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[Continued on next page]

(54) Title: ANTIBACTERIAL COMPOUNDS AGAINST DRUG RESISTANT BACTERIA

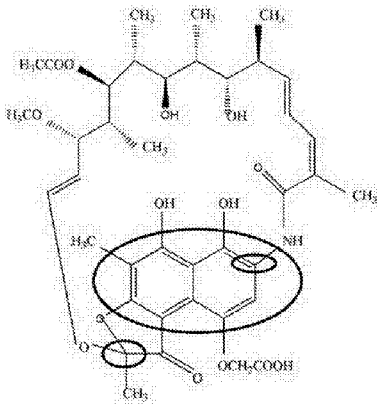

Figure 1: The chemical structure of rifamycin B. The ansa chain joins the naphthoquinone moiety at C-2 and C-12 (circled in black). The naphthoquinone moiety (circled in black) imparts reddish brown colour to the compound.

(57) Abstract: The invention relates to the production of rifamycin analogs against drug resistant bacteria. The invention also relates to antibacterial compounds against drug resistant mycobacteria. The invention also provides for pharmaceutical composition comprising antibacterial compound against drug resistant bacteria, particularly mycobacteria. The invention also provides for use of antibacterial compounds for the treatment of disease caused by bacteria and in particular mycobacteria.

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ANTIBACTERIAL COMPOUNDS AGAINST DRUG RESISTANT BACTERIA

FIELD OF INVENTION

The present invention relates to the production of rifamycin analogsagainst drug resistant bacteria. The present invention further also relates to antibacterial compounds against drug resistant mycobacteria. The present invention also provides for pharmaceutical composition comprising antibacterial compound against drug resistant bacteria, particularly mycobacteria. The present invention also provides for use of antibacterial compounds for the treatment of disease caused by bacteria and in particular mycobacteria.

BACKGROUND OF THE INVENTION

Amycolatopsis mediterranei S699 (ATCC 13685) is an actinobacterium that produces an important antibiotic rifamycin B **(Figure 1)**. Semisynthetic derivatives of rifamycin B are in use against *Mycobacterium tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis (TB) and leprosy, respectively. These derivatives are also used against a variety of other organisms including AIDS related mycobacteria. Rifamycin B and rifamycins in general belong to the ansamycin class of antibiotics and are characterized by a naphthalene moiety spanned by an aliphatic chain like the handle of a basket **(Figure 1)**. This aromatic core imparts reddish brown colour to the rifamycin molecule. The carbon skeleton in rifamycin B of *A. mediterranei* S699 is built from two acetate and eight propionate units and a starter unit 3-amino-5-hydroxybenzoic acid (AHBA). Rifamycin B is a native and stable molecule and acts as the starting material for the synthesis of semisynthetic derivatives including rifamycin S , rifamycin SV, rifampicin, rifabutin, rifapentine and rifaximin (that are in clinical use). In addition, strain S699 has been subjected to classical strain improvement program since 1960 and successors of this strain are in use for production of rifamycin B. Currently, improved industrial strains produce around 15 g/l to 24 g/l of rifamycin B.

The semisynthetic derivatives of rifamycin B, as mentioned earlier, were developed to improve the pharmacokinetics of the molecule **(Figure 2)**. Rifampicin (3-[4-methylpiperazinylimino-methyl] rifamycin SV) was first approved for clinical use in Italy, in 1968. It gained approval by Food and

Drug Administration in 1971, in USA [Sensi,P.*et al.* (1983). *Farmac. Ed. Sc.* Vol. 14, p.146-147]. It was the first semisynthetic derivative of rifamycin B and has been extensively used for the cure of tuberculosis. It binds to the RNA polymerase of the *M. tuberculosis* and blocks the extension of RNA chain.

Another derivative of rifamycin B is Rifabutin (4-N-isobutylspiropiperidyl rifamycin S). It is marketed under the name Mycobutin. It is characterized as being effective against a few strains resistant to rifampicin and has a high activity against *Mycobacterium avium* complex associated with AIDS.

Rifamixin (4-deosy-3'-bromopyrido-[1', 2'-1, 2] imidiazo [5, 4-c]-rifamycin SV), rifamycin B derivative is virtually unabsorbed in the intestine post oral administration. This property makes it favorable for treatment of local conditions within gastrointestinal pathogens.

Rifapentine (3{[(4-cyclopentyl-1-piperazinyl) imino] methyl} rifamycin SV) possess activity against mycobacteria and a pharmacokinetic profile which allows long lasting action. It is marketed under the name Prifith by Sanofi Aventis. It was approved for treatment of tuberculosis by Food and Drug administration in 1998.

A. mediterranei, the producer of rifamycin, has an interesting history. It was isolated from a soil sample from a pine arboretum at an altitude of 200 m, about 50 m off the shore of St. Raphael, France. Due to morphological and biochemical similarities, this isolate was classified as *Streptomyces mediterranei* [Margalith, P. and Beretta, G. (1960). *Mycopathol. Mycol. Appl.* Vol.13, p.321-330]. Later it was reclassified as *Amycolatopsis mediterranei* [Lechevalier *et al.* (1986). *Int. J. Sys. Bacteriol.*Vol. 36, p. 29-37](reference needed). Lal and co-workers developed a series of cloning vectors (pRL series) which can be used for transformation and cloning of several strains of *A. mediterranei*[Lal, R., US5985560A(1999); Lal, R.*et al.* (1991). *Appl. Environ. Microbiol.*Vol.57, p.665-671; Dhingra, G.*et al.* (2003).*Ind.Microbiol. Biotecnol.*Vol.30, p.195-204]

It is pertinent to mention that the synthesis of erythromycin (ery), rapamycin (rap) and rifamycin (rif) is mediated through modular polyketide synthase (multienzyme complexes) also known as type

I PKSs which build these macrocyclic polyketides by condensation of acetate and propionate units. The type I PKSs have a collinear architecture and are composed of modules, each with catalytic domains that determine the order of substrate selection for basic chain assembly and subsequent modification of β–keto esters (polyketides). A type I PKS module generally consists of ketosynthase (KS), acyl transferase (AT) and acyl carrier protein (ACP) domains which form the minimal PKS. Optionally the domains ketoreductase (KR), dehydratase (DH) or enoyl reductase (ER) (also known as reductive domains) may be present to bring about modifications in the ketoester chain. The starter unit (acetyl CoA) is loaded onto the ACP, catalysed by the AT domain of the starter module. The extending chain is handed over from the ACP of the previous module to the KS of the current module and is catalyzed by the KS domain. AT domain selects the type of extender unit to be added to the growing polyketide chain, KS catalyses the condensation reaction and ACP tethers the growing polyketide chain between successive condensation and accepts extender unit from AT in preparation for the next condensation reaction. The growing polyketide is modified by the reductive loop. The KR domain reduces the β–keto group to a hydroxyl group. The DH domain eliminates a molecule of water resulting in anα–β double bond and the ER domain converts the α– β double bond to a saturated bond. The carbon skeleton is then released from the PKS by the hydrolytic action of a TE domain **(Figure 3)**. While the presence of ACP, AT and KS is essential in a module the other domains such as KR, DH, ER are optional and may or may not present in a module.

After the discovery of *ery*PKS and *rap*PKS gene cluster, the entire 90 kbp *rif*PKS gene cluster responsible for the biosynthesis of rifamycin B was cloned from *A. meditteranei* S699 [August, P. R.*et al.*(1998). *Chem Biol.* Vol. 5, p. 69-71.] The biosynthetic gene cluster is divided into four regions. Region II (52kb) is the largest region and encodes the modular PKSs. This region is divided into five ORFs- *rif*A, *rif*B, *rif*C, *rif*D and *rif*E. The ORFs comprises of 10 modules, which are collinearly arranged in accordance with their function in biosynthesis, and catalyze ten successive rounds of polyketide chain elongation to build an undecaketide **(Figure 4)**. The gene *rif*A consists of modules 1-3, *rif*B of modules 4-6, *rif*C of module 7, *rif*D of module 8 and *rif*E of modules 9-10. The domain order in the modules is KS-ketosynthase, AT-acyl transferase (optional reductive domains-DH-dehydratase and KR-ketoreductase) and ACP-acyl carrier protein **(Figure 5)**. The domain ER is absent in *rif*PKS. The *rif*A gene is preceeded by a loading unit, which activates the starter unit to initiate the polyketide chain formation. There are two classes of AT domains in *rif*PKS. The AT

domains found in modules 2 and 9 catalyze the incorporation of acetate extender units. The other eight modules have AT domains which catalyze the incorporation of propionate extender units into the growing polyketide chain. Another gene, *rif*F, present immediately downstream to *rif*E, is translationally coupled to it. The role of RifF is to cyclise and simultaneously release polyketide chain from the ACP domain of module 10 [Stratmann, A.*et al.* (1999).*Microbiol.*Vol.145, p.3356-3375].

The formation of naphthalene moiety occurs between the third and fourth chain elongation step and is not a post PKS modification.This step was crucial in designing the swapping strategy followed in this invention.

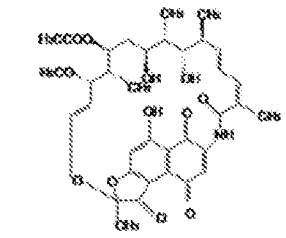
The use of rifamycin derivatives especially of rifampicin in the clinics resulted in a considerable fall of mortality rate due to TB. However a combination of poor compliance and poor medical supervision has resulted in the emergence of multiple drug resistance (MDR) strains of *Mycobacterium tuberculosis*. In accordance to the 2011 WHO report - Tuberculosis Control in the South East Asia Region, India documented 2.3% cases of MDR-TB among the new TB cases reported. The structural complexity of rifamycin B, as mentioned earlier, limits the use of chemical tools only to C-3 and C-4 of the molecule. This reduces the number of permutations and combinations of the altered molecules that can be generated through chemical synthesis. There is a compelling need to produce more analogs of rifamycin for effective and economical cure for MDR-TB.

The semi-synthetic derivatives of rifamycin B, such as rifampicin and rifabutin, have been widely used in the cure of tuberculosis (*M. tuberculosis*), leprosy (*M. leprae*) and AIDS related mycobacterial infections. Rifampicin was first introduced into the market in 1968. Since then, it has been widely used to cure tuberculosis. However, a combination of poor compliance and poor medical supervision has resulted in the emergence of multiple drug resistant (MDR) strains of *M. tuberculosis*. The pharmaceutical companies and the medical world are in the lookout for economical drugs which would be effective against MDR-TB. The complexity of the chemical structure of rifamycin B allows chemical alteration as mentioned above only at the C-3 and C-4 of the aromatic core by chemical modifications that have been fully utilized.

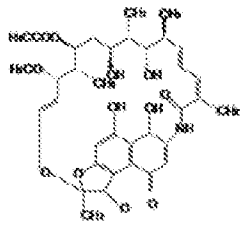
Another option is combinatorial biosynthesis, which involves interchanging/deleting/adding the modules/domains to the existing PKS gene cluster within the rifamycin producing organisms to create unnatural set of genes which may produce molecules of interest. The polyketide synthase gene clusters which have collinear arrangement can be shuffled in this manner and has been demonstrated to produce erythromycin analogs. Although extensive work has been done in this direction on erythromycin molecules (literature cited in previous section), no reports were available for the manipulation of *rif*PKS. Therefore there is need in the art to develop molecules of interest with better and higher degree to effect in treating TB and also to develop such new molecules to which the TB bacterial is not resistant.

SUMMARY OF THE INVENTION

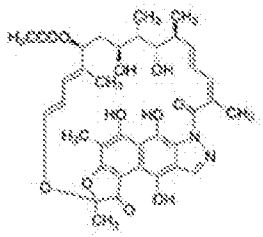
Accordingly, the main embodiment of the present invention relates to antibacterial compounds and/or salts thereof having following chemical structure:

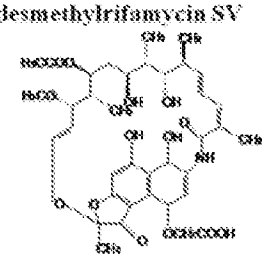


24- desmethylrifamycin S



24- desmethylrifamycin SV





24- desmethylrifamycin B

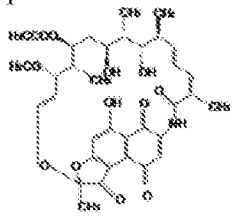
24-desemthylrifampicin and their salts thereof.

Another embodiment of the present invention relates to antibacterial compounds of the present invention which are useful against infection or a disease caused by bacteria.

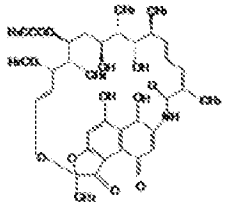
Another embodiment of the present invention relates to antibacterial compounds of the present invention which are useful against infection or a disease caused by *Mycobacterium* species.

Another embodiment of the present invention relates to antibacterial compounds of the present invention which are useful against infection or a disease caused by *Mycobacterium* species, where *Mycobacterium* species selected are *Mycobacterium tuberculosis* and MDR strains of *M. tuberculosis*.

Yet another embodiment of the present invention relates to a pharmaceutical composition comprising antibacterial compounds and/or salts thereof having following chemical structure:

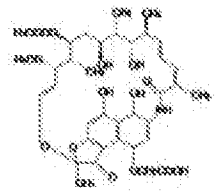


24- desmethylrifamycin S

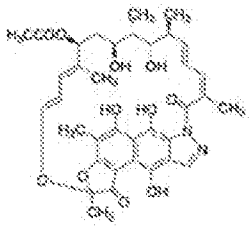


24- desmethylrifamycin SV

24-desmethylrifampicin

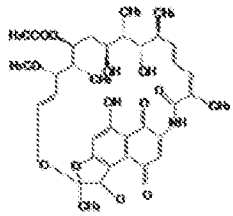


24- desmethylrifamycin B

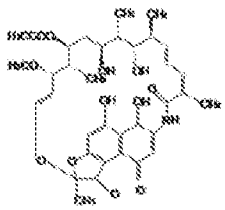


along with their pharmaceutical acceptable carriers.

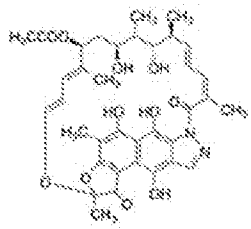
Yet another embodiment of the present invention relates to a method of treatment comprising administering to a patient an antibacterial compounds and/or salts thereof having following structure:



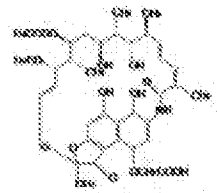
24- desmethylrifamycin S



24- desmethylrifamycin SV

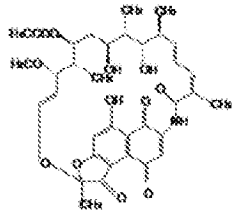


24-desmethylrifampicin

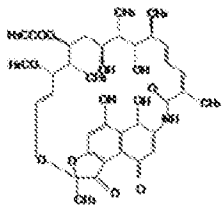


24- desmethylrifamycin R

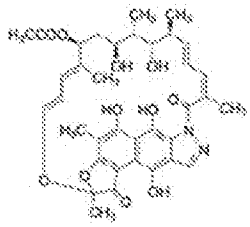
Yet another embodiment of the present invention relates to use of antibacterial compounds and/or salts thereof having following chemical structure for treatment of disease or an infection.



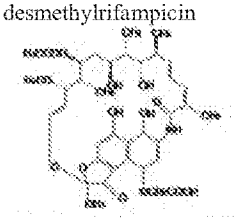
24- desmethylrifamycin S



24- desmethylrifamycin SV



24-desmethylrifampicin



24- desmethylrifamycin B

for the preparation of a medicament.

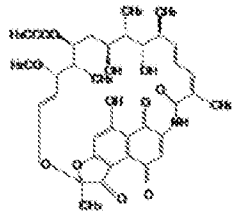
Another embodiment of the present invention relates to use of a medicament comprising antibacterial compounds of the present invention for the treatment of a disease or an infection.

Yet another embodiment of the present invention relates to use of a medicament comprising antibacterial compounds of the present invention for the treatment of a diseases or an infection caused by bacteria.

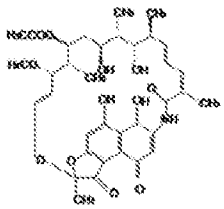
Another embodiment of the present invention relates to novel strain of *Amycolatopsis mediterrranei* S699#34 comprising a gene *rap*AT2 region.

Yet another embodiment of the present invention relates to novel strain of *Amycolatopsis mediterrranei* S699#34 wherein *rif*AT6 region is swapped with *rap*AT2 region.

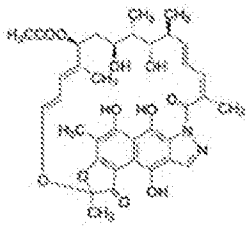
Another embodiment of the present invention relates to novel strain of *Amycolatopsis mediterrranei* S699#34 capable of producing the analogs and derivatives of rifamycin B comprising following chemical structure:



24- desmethylrifamycin S



24- desmethylrifamycin SV



24-desmethylrifampicin

24- desmethylrifamycin B

Another embodiment of the present invention relates a method of preparing novel strain of *Amycolatopsis mediterrranei* S699 comprising *rap*AT2 region in *rif*PKS, said method comprising the steps of :

(a) isolating *rif*AT6 region of *rif*PKS from *Amycolatopsis mediterrranei* S699 and *rap*AT2 region of *Streptomyces hygroscopicus* (Acc. No. DSM-41524);
(b) preparing a vector constructs by swapping *rif*AT6 region of *rif*PKS with *rap*AT2 region of *Streptomyces hygroscopicus* (Acc. No. DSM-41524);
(c) inserting the vector constructs of step (b) in an *Amycolatopsis mediterrranei* S699; and
(d) obtaining a novel strain *Amycolatopsis mediterrranei* S699#34 comprising a *rap*AT2 region of *rif*PKS.

Another embodiment of the present invention relates to a method of preparing novel antibacterial compounds said method comprising the steps of:

(a) culturing cells of *Amycolatopsis mediterrranei* S699#34 comprising *rap*AT2 region in the *rif*PKS region; and
(b) harvesting the cells of *Amycolatopsis mediterrranei* S699#34 to obtain the crude fractions of the products;
(c) purifying the crude products by HPLC;
(d) obtaining antibacterial compounds 24-desmethyrifamycin B and 24-desmethylrifamcin SV.

Another embodiment of the present invention relates to a method of preparing the 24-desmethyrifamycin S derivative of 24-desmethyrifamycin B, said method comprising the steps of :

(a) reacting 24-desmethyrifamycin B in presence of reagents selected from Copper chloride;
(b) carrying the reaction of step (a) overnight at room temperature;
(c) obtaining 24-desmethyrifamycin S

A method of preparing the 24-desmethyrifampicin derivative of 24-desmethyrifamycin B, said method comprising the steps of:

(a) reacting 24-desmethyrifamycin B in presence of Dimethylformamide (DMF) and acetic acid;
(b) adding paraformaldehyde and 1,3,5-trimethyl-hexhydro-1,3,5-triazine to the mixture of step (a);
(c) obtaining 3-methyl-1,3-oxazino (5,6-c)-24-demethylrifamycin'
(d) reacting the compound of step (c) with 1-amino-4-emthyl-piperazine; and
(e) obtaining 24-desmethyrifampicin.

Another embodiment of the present invention relates to a recombinant nucleotide SEQ ID No.1.

Another embodiment of the present invention relates to a bacterial strains comprising nucleotide SEQ ID NO.1.

Another embodiment of the present invention relates to bacterial strain/s as described in the present invention comprising nucleotide SEQ ID NO.1

Another embodiment of the present invention relates to bacterial strain as described in the present invention, wherein bacterial strain is capable of producing rifamycin analogues.

Another embodiment of the present invention relates to vector constructs comprising recombinant nucleotide sequence ID No.1.

Yet another embodiment of the present invention relates to vector constructs, wherein vector constructs are pAT6E and pAT6F.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood when the following detailed description is read with reference to the accompanying drawings in which like characters represent like parts throughout the drawings, wherein:

Figure 1: Chemical structures of Rifamycin B.

Figure 2: Semi-Synthetic Derivatives of rifamycin B.

Figure 3: Schematic representation of processive mechanism of type 1 PKS.

Figure 4: Synthesis of the hypothetical intermediate by rifamycin PKS of region II

Figure 5: Organization of the enzyme domain in *rif* PKS gene cluster.

Figure 6: Strategy for constructing functional cassette by swapping *rif*AT6 with *rap*AT2.

Figure 7: Schematic representation of the strategy to showswapping *rif*AT6 with *rap*AT2.

