigella, Legionella, Proteus, Enterobacter, Pantoea, Helicobacter, Aeromonas, Citrobacter, Burkholderia, Vibrio, Bordetella, Campylobacter, Chlamydia, Klebsiella, Cronobacter, Serratia, Acinetobacter,*

Morganella, Lawsonia, Actinobacillus, Francisella, Erwinia, Ralstonia, Rhizobium and *Xanthomonas*.

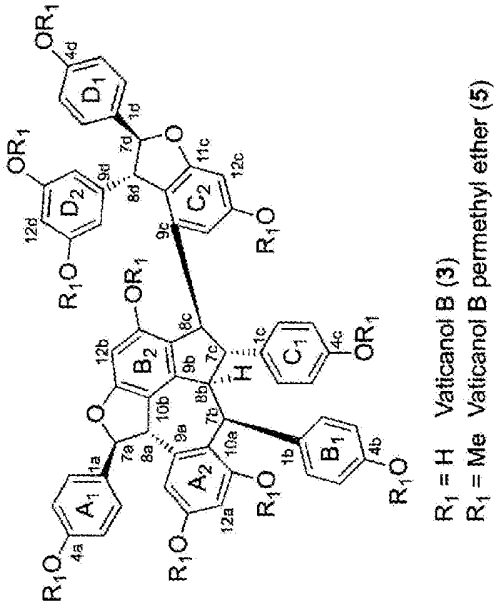
24. The compound for use of any one of claim 21 to claim 23 wherein the multicellular organism is selected from the group consisting of a plant, a fungus and an animal.

25. The compound for use of claim 24 wherein the multicellular organism is an animal selected from the group consisting of birds, fish, reptiles and mammals.

26. Use of a compound of Formula I, or a pharmaceutically acceptable salt thereof, in the manufacture of a medicament for use in the treatment and/or prevention of an infection caused by a Gram-negative bacterium in a multicellular organism.

27. A method of modulating the activity of a type three secretion system in a Gram-negative bacterium comprising the step of exposing the Gram-negative bacterium to a compound as described for the first aspect, or a pharmaceutically acceptable salt thereof.

28. The method of claim 27 wherein the step of modulating the activity is an inhibition of the activity of the type three secretion system in the Gram-negative bacterium.


R1 = H Vaticanol B (3)
R1 = Me Vaticanol B permethyl ether (5)

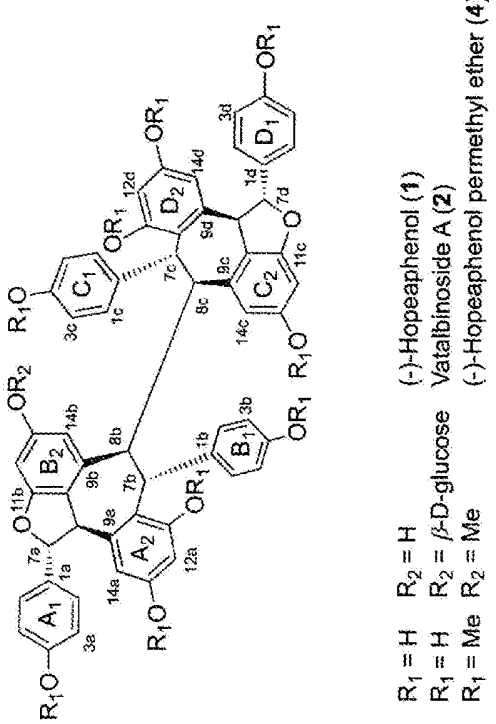

R1 = H R2 = H (-)-Hopeaphenol (1)
R1 = H R2 = β-D-glucose Vatalbinoside A (2)
R1 = Me R2 = Me (-)-Hopeaphenol permethyl ether (4)

FIG 1

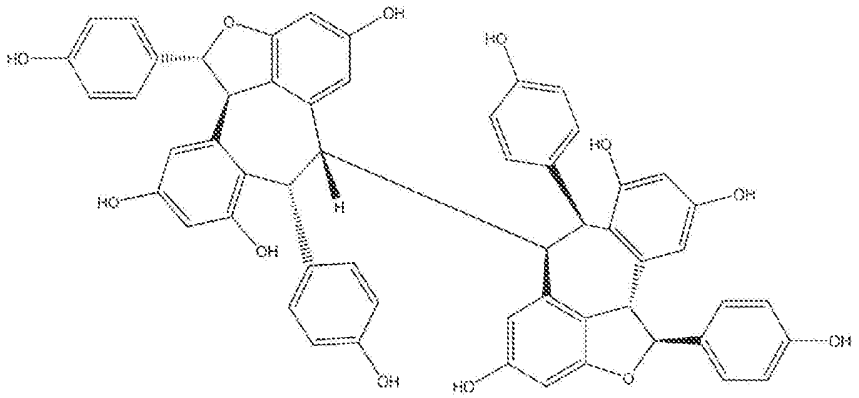

HO
OH
HO
OH
H
OH
HO
HO
OH
HO
OH
OH

FIG 2

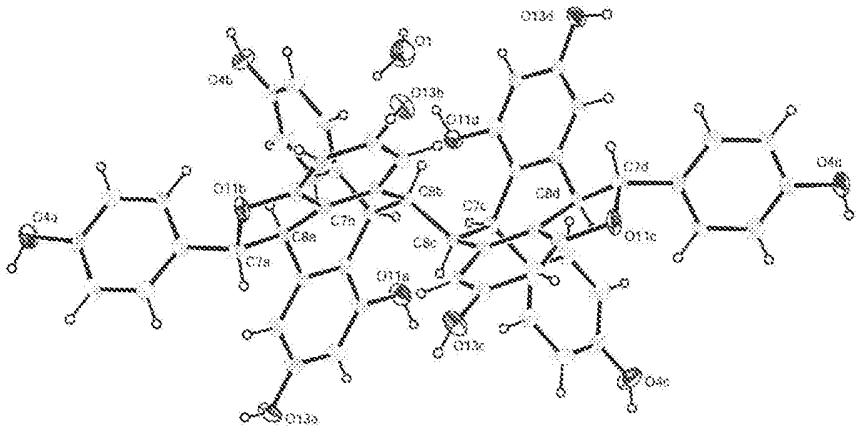

O13d
O1
O4b
O13b
O11d
C7d
O4d
O11b
C8b
C7c
C8d
C7b
C8a
C8c
O11c
O4a
C7a
O11a
O13c
O4c
O13a

