Antimicrobial Compounds, Methods of Production and Uses Thereof

Technical Field

The present invention relates, in general terms, to antimicrobial compounds, their methods of production and uses thereof.

Background

Natural products (NPs) are highly diverse 3D molecules with high efficacy for applications in medicine, agriculture and food. Within commercial drugs, an estimated 50% of non-biologics are NP, NP-derived or NP mimics. Although there is enormous biosynthetic potential in actinomycetes for production of novel and bioactive natural compounds, these microbes are often silent under lab conditions. For example, through classical screening, model *Streptomyces* organism, *Streptomyces coelicolor* was thought to only have 3 NPs, however with genomics and further engineering, it now can produce 17 unique NPs, with potential for upwards of 20 biosynthetic gene clusters (BGCs). As genomic information accumulates, the diversity and numbers of identified BGCs have also rapidly increased – an estimated 33K putative BGCs were uncovered from 1.1K bacterial genomes. Silent gene clusters have recently become a considerable natural chemical diversity resource to be exploited. To tap into these resources, there are various strategies to activate and upregulate NP biosynthesis. With increasing ease of genetic engineering, genetic based strategies can be an efficient route to activating silent gene clusters.

However, even with the various genetic strategies, it is still challenging to identify NPs that are useful. It is also challenging to biosynthesise specific NPs. It is also challenging to derivatise NPs with specific functionalities and stereochemistries.

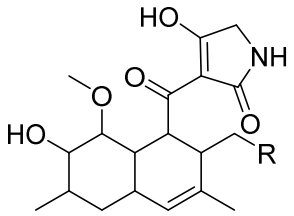
It would be desirable to overcome or ameliorate at least one of the above-described problems.

Summary

The present invention provides methods of producing tetramic acids and the tetramic acids thereof. In particular, application of genetic-based activation to upregulate production of two tetramic acid compounds **1** (BE 54476-A) and **2** (BE 54476-B) (**Figure 1**) is demonstrated. Surprisingly, these upregulated compounds not only have anti-bacterial activity against *Staphylococcus aureus* but also demonstrated bioactivity

towards *Acinetobacter baumannii*. These compounds are believed to be the first example of tetramic acid type compounds with Gram-negative bioactivity (Figure 1).

The present invention provides a method of biosynthesizing an antimicrobial compound of Formula (I):



(I)

wherein R is optionally substituted alkyl;
the method comprising:

a) culturing a microbial cell that is engineered to overexpress fatty-acyl CoA synthase (FAS) or RedD; and

b) isolating the antimicrobial compound of Formula (I) that is produced by the microbial cell;

wherein the biosynthesis of antimicrobial compound of Formula (I) is upregulated by at least about 2-fold compared to a tetramic acid analogue derived from a wild type microbial cell.

The method allows for an upregulation of the antimicrobial compound of Formula (I) such that at least a sufficient amount can be extracted.

In some embodiments, the microbial cell is engineered by introducing a FAS or a RedD expression cassette into the genome of the microbial cell.

In some embodiments, the FAS or RedD expression cassette is integrated into the genome of the microbial cell via an integration plasmid.

In some embodiments, the microbial cell is a bacterium. In some embodiments, the bacterium is *Streptomyces sp*.

In some embodiments, the method further comprises a step before the culturing step (step a) of inoculating the engineered microbial cell in a seed media.

In some embodiments, the seed media is SV2 seed media.

In some embodiments, the engineered microbial cell is inoculated for at least 4 days.

In some embodiments, the engineered microbial cell is cultured in a media comprising glycerol and/or starch. In some embodiments, the media is CA07LB (glycerol-based) and/or CA10LB (starch-based).

In some embodiments, the engineered microbial cell is cultured for at least 7 days.

In some embodiments, the engineered microbial cell is co-cultured with inducer strain. In some embodiments, the inducer strain is a mycolic acid bacteria such as *Rhodococcus* sp.

In some embodiments, a volume ratio of inducer strain to engineered microbial cell is about 1:2 to about 1:5. In some embodiments, a volume ratio of inducer strain to engineered microbial cell is about 1:3.

In some embodiments, the method further comprises a step before the isolating step (step b) of freeze drying the culture.

In some embodiments, the antimicrobial compound of Formula (I) is isolated from the microbial cell culture medium.

In some embodiments, the antimicrobial compound of Formula (I) is an antibacterial compound.

In some embodiments, the antimicrobial compound of Formula (I) is characterised by an antibacterial activity against Gram-positive bacteria and Gram-negative bacteria.

In some embodiments, the Gram-positive bacteria is *S. aureus.*

In some embodiments, the antimicrobial compound of Formula (I) is characterised by a minimum inhibitory concentration (MIC_{50}) against Gram-positive bacteria of less than about 20 μM, less than about 15 μM, or less than about 8 μM.

In some embodiments, the antimicrobial compound of Formula (I) is characterised by a minimum bactericidal concentrations (MBC_{50}) against Gram-positive bacteria of less than about 80 μM, less than about 65 μM, or less than about 22 μM.

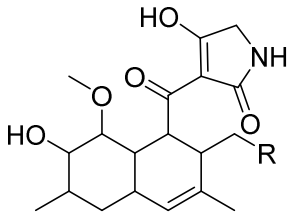
In some embodiments, the Gram-negative bacteria is *A. baumannii.*

In some embodiments, the antimicrobial compound of Formula (I) is characterised by a

minimum inhibitory concentration (MIC_{50}) against Gram-negative bacteria of less than about 20 μM, less than about 10 μM, or less than about 7 μM.

In some embodiments, the antimicrobial compound of Formula (I) is characterised by a IC_{50} against cancer cells of less than about 50 μM or less than about 35 μM.

The present invention also provides an antimicrobial compound of Formula (I):



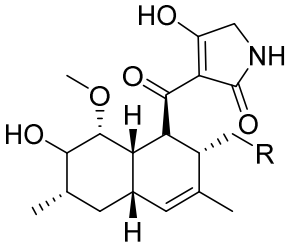
(I)

wherein R is optionally substituted alkyl.

It was found that the absence of a methyl group on the tetramic acid moiety can provide bioactivity against Gram-negative bacteria.

In some embodiments, R is optionally substituted methyl, optionally substituted ethyl or optionally substituted propyl.

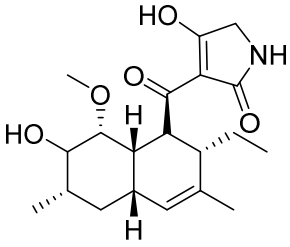
In some embodiments, the antimicrobial compound of Formula (I) is a compound of Formula (Ia):

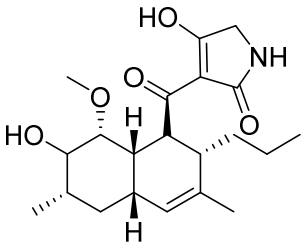


(Ia)

wherein R is optionally substituted alkyl.

In some embodiments, the antimicrobial compound is selected from:





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The present invention also provides a pharmaceutical composition comprising a compound of Formula (I) or a pharmaceutically acceptable salt, solvate or prodrug

thereof.

The present invention also provide a method of treating a microbial disease or condition, the method comprising administering an antimicrobial compound of Formula (I) or a pharmaceutically acceptable salt, solvate or prodrug thereof to the subject in need thereof.

In some embodiments, the microbial disease or condition is selected from a disease or condition caused by *S. aureus* and/or *A. baumannii*. Such disease or conditions may be skin and soft tissue infections (such as abscess), blood infections, pneumonia, bone and joint infection, urinary tract infection, pneumonia, or an infection caused by an open wound.

Brief description of the drawings

Embodiments of the present invention will now be described, by way of non-limiting example, with reference to the drawings in which:

Figure 1 shows structures of selected known tetramic acid containing natural products and Compound 1 and 2.

Figure 2 shows activation in *Streptomyces sp*. A58051. (A) Yield of compound 1 and 2 of various mutants under CA10LB and CA07LB fermentation. A100020, and A100023 are FAS overexpression strains. A100292 is RedD overexpression strain. (B) Total metabolites produced in RedD, FAS mutant compared to WT. Number of metabolites are given in each section in brackets, metabolites in the overlapped sections are produced under two or more regulators. Only metabolite signals that are at least 10^5 base peak abundance are considered here. (List of metabolites in Supplementary) (C) Comparison of unique metabolites produced in wild type (WT) FAS and RedD integrated strains under CA10LB media conditions.