Figure 8: Phenotypic appearance of cultures showing Single Crossover clones.

Figure 9: Gel Electrophoresis profile for Single Crossover Clones.

Figure 10: Phenotypic appearance of cultures showing Double Crossover Clones.

Figure 11: Gel Electrophoresis profile of Double Crossover Clones.

Figure 12: The LC-ESI-MS profile of analog desmethylrifamycin B extracted from DCO.

Figure 13: NMR of rifamycin B and its analog.

Figure 14: Structures of 24-desmethylrifamycin B, 24-desmethylrifamycin S and 24-desmethylrifamycin SV

Figure 15: Crystal structure of 24-desmehtylrifamycin S and drug interaction with RNA polymerase A.

Figure 16:ESI-MS spectra of (a) Rifamycin S; (b) 24-Desmethylrifamycin S; (c) Rifampicin; (d) 24-desmethylrifampicin

Figure 17:NMR spectra of (a) 24-desrifamethylrifampicin; (b) chemically synthesized rifampicin and (c) commercially available rifampicin

Figure 18: Comparative MS/MS analysisof (a) 240desmethylrifampicin and (b) Rifampicin.

Figure 19: Characteristic MS fragments of rifampicin and 24-desmethylrifampicin in ESI negative ion mode.

Figure 20: Antibacterial assay of new rifamycin analogs against various bacterial species.

Figure 21: Antibacterial assay of activity of rifampicin, rifamycin S, 24-desmethylrifampicin, 24-desmethylrifamycin S.

Figure 22: Nucleotide sequence ID No.1

DETAILED DESCRIPTION

While the invention is susceptible to various modifications and/or alternative processes and/or compositions, specific embodiment thereof has been shown by way of example in the drawings/figures and tables and will be described in detail below. It should be understood, however that it is not intended to limit the invention to the particular processes and/or compositions disclosed, but on the contrary, the invention is to cover all modifications, equivalents, and alternative falling within the spirit and the scope of the invention as defined by the appended claims.

The graphs, tables, formulas and protocols have been represented where appropriate by conventional representations in the drawings, showing only those specific details that are pertinent to understanding the embodiments of the present invention so as not to obscure the disclosure with details that will be readily apparent to those of ordinary skill in the art having benefit of the description herein.

The following description is of exemplary embodiments only and is not intended to limit the scope, applicability or configuration of the invention in any way. Rather, the following description provides a convenient illustration for implementing exemplary embodiments of the invention. Various changes to the described embodiments may be made in the function and arrangement of the elements described without departing from the scope of the invention.

The terms "comprises", "comprising", or any other variations thereof, are intended to cover a non-exclusive inclusion, such that one or more processes or composition/s or systems or methods proceeded by "comprises... a" does not, without more constraints, preclude the existence of other processes, sub-processes, composition, sub-compositions, minor or major compositions or other elements or other structures or additional processes or compositions or additional elements or additional features or additional characteristics or additional attributes.

Definitions:

For the purposes of this invention, the following terms will have the meaning as specified therein:

As used herein, the terms *"Combinatorial approach or combinatorial biosynthesis"* when used in the context of the present invention refers togenetic manipulation of *rif*PKS gene cluster by swapping of *rif*AT6 domain of *rif*PKS gene cluster *A. mediterranei* S699 with *rap*AT2 domain of *rap*PKS gene cluster of *Streptomyces hygroscopicus*.

As used herein, the terms *"Vectors/Cloning vectors"* when used in the context of the present invention refers to development of series of vectors pAT6A, pAT6B, pAT6C, pAT6D, pAT6E, and pAT6F. pAT6F is a non-replicative plasmid which was transformed into *A. mediterranei* S699.

As used herein, the terms *"Derivatives"* when used in the context of the present invention refers toderivatives obtained or prepared from 24-desmethylrifampicin B. A few exemplified derivatives are 24-desmethylrifampicin,24-desmethylrifamycin S and 24-desmethylrifamycin SV.

As used herein, the terms *"Analogues"* when used in the context of the present invention refers toanalogs of rifamycin B, which are prepared by swapping*rif*AT6 domain with *rap*AT2 domain or contains *rif*AT6 domain replaced by *rap*AT2 domain. One such exemplary analog is 24-desmethylrifamycin B.

As used herein, the terms *"Mutant/s or mutant strain/s"* when used in the context of the present invention refers tostrain/s*A. mediterranei*in which *rif*AT6 domain is replaced with *rap*AT2 domain of the PKS-1 system and that are capable of producing analogs and derivatives of rifamycin B. For example: *Amycolatopsis mediterranei* #34 which produces analog such as 24-desmethylrifamycin B, etc.

As used herein, the term *"Antibacterial compound/s"* when used in the context of the present invention refers to compounds which are capable of inhibiting growth of bacteria or the infection

caused by bacterial or the disease caused by bacteria or disease condition caused by bacterial belonging to category of Actinobacteria or any gram negative bacteria or gram positive bacteria. In particular the antibacterial compounds used in the context of the present invention referto antibacterial compounds which are effective in inhibiting the growth of bacteria belonging to *Mycobacerium* species. Further theantibacterial compounds used in the context of the present invention referto those antibacterial compounds which are effective in inhibiting any disease or or any infection or disease condition caused by bacteria belonging to *Mycobacerium* species.

As used herein, the term *"Multi-Drug Resistance/MDR"* when used in the context of the present invention refers to a condition enabling disease-causing bacteriato resist distinct antimicrobials or antibacterial compounds i.e. chemicals of a wide variety of structure and function targeted at eradicating the bacteria. In particular MDR in the context of the present invention refers to conditions enabling disease-causing*Mycobacerium* species to resist distinct antimicrobials or antibacterial compounds wherein the such antimicrobials or antibacterial compounds are commonly used antibiotic drugs or commercially available antimicrobials or antibacterial compounds.

As used herein, the term *"salts thereof"* when used in the context of the present invention refers any salt, esters, polymorphs, pure forms, isomers, mixtures of isomers, complexes and any other derivatives or analogs of 24-desmethyrifamycin B, 24-desmethyrifamycin S, 24-desmethyrifamycin SV and 24-desmethyrifampicin. More particulars salts thereof such as salt, esters, polymorphs, pure forms, isomers, mixtures of isomers, complexes and any other derivatives should at least comprise of 24-desmethyl- form or structure i.e. loss of methyl group at the position 24 in analogs, derivatives, salt, esters, polymorphs, pure forms, isomers, mixtures of isomers, complexes of 24-desmethyrifamycin B, 24-desmethyrifamycin S, 24-desmethyrifamycin SV and 24-desmethyrifampicin.

The present invention relates to antibacterial compounds which could be effective against bacterial disease or infection. More particularly the present invention relates to the use of antibacterial compounds which can be effectively used against the infection or disease caused by bacteria group

of gram positive bacteria falling under the category of Actinobacteria. The antibacterial compounds of the present invention are useful against multi-drug resistant bacteria. The antibacterial compounds of the present invention are useful against such as *Mycobacterium* species *Staphylococcus* speciesor strains, *Bacillus* species or strains, *Pseudomonas* species or strains and *E.Coli* strains

Further the present invention relates antibacterial compounds which are useful against the infection or disease caused by *Mycobacterium* species. In particular these antibacterial compounds are effective against *Mycobacterium tuberculosis*, *Mycobacterium lepraeand Mycobacterium smegmatis*. Another aspect of the present invention provides for antibacterial compounds which are useful against multiple drug resistant strains of Mycobacteria species.

Rifamycin was isolated as a congeners of rifamycin complex [SensiP.*et al.* (1983). *Farmac. Ed. Sc.* Vol. 14, p. 146-147] and the*rif*PKS was completely sequenced in 1998 [August, P. R.*et al.* (1998)*Chem. Biol.*Vol. **5**, p. 69-79]. Since then a number of investigations have been carried out to elucidate the functions of the gene cluster and structure and action of rifamycin B. However, unique nature of *rif* PKS in the production of rifamycin B hindered the progress in the production of rifamycin B analogs for long. Prior to this it was difficult to genetically manipulate *rif*PKS of *A. mediterranei* as efficient genetic techniques and cloning vectors were not available for *A. mediterranei.* Since one of the inventors of the present invention has already developed a series of cloning vectors (pRL series) which can be used for transformation and cloning of several strains of *A. mediterranei*[Lal, R., US 5985560A(1999); Lal, R.*et al.* (1991). *Appl. Environ. Microbiol.*Vol.57, p.665-671; Dhingra, G.*et al.* (2003).*Ind.Microbiol. Biotecnol.*Vol.30, p.195-204] and the electrotransformation method so developed has been used to manipulate *rif*PKS gene cluster for production of rifamycin analogs. The manipulations of *rif*PKS was also based on the fact that themodular nature of polyketide synthases of erythromycin (*ery*PKS) and rapamycin (*rap*PKS) that have generated a considerable interest in the past for the production of novel bioengineered polyketides. While *ery*PKS and *rap*PKS were cloned earlier, the cloning and characterizationof the rifamycin biosynthetic gene cluster or *rif*PKS in 1998 [August, P.R.*et al.* (1998).*Chem. Biol.*Vol. **5**, p.69-79] opened up the possibilities of manipulating *rif*PKS gene cluster for the production of rifamycin analogs by using similar approaches that have been used for *ery*PKS. In this present

invention combinatorial approach was used in which rifamycinpolyketide synthase (*rif*PKS) cluster of *A.meditteranei* S699 has been genetically manipulated to produce analogs of rifamycin B.

Although many derivatives of rifamycin have been synthesized by chemical methods in the past forty years, only a handful of rifamycin derivatives are currently used for curing tuberculosis. Most rifamycin derivatives, including those used in the clinics, are chemically modified at the C-3 and/or the C-4 positions of the naphthalene moiety. Chemical modifications of other parts of the compound appeared to be difficult due to the complexity of the molecule. In addition, X-ray crystallography studies of Thermusaquaticus RNAP complexed with rifampicin revealed that the four free hydroxy groups in the molecule are important for RNAP binding [Campbell, E. A. *et al.* (2001).*Cell.* Vol. 104, p. 901-912]). Therefore, modifications of these hydroxyl groups are undesirable. In view of the aforesaid the present invention is unique such that it has designed a strategy to develop novel analogs and derivatives in which modifications take place in the polyketide backbone.

In the present invention the mutants of *A. mediterranei* S699 were developed for production of the analog of rifamycin B, 24-desmethylrifamycin B, is also proof of concept of combinatorial biosynthesis that sets stage for the production of variety of analogs of rifamycin B by using this approach. Derivatives of this analog are effective against MDR strains of *M. tuberculosis*. More importantly this invention provides a proof of concept for the first time that *rif*PKS gene cluster can be manipulated beyond module 4 by combinatorial approaches for the production of rifamycin B analogs. The mutant strain can now be improved for commercial use.

Accordingly the present invention provides for combinatorial biosynthesis strategy for manipulating *rif*PKS of *A. mediterranei* S699. Thus in the present invention strategy devised allowed swapping the acyltransferase domain of sixth module (AT6) of *rif*PKS(that recruits propionate) with that of acyltransferase domain of the second module of *rap*PKS (*rap*AT2 from *Streptomyces hygroscopicus* (having Accession number: X86780.1 and DSM Culture Collection- DSM 41524) (which recruits acetate) onto the growing chain. The swapping of these regions resulted in development and production of a produced a new analog of rifamycin B which is 24-desmethylrifamycin B. Further

the present invention also devised strategy to develop the derivatives of 24-desmethylrifamycin B. Thus the new analogs and the derivatives developed were found to effective against variety of bacterial species **(Figures 20-21)**. More specifically these new analogs and derivative were found to be effective against MDR strains of *M. tuberculosis*.

In spite of the known fact that complex polyketide systems like *rif*PKS are known to be intrinsically less amendable to pathway engineering, either due to inflexibility of the downstream enzymes to accept modified substrates or incompatibility in architectural modularity of the modified PKS systems [Khosla, C. *et al.*(2009).*Curr.Opin, Chem.. Biol.*Vol. 13,p. 135-143]. The present invention not only identifies the ways to unravel and untwine the existing drawback of rifPKS system but arrives at the analogs and derivatives which are highly effective against the known and/or resistant strains of mycobacteria. Thus to resolve the existing problem of un-amendable nature of *rif*PKS system the steps involved in the synthesis of rifamycin polyketide backbone, especially napthaquionone ring, the target was the *rif*AT6 region of the *rif*PKS gene. This *rif*AT6 region of the *rif*PKS gene cluster was swapped with *rap*AT2 using domain-replacement strategy. This resulted in a new rifamycin analog i.e. 24-desmethylrifamycin B and further its derivatives namely 24-desmethylrifamycin S, 24-desmethylrifamycin SV and 24-desmethylrifampicin.

These analogs of 24-desmethylrifamycin B and its derivatives; 24-desmethylrifamycin SV, 24-desmethylrifamycin S& 24-desmethylrifampicin, were not only produced in comparable amounts but also showed stronger antibacterial activity against *Staphylococcus aureus, Mycobacterium smegmatis, Bacillus subtillis, Pseudomonas aeruginosa, Escherichia coli.*

The present invention also provides for mutant strains of *A. mediterranei* S699 which are capable of producing the analog/s of rifamycin B, i.e. 24-desmethylrifamycin B. The present invention also provides for method developing the mutant strains of *A. mediterranei* S699. It has been found in the present invention that the strains of *A. mediterranei* S699 were found to be quite stable in the multiplication and also in the expression of the analogs of the rifamycin B. The analog production from these mutants has been carried out at a laboratory scale. The production yield was about 50 mg/L of the analog 24-desmethylrifamycin B.

The NMR studies of the analogs produced from the mutant strains of *A. mediterranei* S699 show that there is significant difference in the NMR profiles of rifamycin B and 24-desmethylrifamycin B **(Figure 13)**. The NMR profiles show that 24-desmethylrifamycin B has quasi-molecular ions of *m/z* 740 and that of 24-desmethylrifamycin SV is *m/z* 682, which is 14 atomic mass unit less than the rifamycin B and rifamycin SV.

The rifamycin resistance is associated with genetic alterations in an 81-bp region of the rpoB gene encoding the DNA-dependent RNAP β-subunit(Williams *et al.* 1998. Mycobacterium tuberculosis.Antimicrob.Agents.Chemother., Vol.42, pages 1853-1857).The present invention uses rifampicin-resistant *M. tuberculosis* strains, OSDD 321 and OSDD 206, which contain S531L mutation, and OSDD 55, which has H526T mutation in their RNAP β-subunit. The mutations were confirmed in OSDD strains. Based on the sequence data, the present invention finds that the drug resistant mutations disrupt hydrogen bonding in the polyketideansa chain and a possible salt bridge **(Figure 15b)**. The loss of the methyl group in 24-desmethylrifampicin, however, may lead to conformational changes in the ansa chain that allow for more flexibility of the compound to bind mutated RNAPs. Accordingly, it is believed that, in addition to favourable partition coefficient, the flexibility arising from difference in gauche, syn pentane, and eclipsing interactionsof the ansa chain and the changes in the hydrogen bonding network associated with the conformational changes **(Figure 15c)** allow for increase binding affinities in both the wild type and drug resistant strains. This conceivable conformational flexibility may also be connected to the unsettled 1H and 13C NMR spectra of 24-desmethylrifampicin. In view of the aforesaid the present invention provides the most unexpected and unique finding in the present invention is the activity of 24-desmethylrifampicin against rifampicin sensitive and rifampicin resistant strains of *M.tuberculosis*. 24-desmethylrifampicin showed stronger activity against rifampicin resistant strains of *M. tuberculosis*. Thus this finding of the present invention opens a complete hope for the patients suffering from Tuberculosis where the mycobacteria has become resistant to the existing rifampicin drug or related drugs.These results were corroborated with computational free energy perturbation (FEP) studies that demonstrated that RNAP prefers 24-desmethylrifamycin B by1.2 kcal/mol than

rifamycin B **(Table 1).** This is significant, because this compound can now be developed as a promising lead to cure MDR-TB.

Table 1. Computed free energy perturbation (FEP) studies of 24-desmethylrifamycin B with wild-type RNAP.

* All energies in kcal/mol.

	Protein Affinity ΔG	**Solvent Affinity ΔG**	**Binding Affinity ΔG**	**Relative Binding ΔG***
Rifamycin B	-36.2	-28.4	-7.8	0.0
24-Desmethyl-rifamycin B	-35.0	-26.0	-9.0	-1.2

Thus the present invention demonstrate creation of novel analogs and derivatives of rifamycin B by showing for the first time that the rifPKS is amenable to domain replacement modification, leading to the formation of new rifamycin analog.

The present invention provides for a unique recombinant sequence ID No.1 **(Figure 22)** which is capable of producing analogues of rifamycin. The present invention also provides for vector construct comprising the recombinant sequence ID No.1.

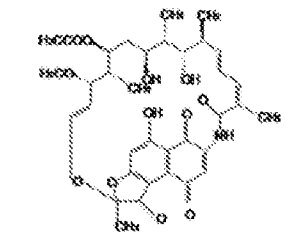
The present invention provides for novel strain of *Amycolatopsis mediterranei* S699 #34 carrying the nucleotide sequence ID No.1. More specifically the novel strain is having the reference number as *Amycolatopsis mediterranei* S699 #34 carrying the recombinant SEQ ID No.1 **(Figure 22)**, which provides the unique characteristic to the strains and this unique characteristic allows the novel strains to express or produce rifamycin analogues, such as 24-desmethylrifamycin B and 24-desmethylrifamycin S.

The present invention also provides for pharmaceutical compositions of the antibacterial compounds and their salts thereof described in the present invention which are effective against the bacterial

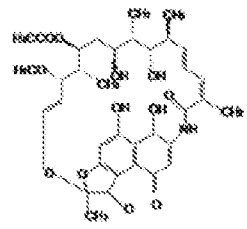
species, particularly mycobacteria. The pharmaceutical composition of the present invention isintended for parenteral and oral administration. Preferably, the pharmaceutical composition described as herein in the present invention can be administered parenterally for example, intravenously, subcutaneously, intradermally or intramuscularly. The present invention also provides for agents which function as "pharmaceutically acceptable excipient", wherein the term "pharmaceutically acceptable excipient" means a pharmaceutically acceptable formulation carrier, solution or additive to enable the delivery, dissolution or suspension of the antibacterial compounds herein as described. The pharmaceutical composition of the present invention may also contain pharmaceutically accepted auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like. The pharmaceutical composition of the present invention may also contain pharmaceutically acceptable carriers, for example adjuvants, etc. The pharmaceutical compositions of this invention may also be administered in any convenient form, for example tablet, capsule, injection, granule or powder form, e.g. in a sachet.

The present invention will be explained further with reference to non-limiting embodiments of the invention.

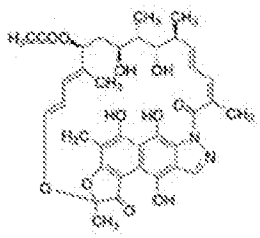
Accordingly, the main embodiment of the present invention relates to antibacterial compounds having following chemical structure:

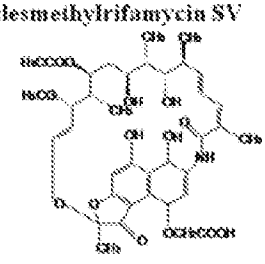


24- desmethylrifamycin S



24- desmethylrifamycin SV





24- desmethylrifamycin B

24-desemthylrifampicin

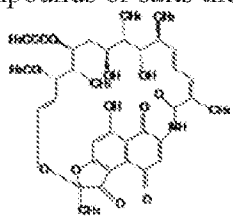
and their salts thereof.

Another embodiment of the present invention relates to antibacterial compounds of the present invention which are useful against infection or a disease caused by bacteria.

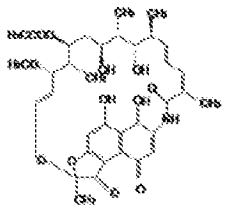
Another embodiment of the present invention relates to antibacterial compounds of the present invention which are useful against infection or a disease caused by *Mycobacterium* species.

Another embodiment of the present invention relates to antibacterial compounds of the present invention which are useful against infection or a disease caused by *Mycobacterium* species, where *Mycobacterium* species selected are *Mycobacterium tuberculosis* and MDR strains of *M. tuberculosis*.