FIG 3

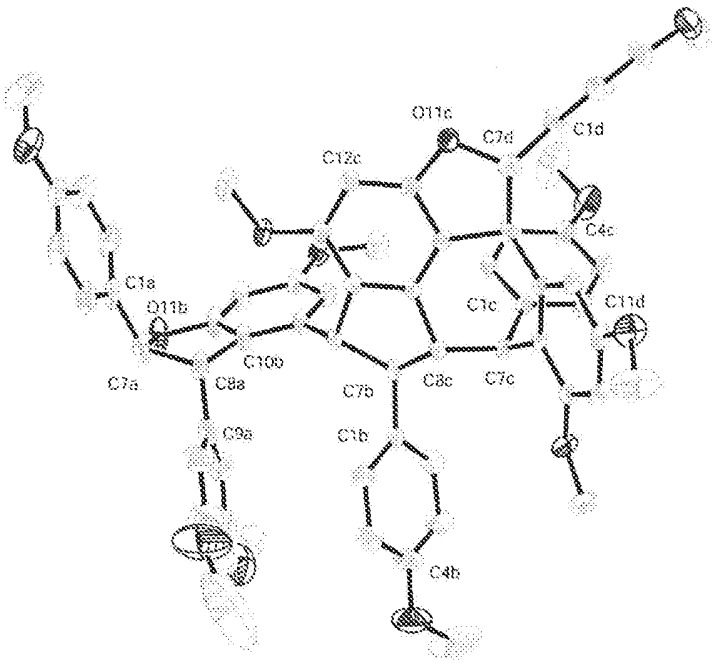

O11c
C7d
C1d
C12c
C4c
C1a
O11b
C1c
C11d
C10b
C7a
C8a
C7b
C8c
C7c
C9a
C1b
C4b

FIG 4

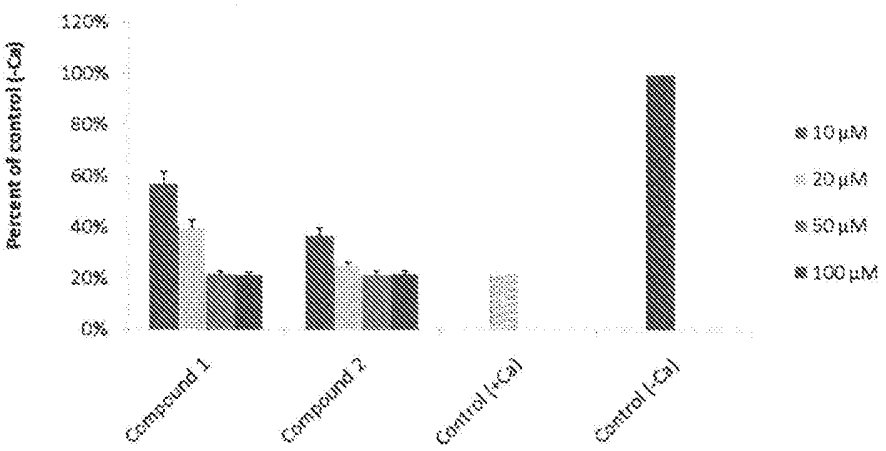

Percent of control (-Ca)
120%
100%
80%
60%
40%
20%
0%
10 µM
20 µM
50 µM
100 µM
Compound 1
Compound 2
Control (+Ca)
Control (-Ca)

FIG 5A

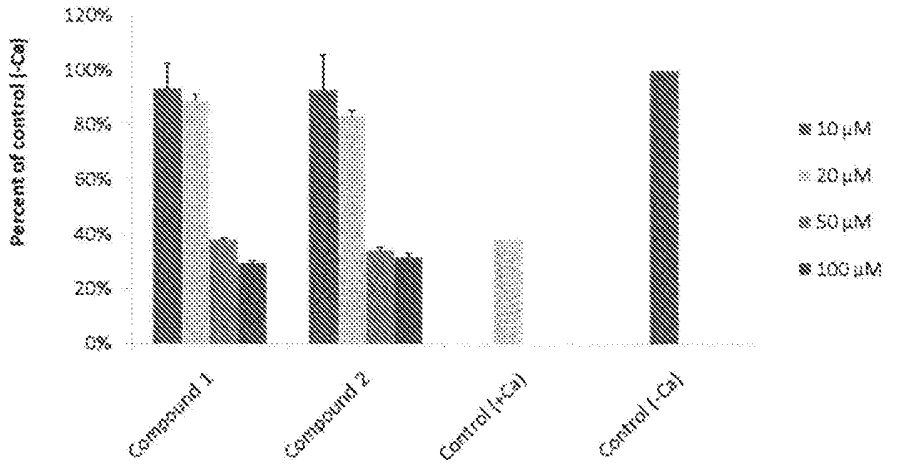

120%
100%
80%
60%
40%
20%
0%
Percent of control (-Ca)
10 µM
20 µM
50 µM
100 µM
Compound 1
Compound 2
Control (+Ca)
Control (-Ca)

FIG 5B

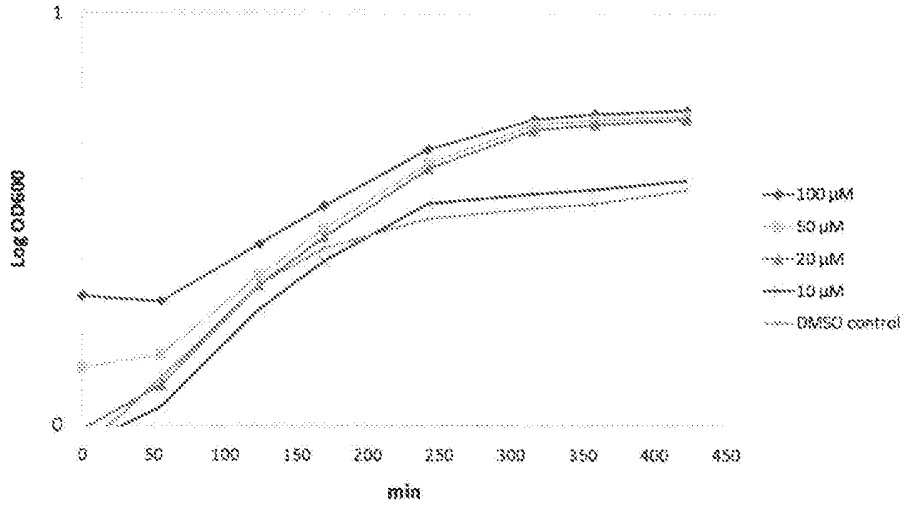

1
0
Log OD600
0
50
100
150
200
250
300
350
400
450
min
100 µM
50 µM
20 µM
10 µM
DMSO control