Figure 3 shows co-culture fermentation: Fold change with respect to wild type in CA07LB for mutant alone, mutant in a co-culture with inducer strain, *Rhodococcus sp*. T5718.

Figure 4 shows chemical structures of the tetramic acid analogs, Compound 1 and 2 (relative configurations of the stereocenters were determined via NOESY correlations as shown in 3C). (B) Selected COSY and HMBC correlations of Compound 1 and 2. (C) Selected NOESY correlations of Compound 1 and 2.

Figure 5 shows the integration of overexpression cassette for SCO6196 (AMP-binding domain-containing protein) into *Streptomyces sp*. A58051.

Figure 6 shows dose response curve against *K. aerogenes* (ATCC® 13048™), *P. aeruginosa* (ATCC® 9027™) *A. fumigatus* (ATCC® 46645™). **A)** Compound **1**, **B)** Compound **2**.

Figure 7 shows dose response curve against *S. aureus* Rosenbach (ATCC® 25923™) (SA25923), A) Compound 1, B) Compound 2.

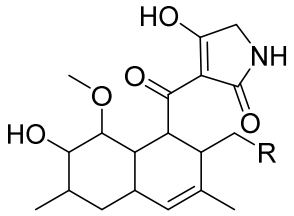
Figure 8 shows dose response curve against *A. baumannii* (ATCC® 19606™) (ACB19606), A) Compound 1, B) Compound 2.

Figure 9 shows dose response curve against the human lung carcinoma cells A549 (ATCC® CCL-185™) (A549), A) Compound 1, B) Compound 2.

Detailed description

Antimicrobial compound refers to an agent which can be used mainly against microorganisms by either killing the microorganism and/or inhibit them from growing further. Microorganisms include bacteria, protozoa, algae, and fungi. Corollary, antibacterial compound refers to an agent which can be used mainly against bacteria by either killing the bacteria or inhibit them from growing further.

The present invention provides a method of producing an antimicrobial compound of Formula (I):



(I)

wherein R is optionally substituted alkyl;

the method comprising:

a) culturing a microbial cell that is engineered to overexpress fatty-acyl CoA synthase (FAS) or RedD; and

b) isolating the antimicrobial compound of Formula (I) that is produced by the microbial cell.

The microbial cells may comprise a cellular or an enzymatic pathway to produce intracellular triacylglycerols. In some embodiments, the microbial cells are configured to upregulate intracellular triacylglycerols in the microbial cells. The method allows for an upregulation of the antimicrobial compound of Formula (I) such that at least a sufficient amount can be extracted.

Fatty-acyl CoA synthase (FAS) or RedD acts to upregulate intracellular triacylglycerols

pool in microbial cell which led to upregulation of secondary metabolite production. This in turn increases the production of the antimicrobial compounds of Formula (I).

The microbial cells may be characterised by an enzymatic pathway which synthesises tetramic acids and/or analogs (including compound of Formula (I)). For example, the enzymatic pathway may include polyketide synthase, cyclases, reductases and/or nonribosomal peptide synthetases. In some embodiments, the microbial cells are engineered to synthesis compound of Formula (I) via an enzymatic pathway. However, the yield of the compound may be low. Accordingly, the microbial cells may be engineered to increase the yield of tetramic acids. In some embodiments, the microbial cell is engineered by introducing a fatty-acyl CoA synthase (FAS) or a RedD expression cassette into the microbial cell. In some embodiments, the microbial cell is engineered by introducing a fatty-acyl CoA synthase (FAS) or a RedD expression cassette into the genome of the microbial cell. An expression cassette is a distinct component of vector DNA consisting of a gene and regulatory sequence to be expressed by a transfected cell. In each successful transformation, the expression cassette directs the cell's machinery to make RNA and protein(s). Some expression cassettes are designed for modular cloning of protein-encoding sequences so that the same cassette can easily be altered to make different proteins. The expression cassette (and its transgene) may be transcribed and/or translated by it merely being taken up by the microbial cell, or the expression cassette may be integrated into the genome of the microbial cell. In this regard, the antimicrobial compounds may be produced as long as FAS and RedD are expressed. As used herein, "expression cassette" and "overexpression cassette" are used interchangeably.

In some embodiments, the FAS or RedD is overexpressed. In some embodiments, the FAS or RedD overexpression cassette is integrated into the genome of the microbial cell via an integration plasmid. Integrative plasmids are in most cases suicide vectors, that is, vectors that are unable to replicate in the destination host and therefore must either integrate or disappear, and hence, any plasmid that can be efficiently transferred into the recipient may be used.

In some embodiments, the integration plasmid is pSET152.

In some embodiments, the microbial cell is a bacterium. In some embodiments, the bacterium is *Streptomyces sp*. In some embodiments, the bacterium is *Actinomycetes*. Other microbial cells may also be used, such as a heterologous host. Heterologous expression refers to the expression of a gene or part of a gene in a host organism which does not naturally have this gene or gene fragment. Insertion of the gene in the

heterologous host may be performed by recombinant DNA technology.

In some embodiments, the method further comprises a step before the culturing step (step a) of inoculating the engineered microbial cell in a seed media. In some embodiments, the seed media is SV2 seed media. SV2 seed media may comprise glucose 15 g/L, glycerol 15 g/L, soya peptone 15 g/L, calcium carbonate 1 g/L, and may be at pH 7.0.

In some embodiments, the engineered microbial cell is inoculated for at least 4 days. In other embodiments, the engineered microbial cell is inoculated for at least 5 days or 6 days. Inoculation introduces the microorganisms into environments where they will grow and reproduce.

In some embodiments, the engineered microbial cell is cultured in a media comprising glycerol and/or starch. It is believed that glycerol may be channelled to glycerol-3-phosphate and TAGs, thus increasing the flux towards compound production. In some embodiments, the media is CA07LB (glycerol-based) and/or CA10LB (starch-based).

A microbiological culture, or microbial culture, is a method of multiplying microbial organisms by letting them reproduce in predetermined culture medium under controlled laboratory conditions.

In some embodiments, the engineered microbial cell is cultured for at least 7 days. In other embodiments, the engineered microbial cell is cultured for at least 8 days, 9 days or 10 days.

In some embodiments, the engineered microbial cell is co-cultured with inducer strain. The inducer strain is a substance that is capable of activating the growth of the engineered microbial cell. For example, mycolic acid can be used to activate the growth of the engineered microbial cell. In some embodiments, the inducer strain is a mycolic acid containing bacteria such as *Rhodococcus* sp.

In some embodiments, a volume ratio of inducer strain to engineered microbial cell is about 1:2 to about 1:5. In some embodiments, a volume ratio of inducer strain to engineered microbial cell is about 1:3.

In some embodiments, the method further comprises a step before the isolating step (step b) of freeze drying the culture. In this way, the engineered microbial cell may be lyophilised into a powder.

In some embodiments, the antimicrobial compound is isolated from the microbial cell culture medium. In some embodiments, the antimicrobial compound is extracted from the culture medium using an organic solvent. In some embodiments, the antimicrobial compound is extracted from the culture medium using methanol.

In some embodiments, the antimicrobial compound is upregulated by at least about 2 fold compared to a tetramic acid analogue derived from a wild type microbe. The tetramic acid analogue derived from a wild type microbe may be BE-54476. In other embodiments, the upregulation is at least about 3 fold, 4 fold, 5 fold, 6 fold, 8 fold, 10 fold, 12 fold, 14 fold, 16 fold, 18 fold or 20 fold.

In some embodiments, the antimicrobial compound is an antibacterial compound.

In some embodiments, the antimicrobial compound is characterised by an antibacterial activity against Gram-positive bacteria and Gram-negative bacteria. In some embodiments, the Gram-positive bacteria is *S. aureus.* In some embodiments, the Gram-negative bacteria is *A. baumannii.*

In some embodiments, the antimicrobial compound is characterised by a minimum inhibitory concentration (MIC_{50}) against Gram-positive bacteria of less than about 20 μM. In some embodiments, the antimicrobial compound is characterised by a minimum inhibitory concentration (MIC_{50}) against Gram-positive bacteria of less than about 15 μM. In some embodiments, the antimicrobial compound is characterised by a minimum inhibitory concentration (MIC_{50}) against Gram-positive bacteria of less than about 8 μM.