Yet another embodiment of the present invention relates to apharmaceutical composition comprising antibacterial compounds or salts thereof having following chemical structure:

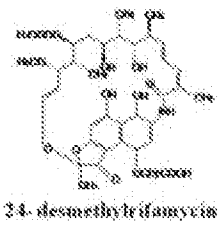


24- desmethylrifamycin S

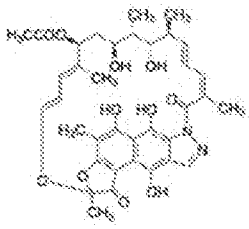


24- desmethylrifamycin SV

24-desmethylrifampicin



24- desmethylrifamycin B



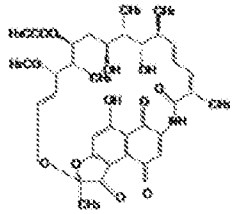
along with their pharmaceutical acceptable carriers.

Another embodiment of the present invention relates to a pharmaceutical composition comprising antibacterial compounds of the present invention which are useful against infection or a disease caused by bacteria.

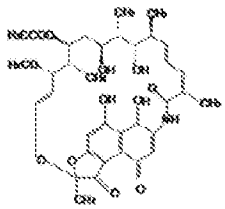
Another embodiment of the present invention relates to a pharmaceutical composition comprising antibacterial compounds of the present invention which are useful against infection or a disease caused by *Mycobacterium* species.

Yet another embodiment of the present invention relates to pharmaceutical composition comprising antibacterial compounds of the present invention which are useful against infection or a disease caused by *Mycobacterium tuberculosis* and MDR strains of *M. tuberculosis*.

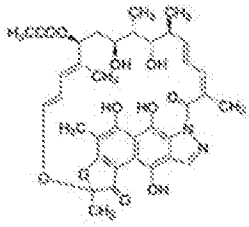
Yet another embodiment of the present invention relates to a method of treatment comprising administering to a patient an antibacterial compounds or salts thereof having following structure:



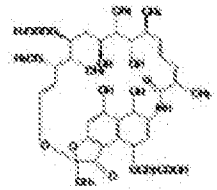
24- desmethylrifamycin S



24- desmethylrifamycin SV

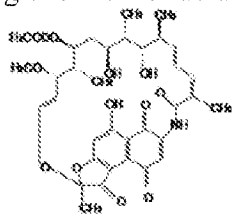


24-desmethylrifampicin

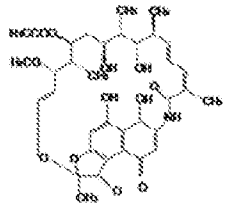


24- desmethylrifamycin B

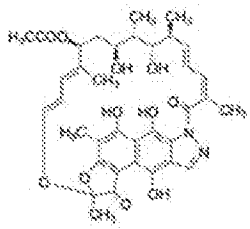
Yet another embodiment of the present invention relates to use of antibacterial compounds or salts thereof having following chemical structure for treatment of disease or an infection.



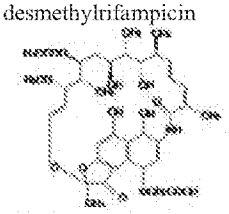
24- desmethylrifamycin S



24- desmethylrifamycin SV



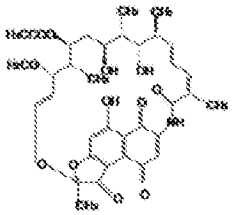
24-desmethylrifampicin



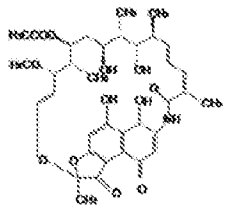
24- desmethylrifamycin B

for the preparation of a medicament.

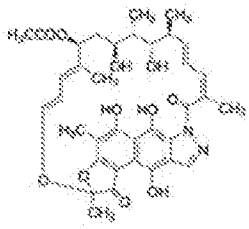
Yet another embodiment of the present invention relates to use of antibacterial compounds or salts thereof having following chemical structure:



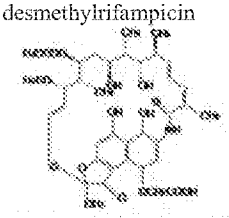
24- desmethylrifamycin S



24- desmethylrifamycin SV



24-desmethylrifampicin



24- desmethylrifamycin B

for the preparation of a medicament.

Another embodiment of the present invention relates to use of a medicament comprising antibacterial compounds of the present invention for the treatment of a disease or an infection.

Yet another embodiment of the present invention relates to use of a medicament comprising antibacterial compounds of the present invention for the treatment of a diseases or an infection caused by bacteria.

Another embodiment of the present invention relates to use of medicament comprising antibacterial compounds of the present invention for the treatment of a diseases or an infection caused by caused by *Mycobacterium* species.

Yet another embodiment of the present invention relates to use of medicament comprising antibacterial compounds of the present invention as claimed in claim 10 or 13, for the treatment of a diseases or an infection caused by *Mycobacterium* species, *Mycobacterium tuberculosis* and MDR strains of *M. tuberculosis*.

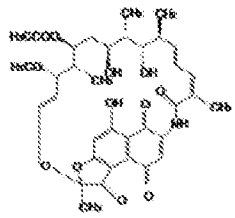
Another embodiment of the present invention relates to a method of preparing a pharmaceutical composition comprising antibacterial compounds of the present invention along with pharmaceutical acceptable carrier/s.

Another embodiment of the present invention relates to novel strain of *Amycolatopsis mediterranei* S699 #34 comprising a gene *rap*AT2 region.

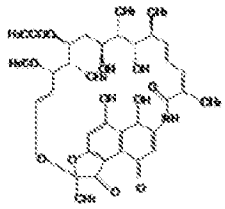
Yet another embodiment of the present invention relates to novel strain of *Amycolatopsis mediterranei* S699 #34 wherein *rif*AT6 regionis swapped with *rap*AT2 region.

Yet another embodiment of the present invention relates to novel strain of *Amycolatopsis mediterranei* S699 #34 capable of producing novel analogs and derivatives of rifamycin B such as herein described.

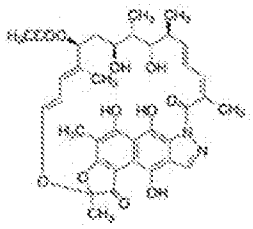
Another embodiment of the present invention relates to novel strain of *Amycolatopsis mediterranei* S699 #34 capable of producing the analogs and derivatives of rifamycin B comprising following chemical structure:



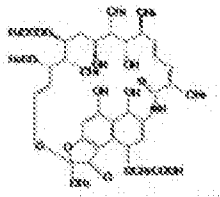
24- desmethylrifamycin S



24- desmethylrifamycin SV



24-desmethylrifampicin



24- desmethylrifamycin B

Another embodiment of the present invention relates a method of preparing novel strain of *Amycolatopsis mediterranei* S699 #34 comprising *rap*AT2 region in *rif*PKS, said method comprising the steps of :

(a) isolating *rif*AT6 regionof *rif*PKS from *Amycolatopsis mediterranei* S699 and *rap*AT2 region of *Streptomyces hygroscopicus* ((Acc. No. DSM-41524);
(b) preparing a vector constructs by swapping *rif*AT6 regionof *rif*PKS with *rap*AT2 region of *Streptomyces hygroscopicus* (Acc. No. DSM-41524);
(c) inserting the vector constructs of step (b) in an *Amycolatopsis mediterrranei* S699; and
(d) obtaining a novel strain *Amycolatopsis mediterranei* S699 #34 comprising a *rap*AT2 region of *rif*PKS.

Another embodiment of the present invention relates to a method of preparing novel antibacterial compounds said method comprising the steps of:

(a) culturing cells of *Amycolatopsis mediterranei* S699 #34 comprising *rap*AT2 region in the *rif*PKS region; and

(b) harvesting the cells of *Amycolatopsis mediterranei* S699 #34 to obtain the crude fractions of the products;
(c) purifying the crude products by HPLC;
(d) obtaining antibacterial compounds 24-desmethyrifamycin B and 24-desmethylrifamcin SV.

Another embodiment of the present invention relates to a method of preparing the 24-desmethyrifamycin S derivative of 24-desmethyrifamycin B, said method comprising the steps of :

(a) reacting 24-desmethyrifamycin B in presence of reagents selected from Copper
chloride;
(b) carrying the reaction of step (a) overnight at room temperature;
(c) obtaining 24-desmethyrifamycin S

A method of preparing the 24-desmethyrifampicin derivative of 24-desmethyrifamycin B, said method comprising the steps of :

(a) reacting 24-desmethyrifamycin B in presence of Dimethylformamide (DMF) and acetic acid;
(b) adding paraformaldehyde and 1,3,5-trimethyl-hexhydro-1,3,5-triazine to the mixture of step (a);
(c) obtaining 3-methyl-1,3-oxazino (5,6-c)-24-demethylrifamycin'
(d) reacting the compound of step (c) with 1-amino-4-emthyl-piperazine; and
(e) obtaining 24-desmethyrifampicin.

Another embodiment of the present invention relates to a recombinant nucleotide SEQ ID No.1.

Another embodiment of the present invention relates to a bacterial strain/s of the present invention comprising nucleotide SEQ ID NO.1

Another embodiment of the present invention relates to *Amycolatopsis mediterrranei* S699 #34 strain comprising nucleotide SEQ ID NO.1

Another embodiment of the present invention relates to bacterial strain of the present invention, wherein bacterial strain is capable of producing rifamycin analogues.

Another embodiment of the present invention relates to *Amycolatopsis mediterrranei* S699 #34 strain, wherein bacterial strain is capable of producing rifamycin analogues.

Another embodiment of the present invention relates to a recombinant nucleotide SEQ ID No.1, capable of expressing novel analogues of rifamycin.

Another embodiment of the present invention relates to a recombinant nucleotide sequence of the present invention, wherein the analogues are 24-desmethyrifamycin B and 24-desmethyrifamycin S.

Another embodiment of the present invention relates to vector constructs comprising recombinant nucleotide sequence ID No.1.

Yet another embodiment of the present invention relates to vector constructs, wherein vector constructs are pAT6E and pAT6F.

Another embodiment of the present invention relates to a bacterial strain comprising the vector constructs as described in the present invention.

Another embodiment of the present invention relates to *Amycolatopsis mediterrranei* S699#34 strain comprising vector constructs as described in the present invention.

Another embodiment of the present invention relates *Amycolatopsis mediterrranei* S699#34 strain carrying a pAT6F vector wherein the pAT6F vector comprises of a nucleotide sequence No.1.

Another embodiment of the present invention relates to a bacterial strain capable of carrying pAT6F vector wherein the pAT6F vector comprises of a nucleotide sequence No.1.

Another embodiment of the present invention relates to a microbial strain capable of carrying pAT6F vector wherein the pAT6F vector comprises of a nucleotide sequence No.1.

Another embodiment of the present invention relates to a microbial strain capable of expressing nucleotide sequence No.1 and/or its products thereof.

Though there have been no reports on the manipulation of the backbone of rifamycin B by combinatorial biosynthesis, yet there are reports of combinatorial biosynthesis being attempted on other polyketide synthase genes. A notable success was achieved in altering the rifamycin B molecule by using combinatorial approach as evident from the description of the present invention.

The following description is of exemplary embodiments only and is not intended to limit the scope, applicability or configuration to the invention in any way, Rather, the following description provides a convenient illustration for implementing exemplary embodiments of the invention various changes

to the described embodiments may be made in the functions and arrangement of the elements described without departing from the scope of the invention,

EXAMPLES

Example 1

Genetic manipulation of *A. mediterranei* S699.

Routine genetic procedures such as genomic DNA isolation (according to CTAB- Cetyltrimethyl Ammonium Bromide, method), plasmid isolation (Promega DNA purification kit), and restriction endonuclease digestion were carried out by standard techniques. A plasmid pAT6F was constructed that was transformed (using BioRadGenePulser) into *A. mediterranei* S699 using the method described previously [Dhingra, G. *et al.* (2003). *J. Ind. Microbiol.Biotechnol.*Vol. 13, p. 195-204] and the electroporated mixture was plated on YMG agar plates containing yeast extract 4 g, malt extract 10 g and glucose 4 g per litre (pH 7.2), that were overlaid with soft agar (5 g/L, DifcoTM agar, granulated) containing 500 µg/mL of erythromycin to confer resistance after incubation of 12-16 h. The transformants obtained after 5-7 days and were grown in YMG medium containing 50µg/mL erythromycin (Sigma Aldrich) to confirm resistance. The integration of pAT6F in these single crossover (SCO) clones was confirmed by Southern blot hybridization. In order to affect replacement of *rif*AT6 with that of *rap*AT2, these SCOs were cultured for 3-4 rounds in YMG medium without erythromycin. The cells were plated both on YMG agar plate with and without erythromycin pressure and colonies that were antibiotic sensitive were selected. The double cross-over (DCO) clones were further confirmed by Southern blot hybridization. For Southern hybridization, the genomic DNA was immobilized on a Hybond N+ membrane (Amersham Biosciences). Hybridization was performed at 65°Cfor 12h [α-^{32}P]-labeled DNA probes. For non-radioactive methods, hybridization was carried out using DIG-labeled DNA probe at 65°C. Stringency washes were done with 5x SSC, 2x SSC, 1x SSC and 0.1x SSC at 65°C. The DCO clones obtained contained the recombinant nucleotide sequence ID No.1 **(Figure 22)**. The *A. mediterranei* S699 clone as described and obtained comprised of recombinant nucleotide sequence ID No.1 was provided a (internal) reference number *Amycolatopsis mediterrranei* S699#34.

Construction of primers for PCR I and PCR II

The flanking region of AT6 also called PCR I (41862- 43533bp) located upstream of *rif*AT6 was amplified using oligoprimers, primers I and II (Table 1) as forward and reverse primers respectively. The restriction sites *Xba*I and *Bal*I were introduced at 5' ends of the primer I and II respectively in order to facilitate the cloning of PCR I in the *Sma*I site of the pUC 18. These sites were subsequently used for cloning.

Similarly PCR II (44488-45989bp) located downstream of *rif*AT6 was amplified using the primer pairs primer III and primer IV **(Table 2)**. *Avr*II site was introduced in primer III and *Xba*I in primer IV at the 5' end for subsequent cloning in pUC 18.

Table 2: The sequence of the primers used to amplify PCR I and PCR II.

Primer I	*Xba* I (41862-41892 of *rif*PKS cluster) 5'-CTCTAGAAGGCGCTCGCCCGGCACCTGCGCGACGAACT-3'
Primer II	*Bal* I (43513-43533 of *rif*PKS cluster) 5'-CTGGCCAGGGAAGACCCAGACGAGCTTG-3'
Primer III	*Avr*II (45963- 44513 of *rif*PKS cluster) 5'-CCCTAGGGCGGACCGGCCGGGTCGACCTGCCGA-3'
Primer IV	*Xba* I (45963-45989 of *rif*PKS cluster) 5'-CTCTAGAGGTGCCCAGCGATCCGGCGCCCGAGA-3'

Primers for PCR I

Primers for PCR II

Additional nucleotides inserted to generate the restriction site in all primers have been indicated in red. The nucleotide sequences of restriction enzyme sites generated in forward primers of PCR I and PCR II have been highlighted in light blue and reverse primers in yellow.

Example 2

Construction of pAT6A, pAT6B and pAT6C

PCR I and PCR II products were generated from the *rif*PKS on cosmid clone no. 13 Ligation of polnucleotide kinase treated PCR I or PCR II fragment at *Sma*I site of linearised and dephosphorylated pUC18 was then carried out. Subsequently, the ligation mix was transferred into *E. coli* JMI0I. The plasmids containing PCR I and PCR II in pUC 18 were named pAT6A and pAT6B, respectively. In the plasmid 'pAT6A', PCR I was ligated in the orientation reverse to one that was required for subsequent cloning. According to the required orientation *Xba*I site of PCR I should be adjacent to *Nde*I site of plasmid, however the clones obtained had *Xba*I site away from *Nde*I site of the plasmid. In order to reverse the orientation the PCR I was excised with *Eco*RI/ *Hind*III double digestion and introduced in pUC19 to finally get the plasmid, pAT6C.

Example 3

Construction of pAT6D

The plasmid pMO2 [Olinyk, M.*et al.*(1996). *Chem. Biol.* Vol. 3, p. 833-839]) which has acyl transferase of module 2 (AT2) of rapamycin biosynthetic gene cluster from *Streptomyces hygroscopicus* cloned in it, was digested with *Avr*II / *Hind*III, to release the *rap*AT2 domain. The plasmid pAT6B was also digested with *Avr*II/ *Hind*III and *rap*AT2 and linearized pAT6B were ligated and ligation mix was transferred in *E.coli* JM101 and clone containing right insert were selected. These constructs was named as pAT6D.

Example 4

Construction of pAT6E

PCR I was excised by digesting pAT6C with *BalI*/ *Nde*I and simultaneously pAT6D was digested with *BalI*/ *Nde*I. Both inserts from pAT6C and linearized pAT6D were ligated and ligation mix was transferred in *E.coli* JM101. The constructs so obtained was named as pAT6 E.

Example 5

[The strategy to develop the functional cassette (pAT6F- 8.3kb) for swapping (Figure 6)]:
Construction of the final cassette pAT6F

The plasmid pAT6E was digested with *Xba*I to release PCR I-*rap*AT2-PCR II fragment (approx. 4 kb) and cloned in *Xba*I digested vector pIJ4026, containing erythromycin resistance gene. pIJ4026(provided by M. J. Bibb, John Innes Institute, Norwich) (**Figure6**) is an *E. coli* plasmid but contains the *erm*E gene that confers resistance to erythromycin and is expressed only in *A. mediterranei* but not in *E. coli.* The final construct comprises of 3.85 fragment consisting of PCR I, *rap*AT2 and PCRII fragments in pIJ4026 was named pAT6F. The 3.85 kb fragment designated as SEQ ID NO.1 **(Figure 22)**.

This plasmid pAT6F was transformed by electroporation (7.5KV/Cm, 1000Ω, 25μF) into *A. mediterranei* S699. The single cross over clones were selected under erythromycin pressure in GYM agar media (Glucose 4g/l; Malt Extract 10g/l; Yeast Extract 4g/l). The single cross over (SCO) clones which did not show brown pigmentation (due to absence of rifamycin production as the biosynthetic pathway was blocked by the integration of pAT6F into the chromosome through homologous recombination were selected (**Figure 7 & 8**). The plasmid pTA6F contained the nucleotide sequence ID No.1 as described in the present invention and represented in **Figure 22.**

For genotypic confirmation, the DNA from these single crosses over (S.C.O.) clones were hybridized with plasmid (pIJ4026). Four SCO clones (1-5, 2-2, 6-1, 6-2) showed positive hybridization signal **(Figure 9).** The positive SCO clone 2-2, in order to select double cross overs (D.C.O.), was further cultured for 3-4 generations without erythromycin pressure. The erythromycin sensitive double crossover (D.C.O.) clones, which had the ability to produce rifamycin (brown pigmentation resumed), were selected. These clones had *rif*AT6 swapped with *rap*AT2 (**Figure 7**). The D.C.O.s obtained **(Figure 10)** were once again checked at genetic level for the presence of AT2 domain instead of AT6 by Southern hybridization and DNA sequencing. Four clones showed

positive signals with *rap*AT2 gene (**Figure 11**). The D.C.O clones which contained the recombinant sequence ID No. 1 were designated as *Amycolatopsis mediterranei* S699#34.

The expected rifamycin analogs produced by these DCO clones were analyzed by LC ESI-MS. Only three of these positive clones (#3, #34 and #36) produced the expected analog (**Figure 12**). The results were confimed by ^{1}H NMR (**Figure 13**). The molecular weight of the analog was found to be 741, which is 14 amu (atomic mass unit) less than the molecular weight of the native rifamycin B (755).

Spores of the mutant strain were initially grown on a shaker in YMG medium for 3 days at 30°C and 200 rpm. The seed culture was then used to inoculate (10%, v/v) YMG medium (10 x 100 mL) in 500 mL flasks. After incubation for 10 days under the same conditions, the cultures were centrifuged, the pooled supernatants acidified to pH 3 with 1N HCl, and the metabolites extracted with ethyl acetate (2 x 1 L). The crude extracts of rifamycin-related compounds were subjected to silica gel chromatography using $CHCl_3$-MeOH/5%NH_4OH (10:1 and then 8:1) as a mobile phase. Fractions containing the products were pooled and dried using rotary evaporator. The product obtained was further purified using HPLC [YMC-ODS-A, 250 x 10 mm, CH_3CN-$HCOONH_4$ (0.05 M) (60:40), flow rate 2 mL/min, 254 nm]. The product was then desalted using Sephadex LH-20 column with MeOH as eluent to give 24-desmethylrifamycin B (20 mg) and 24-desmethylrifamycin SV (8 mg).