FIG 6A

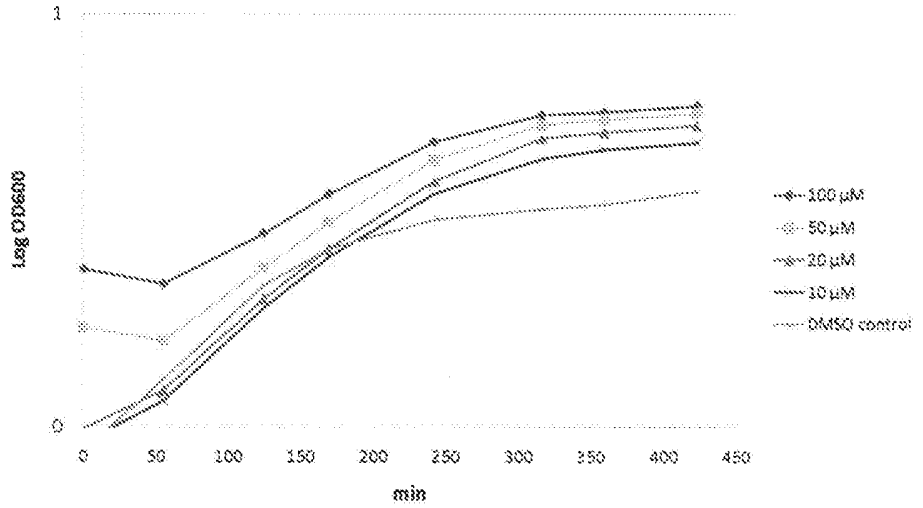

1
0
Log OD600
0
50
100
150
200
250
300
350
400
450
min
100 µM
50 µM
20 µM
10 µM
DMSO control

FIG 6B

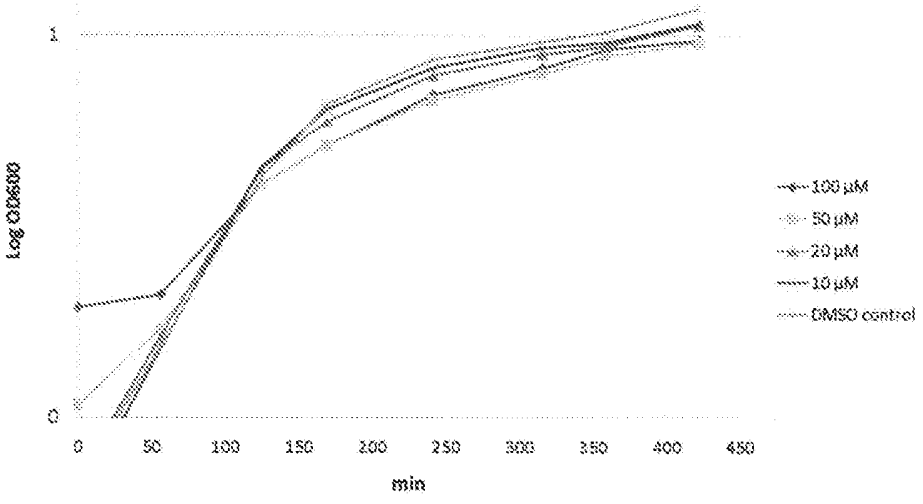

1
0
Log OD600
0
50
100
150
200
250
300
350
400
450
min
100 µM
50 µM
20 µM
10 µM
DMSO control

FIG 7A

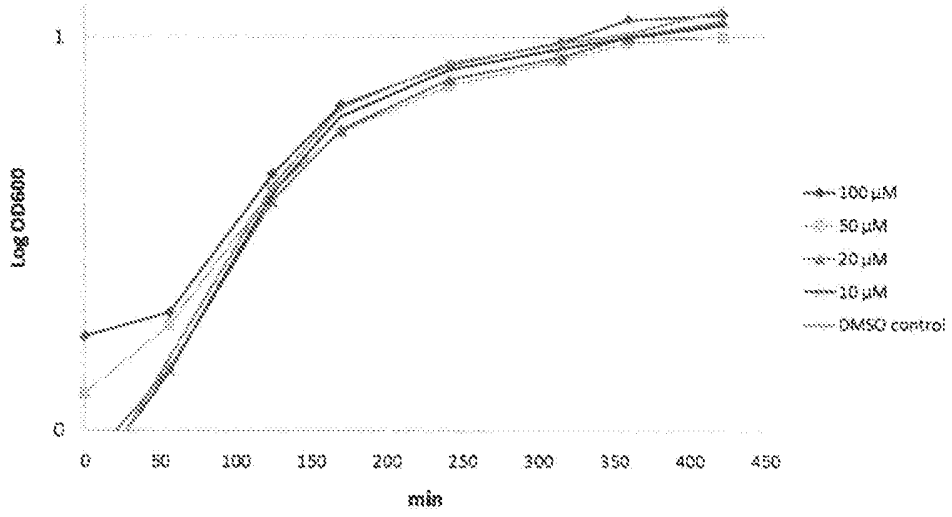

1
0
Log OD600
0
50
100
150
200
250
300
350
400
450
min
100 µM
50 µM
20 µM
10 µM
DMSO control