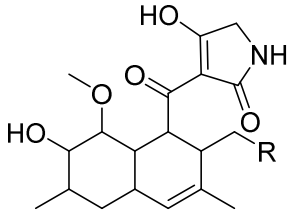
In some embodiments, the antimicrobial compound is characterised by a minimum bactericidal concentrations (MBC_{50}) against Gram-positive bacteria of less than about 80 μM. In some embodiments, the antimicrobial compound is characterised by a minimum bactericidal concentrations (MBC_{50}) against Gram-positive bacteria of less than about 65 μM. In some embodiments, the antimicrobial compound is characterised by a minimum bactericidal concentrations (MBC_{50}) against Gram-positive bacteria of less than about 22 μM.

In some embodiments, the antimicrobial compound is characterised by a minimum inhibitory concentration (MIC_{50}) against Gram-negative bacteria of less than about 20 μM. In some embodiments, the antimicrobial compound is characterised by a minimum inhibitory concentration (MIC_{50}) against Gram-negative bacteria of less than about 10 μM. In some embodiments, the antimicrobial compound is characterised by a minimum

inhibitory concentration (MIC_{50}) against Gram-negative bacteria of less than about 7 μM.

In some embodiments, the antimicrobial compound of Formula (I) is characterised by a IC_{50} against cancer cells of less than about 50 μM. In some embodiments, the antimicrobial compound of Formula (I) is characterised by a IC_{50} against cancer cells of less than about 35 μM.

In some embodiments, the method of producing an antimicrobial compound of Formula (I):



(I)

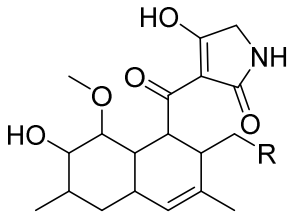
wherein R is optionally substituted alkyl;
the method comprising:

a) integrating a fatty-acyl CoA synthase (FAS) expression cassette or a RedD expression cassette into a microbial cell in order to form an engineered microbial cell;

b) culturing the engineered microbial cell; and

c) isolating the antimicrobial compound that is produced by the microbial cell.

In some embodiments, the method of producing an antimicrobial compound of Formula (I):



(I)

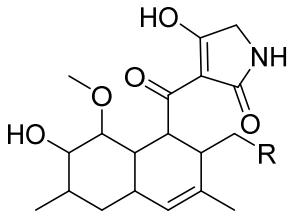
wherein R is optionally substituted alkyl;
the method comprising:

a) integrating a fatty-acyl CoA synthase (FAS) expression cassette or a RedD expression cassette into a genome of a microbial cell in order to form an engineered microbial cell;

b) culturing the engineered microbial cell; and

c) isolating the antimicrobial compound that is produced by the microbial cell.

In some embodiments, the method of producing an antimicrobial compound of Formula (I):



(I)

wherein R is optionally substituted alkyl;

the method comprising:

a) integrating a fatty-acyl CoA synthase (FAS) expression cassette or a RedD expression cassette into a genome of a microbial cell in order to form an engineered microbial cell;

b) culturing the engineered microbial cell;

c) lyophilising the engineered microbial cell of step b); and

d) isolating the antimicrobial compound that is produced by the microbial cell.

The present invention also provides a method of large scale production of antimicrobial compounds. The present method may be used in the laboratory to isolate antimicrobial compounds at a scale of at least about 2 or 3 mg/L. Under co-culture conditions, up to 10-fold improvement in production of the compounds can be observed which is equivalent to a yield of up to about 20 mg/L. The engineered microbial cell may be co-cultured with an inducer strain, such as *Rhodococcus sp*..

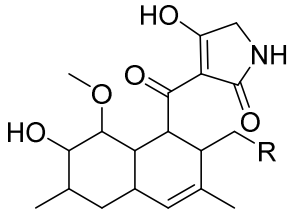
In some embodiments, the method comprising:

a) culturing a microbial cell that is engineered to overexpress fatty-acyl CoA synthase (FAS) or RedD; and

b) isolating the antimicrobial compound of Formula (I) that is produced by the microbial cell;

wherein the microbial cell is cultured under co-culture in the presence of an inducer strain.

The present invention also provides an antimicrobial compound of Formula (I), comprising:



(I)

wherein R is optionally substituted alkyl.

It was found that the absence of a methyl group on the tetramic acid moiety can provide

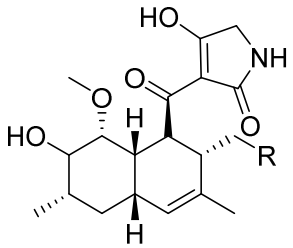
bioactivity against Gram-negative bacteria.

In some embodiments, R is optionally substituted methyl, optionally substituted ethyl, or optionally substituted propyl. In some embodiments, R is methyl, ethyl or propyl.

In this specification "optionally substituted" is taken to mean that a group may or may not be further substituted or fused (so as to form a condensed polycyclic group) with one or more groups selected from hydroxyl, acyl, alkyl, alkoxy, alkenyl, alkenyloxy, alkynyl, alkynyloxy, amino, aminoacyl, thio, arylalkyl, arylalkoxy, aryl, aryloxy, carboxyl, acylamino, cyano, halogen, nitro, phosphono, sulfo, phosphorylamino, phosphinyl, heteroaryl, heteroarylalkyl, heteroaryloxy, heterocyclyl, heterocyclylalkyl, heterocyclyloxy, oxyacyl, oxime, oxime ether, hydrazone, oxyacylamino, oxysulfonylamino, aminoacyloxy, trihalomethyl, trialkylsilyl, pentafluoroethyl, trifluoromethoxy, difluoromethoxy, trifluoromethanethio, trifluoroethenyl, mono- and di-alkylamino, mono-and di-(substituted alkyl)amino, mono- and di-arylamino, mono- and di-heteroarylamino, mono- and di-heterocyclyl amino, and unsymmetric di-substituted amines having different substituents selected from alkyl, aryl, heteroaryl and heterocyclyl, and the like. For instance, an "optionally substituted amino" group may include amino acid and peptide residues.

In some embodiments, the optional substituent on R is one or more groups selected from hydroxyl, acyl, alkoxy, alkenyl, alkenyloxy, alkynyl, alkynyloxy, amino, aminoacyl, arylalkyl, arylalkoxy, aryl, aryloxy, carboxyl, cyano, halogen, nitro, phosphorylamino, heteroaryl, heteroarylalkyl, heteroaryloxy, heterocyclyl, heterocyclylalkyl, and heterocyclyloxy.

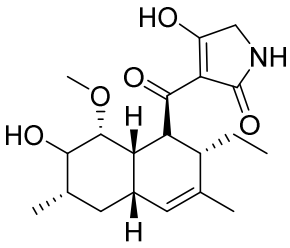
In some embodiments, the antimicrobial compound of Formula (I) is a compound of Formula (Ia):

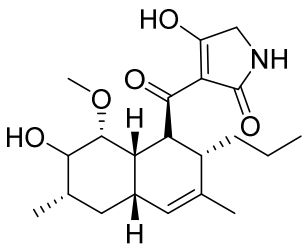


(Ia)

wherein R is optionally substituted alkyl.

In some embodiments, the antimicrobial compound is selected from:





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The present invention also provides a pharmaceutical composition comprising a compound of Formula (I) or a pharmaceutically acceptable salt, solvate or prodrug thereof.

The present invention also provide a method of treating a microbial disease or condition, the method comprising administering an antimicrobial compound of Formula (I) or a pharmaceutically acceptable salt, solvate or prodrug thereof to the subject in need thereof.

The present invention provides a use of an antimicrobial compound of Formula (I) or a pharmaceutically acceptable salt, solvate or prodrug thereof in the manufacture of a medicament for treating a microbial disease or condition.

The present invention also provides an antimicrobial compound of Formula (I) or a pharmaceutically acceptable salt, solvate or prodrug thereof for use in treating a microbial disease or condition.

In some embodiments, the microbial disease or condition is selected from a disease or condition caused by *S. aureus* and/or *A. baumannii*. Such disease or conditions may be skin and soft tissue infections (such as abscess), blood infections, pneumonia, bone and joint infection, urinary tract infection, pneumonia, or an infection caused by an open wound.

The present invention also provide a method of treating cancer, the method comprising administering an antimicrobial compound of Formula (I) or a pharmaceutically acceptable salt, solvate or prodrug thereof to the subject in need thereof.

The present invention provides a use of an antimicrobial compound of Formula (I) or a pharmaceutically acceptable salt, solvate or prodrug thereof in the manufacture of a medicament for treating cancer.