24-desmethylrifamycin B: ^{1}H NMR (700 MHz, D_2O, CryoProbe): δ 6.69 (s, 1H, H-3), 6.34 (d, 1H, J = 12 Hz, H-29), 5.98 (d, J = 11 Hz, 1H, H-17), 5.75 (dd, J = 15 Hz, 11 Hz, 1H, H-18), 5.21 (dd, J = 15 Hz, 10 Hz, 1H, H-19), 5.05 (m, 2H, H-25, H-28), 4.50 (d, J = 17 Hz, 1H, -CH_2-COOH), 4.42 (d, J = 17 Hz, 1 H, -CH_2-COOH), 3.38 (bd, J = 10 Hz, 1H, H-23), 3.22 (m, 2H, H-21 and H-27), 3.22 (s, 3H, H-37), 2.11 (s, 3H, H-36), 2.08 (s, 3H, H-14), 1.98 (m, 2H, H-20 and H-26), 1.70 (s, 3H, H-13), 1.53 (m, 2H, H-22 and H-24), 1.28 (t, J = 12 Hz, 1H, H-24), 0.92 (d, J= 6.5 Hz, 3H, H-31), 0.85 (d, J = 7 Hz, 3H, H-32), 0.72 (d, J = 6.5 Hz, 3H, H-34). ^{13}C NMR (175 MHz, CD_3OD, CryoProbe): δ_C 191.7, 176.7, 174.7, 168.1, 144.7, 142,1, 141.5, 131.5, 126.5, 126.4, 119.4, 117.8, 113.7, 112.8, 112.1, 109.6, 104.4, 101.1, 80.5, 73.9, 72.4, 71.5, 68.8, 54.9, 48.8, 41.4, 39.9, 35.9, 32.2, 21.8, 20.6, 20.5, 15.4, 9.5, 9.5, 6.9. (-) -HR-ESI-TOF-MS *m/z*740.2939 (calcd for $C_{38}H_{46}NO_{14}$[M- H]$^-$: 740.2918).

24-Desmethylrifamycin SV: ^{1}H NMR (700 MHz, CD_3OD, CryoProbe): ^{1}H NMR (700 MHz, CD_3OD): δ6.42 (s, 1H), 6.31 (bd, J = 12 Hz, 1H), 5.95-5.89 (m, 2H), 5.51 (s, 1H), 5.20 (bt, J = 12 Hz, 1H), 5.14 (bd, J = 9 Hz, 1H), 3.22 (s, 3 H), 3.17 (t, J = 10 Hz, 1H), 2.13 (s, 3H), 2.12-2.11 (m, 2H), 2.01 (s, 3H), 1.96 (s, 3H), 1.67 (s, 3H), 1.54-1.49 (m, 4H), 0.93 (d, 6H), 0.78 (d, J = 5 Hz, 3H). HRMS (ESI-TOF) *m/z* (calcd for $C_{36}H_{42}NO_{12}[M- H]^-$: 682.2707).

Example 6

Conversion of 24-desmethylrifamycin B to 24-desmethylrifamycin S

24-desmethylrifamycin B (8 mg, 0.0107 mmol) was dissolved in MeOH-H_2O (10:1, 5mL) containing $CuCl_2$ (0.1 mM). The reaction mixture was stirred at RT overnight to convert 24-desmethylrifamycin B to 24-desmethylrifamycin S. The mixture was acidified to pH 3 and the product was extracted with ethyl acetate (3 x 5mL). The extract was subjected to silica gel column using $CHCl_3$-MeOH (10:1) as eluent to give 24-desmethylrifamycin S (6 mg).

The product was analyzed by (-)-ESI-MS (m/z of 680 [M-H]-, indicating the lack of the glycolate moiety and the presence of a naphthoquinone unit in the molecule) and 1H NMR spectrum. Interestingly, during storage in CDCl3 at -20°C, transparent red brown orthorhombic crystals were formed that revealed a dimeric form of 24-desmethylrifamycinS coordinating with Ca2+ through C-1, C-8, C-21, and C-23 oxygen atoms **(Figure 15a)**.

^{1}H NMR (300 MHz, CD_3OD): 7.85 (s, 1H), 7.04 (m, 1H), 6.88 (bd, J = 11 Hz, 1H), 6.59 (m, 1H), 5.23 (dd, J = 12.6 Hz, 9.7 Hz), 4.53 (bt, J = 8 Hz, 1H), 4.22 (bd, J = 10 Hz, 1 H), 3.80 (bd, J = 10 Hz, 1H), 3.48 (s, 3H), 2.50 (s, 3H), 2.35 (s, 3H), 2.33 (s, 3H), 2.00 (s, 3H), 1.62 (m, 3 H), 1.41 (d, J = 7 Hz, 3H), 1,29 (d, J = 6.8 Hz, 3H), 0.67 (d, J = 7 Hz, 3H). HRMS (ESI-TOF) *m/z*680.2730 (calcd for $C_{36}H_{42}NO_{12}[M- H]^-$: 680.2707).

X-ray Crystallography of 24-desmethylrifamycin S.Diffraction intensities were collected at 173(2) K on a Bruker Apex CCD diffractometer using MoK radiation = 0.71073 Å. Space group was determined based on systematic absences. Absorption corrections were applied by SADABS[Sheldrick, G. M. (1998). Bruker/Siemens Area Detector Absorption Correction Program (Bruker AXS, Madison. WI)]. Structures were solved by direct methods and Fourier techniques and refined on F^2 using full

matrix least-squares procedures. All non-H atoms were refined with anisotropic thermal parameters **(Figure 16)**. H atoms were treated in calculated positions in a rigid group model. It was found that solvent water is partially occupied in a position between molecules with an occupation factor of 0.25. H atoms in this solvent water molecule were not taken in consideration. The Flack parameter is 0.00(10). Relatively high value of R_{int}, 0.1271, is related to the fact that diffraction at high angles was very weak and intensity statistics at high angles are poor. All calculations were performed by the Bruker SHELXTL (v. 6.10) package [Sheldrick, G. M. (1998). Bruker/Siemens Area Detector Absorption Correction Program (Bruker AXS, Madison. WI)].

Example 7

Synthesis of 24-desmethylrifampicin

24-desmethylrifamycin S (5mg, 0.0073 mmol) was dissolved in DMF (200 µL) and acetic acid (50µL). After stirring the mixture at 50°C, paraformaldehyde (3mg) and 1,3,5-trimethyl-hexahydro-1,3,5-triazine (8µL) were added. The reaction was stirred at 50°C for 2h until all starting material was converted to 3-methyl-1,3-oxazino(5,6-c)-24-desmethylrifamycin, indicated by a blue spot on TLC. Subsequently, 1-amino-4-methyl-piperazine (8µL) was added to the mixture. The reaction was stirred and monitored by TLC until the disappearance of the blue spot and the formation of 24-desmethylrifampicin. The mixture was diluted with cooled 2% acetic acid (1.5mL) and extracted three times with $CHCl_3$ (2mL). The organic fractions were combined and concentrated to 1mL and further washed 3 times with Brine solution. Organic fractions were combined and dried over anhydrous sodium sulfate then dried under rotary evaporator. Crude fractions were subjected to silica gel chromatography with $CHCl_3$–MeOH in 10:1 & 8:1 ratio as eluent. Fractions containing the product were pooled out and further purified by HPLC [CH_3CN-0.05 M $HCOONH_4$ (60:40)] with (YMC-ODS-A, 250X10 mm ID, 5 microns particle size) column and flow rate 2 mL/min. at 254 nm. Fractions containing 24-desmethylrifampicin were dried to afford the title compound (2.5 mg, 0.0031 mmol) of reddish orange powder of product.

^{1}H NMR (500 MHz, CD_3OD): **(Figure 17)**HRMS (ESI-TOF) *m/z*807.3829 (calcd for $C_{42}H_{55}N4O_{12}$[M-H]$^-$: 807.3816).

To validate the identity of 24-desmethylrifampicin, a comparative MS/MS analysis was carried out **(Figure 18).**The result showed that 24-desmethylrifampicin (*m/z* 807 → 676 → 616 → 490 → 420) and rifampicin(*m/z* 821 → 690 → 630 → 490 → 420) have identical fragmentation patterns, albeit most of the fragments of 24-desmethylrifampicin are 14 atomic mass units less than the corresponding fragments from rifampicin**(Figure 19)**. These fragmentation patterns are also consistent with those previously reported for rifampicin (Prasad, B. and Singh, S. J. (2009) Pharm.Biomed. Anal. Vol.50, pages 475-490).

Example 8

Antibacterial Assay

Antibacterial activity of rifampicin and its analogues and derivatives i.e. 24-desmethyrifamycin B, 24-desmethyrifamycin S, 24-desmethyrifamycin SV and 24-desmethyriampicin also determined by agar diffusion assay.

Mycobacterium smegmatis, *Mycobacterium smegmatis*, *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* were streaked on nutrient agar and incubated overnight at 37 °C. Colonies were transferred to nutrient broth and incubated at 37 °C for 24 h. The growth of the cultures was measured to a proper density at 600 nm(BioRad, SmartSpec 300). Inoculum (1mL) was mixed thoroughly with warm nutrient agar (24 mL) and poured into petri dishes. The agar plates were allowed to solidify and dry for 30 min before assay. Sterile Whatman discs were impregnated with rifampicin and its analogues (20 µL) at concentrations 1mg/mL and dried at room temperature. The discs were placed onto inoculated agar plates and incubated at 37 °C for 24 h. To produce a contrast background of the inhibition zone, 0.25% MTT developing dye (2mL) was added over the plates.

The Antibacterial assays carried out with 24-desmethylrifamycin S and 24-desmethylrifamycin SV against Gram-positive and Gram-negative bacteria such as *Mycobacterium smegmatis*, *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli* (**Figure 20**). The results showed that the new compounds (24-desmethylrifamycin S and 24-desmethylrifamycin SV) derived from new analog 24-desmethylrifamycin B were more active than rifamycin B against *M. smegmatis*. Modelling studies

were performed that suggested that 24-desmethylrifampicin binds to rifamycin resistant RNA polymerase of *M. tuberculosis* better than rifampicin.

Similarly the antibacterial assay was carried out using 24-desmethylrifampicin, 24-desmethylrifamycinSand commercially available rifampicin and rifamycin S against *Mycobacterium smegmatis* and *Staphylococcus aureus*. The results showed that 24-desmethylrifamycin S and 24-desmethylrifampicin are active against *M. smegmatis* and *S. aureus,* comparable to rifamycin S and rifampicin, respectively **(Figure 21).**

Based on the above results further study was carried out the effect of analogs and derivatives of the present invention to study the multidrug resistant strains of *M. tuberculosis*. For this, MDR strains were procured from Open Source Drug Discovery (OSDD; www.osdd.net) and the drug testing was performed at Premas Biotech, Haryana, India, according to WHO guidelines. The drug sensitivity tests were carried out against tworifampicin-sensitive and three-resistant strains of *M. tuberculosis*:OSDD 209 & H37Rv and OSDD 55, OSDD 206 & OSDD 321, respectively **(Table 3).**

Table 3: Comparitive data of the drug Sensitivity assays on various *Mycobacterium tuberculosis* (resistant and sensitive) strains (procured from OSDD) against commercially available rifampicin and the novel compound 24-desmethylrifamycin S & 24-desmethylrifampicin.

***M. tuberculosis* strains**		**Rifampicin (HiMedia)**	**24-desmethyl-Rifamycin S**	**24-desmethyl-Rifampicin**
OSDD 55 (H526T)	**Resistant**	>50	0.1	<0.01
OSDD 206 (S531L)		>50	0.05	0.05
OSDD 321 (S531L)		>50	0.1	0.05
OSDD 209*	**Sensitive**	0.1	<0.01	<0.01
H37Rv*		0.05	<0.01	0.05

* norpoB mutation

Rifampicin (commercially available from HiMedia), 24-desmethylrifampicin and 24-desmethylrifamycin Swere tested against the above mentioned pathogenic strains. The tests were done at various concentrations (0.01 - 50µg/mL) of drugs using BacT/ALERT MB System[Crump, J. A. *et al.* (2011). *J. Clin. Microbiol.* Vol. 49, p. 3054-3057]. The results revealed that 24-desmethylrifamycin

S &24-desmethylrifampicin showed strong antibacterial activity against both rifamycin-sensitive and -resistant strains of *M. tuberculosis*.

Drug sensitivity assays were done by Premas Biotech, Haryana, India, using various concentrations of drugs (0.01 - 1µg/ml). The drug testing was done using MB BacT/ALERT SystemSystem[Crump, J. A. *et al.* (2011). *J. Clin. Microbiol.* Vol. 49, p. 3054-3057], which is a mycobacterial detection system that utilizes a colorimetric sensor and reflectance detector to determine the level of carbon dioxide within the bottle. With the growth of microorganism, there is production of CO_2 resulting in color change of the sensor (at the bottom of the bottle). As the concentration of CO_2 increases there is change in color from green to yellow. The bottle contains a media and MB/BacT Reconstitution fluid, which promotes the growth of mycobacteria. The sample is inoculated into MP BacT/ALERT bottle. The testing is performed with two controls: Direct growth control (DGC) and Proportionate growth control (PGC). DGC involves 0.1mL seed culture into MP BacT/ALERT bottle along with 0.5ml reconstitution fluid. PGC involves 0.5 mL of DGC into MP BacT/ALERT bottle along with 0.5ml reconstitution fluid. Test Bottle involves 0.5 mL seed culture into MP BacT/ALERT bottle along with 0.5 mL reconstitution fluid as well as antibiotic. The test is considered as complete when PGC bottle flags positive.

Free Energy Perturbation Studies

As the 24-desmethylrifamycinanalogsshowed comparable or better activity than rifampicin against both rifampicin-sensitive and –resistant *M. tuberculosis*, we decided to carry out free energy perturbation (FEP) studies using wild type RNAPv [Postma, J. P. M. *et al.* (1982*). Faraday Symp.Chem. Soc.* Vol. 17, p. 55-67; Singh, U. C. *et al.* (1987).*J. Am. Chem. Soc.* Vol. 109, p. 1607-1614]**(Table 4)**.Whereas FEP calculations with rifampicin-resistant RNAP are more desirable, unfortunately, a crystal structure of the mutated holo-RNAP is yet to be elucidated. Rifamycin B and 24-desmethylrifamycin B were chosen in the study on the basis of their potentially lower computational expenses, as rifampicin derivatives are slightly larger. In addition, it has been generally accepted that the C-3 or C-4 side chains do not significantly change the binding of the drug, but appreciably changed the transport properties.

Table 4. Computed free energy perturbation (FEP) studies of 24-desmethylrifamycin B with wild-type RNAP.

Compound	Protein Affinity ΔG	Solvent Affinity ΔG	Binding Affinity ΔG	Relative Binding ΔG*
Rifamycin B	-36.2	-28.4	-7.8	0.0
24-Desmethyl-rifamycin B	-35.0	-26.0	-9.0	-1.2

* All energies in kcal/mol.

The FEP calculations were run with Gromacs 4.5[Pronk, S. *et al.* (2013). *Bioinformatics*. Vol. 29, p. 845-854]with 21 parallel windows from the previously generated structure, and the Bennet acceptance ratio was used to tabulate the free binding energy.The FEP results showed that 24-desmethylrifamycin B has a similar affinity with rifamycin B for wild-type RNAP, but has a lower affinity for water than the latter compound. This considerable decrease in 24-desmethylrifamycin B solvation affinity brings about a greater partition coefficient for protein than rifamycinB, resulting in a better relative binding affinity of 24-desmethylrifamycinB for the RNAP by 1.2 kcal/mol**(Table 4).**

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The State of Oregon Acting By And Through The State Board
of Higher Education on Behalf of Oregon State University

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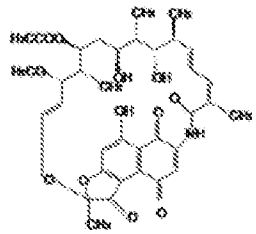
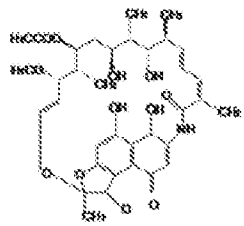
We claim:

1. Antibacterial compounds and/or salts thereof having following chemical structure:



24- desmethylrifamycin S



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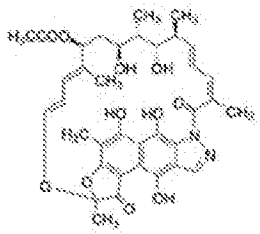
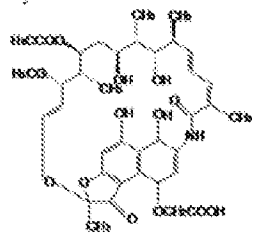


24-desemthylrifampicin



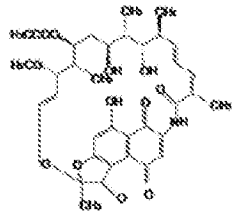
24- desmethylrifamycin B

2. The antibacterial compounds as claimed in claim 1, wherein the antibacterial compounds are useful against infection or a disease caused by bacteria.

3. The antibacterial compounds as claimed in claims 1-2, wherein the antibacterial compounds are useful against infection or a disease caused by *Mycobacterium* species.

4. The antibacterial compounds as claimed in claims 1-3, wherein *Mycobacterium* species selected are *Mycobacterium tuberculosis* and MDR strains of *M. tuberculosis*.

5. A pharmaceutical composition comprising antibacterial compounds and/or salts thereof having following chemical structure: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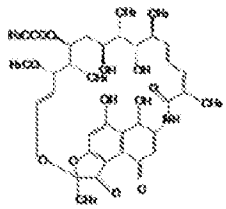
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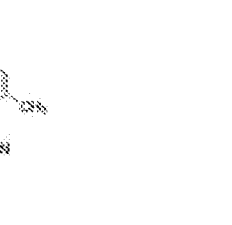
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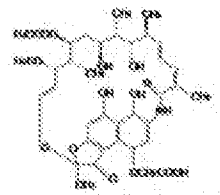

24- desmethylrifamycin B

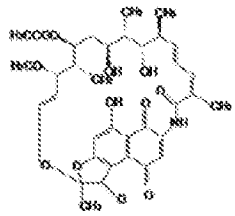
along with their pharmaceutical acceptable carriers.

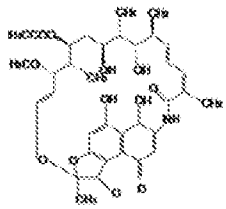
6. The pharmaceutical composition as claimed in claim 5, wherein the antibacterial compounds are useful against infection or a disease caused by bacteria.

7. The pharmaceutical composition as claimed in claims 5-6, wherein the antibacterial compounds are useful against infection or a disease caused by *Mycobacterium* species.

8. The pharmaceutical composition as claimed in claims 5-6, wherein the antibacterial compounds are useful against infection or a disease caused by *Mycobacterium tuberculosis* and MDR strains of *M. tuberculosis*..

9. A method of treatment comprising administering to a patient an antibacterial compounds and/or salts thereof having following structure:


24- desmethylrifamycin S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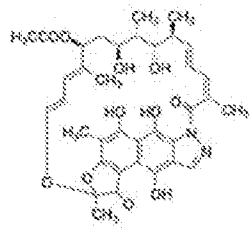
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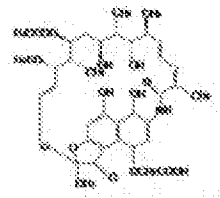
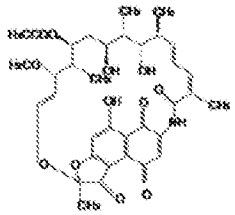

24- desmethylrifamycin B

10. Use of antibacterial compounds or salts thereof having following chemical structure and/or salts thereof for treatment of disease or an infection.



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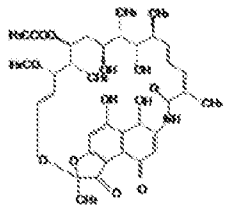

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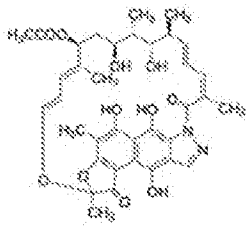

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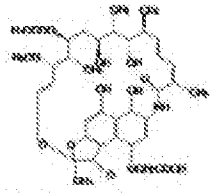
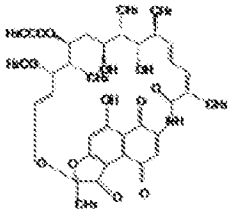


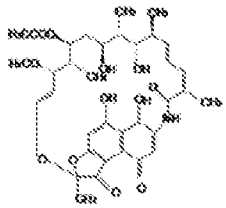
24- desmethylrifamycin B

for the preparation of a medicament.