FIG 7B

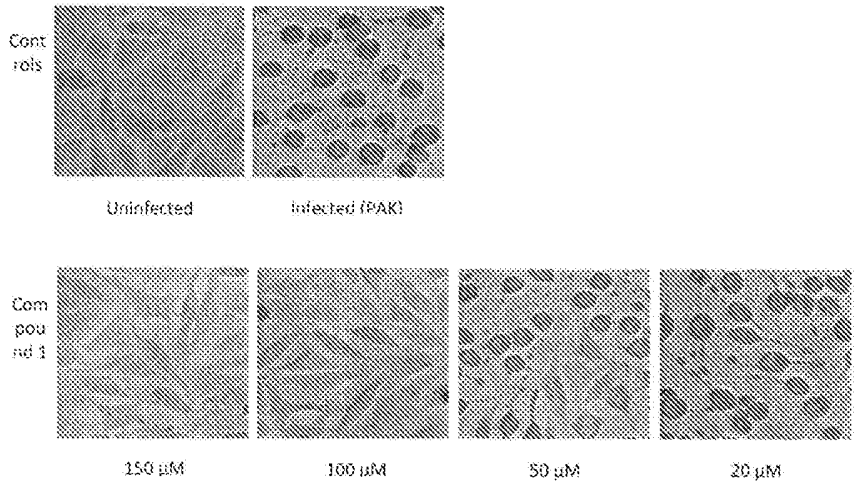

Cont rols
Uninfected
Infected (PAK)
Com pou nd 1
150 µM
100 µM
50 µM
20 µM

FIG 8A

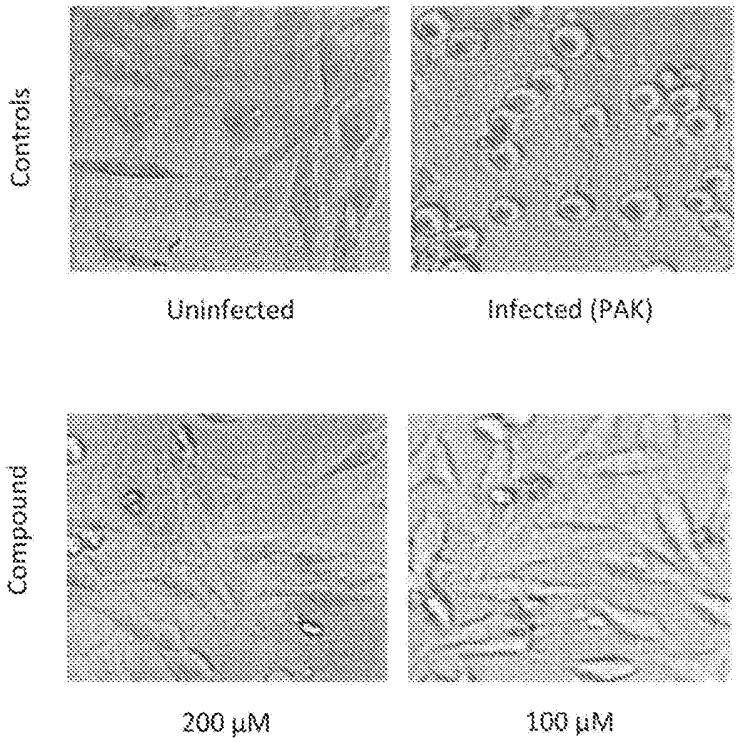


FIG 8B

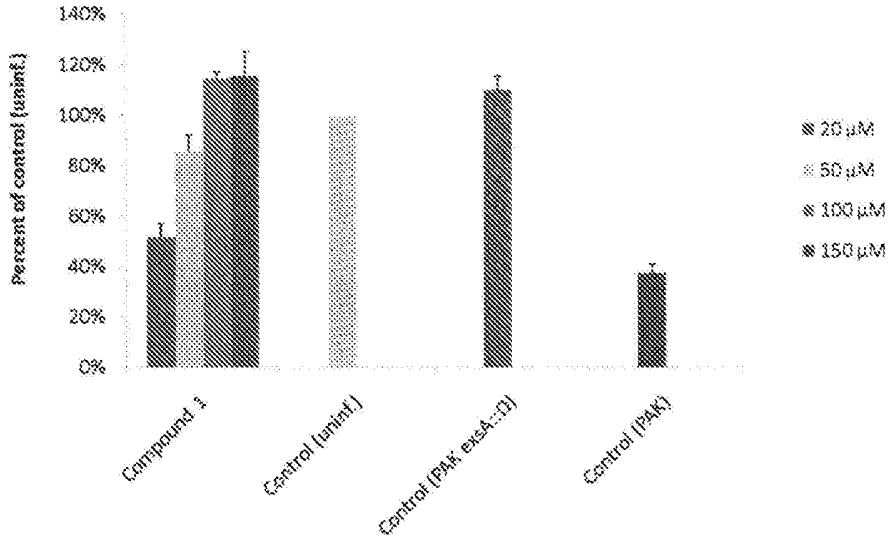

140%
120%
100%
80%
60%
40%
20%
0%
Percent of control (uninf.)
20 µM
50 µM
100 µM
150 µM
Compound 1
Control (uninf.)
Control (PAK exoA::Ω)
Control (PAK)

FIG 9

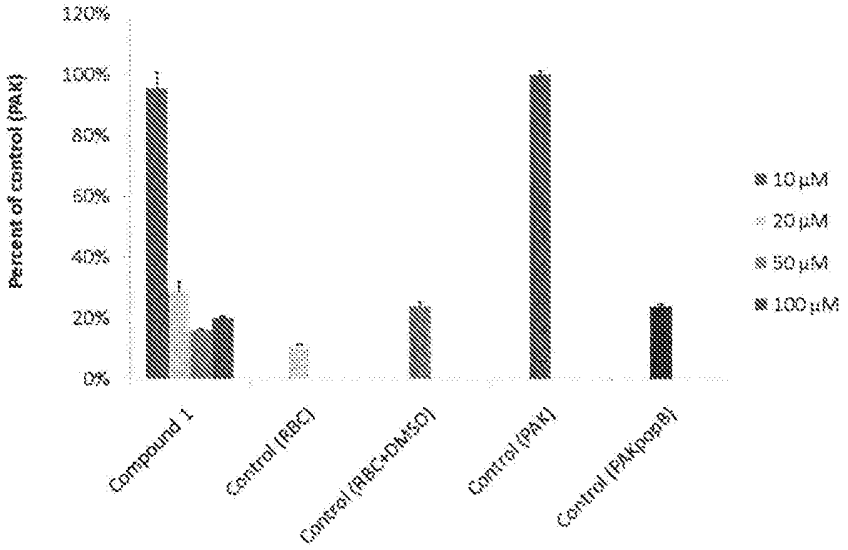

120%
100%
80%
60%
40%
20%
0%
Percent of control (PAK)
10 µM
20 µM
50 µM
100 µM
Compound 1
Control (RBC)
Control (RBC+DMSO)
Control (PAK)
Control (PAKpqsB)