The present invention also provides an antimicrobial compound of Formula (I) or a pharmaceutically acceptable salt, solvate or prodrug thereof for use in treating cancer.

In some embodiments, the cancer is lung carcinoma.

The compound of the invention can be administered to a subject as a pharmaceutically acceptable salt thereof. Suitable pharmaceutically acceptable salts include, but are not limited to salts of pharmaceutically acceptable inorganic acids such as hydrochloric, sulphuric, phosphoric, nitric, carbonic, boric, sulfamic, and hydrobromic acids, or salts of pharmaceutically acceptable organic acids such as acetic, propionic, butyric, tartaric, maleic, hydroxymaleic, fumaric, maleic, citric, lactic, mucic, gluconic, benzoic, succinic, oxalic, phenylacetic, methanesulphonic, toluenesulphonic, benezenesulphonic, salicyclic sulphanilic, aspartic, glutamic, edetic, stearic, palmitic, oleic, lauric, pantothenic, tannic, ascorbic and valeric acids.

Base salts include, but are not limited to, those formed with pharmaceutically acceptable cations, such as sodium, potassium, lithium, calcium, magnesium, ammonium and alkylammonium. In particular, the present invention includes within its scope cationic salts eg sodium or potassium salts, or alkyl esters (eg methyl, ethyl) of the phosphate group.

Basic nitrogen-containing groups may be quarternised with such agents as lower alkyl halide, such as methyl, ethyl, propyl, and butyl chlorides, bromides and iodides; dialkyl sulfates like dimethyl and diethyl sulfate; and others.

It will be appreciated that any compound that is a prodrug of the compound of formula (I) is also within the scope and spirit of the invention. Thus the compound of the invention can be administered to a subject in the form of a pharmaceutically acceptable pro-drug. The term "pro-drug" is used in its broadest sense and encompasses those derivatives that are converted in vivo to the compound of the invention. Such derivatives would readily occur to those skilled in the art. Other texts which generally describe prodrugs (and the preparation thereof) include: Design of Prodrugs, 1985, H. Bundgaard (Elsevier); The Practice of Medicinal Chemistry, 1996, Camille G. Wermuth et al., Chapter 31 (Academic Press); and A Textbook of Drug Design and Development, 1991, Bundgaard et al., Chapter 5, (Harwood Academic Publishers). For example, the amine moiety may be quarternarised or converted into an amide moiety, or the hydroxyl moiety may be converted into an ester moiety.

The compound of the invention may be in crystalline form either as the free compound or as a solvate (e.g. hydrate) and it is intended that both forms are within the scope of the present invention. Methods of solvation are generally known within the art.

The compound of the invention, or a pharmaceutically acceptable salt, solvate or prodrug thereof is administered to the patient in a therapeutically effective amount. As used herein, a therapeutically effective amount is intended to include at least partially attaining the desired effect, or delaying the onset of, or inhibiting the progression of, or halting or reversing altogether the onset or progression of macular degeneration.

As used herein, the term "effective amount" relates to an amount of compound which, when administered according to a desired dosing regimen, provides the desired therapeutic activity. Dosing may occur at intervals of minutes, hours, days, weeks, months or years or continuously over any one of these periods. Suitable dosages may lie within the range of about 0.1 ng per kg of body weight to 1 g per kg of body weight per dosage, such as is in the range of 1 mg to 1 g per kg of body weight per dosage. In one embodiment, the dosage may be in the range of 1 mg to 500 mg per kg of body weight per dosage. In another embodiment, the dosage may be in the range of 1 mg to 250 mg per kg of body weight per dosage. In yet another embodiment, the dosage may be in the range of 1 mg to 100 mg per kg of body weight per dosage, such as up to 50 mg per body weight per dosage.

Suitable dosage amounts and dosing regimens can be determined by the attending physician and may depend on the severity of the condition as well as the general age, health and weight of the patient to be treated.

The compound of the invention may be administered in a single dose or a series of doses. While it is possible for the active ingredient to be administered alone, it is preferable to present it as a composition, preferably as a pharmaceutical composition. The formulation of such compositions is well known to those skilled in the art. The composition may contain any suitable carriers, diluents or excipients. These include all conventional solvents, dispersion media, fillers, solid carriers, coatings, antifungal and antibacterial agents, dermal penetration agents, surfactants, isotonic and absorption agents and the like. It will be understood that the compositions of the invention may also include other supplementary physiologically active agents.

The carrier must be pharmaceutically "acceptable" in the sense of being compatible with the other ingredients of the composition and not injurious to the patient. The compositions may conveniently be presented in unit dosage form and may be prepared

by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

The compound may be injected directly to the eye, and in particular the vitreous of the eye. The compound or composition of the invention can be administered to the vitreous of the eye using any intravitreal or transscleral administration technique. For example, the compound or composition can be administered to the vitreous of the eye by intravitreal injection. Intravitreal injection typically involves administering a compound of the invention or a pharmaceutically acceptable salt, solvate or prodrug in a total amount between 0.1 ng to 10 mg per dose.

Injectables for such use can be prepared in conventional forms, either as a liquid solution or suspension or in a solid form suitable for preparation as a solution or suspension in a liquid prior to injection, or as an emulsion. Carriers can include, for example, water, saline (e.g., normal saline (NS), phosphate-buffered saline (PBS), balanced saline solution (BSS)), sodium lactate Ringer's solution, dextrose, glycerol, ethanol, and the like; and if desired, minor amounts of auxiliary substances, such as wetting or emulsifying agents, buffers, and the like can be added. Proper fluidity can be maintained, for example, by using a coating such as lecithin, by maintaining the required particle size in the case of dispersion and by using surfactants. By way of example, the compound or composition can be dissolved in a pharmaceutically effective carrier and be injected into the vitreous of the eye with a fine gauge hollow bore needle (e.g., 30 gauge, 1/2 or 3/8 inch needle) using a temporal approach (e.g., about 3 to about 4 mm posterior to the limbus for human eye to avoid damaging the lens).

A person skilled in the art will appreciate that other means for injecting and/or administering the compound or composition to the vitreous of the eye can also be used. These other means can include, for example, intravitreal medical delivery devices. These devices and methods can include, for example, intravitreal medicine delivery devices, and biodegradable polymer delivery members that are inserted in the eye for long term delivery of medicaments. These devices and methods can further include transscleral delivery devices.

Other modes of administration including topical or intravenous administration may also be possible. For example, solutions or suspensions of the compound or composition of the invention may be formulated as eye drops, or as a membranous ocular patch, which

is applied directly to the surface of the eye. Topical application typically involves administering the compound of the invention in an amount between 0.1 ng and 10 mg.

The compound or composition of the invention may also be suitable for intravenous administration. For example, a compound of formula (I) or a pharmaceutically acceptable salt, solvate or prodrug thereof may be administered intravenously at a dose of up to 16 mg/m^2.

The compound or composition of the invention may also be suitable for oral administration and may be presented as discrete units such as capsules, sachets or tablets each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste. In another embodiment, the compound of formula (I) or a pharmaceutically acceptable salt, solvate or prodrug is orally administerable.

A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g inert diluent, preservative disintegrant (e.g. sodium starch glycolate, cross-linked polyvinyl pyrrolidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

The compound or composition of the invention may be suitable for topical administration in the mouth including lozenges comprising the active ingredient in a flavoured base, usually sucrose and acacia or tragacanth gum; pastilles comprising the active ingredient in an inert basis such as gelatine and glycerin, or sucrose and acacia gum; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

The compound or composition of the invention may be suitable for topical administration to the skin may comprise the compounds dissolved or suspended in any suitable carrier or base and may be in the form of lotions, gel, creams, pastes, ointments and the like.

Suitable carriers include mineral oil, propylene glycol, polyoxyethylene, polyoxypropylene, emulsifying wax, sorbitan monostearate, polysorbate 60, cetyl esters wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water. Transdermal patches may also be used to administer the compounds of the invention.

The compound or composition of the invention may be suitable for parenteral administration include aqueous and non-aqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bactericides and solutes which render the compound or composition isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The compound or composition may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

Preferred unit dosage composition are those containing a daily dose or unit, daily sub-dose, as herein above described, or an appropriate fraction thereof, of the active ingredient.