11. Use of antibacterial compounds and/or salts thereof having following chemical structure:



24- desmethylrifamycin S



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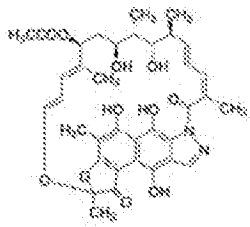

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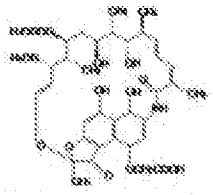


24- desmethylrifamycin B

for the preparation of a medicament.

12. Use of medicament as claimed in claim 10, for the treatment of a disease or an infection.

13. Use of medicament as claimed in claim 10, for the treatment of a diseases or an infection caused by bacteria.

14. Use of medicament as claimed in claim 10, for the treatment of a diseases or an infection caused by caused by *Mycobacterium* species.

15. Use of medicament as claimed in claim 10 or 13, for the treatment of a diseases or an infection caused by *Mycobacterium* species, *Mycobacterium tuberculosis*and MDR strains of *M. tuberculosis*.

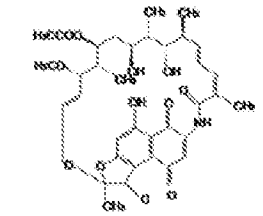
16. Method of preparing a pharmaceutical composition as claimed in 5, said method comprising antibacterial compounds as claimed in claim 1 along with pharmaceutical acceptable carrier.

17. Novel strains of *Amycolatopsis mediterrranei* S699#34 comprising a gene *rap*AT2 region.

18. Novel strains of *Amycolatopsis mediterrranei* S699#34 wherein *rif*AT6 regionis swapped with *rap*AT2 region.

19. Novel strains of *Amycolatopsis mediterrranei* S699#34 capable of producing novel analogs and derivatives of rifamycin B.

20. Novel strains of *Amycolatopsis mediterrranei* S699#34 as claimed in claim 19, wherein the analogs and derivatives of rifamycin B comprise of compounds having following chemical structure:



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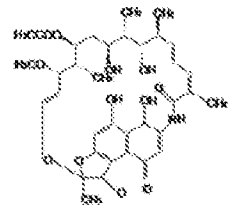

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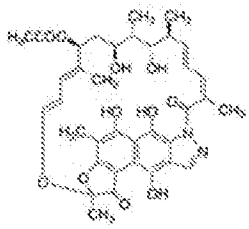

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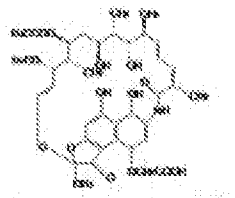

34- desmethylrifamycin S

21. A method of preparing novel strains of *Amycolatopsis mediterrranei* S699 comprising *rap*AT2 regionin *rif*PKS, said method comprising the steps of :

(a) isolating *rif*AT6 regionof *rif*PKS from *Amycolatopsis mediterrranei* S699 and *rap*AT2 region of *Streptomyces hygroscopicus* (Acc. No.: DSM - 41524);

(b) preparing a vector constructs by swapping *rif*AT6 regionof *rif*PKSwith *rap*AT2 region of *Streptomyces hygroscopicus* (Acc. No.: DSM - 41524);

(c) inserting the vector constructs of step (b) in an *Amycolatopsis mediterrranei* S699; and

(d) obtaining a novel strains of *Amycolatopsis mediterrranei* S699#34 comprising a *rap*AT2 region of *rif*PKS.

22. A method of preparing novel antibacterial compounds said method comprising the steps of:
(a) culturing cells of *Amycolatopsis mediterrranei* S699#34 comprising *rap*AT2 region in the *rif*PKS region; and
(b) harvesting the cells of *Amycolatopsis mediterrranei* S699#34 to obtain the crude fractions of the products;
(c) purifying the crude products by HPLC;
(d) obtainingantibacterial compounds 24-desmethyrifamycin B and 24-desmethylrifamcin SV.

23. A method of preparing the 24-desmethyrifamycin S derivative of 24-desmethyrifamycin B, said method comprising the steps of :
(a) reacting 24-desmethyrifamycin B in presence of reagents selected from Copper chloride;
(b) carrying the reaction of step (a) overnight at room temperature;
(c) obtaining 24-desmethyrifamycin S

24. A method of preparing the 24-desmethyrifampicin derivative of 24-desmethyrifamycin B, said method comprising the steps of :
(a) reacting 24-desmethyrifamycin B in presence of Dimethylformamide (DMF) and acetic acid;
(b) adding paraformaldehyde and 1,3,5-trimethyl-hexhydro-1,3,5-triazine to the mixture of step (a);
(c) obtaining 3-methyl-1,3-oxazino (5,6-c)-24-demethylrifamycin'
(d) reacting the compound of step (c) with 1-amino-4-emthyl-piperazine; and
(e) obtaining 24-desmethyrifampicin.

25. A recombinant nucleotide SEQ ID No.1.

26. A bacterial strain comprising nucleotide SEQ ID NO.1.

27. A bacterial strain as claimed in claim 26, wherein bacterial strain is capable of producing rifamycin analogues.

28. A bacterial strain as claimed in claims 26-27, wherein the bacterial strain is *Amycolatopsis mediterrranei* S699#34.

29. A recombinant nucleotide SEQ ID No.1, capable of expressing novel analogues of rifamycin.

30. A recombinant nucleotide sequence as claimed in claim 1, wherein the analogues are 24-desmethyrifamycin B and 24-desmethyrifamycin S.

31. A vector constructs comprising recombinant nucleotide sequence ID No.1.

32. A vector constructs as claimed in claim 29, wherein vector constructs are pAT6E and pAT6F.

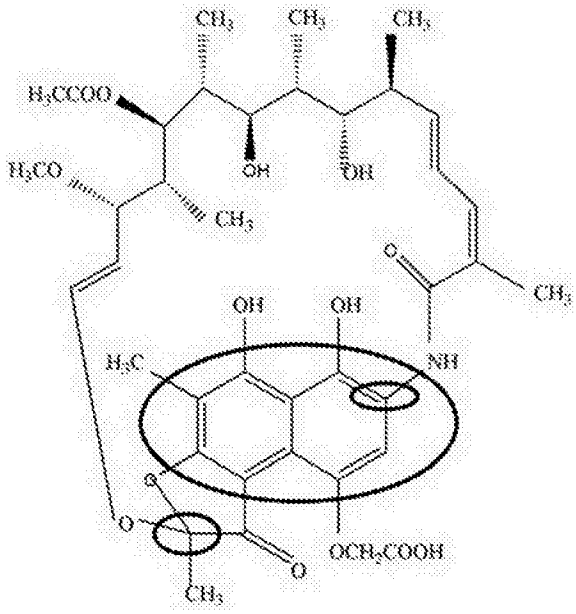

Figure 1: The chemical structure of rifamycin B. The ansa chain joins the naphthoquinone moiety at C-2 and C-12 (circled in black). The naphthoquinone moiety (circled in black) imparts reddish brown colour to the compound.

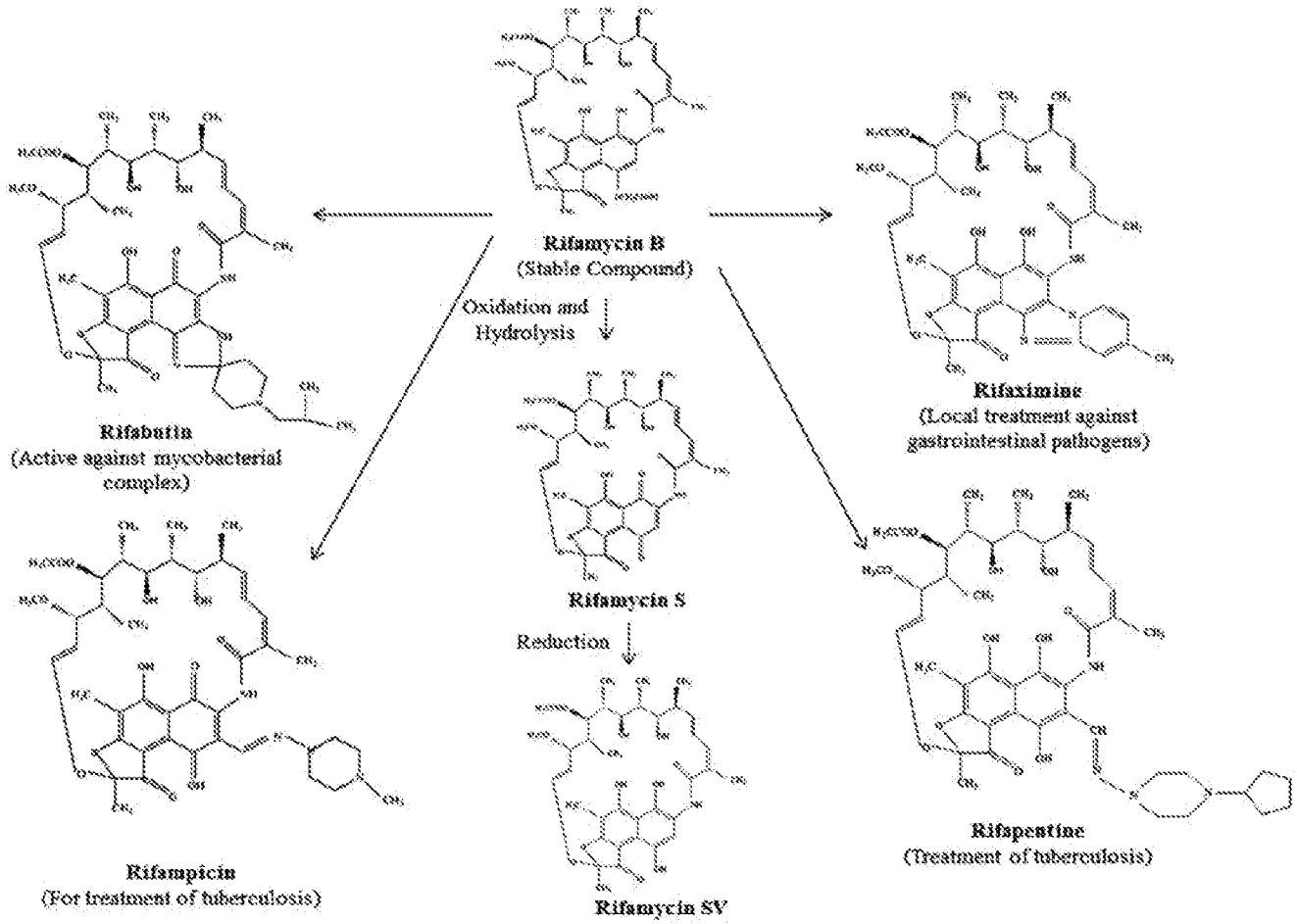

Figure 2: The semisynthetic derivatives of rifamycin B currently available for clinical use in the market. The complexity of the rifamycin molecule allows modification only at the C-3 and C-4 of the carbon skeleton and thus the modifications are limited to these positions of rifamycin B. To our knowledge no further derivatives of rifamycin (except those mentioned in Figure 2) with formidable antibiotic activity could be produced by chemical modifications. This created a bottleneck in the production of rifamycin analogs

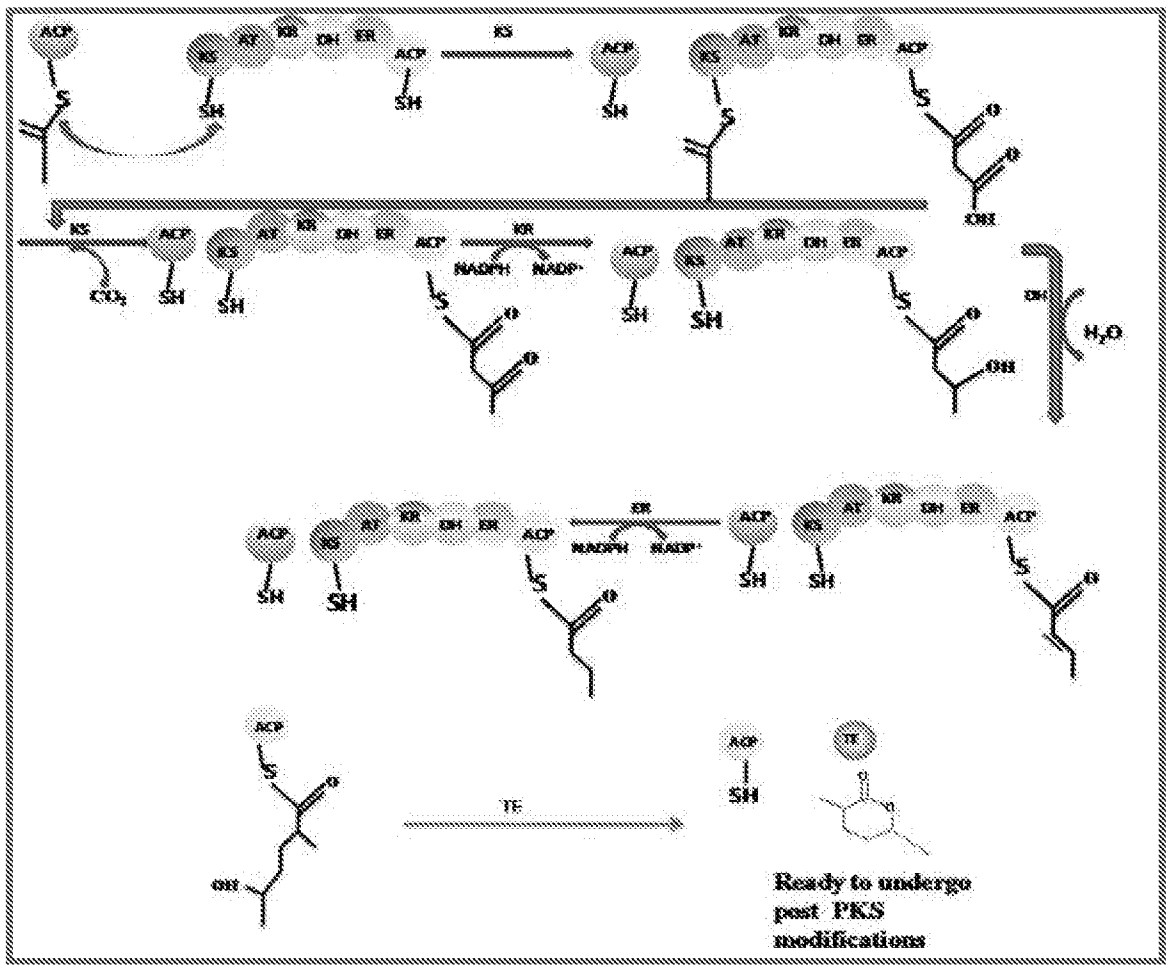

Figure 3: Schematic representation of processive mechanism of type I PKS. The starter unit (acetyl CoA) is loaded onto the ACP catalysed by the AT domain of the starter module. The extending chain is handed over from the ACP domain of the previous module to the KS domain of the current module. The extender unit is loaded onto the current ACP domain catalysed by the AT domain. The KS bound extender unit reacts with ACP bound extender unit via decarboxylation and Claisen's condensation. The growing polyketide chain may optionally be modified by additional domains. The KR domain reduces the β keto group to a hydroxyl group. The DH domain eliminates a molecule of water to give a double bond. ER domain reduces the double bond to a saturated hydrocarbon chain. The carbon skeleton is then released from the PKS by hydrolytic action of the TE domain.

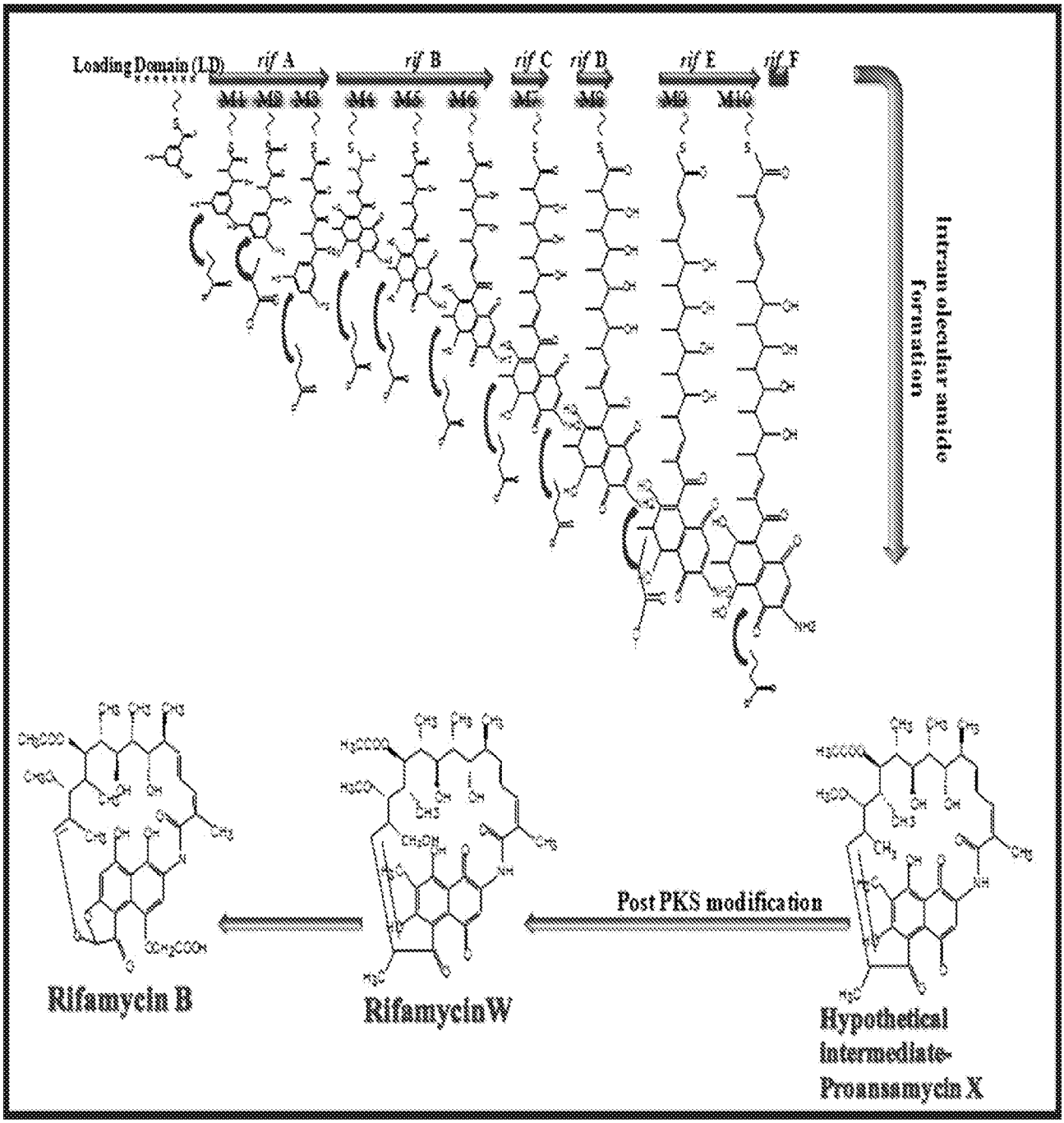

Figure 4: The synthesis of the hypothetical intermediate by rifamycin PKS of region II during the biosynthesis of rifamycin B. The chain is assembled of starter unit (AHBA), 2 acetate and 8 propionate extender units. Rif F which is translationally coupled to RifE and substitutes the function of TE domain. It encodes amide synthase and thus displaces the thioesterase linkage of the assembled polyketide chain. Proansamycin X then undergoes post PKS modifications to form rifamycin B.