FIG 10

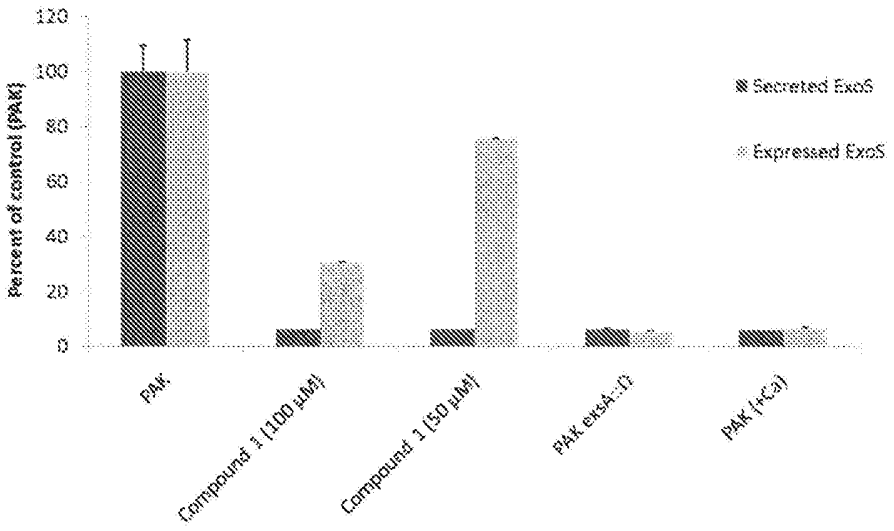

120
100
80
60
40
20
0
Percent of control (PAK)
Secreted ExoS
Expressed ExoS
PAK
Compound 1 (100 µM)
Compound 1 (50 µM)
PAK exsA::Ω
PAK (+Ca)

FIG 11

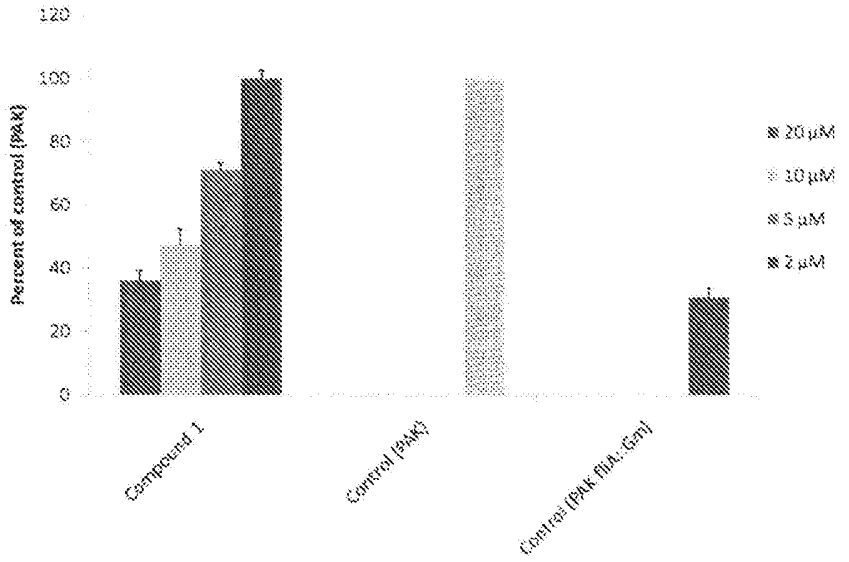

120
100
80
60
40
20
0
Percent of control (PAK)
20 µM
10 µM
5 µM
2 µM
Compound 1
Control (PAK)
Control (PAK flaL::Gm)