It should be understood that in addition to the active ingredients particularly mentioned above, the composition of this invention may include other agents conventional in the art having regard to the type of composition in question, for example, those suitable for oral administration may include such further agents as binders, sweeteners, thickeners, flavouring agents disintegrating agents, coating agents, preservatives, lubricants and/or time delay agents. Suitable sweeteners include sucrose, lactose, glucose, aspartame or saccharine. Suitable disintegrating agents include cornstarch, methylcellulose, polyvinylpyrrolidone, xanthan gum, bentonite, alginic acid or agar. Suitable flavouring agents include peppermint oil, oil of wintergreen, cherry, orange or raspberry flavouring. Suitable coating agents include polymers or copolymers of acrylic acid and/or methacrylic acid and/or their esters, waxes, fatty alcohols, zein, shellac or gluten. Suitable preservatives include sodium benzoate, vitamin E, alpha-tocopherol, ascorbic acid, methyl paraben, propyl paraben or sodium bisulphite. Suitable lubricants include magnesium stearate, stearic acid, sodium oleate, sodium chloride or talc. Suitable time delay agents include glyceryl monostearate or glyceryl distearate.

A detailed description of the workings of the invention is laid out below. In the embodiments that follows, the invention is described in relation to some conditions for

consistency to showcase the present invention. However, the skilled person would understand that the invention is not limited to such.

Strain activation via integration of pletropic regulators

Streptomyces sp. A58051, is from the Natural Organism Library (NOL), A*STAR, Singapore. Based on genomic analysis, it is 77.6% similar to *Streptomyces durhamensis* (GCA_000725475.1). To perturb and activate secondary metabolites within *Streptomyces* sp. A58051, we first used the genetic engineering-based strategy of introducing pleotropic regulators into its genome. In this work, mutants were integrated with an overexpression cassette containing either a fatty acyl-CoA synthase (FAS, SCO6196, https://www.ncbi.nlm.nih.gov/gene/1101637) or the specific pathway regulatory protein (SARP), RedD (SLIV_09220, https://www.ncbi.nlm.nih.gov/protein/672368967). FAS was earlier shown to upregulate intracellular triacylglycerols pool in *Streptomyces* which led to upregulation of secondary metabolite production. Although SARPs are oftentimes pathway specific, RedD and similar homologs have been observed to have global effects on biosynthetic gene clusters within *Streptomyces*. In both FAS and RedD overexpression mutants, we observed the upregulation of two tetramic acid compounds **1** and **2** (**Figure 1**). To compare differences in native and mutant strains, we use chemo-informatics to classify unique metabolites in the extracts, based on peaks of $>10^5$ base peak abundance observed in base peak chromatograms (BPC) from liquid chromatrography mass spectrometry (LC-MS) analyses. These peaks were characterized primarily by their base peak *m/z* and retention time (full details on their processing are in the supporting Information). With the overexpression of FAS (A100020) and RedD (A100292), it would appear that the number of unique metabolite species have almost doubled from 16 to 28 (**Figure 2b**), where FAS has the most significant effect on chemical profile. Eleven new metabolites can be attributed to FAS overexpression (**Figure 2c**).

Upregulation of the tetramic acid compounds were also found to be media dependent. Among the mutant strains, CA10LB (starch-based) fermentation had significant 3- to 15-fold increases in yield with respective to wild type production under identical conditions. Whilst in CA07LB (glycerol-based) only 2-fold production was observed (Figure 2). Depending on the fermentation media conditions and mutants, this strategy led to a 2- to 15-fold change in the production of **1** and **2**, including increased proportion of **2** produced (**Figure 2**). In co-culture with mycolic acid bacteria, *Rhodococcus* sp. T5718, we are also able to further triple the yields in this native strains (**Figure 3**).

Isolation and Structural Characterization of Compounds 1 and 2

A100020 was cultured in 1 L of CA07LB liquid media for 10 days at 28 °C. The MeOH extract obtained was separated using preparative C_{18} HPLC to obtain two new tetramic acid derivatives, compounds **1** and **2** (**Figure 4**).

Compound **1** was isolated as a brown amorphous powder and was assigned the molecular formula $C_{20}H_{29}O_5N$ following analysis of the (–)-HRESIMS data (*m/z* 362.1968 $[M–H]^-$, calcd for $C_{20}H_8O_5N$, 362.1973). The ^1H NMR spectrum of **1** showed the signals corresponding to four methyl groups (δ_H 0.75, 1.04, 1.63, and 3.26), three methylenes (δ_H 1.15/1.65, 1.29, and 3.75), and eight methine protons (δ_H 1.39, 2.12, 2.57, 2.76, 2.99, 3.43, 4.06, and 5.61). The ^{13}C NMR and edited HSQC spectra exhibited twenty resonances including four methyls, three methylenes, eight methines, and five non-protonated carbons. The UV absorptions maxima at 220 and 285 nm were typical for a tetramic acid moiety, which was further supported by the characteristic broad ^{13}C NMR signals at δ_C 104.5, 178.2, 196.0, and 201.8. The broad ^{13}C peaks were most likely due to keto-enol tautomerism of the tetramic acid moiety. The remaining structure of **1** was assigned based on COSY and HMBC data (**Figure 4B**). Analysis of COSY spectrum provided the fragments of H-5/H-6/H_2-7/H-8/H-9/H-10/H-11/H-2/H-3 and H_3-15/H-8 (**Figure 4B)**. These data together with HMBC correlations from H-5 to C-6, C-7, and C-11, from H-10 to C-6 constructed a partial structure of decalin. Further ^1H-^1H COSY spin-system of H-3/H_2-12/H_3-13 along with HMBC cross-peak from H_2-12 to C-3 confirmed the location of CH_3-CH_2- substituent at C-3. In addition, HMBC correlation from H_3-14 to the olefinic carbon at δ_C 135.4 placed a methyl group at C-4. The position of a methoxy at C-10 was evident by HMBC correlation from the singlet methyl resonance at δ_H 3.75 to an oxygenated carbon at δ_C 88.7. The presence of a tetramic acid moiety at C-2 was further confirmed by HMBC correlations from H_2-5' (δ_H 3.75) to C-2' and C-4' and by considering the deshielded chemical shift of H-2 (δ_H 4.06), which was typical of tetramic acids system[16,17]. Finally, a hydroxy group was located at C-9 by considering the molecular formula of **1** and the chemical shift of CH-9 (δ_H/δ_C 3.43/75.1).

The relative configuration of **1** was assigned by analysis of NOESY data (**Figure 4C**) and ^1H-^1H coupling constants, as well as comparison of reported values of NMR data of similar decalin-containing tetramic acid compounds. For instance, the chemical shift of the decalin ring junction signals H-11 (δ_H 2.76) and H-6 (δ_H 2.12) were consistent with other *cis*-decalin tetramic acid analogs, when compared to the *trans*-fused congeners. A large coupling constant of 9.8 Hz between H-9 and H-10 indicated that these protons were axially oriented. NOESY correlations between H-2 and H-9 as well as between H-9 and H-7_{ax} [δ_H 1.15, ddd (12.3, 12.3, 12.3)] suggested these protons to be on the same side of the molecule (*a*-oriented). Finally, further NOESY cross-peaks between H-

6 and H-8 as well as between H-3 and H-11 supported a *β*-configuration of these protons. Hence, the structure of **1** was established as a new tetramic acid derivative.

Compound **2** was assigned the molecular formula $C_{20}H_{31}O_5N$ by (–)-HRESIMS. The ^1H and ^{13}C NMR data as well as UV spectrum of **2** were similar to those of **1**. Detailed analysis of NMR and MS data of **2** suggested that compound **2** has an additional $–CH_2–$ group. 2D NMR data analysis indicated that **2** had a propyl moiety at C-3 instead of an ethyl group in **1**. This was further supported by HMBC correlations from a triplet methyl at δ_H 0.81 (13-CH_3) to C-12 and C-13 as well as COSY correlations for 13-CH_3/H_2-13/H_2-12/H-3. The same relative configuration previously determined for **1** was also assigned for **2** following analysis of NOESY spectrum and ^1H-^1H coupling constants.

Table 1. ^1H and ^{13}C NMR data of tetramic acid analogs, Compound **1** and **2** in MeOH-d_4.