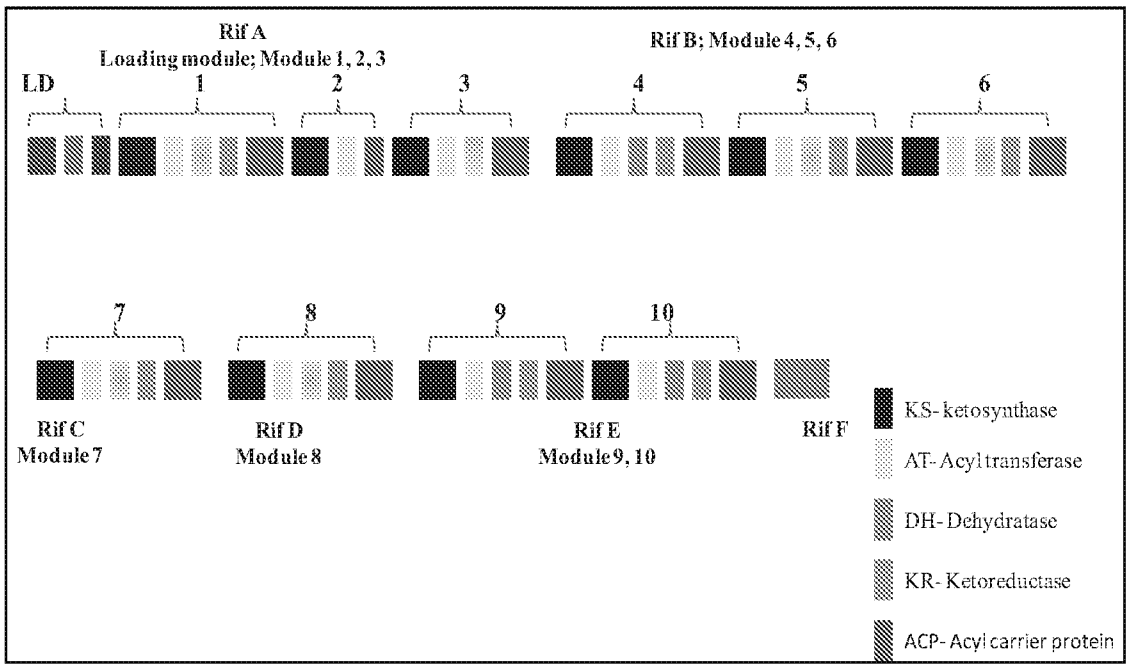

Figure 5: Organization of the enzymatic domain in *rif* PKS gene cluster. The domains are arranged in a collinear fashion to form modules which in turn form the five large ORFS. Rif A has modules 1-3, Rif B modules 4-6, Rif C module 7, Rif D module 8, Rif E modules 9 and 10. Rif F is present downstream of Rif E. The loading unit precedes the module 1. Each domain is colour coded. The shaded boxes represent inactive domains. KR domain of module 3 and DH domains of modules 1, 3, 5, 6, 7 and 8 are inactive. The AT domain of modules 2 and 9 catalyse the incorporation of acetate extender unit whereas the rest of the AT domains incorporate propionate extender units to the growing polyketide chain.

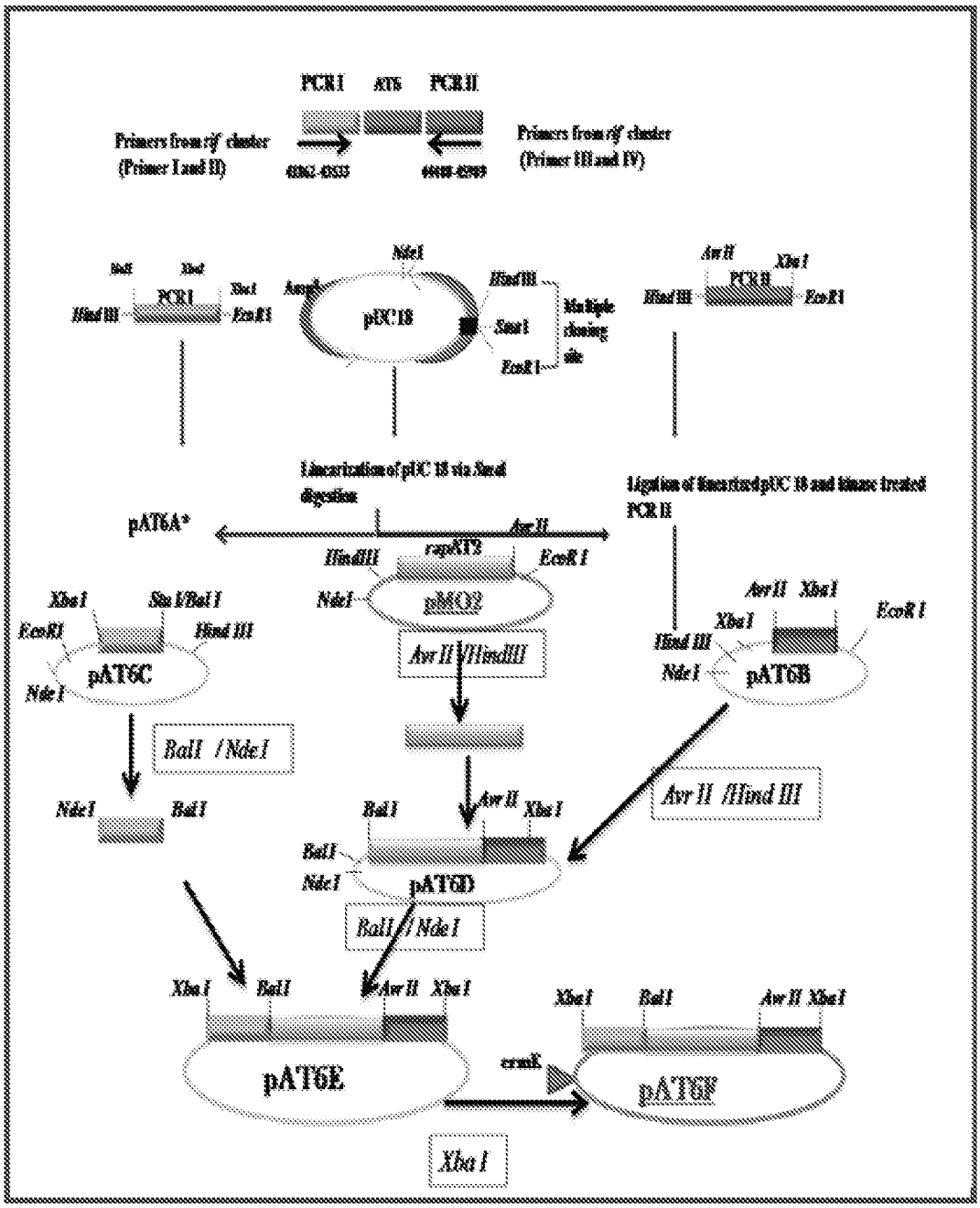

Figure 6: Strategy for construction of functional cassette in the plasmid pIJ4026, which was electroporated into *Amycolatopsis mediterranei* to swap *rif*AT6 with *rap*AT2. The flanking regions immediately upstream (41862-43533bp-PCRI) and downstream (44888-45989bp-PCRII) were amplified using primers pair I, II and primer pair III, IV respectively. PCRI and PCRII were cloned in pUC18 and were named pAT6A* and pAT6B respectively. PCRI was excised from pAT6A* and ligated in pUC19 to get pAT6C. The plasmid pMO2 was digested to release *rap*AT2 which was ligated in the linearized pAT6B to form pAT6D. PCRI from pAT6C was digested and ligated into linearized pAT6D to form the plasmid pAT6E. The entire construct PCRI+*rap*AT2+PCRII was digested from pAT6E and finally cloned in the plasmid pIJ4026, which confers erythromycin resistance to the host organism *Amycolatopsis mediterranei*. This final construct pAT6F was transformed in the wild type strain (*A. mediterranei* S699). pAT6A*- The detailed conversion of pAT6A to pAT6C has not been shown

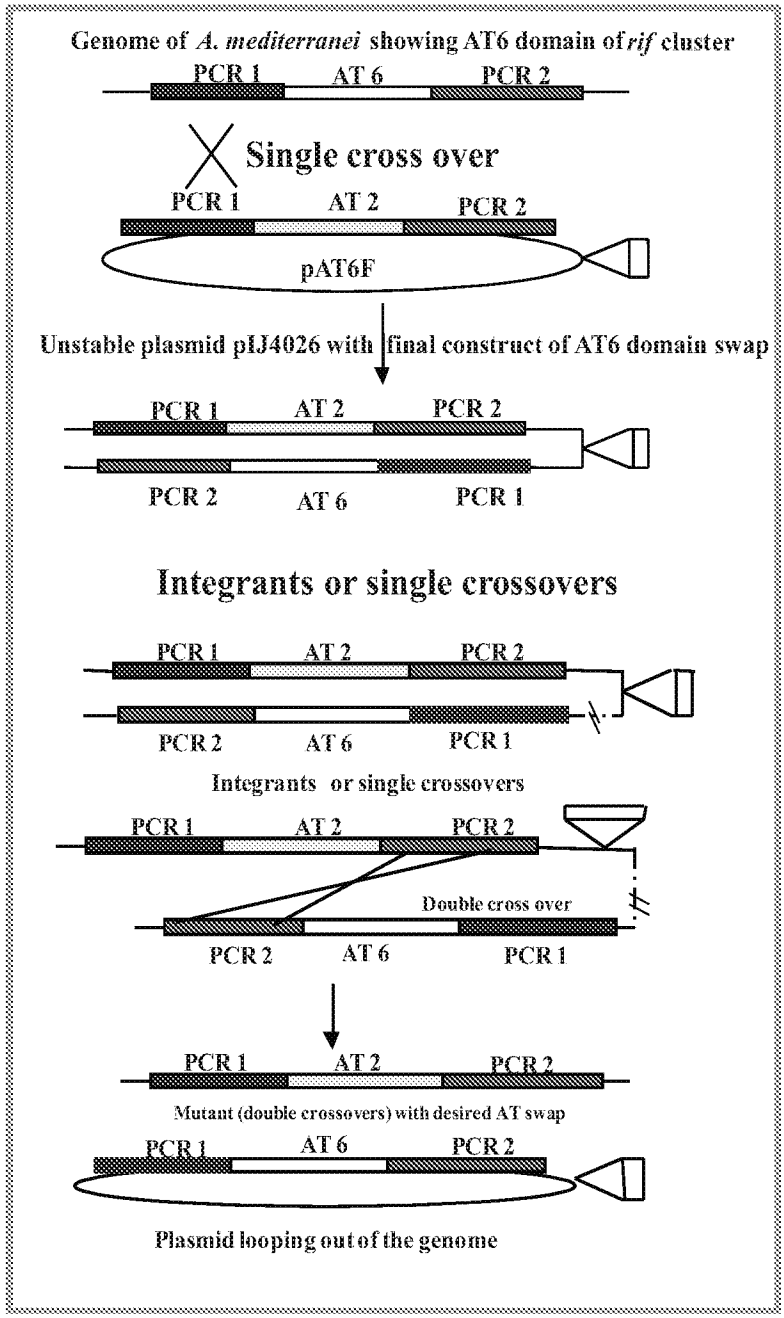

Figure 7: Schematic representation of the strategy to show the swapping of *rif*AT6 with *rap*AT2 by two step homologous recombination leading to the development of A. meditterranei S699 mutants 3, 34 and 36 producing 24-desmethylrifamycin B

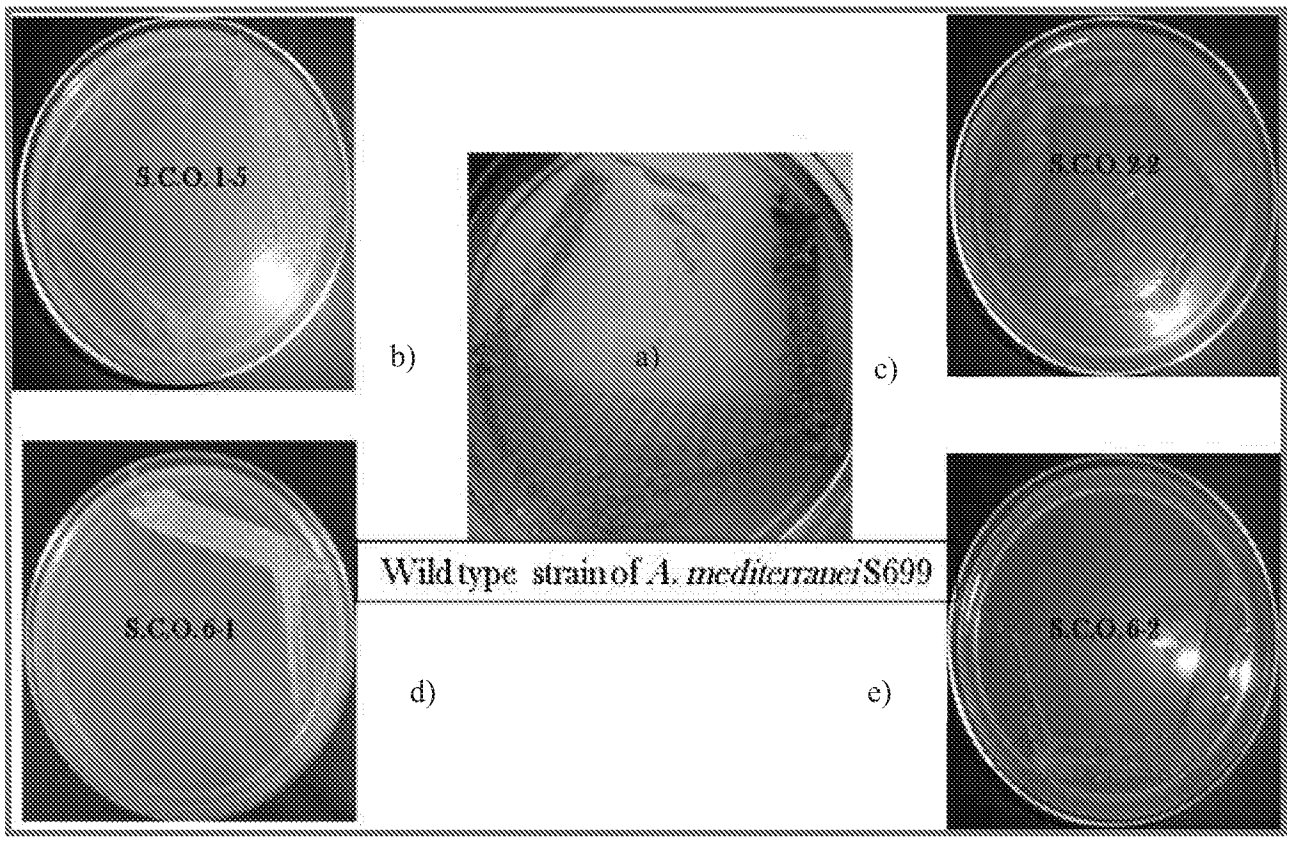

Figure 8: Four single crossover clones (b)1-5,(c) 2-2, (d) 6-1 and (e) 6-2 generated from (a) *A. meditterranei* S699 during the study. No pigmentation is seen around the SCO which indicates absence of rifamycin production due to blocked rifamycin biosynthetic pathway.

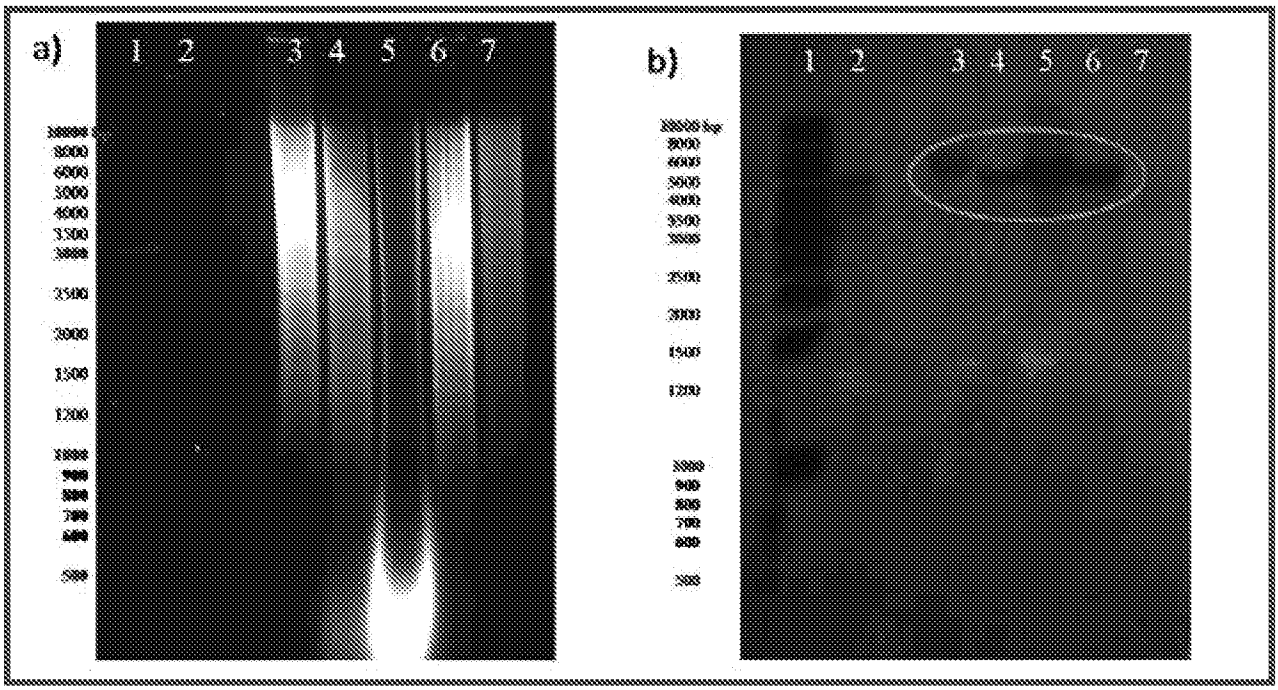

Figure 9: a) Agarose gel electrophoresis profile of *BamH*I digested genomic DNA of S.C.O. clones. Lane 1- Gene ruler (MBI Fermentas # SMO331), Lane 2- *BamH*I digested pIJ4026 (positive control), Lane 3-6- *BamH*I digested S.C.O. 1-5, 2-2, 6-1 and 6-2, Lane 7- *BamH*I digested DNA of *A. mediterranei* S699. b) The corresponding Southern blot hybridization profile of *BamH*I digested DNA of the four S.C.O. clones probed with [α^{32}P]-dATP labeled pIJ4026. Lane 1- Gene ruler (MBI Fermentas # SMO331), Lane 2- *Bam*HI digested pIJ4026 (positive control), Lane 3-6- *BamH*I digested S.C.O. 1-5, 2-2, 6-1 and 6-2, Lane7- *BamH*I digested DNA of *A. mediterranei* S699. Signals were obtained in all four S.C.O. clones but no signal was seen in the *BamH*I digested DNA of *A. mediterranei* S699 (negative control).

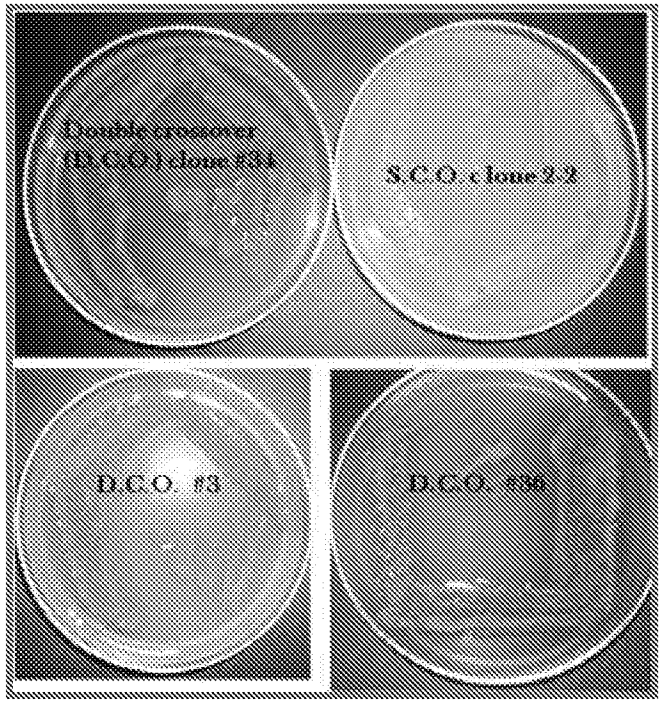

Figure 10: The three positive double crossover clones (#3, #34, and #36) generated from the single cross over (SCO) clone 2-2. Pigmentation is seen around the culture which indicates that rifamycin production is restored after AT6 swapping by AT2.