FIG 12

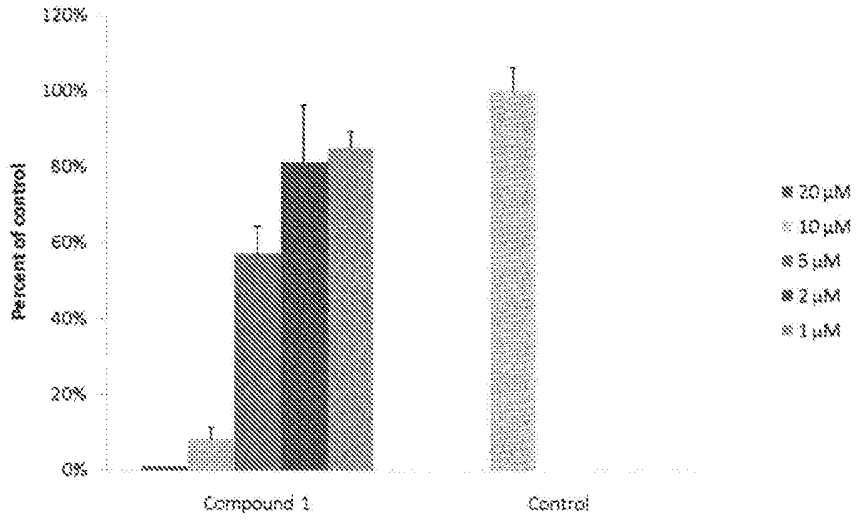


FIG 13

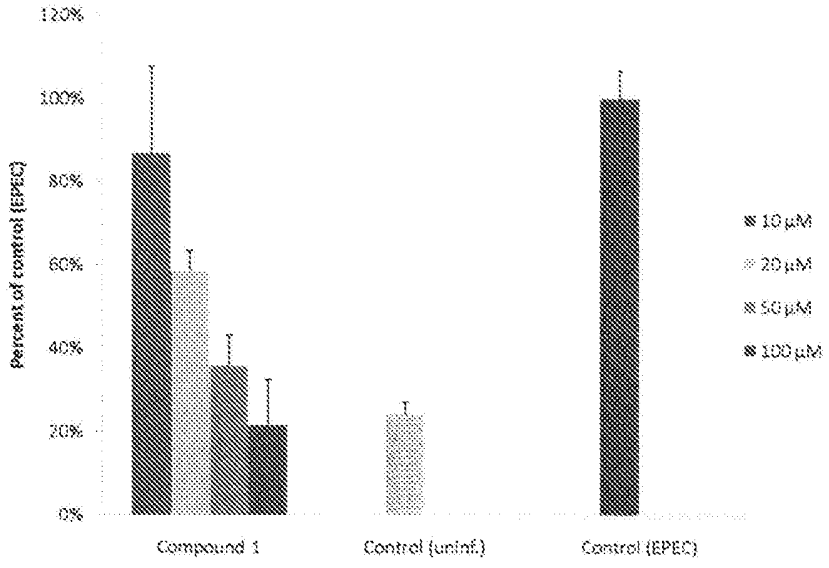


FIG 14A

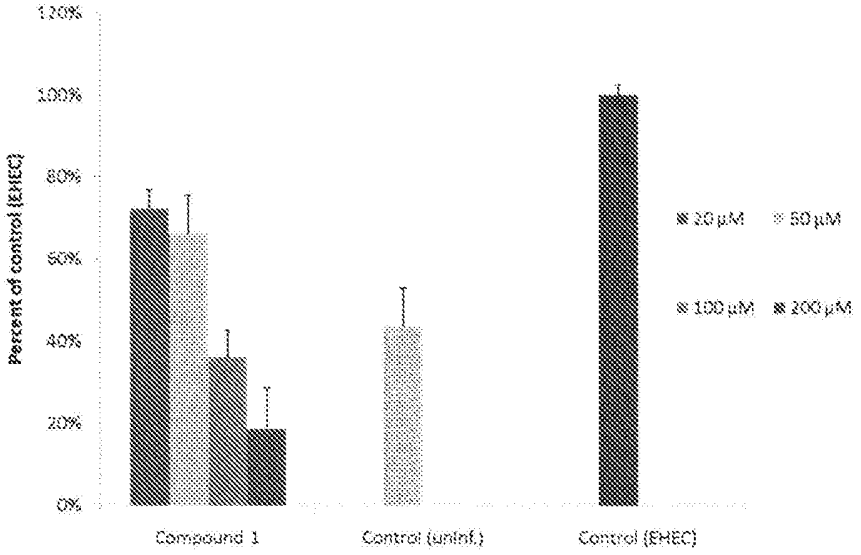


FIG 14B

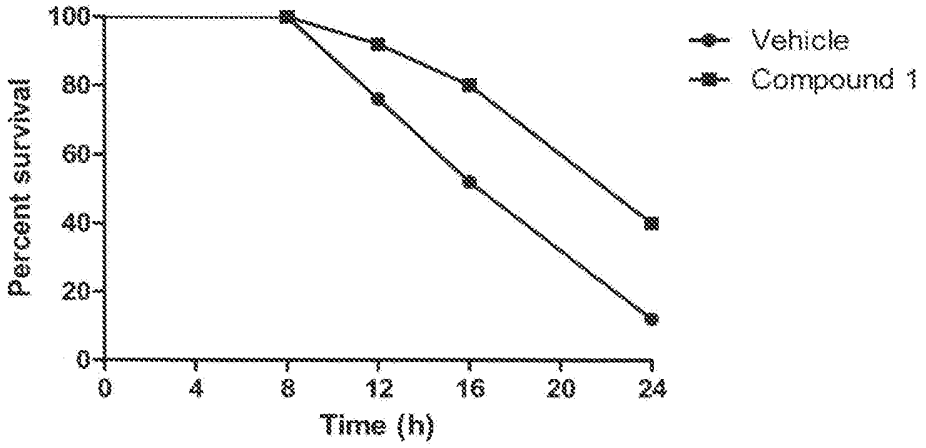


FIG 15

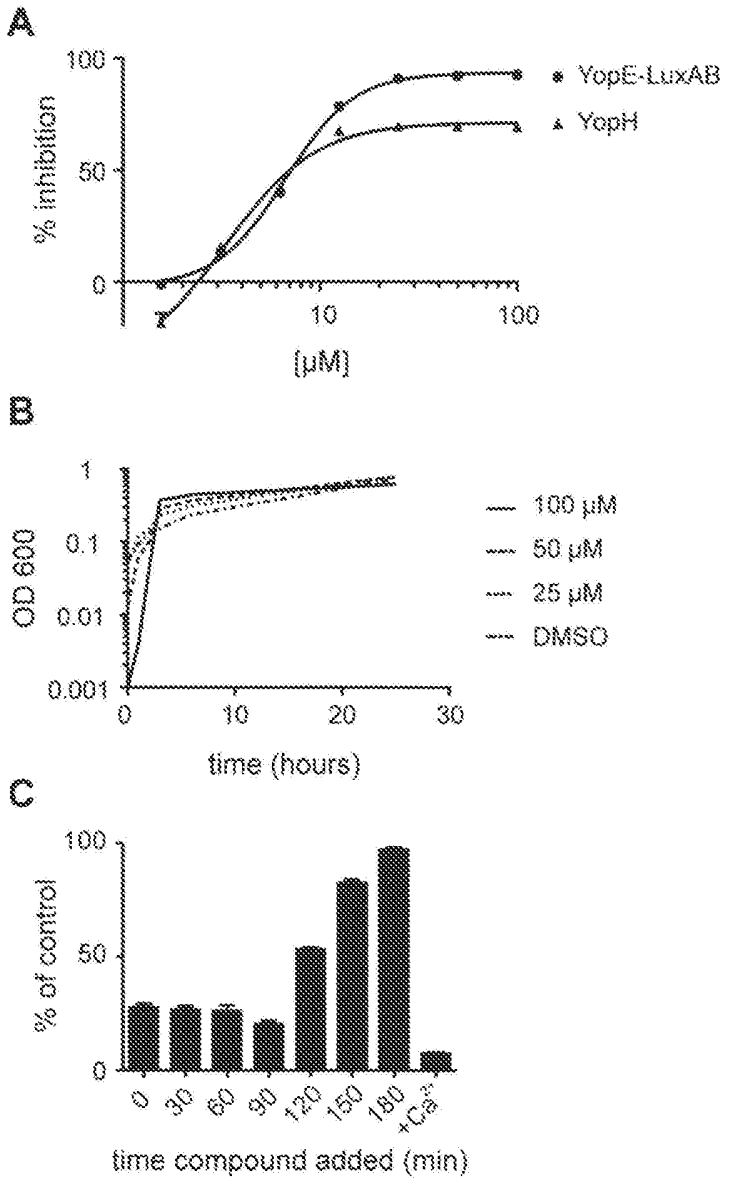

A
100
50
0
% inhibition
10
100
[μM]
YopE-LuxAB
YopH
B
1
0.1
0.01
0.001
OD 600
0
10
20
30
time (hours)
100 μM
50 μM
25 μM
DMSO
C
100
50
0
% of control
0
30
60
90
120
150
180
+Ca2+
time compound added (min)