Pos.	**1**		**2**	
	δ_C, type	δ_H, mult. (*J* = Hz)	δ_C, type	δ_H, mult. (*J* = Hz)
1	201.8,[a] C	–	[b]	–
2	38.9, CH	4.06, dd (12.0)	39.3, CH	4.09, dd (12.3)
3	45.3, CH	2.57, br d (10.0)	44.9, CH	2.53, br d (10.0)
4	135.4, C	–	135.8, C	–
5	129.0, CH	5.61, d (6.0)	128.4, CH	5.56, d (5.8)
6	37.3, CH	2.12, m	37.2, CH	2.10, m
7	38.2, CH_2	1.15, ddd (12.3, 12.3, 12.3); 1.65, m	38.2, CH_2	1.14, ddd (12.9, 12.9, 12.9); 1.63, m
8	38.7, CH	1.39, m	38.9, CH	1.39, m
9	75.1, CH	3.43, m	75.1, CH	3.44, m
10	88.9, CH	2.99, dd (4.3, 9.8)	88.8, CH	2.97, dd (4.4, 9.7)
11	40.8, CH	2.76, ddd (4.3, 4.3,12.0)	40.9, CH	2.74, dt (4.4, 4.4, 12.3)
12	21.9, CH_2	1.29, m	18.2, CH_2	1.48, m
13	8.6, CH_3	0.75, t (7.4)	32.3, CH_2	1.29, m
14	21.1, CH_3	1.63, s	21.2, CH_3	1.63, s
15	18.8, CH_3	1.04, d (6.4)	18.8, CH_3	1.04, d (6.3)
16	58.3, CH_3	3.26, s	58.3, CH_3	3.25, s

2'	178.2,[a] C	–	178.0, C	–
3'	104.5,[a] C	–	[b]	–
4'	196.1,[a] C	–	196.0, C	–
5'	52.9,[a] CH_2	3.75 (2H), br s	52.5, CH_2	3.73 (2H), br s
13-Me	–	–	15.0, CH_3	0.81, t (6.9)

^1H (400 MHz) and ^{13}C (100 MHz) in MeOH-d_4. Assignments based on COSY, NOESY, HSQC and HMBC. Chemical shifts (δ) in ppm. s: singlet; br s: broad singlet; d: doublet; br d: broad doublet; t: triplet, m: multiplet. One proton unless otherwise stated. [a]Broad signal. [b]Not detected.

Bioactivity of Compounds 1 and 2

Compounds **1** and **2** were tested for their antimicrobial activity against a panel of microorganisms consisting of Gram-positive and Gram-negative bacteria, as well as against one fungal strain. Namely, *A. baumannii* (ATCC® 19606™), *K. aerogenes* (ATCC® 13048™), *P. aeruginosa* (ATCC® 9027™), *S. aureus Rosenbach* (ATCC® 25923™) and *A. fumigatus* (ATCC® 46645™).

Both compounds were found to be inactive against Gram-negative bacteria *K. aerogenes* (ATCC® 13048™), *P. aeruginosa* (ATCC® 9027™) and fungal strain *A. fumigatus* (ATCC® 46645™). (**Table 2, Figure 6**). However, compounds **1** and **2** showed activity against *S. aureus Rosenbach* (ATCC® 25923™) (**Table 2, Figure 7**) with minimum inhibitory concentrations (MIC_{50}) of 14.5 μM and minimum bactericidal concentrations (MBC_{50}) of 65.5 μM for compound **1** and MIC_{50} of 7.5 μM, MBC_{50} of 22.3 μM for compound **2**. Interestingly, the compounds were also found to be active against Gram-negative strain *A. baumannii* (ATCC® 19606™) (**Table 2, Figure 8**), with MIC_{50} of 9.8 μM for Compound **1** and MIC_{50} of 6.9 μM for Compound **2**. No MBC activities were observed in antimicrobial testing of *A. baumannii*. It is believed that this is due to different resistance mechanism for these Gram-negative bacteria. Apart from that, both compounds were also tested for their cytotoxicity against the human lung carcinoma cell line A549 (ATCC® CCL-185™) with IC_{50} of 34.4 μM and 46.3 μM respectively (**Table 2, Figure 9**).

Table 2. Bioactivity characterization of compound **1** and **2**

	Compound 1		**Compound 2**	
Target organism (ATCC® number)	**MIC[a] (μM)**	**MBC/MFC[b] (μM)**	**MIC[a] (μM)**	**MBC/MFC[b] (μM)**

*Acinetobacter baumannii (*ATCC® 19606™)	9.8	>100	6.9	>100
Klebsiella aerogenes (ATCC® 13048™)	>100	>100	>100	>100
Pseudomonas aeruginosa (ATCC® 9027™)	>100	-	>100	-
Staphylococcus aureus Rosenbach (ATCC® 25923™)	14.5	65.5	7.5	22.3
Aspergillus fumigatus (ATCC® 46645™)	>100	-	>100	-
Target cell line ATCC® number)		**IC_{50} (μM)**		
A549 Human lung carcinoma cells (ATCC® CCL-185™)	34.4		46.3	

[a] Minimum inhibitory concentration IC_{50} for microbial assay *A. baumannii* (ATCC® 19606™), *K. aerogenes* (ATCC® 13048™), *P. aeruginosa* (ATCC® 9027™), *S. aureus* Rosenbach (ATCC® 25923™) and *A. fumigatus* (ATCC® 46645™)

[b] Minimum bactericidal/fungicidal concentration IC_{50} for microbial assay *A. baumannii* (ATCC® 19606™), *K. aerogenes* (ATCC® 13048™), *P. aeruginosa* (ATCC® 9027™), *S. aureus* Rosenbach (ATCC® 25923™) and *A. fumigatus* (ATCC® 46645™)

Discussion

Emergence of infectious diseases coupled with reduced efficacy of antibiotics due to multidrug resistance is a continuous international problem. Subsequently, besides corrective measures for appropriate antibiotics usage, new agents are required to replace the current frontline antibiotics against infectious diseases. Recently, appearance of multi-drug resistance *Acinetobacter baumannii* has also been of growing concern, especially as they become resistant to last line antibiotics. Here, we demonstrate utilization of genetic activation to upregulate new tetramic acid compounds with surprisingly bioactivities towards *A. baumannii.*

Previously, other structurally similar tetramic acid analogs (**Figure 1**) have been reported to exhibit antimicrobial activity, mainly against Gram-positive bacteria. For instance, equisetin had been described for its activity against *Staphylococcus erythraea* and *Staphylococcus aureus*; ascosalipyrrolidone A was active against *Bacillus megaterium*, *Mycoptypha microsporosum*, and *Microbotyryum violaceum*; BU-4514N and the fungal metabolite altersetin had inhibitory activity against several Gram-positive bacteria. Notably, a similar compound antibiotic BE-54476 in comparison to compounds

1 and **2** was reported to have antibacterial activity against Gram-positive bacteria such as *Bacillus subtilis*, *Enterococcus faecalis*, and *S. aureus*, and possess anti-tumour activity. Surprisingly, the removal of a methyl group (**1** and **2**) is sufficient to present one of the first example of Gram-negative bioactivity within similar scaffolds; significant bioactivity towards *Acinetobacter baumannii*. The chemical structure of compound **1** differs from compound **2** at C-3, featuring an ethyl group as opposed to the propyl moiety in compound **2.** However, bioactivity testing showed that both compounds had similar activities, including antibacterial activity against Gram-positive *S. aureus* and Gram-negative *A. baumannii*, as well as cytotoxicity against human lung carcinoma A549 cells. This indicated that the difference in chemical structure between compounds **1** and **2** did not effect a major difference in their antimicrobial and cytotoxic activities. As an indication of their uniqueness, high resolution MS scans among ~2K actinomycetes within the NOL collection present no other observation of compounds **1** and **2**.

Finally, we also demonstrated multiple strategies to increase tetramic acid yield, including genetic engineering and media optimization. Overexpression of either RedD and FAS were able to improve tetramic acid yields and enable isolation of the compounds. In the wild type strain, media played a significant role in yield improvement. However, this difference was reduced in overexpression mutants. FAS was earlier demonstrated to allow *Streptomyces* to utilize their intracellular triacylglycerols pool towards enhancing biosynthesis of natural products. With the wild type strain, a glycerol based fermentation was shown to be 2- to 4-fold more productive compared to non-glycerol media. We hypothesized that glycerol was channelled to glycerol-3-phosphate and TAGs, thus increasing the flux towards tetramic acid production. However in the presence of FAS overexpression, this pathway towards acyl-CoAs is no longer limiting, resulting in the observed less significant yield differences in the media.