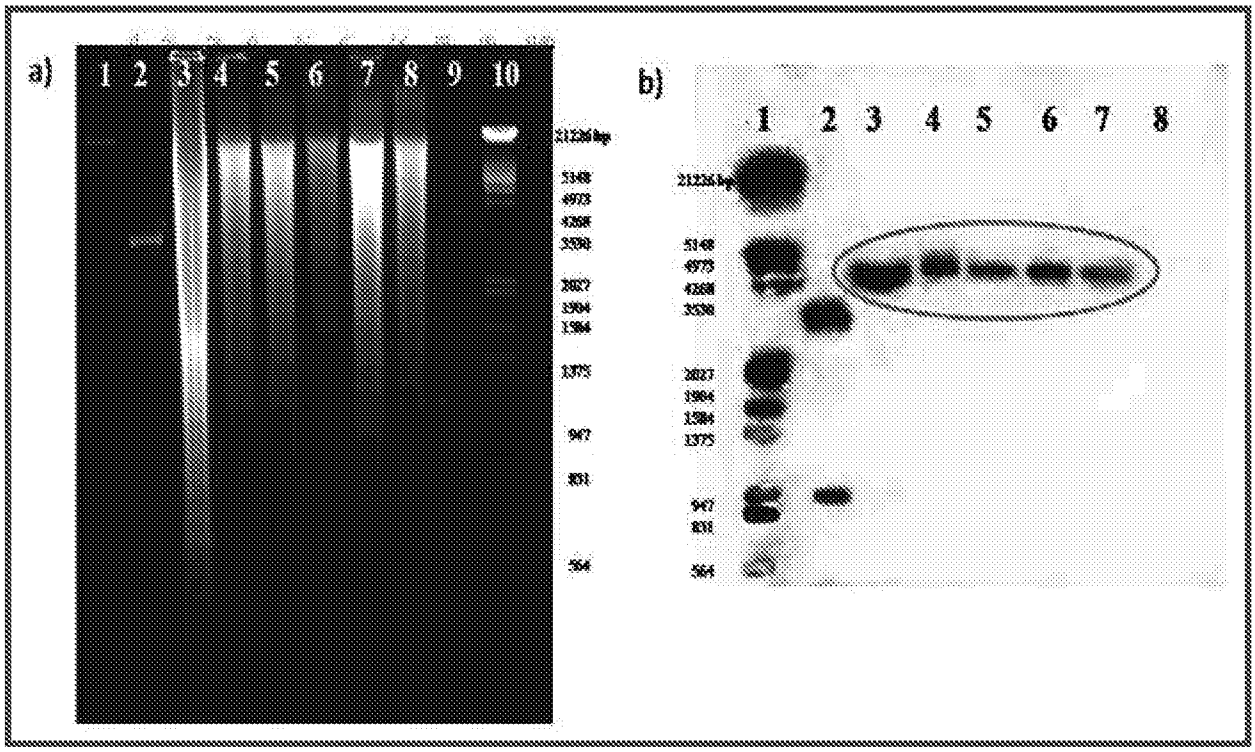

Figure 11: a) Agarose gel electrophoresis profile of *BamH*I digested genomic DNA of D.C.O. clones of S.C.O. 2-2. Lane 1- Marker lambda DNA *EcoR*I/*Hind*III digested (kb) (MBI Fermentas #SMO191), Lane 2- pMO2 digested with *EcoR*I and *Hind*III , Lane 3-7 has S.C.O. 2-2, D.C.O.s #3, #34, #36, and #43 respectively, Lane 8- *A. mediterranei* S699 (wild type), Lane 10- Marker lambda DNA *EcoR*I/*Hind*III digested (kb) (MBI Fermentas #SMO191). b) The corresponding southern blot hybridization profile of *BamH*I digested DNA of the four D.C.O. clones probed with DIG-dUTP labeled *rap*AT2. Lane 1- Marker lambda DNA *EcoR*I/*Hind*III digested (kb) (MBI Fermentas #SMO191), Lane 2- pMO2 digested with *EcoR*I and *Hind*III , Lane 3-7 has S.C.O. 2-2, D.C.O.s #3, #34, #36, and #43 respectively, Lane 8- *A. mediterranei* S699 (wild type), Lane 10- Marker lambda DNA *EcoR*I/*Hind*III digested (kb) (MBI Fermentas #SMO191). Positive signals are obtained in S.C.O. 2-2 and all the D.C.O. clones (encircled in red)

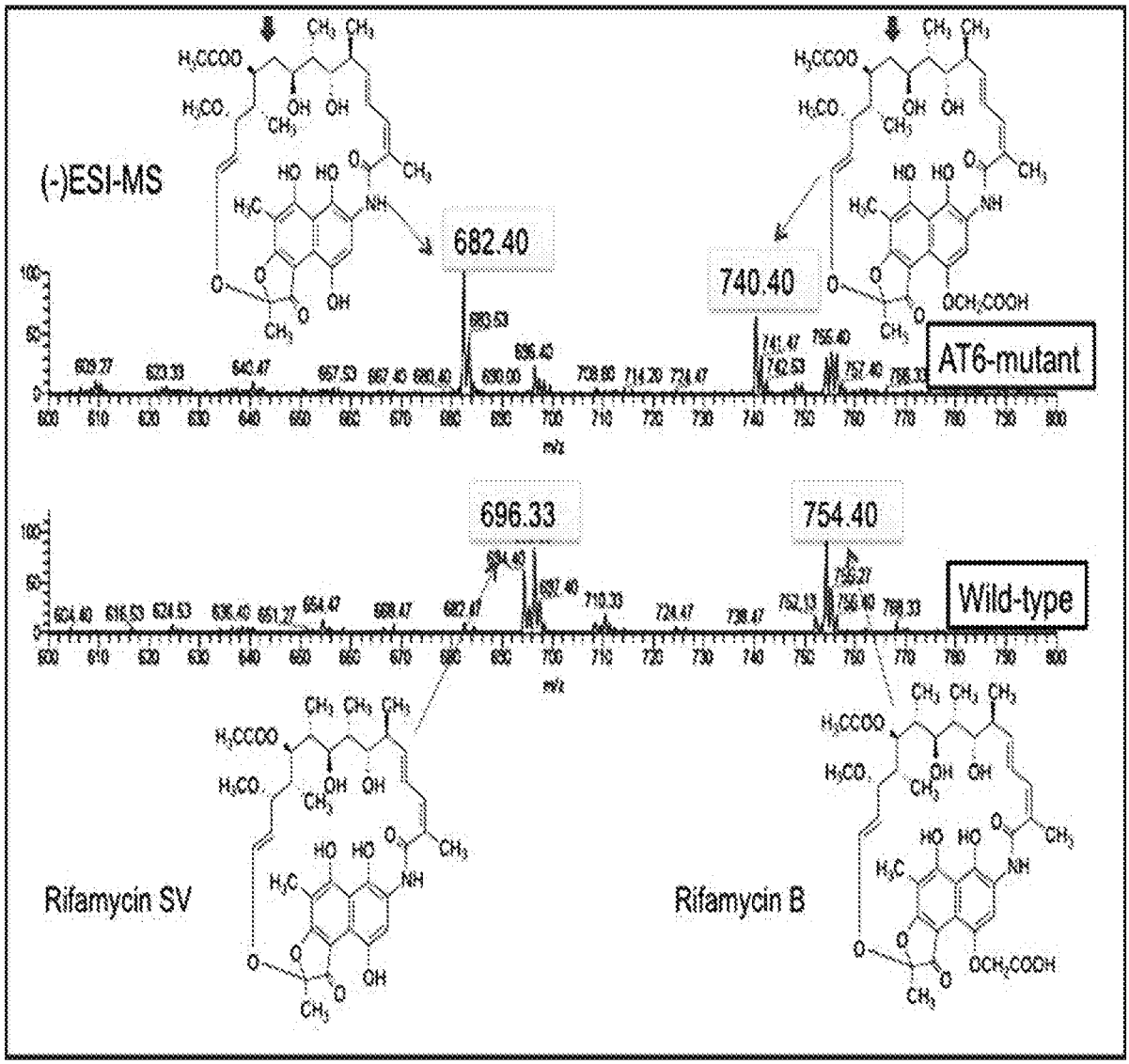

Figure 12: The LC-ESI-MS profile of the analog 24-desmthylrifamycin B extracted from DCO #36 and rifamycin B, the native molecule. Also highlighted are the peaks of rifamycin SV, an intermediate in the rifamycin B biosynthetic pathway along with its counterpart demethylrifamycin SV.

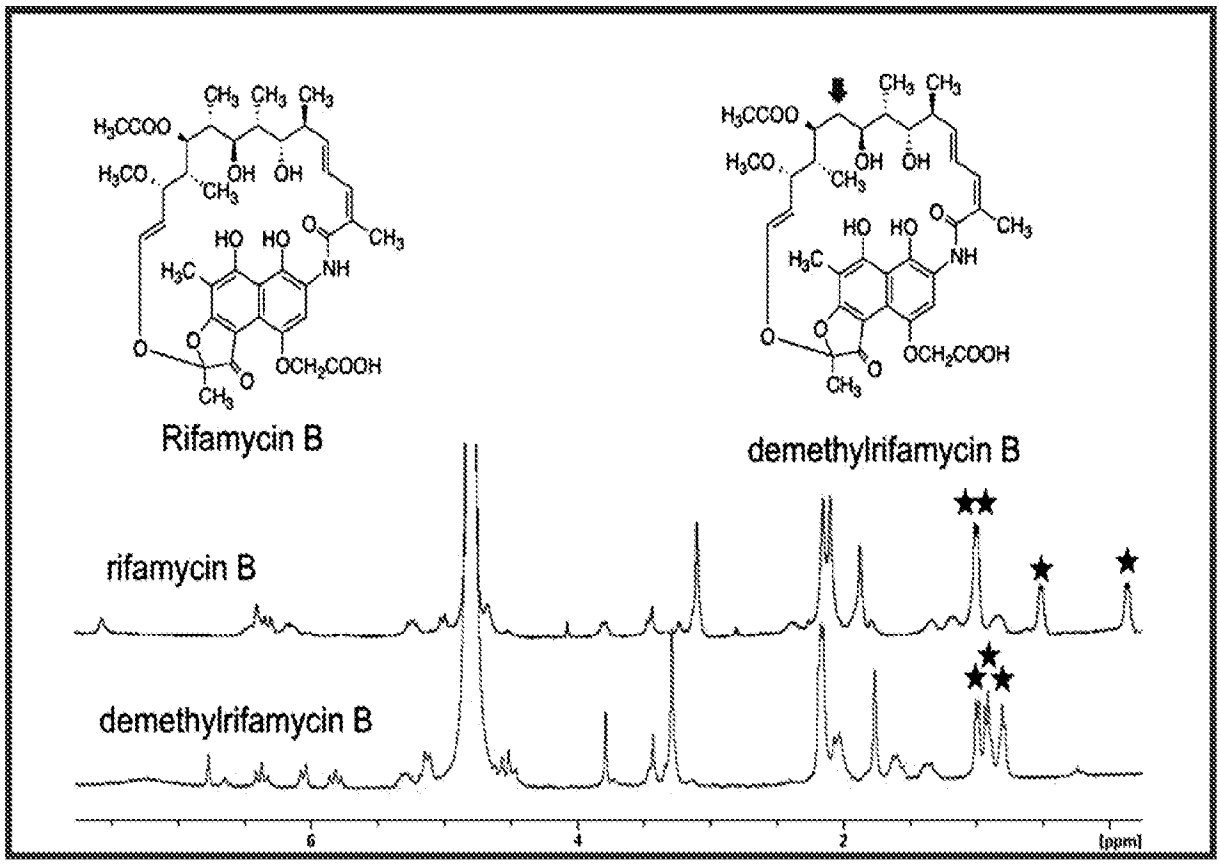

Figure 13: The NMR results of rifamycin B and its analog. The analog shows the absence of a group of signals for methyl group. This is due to the loss of the methyl pendant at C-33.

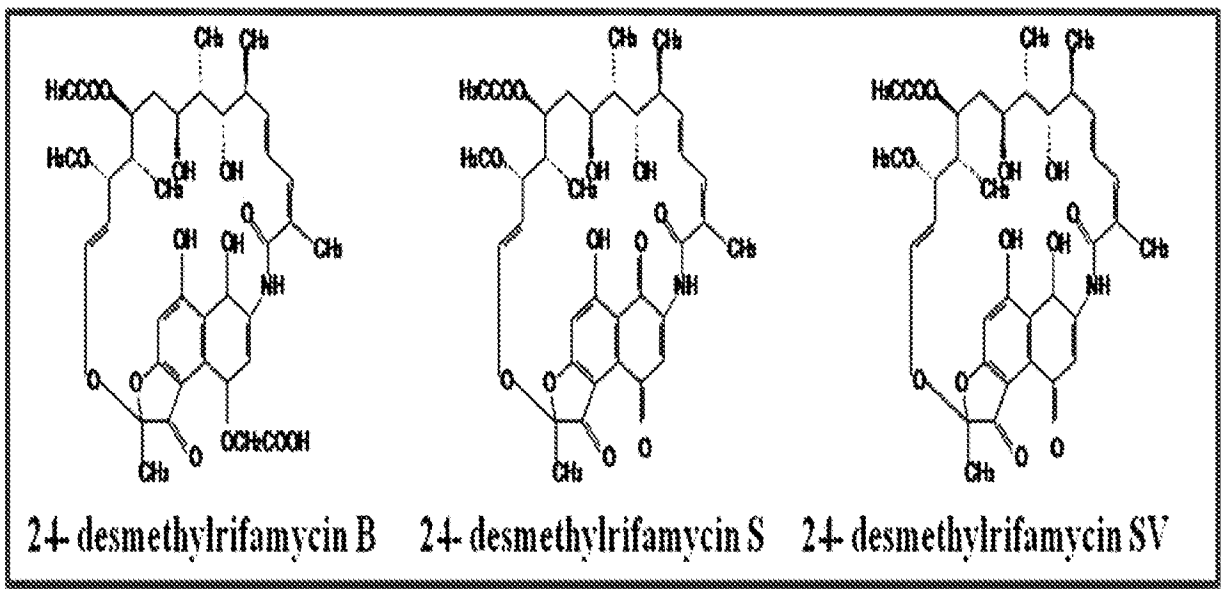

Figure 14: The structures of 24-desmethylrifamycin B, 24-desmethylrifamycin S and 24-desmethylrifamycin SV

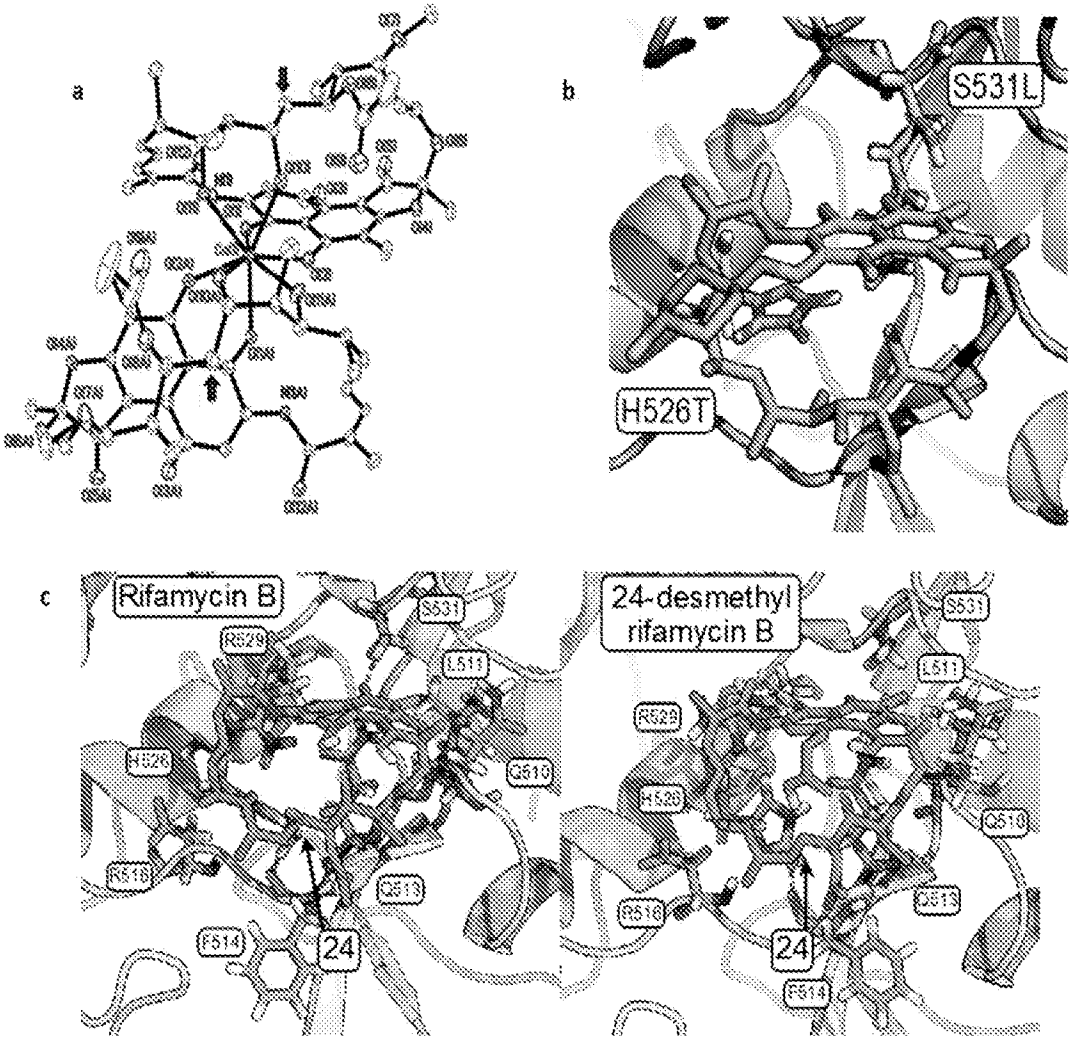

Figure 15. Crystal structure of 24-desmethylrifamycin S and drug interaction with RNA polymerase (a) X-ray crystal structure of 24-desmethylrifamycin S dimer in complex with Ca^{2+}. Broad red arrows indicate the missing methyl groups. (**b**) RNAP drug resistant mutations, Ser to Leu and His to Thr shown, disrupt hydrogen-bonding networks and induce steric interactions. (**c**) Comparison of rifamycin B and 24-desmethylrifamycin B in the binding pocket with explicit polar interactions shown.

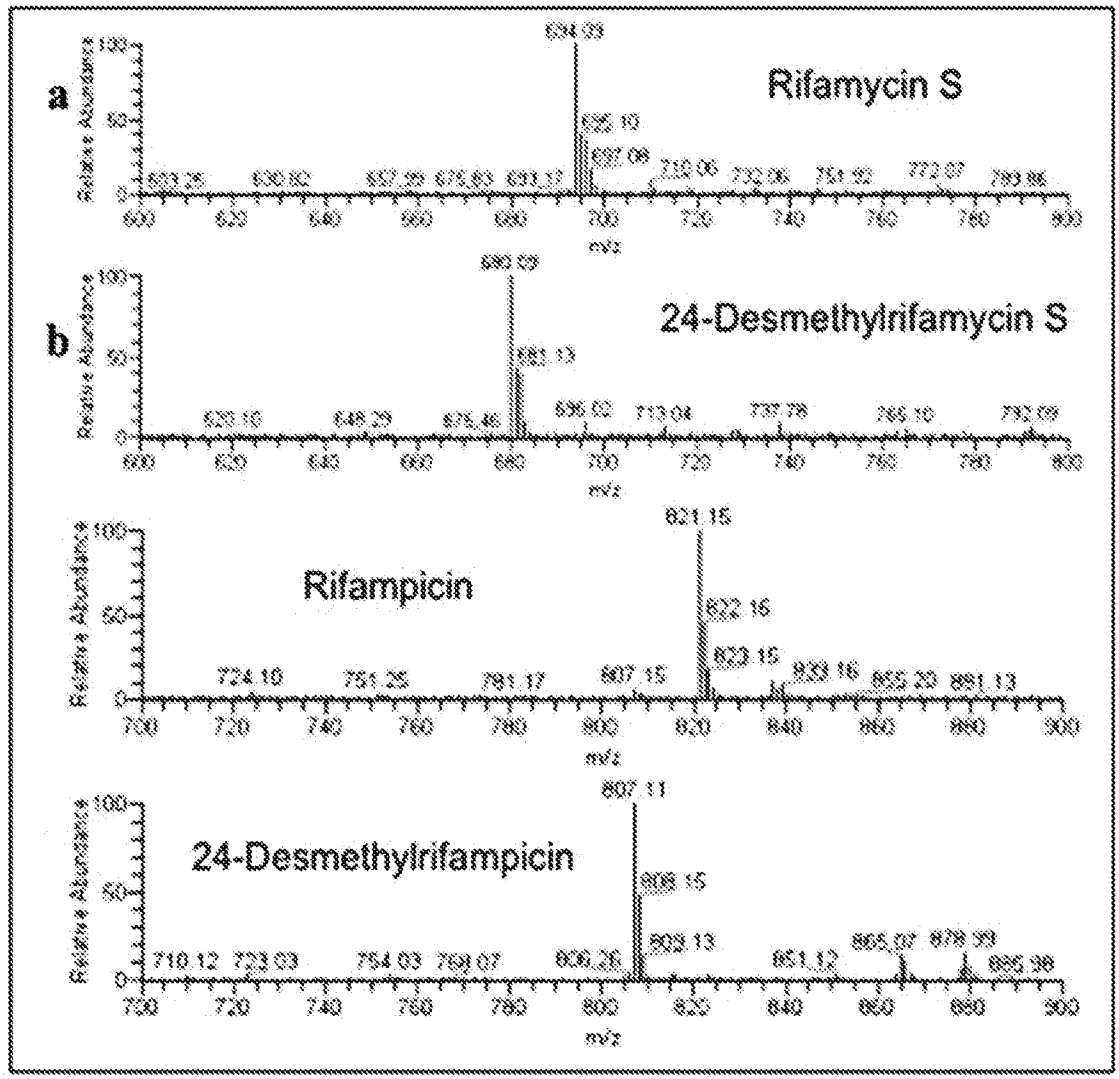

Figure 16. (-)-ESI-MS spectra of (a) Rifamycin S, (b) 24-Desmethylrifamycin S, (c) Rifampicin, (d) 24-Desmethylrifampicin.