FIG 16

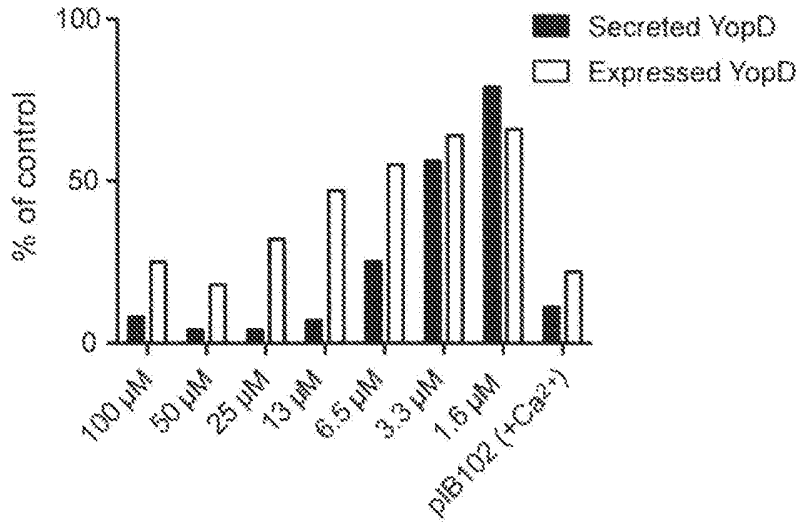

100
50
0
% of control
Secreted YopD
Expressed YopD
100 µM
50 µM
25 µM
13 µM
6.5 µM
3.3 µM
1.6 µM
pIB102 (+Ca2+)

FIG 17

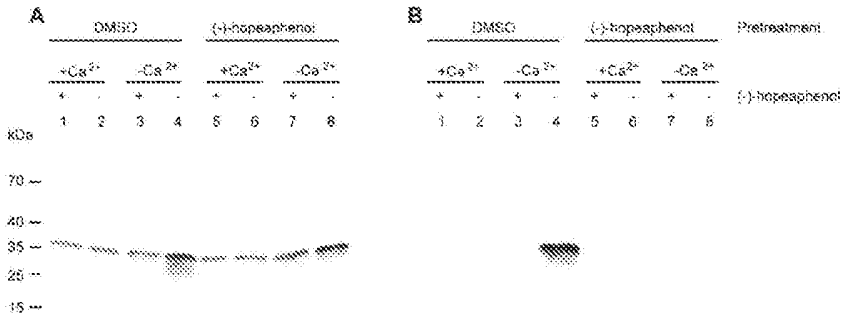

A
DMSO
(-)-hopeaphenol
+Ca2+
-Ca2+
1 2 3 4 5 6 7 8
kDa
70
40
35
25
15
B
DMSO
(-)-hopeaphenol
Pretreatment
(-)-hopeaphenol
1 2 3 4 5 6 7 8

FIG 18

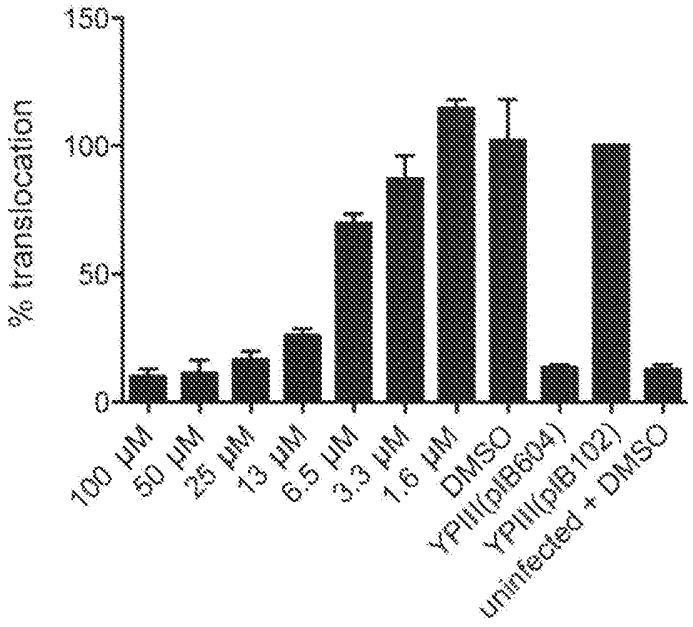

% translocation
150
100
50
0
100 µM
50 µM
25 µM
13 µM
6.5 µM
3.3 µM
1.6 µM
DMSO
YPIII(pIB604)
YPIII(pIB102)
uninfected + DMSO

FIG 19

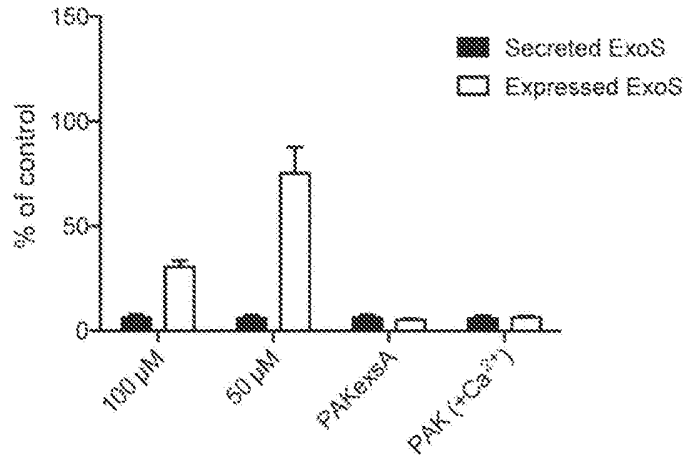

% of control
150
100
50
0
Secreted ExoS
Expressed ExoS
100 µM
50 µM
PAKexsA
PAK (+Ca2+)