Overall, a combination of strategies has been used to perturb the regulation of antibiotics production. In this work, seemingly unrelated regulators were sufficient to increase production of a low yielding but bioactive compound. Due to this increase, we were able to uncover new tetramic acid analogs with new activity towards *A. baumannii.*

Materials and methods

Overexpression cassettes

Overexpression FAS cassette consist of *kasO*p*-FAS (accession code: WP_011030732). Overexpression RedD cassette consists of *kasO*p*-RedD (accession code: AIJ12849.1). *kasO*p* is a strong constitutive promoter. The integration plasmid was derived by

inserting the overexpression cassette into pSET152. Integration is mediated by *att*P site of the *Streptomyces* phage ΦC31. The completed plasmid was conjugated into *Streptomyces* sp A58051 from the Natural Organism Library collection (SIFBI, NPL) and genetically integrated mutants were screened and sequenced (Supplementary Figure). FAS mutants are labelled as A100020 and A100023 and RedD integration mutant is A100292 (Figure 2).

Fermentation and extraction

Wild type *Streptomyces* and edited mutants were cultured on ISP2 plates [malt extract broth 10 g/L, Bacto yeast extract 4 g/L, glucose 4 g/L, 20 g/L agar Bacto] at 30 ^{o}C for 5 days. Three agar plugs of 5 mm diameter from the culture plate was then used to inoculate into 4 x 250 mL Erlenmeyer flasks each containing 50 mL SV2 seed media [glucose 15 g/L, glycerol 15 g/L, soya peptone 15 g/L, calcium carbonate 1 g/L, pH 7.0] and incubated for 4 days at 30 ^{o}C, with shaking at 200 rpm.

A volume of 2.5 mL of the homogenized seed cultures were then inoculated into 250 mL Erlenmeyer flasks each containing 50 mL of ferment medium, CA07LB [glycerol 15 g/L, oatmeal 30 g/L, yeast extract 5 g/L, potassium dihydrogen phosphate 5 g/L, disodium hydrogen phosphate dodecahydrate 5 g/L, magnesium chloride hexahydrate 1 g/L] or CA10LB [soluble starch 20 g/L, soybean flour 15 g/L, potassium dihydrogen phosphate 3 g/L, disodium hydrogen phosphate dodecahydrate 2 g/L, magnesium sulphate heptahydrate 0.5 g/L, trace salt solution 1 mL/L (iron(II) heptahydrate 2 g/L, manganese chloride tetrahydrate 2 g/L, zinc sulfate heptahydrate 2 g/L, copper(II) sulfate pentahydrate 2 g/L, cobalt(II) chloride hydrate 2 g/L, pH 7.2]. All the cultures were fermented at 30 ^{o}C for 9 days shaking at 200 rpm with 50 mm throw.

At the end of the incubation periods, cultures were freeze dried. The lyophilized samples were extracted overnight with methanol. The extract mixture was passed through cellulose filter paper (Whatman Grade 4, 1004-185) and the filtrate was then dried using rotary evaporator.

Large scale fermentation and extraction

A100020 mutant was grown on Bennet's agar (Himedia, M694) plates at 28^{o}C for 5 days. Three agar plugs of 5mm diameter from the culture plate was then used to inoculate into 4 x 250 mL Erlenmeyer flasks each containing 50 mL of SV2 seed media and incubated for 4 days at 28^{o}C, with shaking at 200 rpm.

A volume of 2.5 mL of the homogenized seed cultures were then inoculated into 250 mL Erlenmeyer flasks each containing 50 mL of ferment medium, CA07LB. All the cultures were fermented at 28°C for 10 days shaking at 200 rpm with 50 mm throw.

For co-culture fermentation with *Rhodococcus* sp. T5718 (Natural Organism Library, A*STAR), seed cultures of inducer strain and A100020 (FAS mutant strain) was inoculated at volume ratio of 1:3 (0.8mL: 2.5mL) into 50 mL CA07LB, 200 rpm shaking at 30 °C for 7 days.

At the end of the incubation periods, cultures were harvested and freeze dried. The lyophilized cultures were extracted overnight with methanol. The extract mixture was passed through cellulose filter paper (Whatman Grade 4, 1004-185) and the filtrate was then dried using rotary evaporator.

Analysis between mutants

The extracts were analysed on an Agilent 1290 Infinity LC System coupled to an Agilent 6540 accurate-mass quadrupole time-of-flight (QTOF) mass spectrometer. 5 µL of extract was injected onto a Waters Acquity UPLC BEH C_{18} column, 2.1 x 50 mm, 1.7 µm. Mobile phases were water (A) and acetonitrile (B), both with 0.1 % formic acid. The analysis was performed at flow rate of 0.5 mL/min, under gradient elution of 2% B to 100% B in 8 min. Both MS and MS/MS data were acquired in positive and/or negative electrospray ionization (ESI) mode. The typical QTOF operating parameters were as follows: sheath gas nitrogen, 12 L/min at 325 °C; drying gas nitrogen flow, 12 L/min at 350 °C; nebulizer pressure, 50 psi; nozzle voltage, 1.5 kV; capillary voltage, 4 kV. Lock masses in positive ion mode: purine ion at *m/z* 121.0509 and HP-0921 ion at *m/z* 922.0098.

Data analysis

Compounds were detected based on peaks identified in base peak chromatograms (BPC) from LC-MS data. ESI (+ve/-ve) mass spectral data at the apex of detected peaks were sampled and corrected for background noise by deducting the nearest baseline mass spectra. An in-house algorithm was developed and employed to detect and do background correction on peaks from BPC data. Detected peaks were then characterized with 4 major parameters: (1) base peak m/z, (2) retention time, (3) molecular ion peak and (4) number of m/z peaks exceeding 50,000 abundance. Peaks were considered to be identical if they fulfilled 2 criteria: (1) base peak m/z within 0.02 m/z and (2) retention time within 0.2 min.

Compound isolation

The dried extracts obtained from 1 L of fermentation were combined and partitioned with 240 mL CH_2Cl_2/MeOH/H_2O in a ratio of 1:1:1. The aqueous MeOH layer was washed with 80 mL CH_2Cl_2 (x2) and dried under reduce pressure via Buchi rotary evaporator. The dried crude extract (6 g) was soaked and resuspended with 20 mL MeOH, sonicated for 5 min and centrifuged to separate the insoluble from the soluble. The supernatants were transferred to 50 mL round bottom flaks and dried using Buchi rotary evaporator. The dried enriched samples (427 mg) were dissolved in 2.5 mL MeOH, centrifuged and the supernatants were then subjected to C_{18} reversed-phase preparative HPLC purification by the following condition (solvent A: H_2O + 0.1% HCOOH, solvent B: MeCN + 0.1% HCOOH; flow rate: 30 mL/min, gradient conditions: 90:10 isocratic for 5 min; followed by 10% to 45% of solvent B over 15 min, 45% to 75% of solvent B over 38 min, 75% to 100% of solvent B over 2 min, and finally isocratic at 100% of solvent B for 12 min) to give 3.5 mg of Compound **1** and 2.3 mg of Compound **2**.

General Chemistry Experimental Procedures

JASCO P-2000 digital polarimeter was used for specific rotations measurement. NMR spectra were collected using Bruker DRX-400 NMR spectrometer with Cryoprobe. 5-mm BBI (^{1}H, G-COSY, multiplicity-edited G-HSQC, and G-HMBC spectra) or BBO (^{13}C spectra) probe heads equipped with z-gradients. The ^{1}H and ^{13}C NMR chemical shifts were referenced to the residual solvent peaks for MeOH-d_4 at δ_H 3.31 and δ_C 49.0 ppm. Agilent 1260 Infinity Preparative-Scale LC/MS Purification System and Agilent 6130B single quadrupole mass spectrometer with Agilent 5 Prep-C_{18} column (100 x 30 mm, 5 µm, 100 Å) was used to perform preparative HPLC experiment. Agilent UHPLC 1290 Infinity coupled to Agilent 6540 accurate-mass quadrupole time-of-flight (QTOF) mass spectrometer equipped with a splitter and an ESI source was used to conduct HPLC-LCMS. For over 8.6 min, under standard gradient condition of 98% water with 0.1% formic acid to 100% acetonitrile with 0.1% formic acid, the analysis was performed with a Acquity UPLC BEH C_{18} 2.1 x 50 mm, 1.7 µm column at flow rate of 0.5 mL/min. All solvents for chromatography, specific rotations, and UV were Fisher Chemical HPLC or LCMS grade.