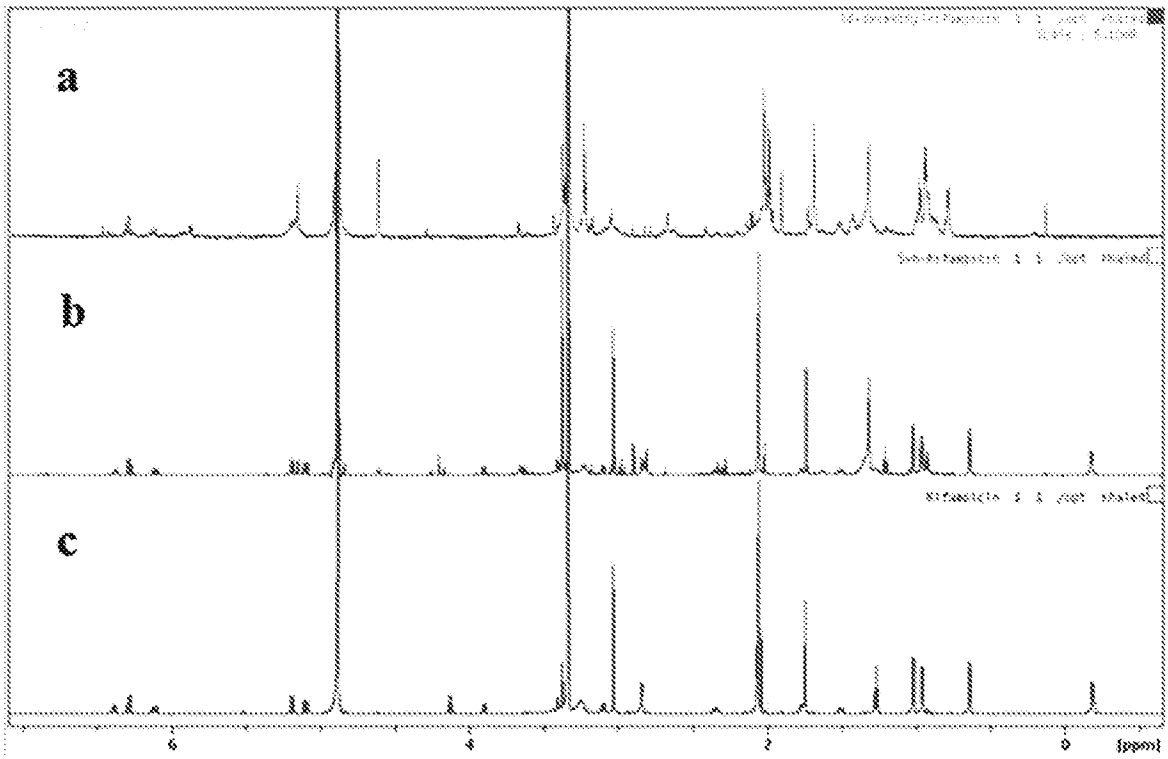

Figure 17. ^{1}H NMR spectra of: **(a)** 24-desmethylrifampicin, **(b)** chemically synthesized rifampicin, **(c)** commercially available rifampicin.

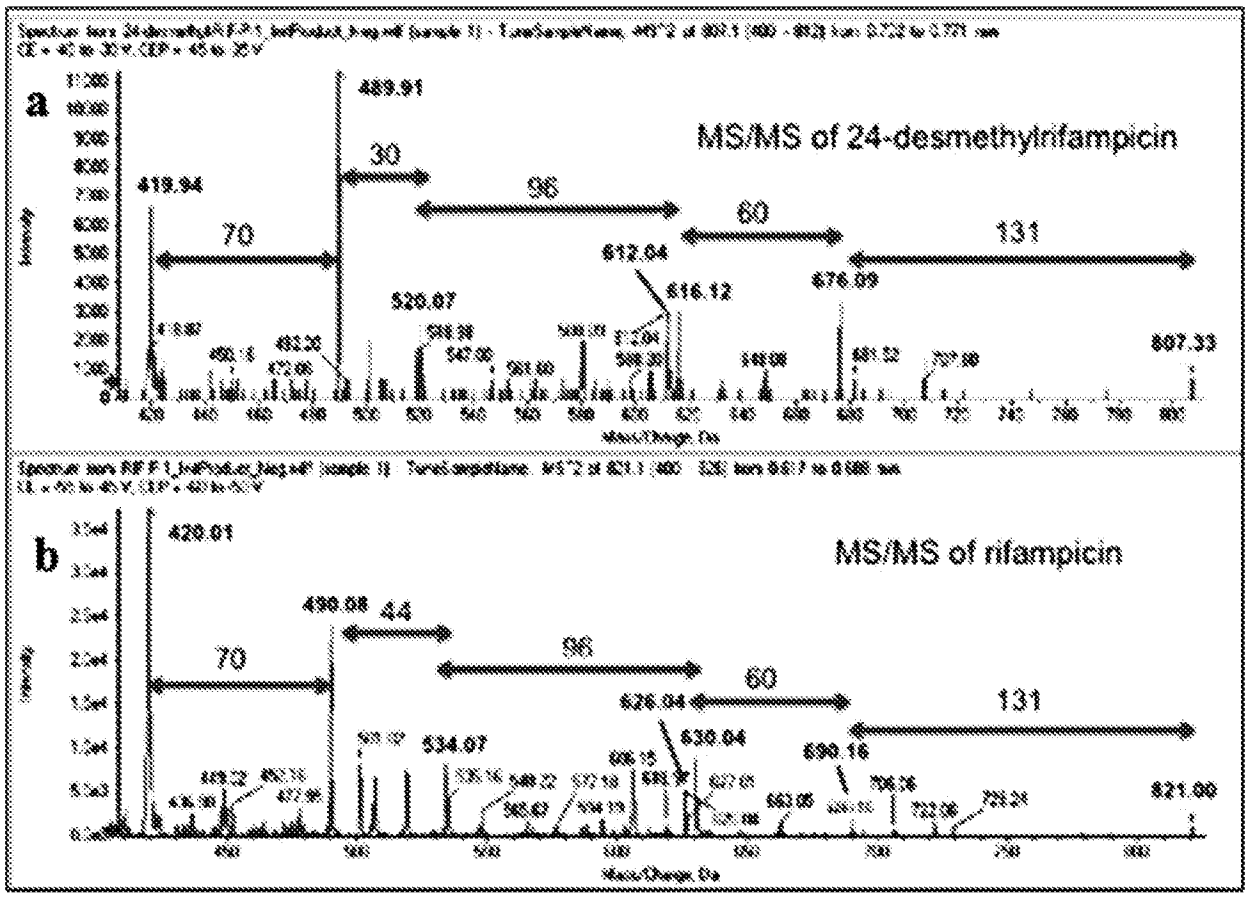

Figure 18. Comparative MS/MS Analysis of **(a)** 24-desmethylrifampicin and **(b)** Rifampicin

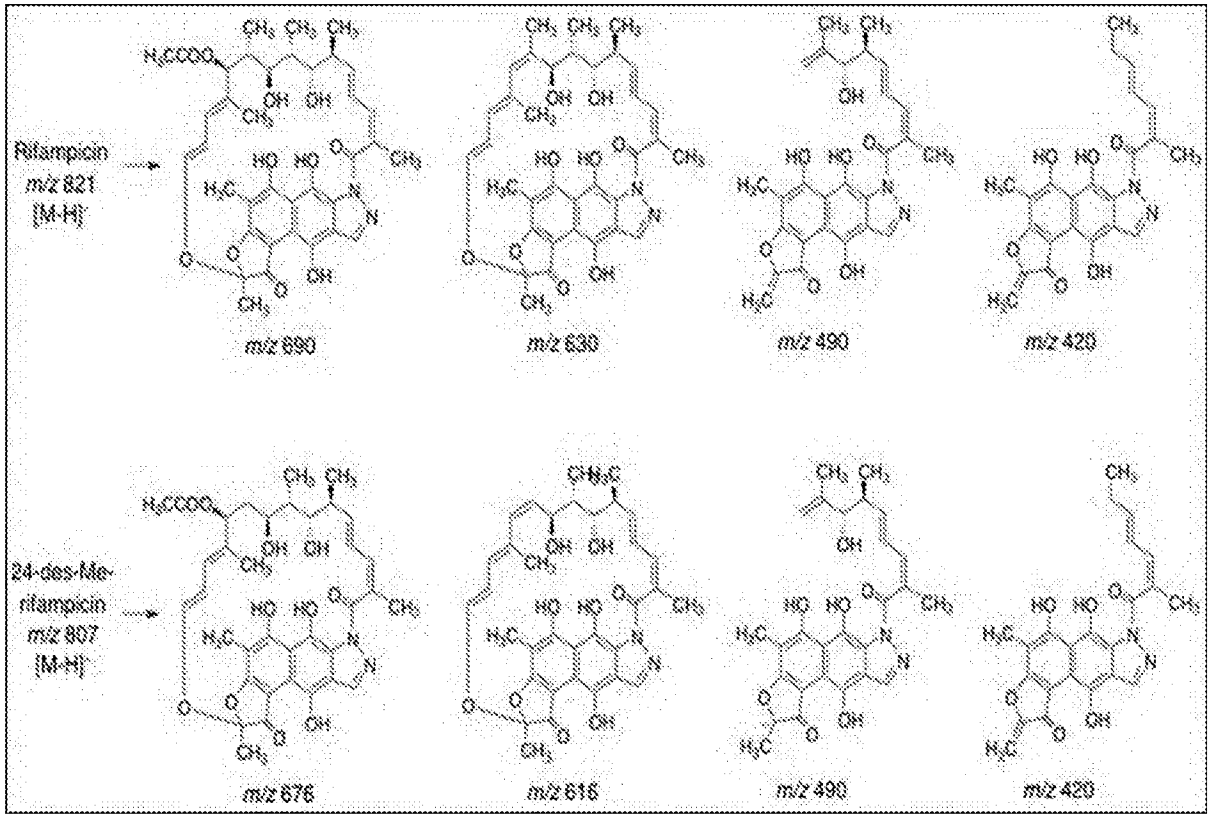

Figure 19. Characteristic MS fragments of rifampicin and 24-desmethylrifampicin in ESI negative ion mode.

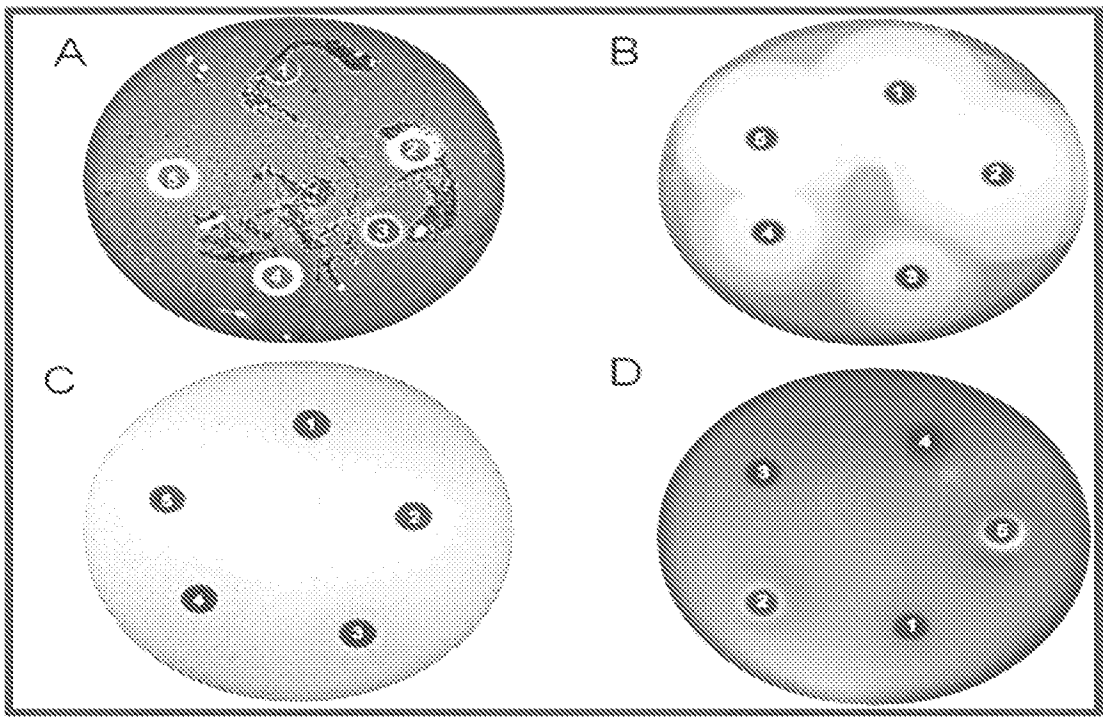

Figure 20: The results of the antibacterial activity of the new rifamycin analogs. (A) *M. smegmatis*, (B) *S. aureus*, (C) *B. subtilis* and (D) *E. coli*. Discs were loaded with 20 µL of 1 mg/mL of each compound: (1) rifamycin B, (2) rifamycin SV, (3) 24-desmethylrifamycin SV, (4) 24-desmethylrifamycin S and (5) rifampin.

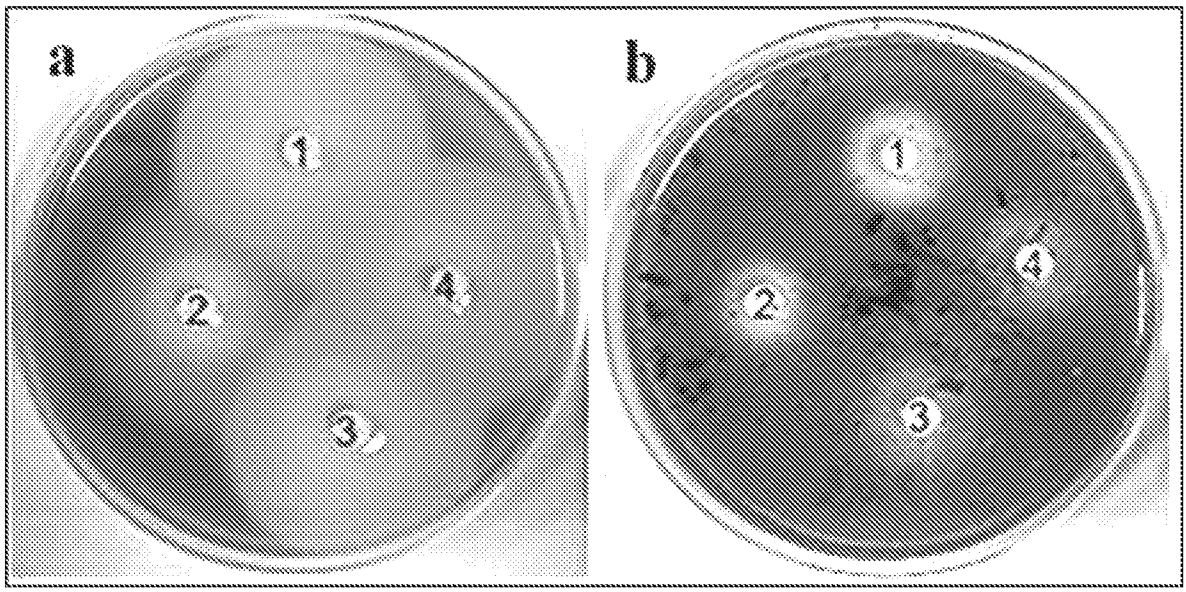

Figure 21. Agar diffusion assay of rifamycin S, rifampicin, 24-desmethylrifamycin S and 24-desmethylrifampicin against: (a) *Staphylococcus aureus*,(b) *Mycobacterium smegmatis*. (1) rifamycin S, (2) rifampicin, (3) 24-desmethylrifamycin S, (4) 24-desmethylrifampicin.

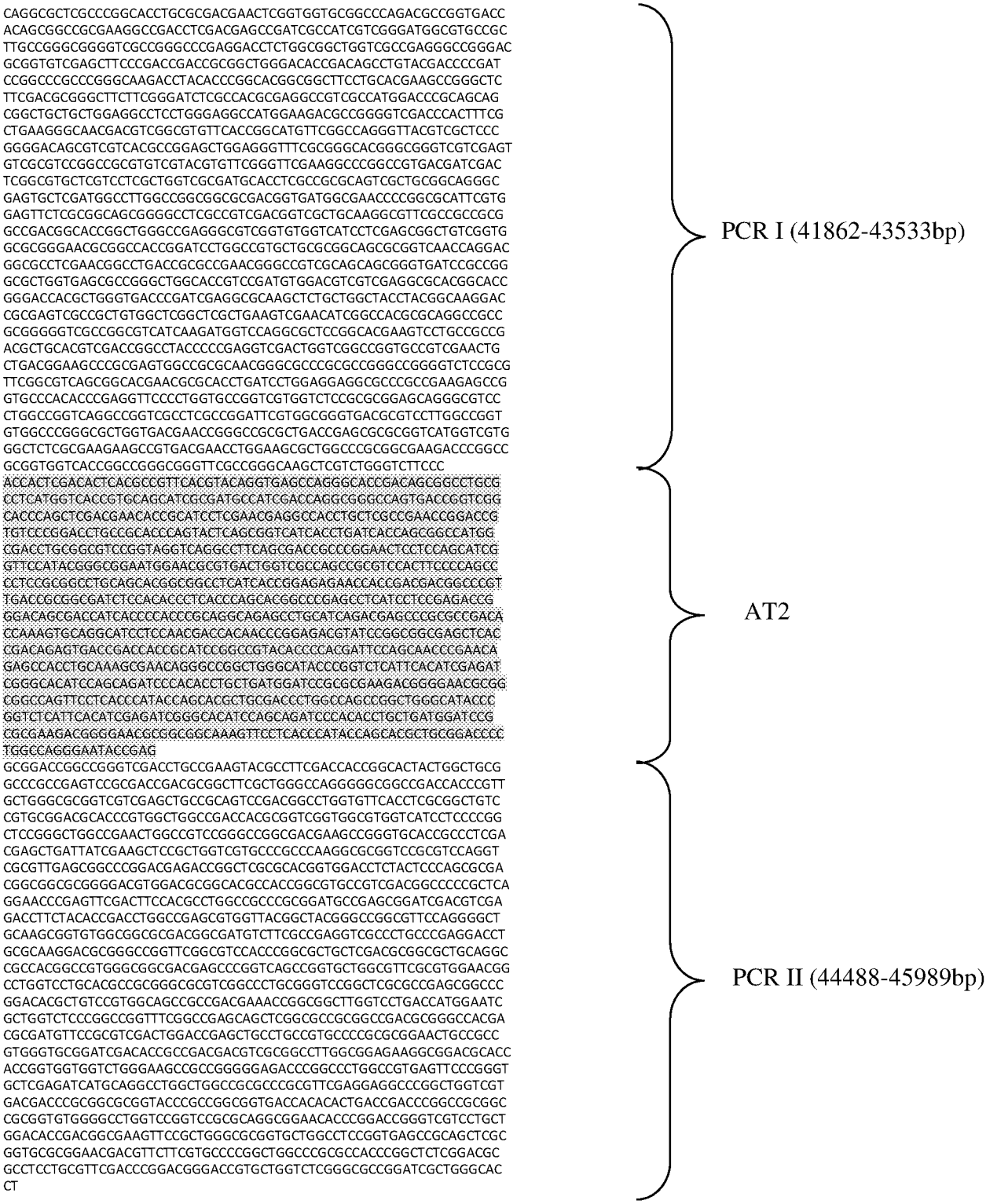

Figure 22. Nucleotide sequence ID No.1