FIG 20

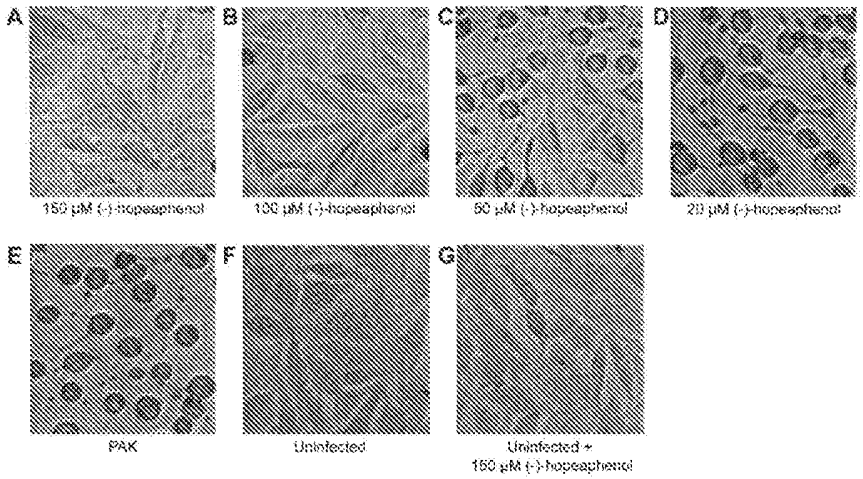

A
B
C
D
150 µM (-)-hopeaphenol
100 µM (-)-hopeaphenol
50 µM (-)-hopeaphenol
25 µM (-)-hopeaphenol
E
F
G
PAK
Uninfected
Uninfected +
150 µM (-)-hopeaphenol

FIG 21

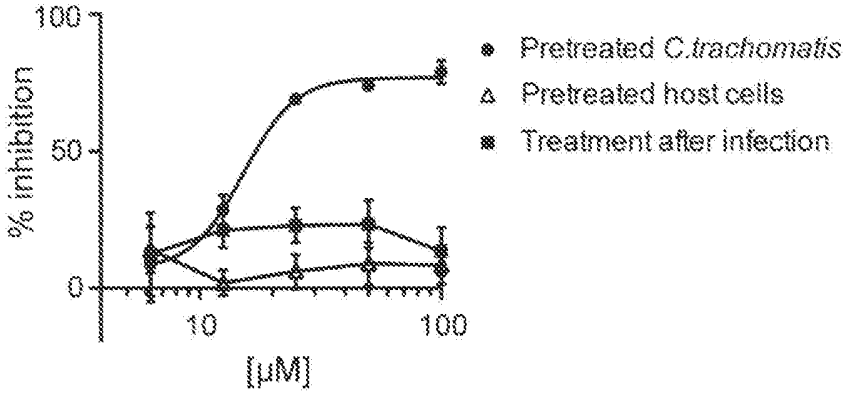

100
50
0
% inhibition
10
100
[µM]
Pretreated C.trachomatis
Pretreated host cells
Treatment after infection

FIG 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2014/000285

A. CLASSIFICATION OF SUBJECT MATTER

A61K 31/55 (2006.01) C07D 307/77 (2006.01) C07D 313/02 (2006.01) A61P 31/04 (2006.01)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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)

STN (CA): structure search based on formula I & keywords (microbe, bacteria & like terms).
STN (WPIDS, Medline, CAPlus) & keywords: hopeaphenol, 17912-85-5, Vatalbinoside, Vaticanol B, neohopeaphenol A, Isohopeaphenol, microbe, bacteria & like terms
Patentscope: Applicant and Inventor search
Google Patents: hopeaphenol, Vatalbinoside, Vaticanol B, neohopeaphenol A, Isohopeaphenol, composition

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] 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
20 May 2014	20 May 2014
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au Facsimile No.: +61 2 6283 7999	Corrina Parker AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262223661

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation).	DOCUMENTS CONSIDERED TO BE RELEVANT	**PCT/AU2014/000285**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0998924 A1 (MEIJI MILK PRODUCTS COMPANY LIMITED) 10 May 2000 paragraphs [0001], [0007], [0008], [0014]; claim 2	17,19-25
X	CN 101543489 A (INSTITUTE OF MATERIA MEDICA, CHINESE ACADEMY OF MEDICAL SCIENCES) 30 September 2009 abstract; page 5, summary of invention, third paragraph; bottom page 8 – top page 9; claim 1	17-25
X	TABATA, Y., ET AL, "Vaticanol B, a resveratrol tetramer, regulates endoplasmic reticulum stress and inflammation". *Am J Physiol Cell Physiol*, 2007, vol.293 pages C411–C418 abstract	21-25
X	ZGODA-POLS, J.R., ET AL, "Antimicrobial Resveratrol Tetramers from the Stem Bark of *Vatica oblongifolia* ssp. *oblongifolia*". *Journal of Natural Products*, 2002, vol. 65, pages 1554-1559 abstract	21-25
X	WO 2001/091764 A2 (ISTITUTO DI NEUROBIOLOGIA E MEDICINA MOLECOLARE DEL CNR AND ISTITUTO AGRARIO DI S. MICHELE ALL' ADIGE) 06 December 2001 abstract; page 8; page 14, lines 28-30; claims 21, 22, 36	17-25
X Y	WIBOWO, A., ET AL, "Malaysianol B, an oligostilbenoid derivative from *Dryobalanops lanceolata*". *Fitoterapia*, 2012, vol. 83, pages 1569-1575 abstract; page 1570, paragraph 2.4; table 2 abstract; page 1570, paragraph 2.4; table 2	21-25 1-16,26
X	LIU, J.Y., ET AL, "New Resveratrol Oligomers from the Stem Bark of *Hopea hainanensis*". *Helvetica Chimica Acta*, 2005, vol. 88, pages 2910-2917 abstract	21-25
X	ATUN, S., ET AL, "Resveratrol Derivatives from Stem Bark of *Hopea* and Their Biological Activity Test". *Journal of Physical Science*, 2008, vol. 19, no. 2, pages 7-21 abstract	21-25
X Y	CN 101810598 B (INDUSTRIAL TECHNOLOGY RESEARCH INSTITUTE) 25 July 2012 abstract; paragraph [0013] abstract; paragraph [0013]	17,19-25 1-16,26

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2014/000285**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
EP 0998924 A1	10 May 2000	CA 2296478 A1	04 Feb 1999
		WO 9904776 A1	04 Feb 1999
		JP H1135454 A	09 Feb 1999
CN 101543489 A	30 Sep 2009	CN 101543489 B	30 Jan 2013
WO 2001/091764 A2	06 Dec 2001	AU 6054201 A	11 Dec 2001
		EP 1292320 A2	19 Mar 2003
		EP 1292320 B1	16 Nov 2005
		IT RM20000294 A1	30 Nov 2001
		IT 1317034 B1	26 May 2003
		US 2004014682 A1	22 Jan 2004
		US 2008070851 A1	20 Mar 2008
CN 101810598 B	25 Jul 2012	CN 101810598 B	25 Jul 2012

End of Annex

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.