Chemical structural data

Compound **1.** Brown amorphous powders; $[\alpha]_D^{23}$+50.8 (c 0.5, MeOH); UV (MeCN/H_2O) λ_{max} (%) 220 (100%), 285 (77%) nm; (–)-HRESIMS: *m/z* 362.1968 $[M-H]^-$ (calcd for $C_{20}H_{28}O_5N$, 362.1973); ^{1}H and ^{13}C NMR data, see **Table 1**.

Compound **2**: Brown amorphous powders; $[\alpha]_D^{23}$+28 (c 0.2, MeOH); UV (MeCN/H_2O) λmax (%) 222 (100%), 286 (83%) nm; (–)-HRESIMS: *m/z* 376.2132 $[M-H]^-$ (calcd for $C_{20}H_{30}O_5N$, 376.2129); ^{1}H and ^{13}C NMR data, see **Table 1**.

Biological assays

The minimum inhibition concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of the isolated compounds against a panel of microbial pathogens were determined using the microbroth dilution method. This is done according to the Clinical Laboratory Standards Institute (CLSI) guidelines, with slight modifications. Antibacterial assays were carried out with *Acinetobacter baumannii* (ATCC® 19606™), *Klebsiella aerogenes* (ATCC® 13048™), *Pseudomonas aeruginosa* (ATCC® 9027™) and *Staphylococcus aureus* Rosenbach (ATCC® 25923™) at 5×10^5 cells/mL. Antifungal assay was performed with *Aspergillus fumigatus* (ATCC® 46645™) at 2.5×10^4 spores/mL. Standard inhibitors Gentamicin (Gibco) and Amphotericin (Sigma-Aldrich) were used as the assay controls respectively for the antibacterial and antifungal assay testing. For MIC determination, the bacterial cells were incubated together with the isolated compounds at 37 °C for 24 hours; and at 25 °C for 72 hours for the fungal spores. Optical density at 600 nm was then measured using a microplate reader (Tecan Infinite® M1000 Pro) to assess the inhibitory effect of the compounds on microbial growth. Following that, MBC/MFC was then evaluated by transferring 5 µL of the treated culture into fresh media in 384-well microtitre plates. The plates were incubated under the same conditions, and MBC/MFC was determined by measuring the optical density at 600 nm. All assays were performed in triplicates to ensure reproducibility.

For mammalian cell cytotoxicity assay, A549 human lung carcinoma cells (ATCC® CCL-185™) were seeded at 3.3×10^4 cells/mL in a 384-well microplate. The cells were treated with the compounds for 72 hours and incubated at 37 °C in the presence of 5% CO_2. Standard inhibitor Puromycin (Sigma-Aldrich) was used as the assay control for cytotoxicity testing. To assess the cytotoxic effect of the compounds on the cells, the microplates were incubated with PrestoBlue™ cell viability reagent (ThermoFisher Scientific, USA) for 2 hours, followed by fluorescence reading at excitation 560 nm and emission 590 nm. The analysis of antimicrobial and cytotoxicity activity for their IC_{50} values were carried out with the GraphPad Prism program (GraphPad Software, CA).

It will be appreciated that many further modifications and permutations of various aspects of the described embodiments are possible. Accordingly, the described aspects are intended to embrace all such alterations, modifications, and variations that fall within the spirit and scope of the appended claims.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word “comprise”, and variations such as “comprises” and “comprising”,

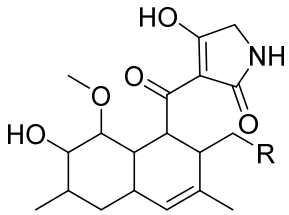
will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Throughout this specification and the claims which follow, unless the context requires otherwise, the phrase "consisting essentially of", and variations such as "consists essentially of" will be understood to indicate that the recited element(s) is/are essential i.e. necessary elements of the invention. The phrase allows for the presence of other non-recited elements which do not materially affect the characteristics of the invention but excludes additional unspecified elements which would affect the basic and novel characteristics of the method defined.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Claims

1. A method of biosynthesizing an antimicrobial compound of Formula (I):



(I)

wherein R is optionally substituted alkyl;
the method comprising:
a) culturing a microbial cell that is engineered to overexpress fatty-acyl CoA synthase (FAS) or RedD; and
b) isolating the antimicrobial compound of Formula (I) that is produced by the microbial cell;
wherein the biosynthesis of antimicrobial compound of Formula (I) is upregulated by at least about 2-fold compared to a tetramic acid analogue derived from a wild type microbial cell.

2. The method according to claim 1, wherein the microbial cell is engineered by introducing a FAS or a RedD expression cassette into the genome of the microbial cell; or
wherein the FAS or RedD expression cassette is integrated into the genome of the microbial cell via an integration plasmid.

3. The method according to claim 1 or 2, wherein the microbial cell is a bacterium.

4. The method according to any one of claims 1 to 3, further comprising a step before the culturing step (step a) of inoculating the engineered microbial cell in a seed media.

55. The method according to claim 4, wherein the engineered microbial cell is inoculated for at least 4 days.

6. The method according to any one of claims 1 to 5, wherein the engineered microbial cell is cultured in a media comprising glycerol and/or starch.

7. The method according to any one of claims 1 to 6, wherein the engineered microbial cell is cultured for at least 7 days.

8. The method according to any one of claims 1 to 7, wherein the engineered microbial cell is co-cultured with inducer strain.

9. The method according to claim 7 or 8, wherein a volume ratio of inducer strain to engineered microbial cell is about 1:2 to about 1:5.

10. The method according to any one of claims 1 to 9, further comprising a step before the isolating step (step b) of freeze drying the culture.

11. The method according to any one of claims 1 to 10, wherein the antimicrobial compound of Formula (I) is isolated from the microbial cell culture medium.

12. The method according to any one of claims 1 to 11, wherein the antimicrobial compound of Formula (I) is characterised by at least one of the following:

a) a minimum inhibitory concentration (MIC_{50}) against Gram-positive bacteria of less than about 20 μM;

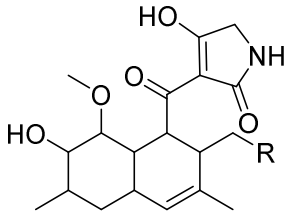
b) a minimum bactericidal concentrations (MBC_{50}) against Gram-positive bacteria of less than about 80 μM;

c) a minimum inhibitory concentration (MIC_{50}) against Gram-negative bacteria of less than about 20 μM; and

d) a IC_{50} against cancer cells of less than about 50 μM.

13. The method according to any one of claims 1 to 12, wherein the Gram-negative bacteria is *A. baumannii.*

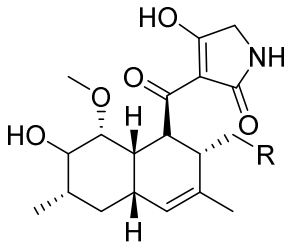
14. An antimicrobial compound of Formula (I):



(I)

wherein R is optionally substituted alkyl.

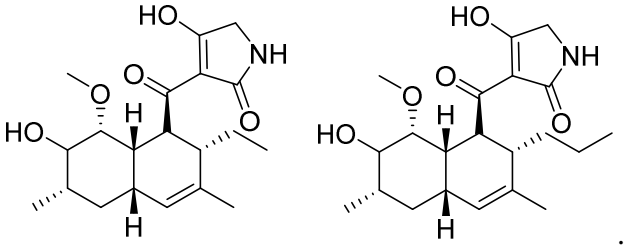
15. The antimicrobial compound according to claim 14, wherein the antimicrobial compound of Formula (I) is a compound of Formula (Ia):



(Ia)

wherein R is optionally substituted alkyl.

16. The antimicrobial compound according to claim 14 or 15, selected from:



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17. A pharmaceutical composition comprising a compound of Formula (I) according to any one of claims 14 to 16 or a pharmaceutically acceptable salt, solvate or prodrug thereof.

18. Use of an antimicrobial compound of Formula (I) according to any one of claims 14 to 16 or a pharmaceutically acceptable salt, solvate or prodrug thereof in the manufacture of a medicament for treating a microbial disease or condition.

19. The use according to claim 18, wherein the microbial disease or condition is selected from a disease or condition caused by *S. aureus* and/or A*. baumannii*.

20. The use according to claim 18 or 19, wherein the microbial disease or condition is selected from skin and soft tissue infection, blood infection, pneumonia, bone and joint infection, urinary tract infection, pneumonia, or an infection caused by an open wound.