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(71) Applicant: UNIVERSITY OF FLORIDA RESEARCH  
FOUNDATION, INC. [US/US]; 223 Grinter Hall,  
Gainesville, FL 32611 (US).

(72) Inventors: WANG, Nian; 700 Experiment Station Road,  
Lake Alfred, FL 33850 (US). AKULA, Nagaraju; 700 Ex-  
periment Station Road, Lake Alfred, FL 33850 (US).

(74) Agents: SALIWANCHIK, David, R. et al.; Saliwanchik,  
Lloyd & Eisenschenk, PO Box 142950, Gainesville, FL  
32614-2950 (US).

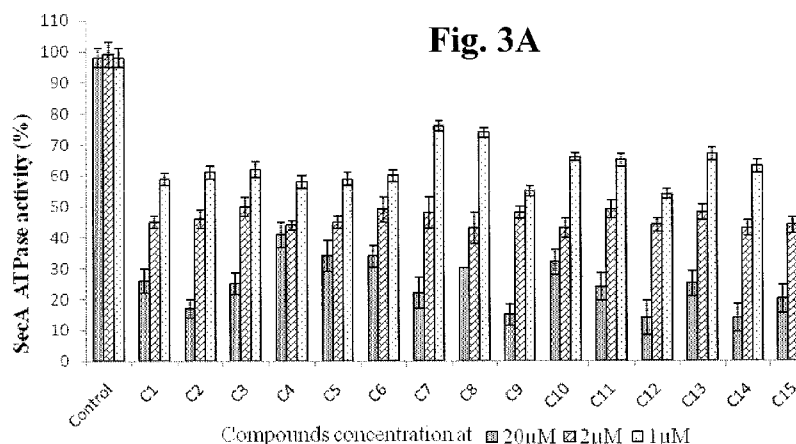
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(54) Title: ANTIMICROBIAL COMPOUNDS AND THEIR USE IN TREATING PLANT DISEASE



(57) Abstract: The subject invention is directed toward antimicrobial agents and methods of treating and preventing infection by pathogenic microorganisms and endophytic microorganisms in a plant through the use of SecA inhibitors.

## DESCRIPTION

## ANTIMICROBIAL COMPOUNDS AND THEIR USE IN TREATING PLANT DISEASE

## BACKGROUND OF INVENTION

Bacterial plant pathogens pose unique problems for disease control. One primary control strategy for bacterial diseases is based on excluding the pathogen through the use of disease free seed, or quarantine and eradication if bacterial pathogens are introduced into an area.

There are only a few chemical control agents for established bacterial diseases, and their use is often limited because of phytotoxicity or pathogen mutations resulting in resistance to the agent. Also, commonly applied protective copper compounds (for example sulfates or oxides) have limited benefit in controlling bacterial diseases because of their poor penetration into plant tissues where bacteria establish themselves and, again, mutations provide bacteria with resistance to these materials.

Unlike the control of disease outbreaks in annual crops that can be remediated in subsequent years through sanitation and the use of bacteria-free seed stocks, replanting of perennial crops such as citrus involves high capital costs to establish the planting, and several years after planting before production is initiated.

Established bacterial diseases such as those caused by *Candidatus Liberibacter* species (citrus greening or Huanglongbing, psyllid yellows and tomato, or purple top and zebra chip of potatoes, etc.) that survive in alternate host plants in the environment and are disseminated by insect vectors that commonly infect throughout the plant life cycle are very difficult to contain because of the wide dissemination range of the insect vector and the long lag time for symptom expression (Bove, J. M. (2006) Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *Journal of Plant Pathology* 88:7-37.). Unfortunately, recent attempts to culture the organism were met with limited success (Sechler, A., Schuenzel, E. L., Cooke, P., Donnua, S., Thaveechai, N., Postnikova, E., Stone, A. L., Schneider, W. L., Damsteegt, V. D. and Schaad, N. W. (2009). Cultivation of ‘*Candidatus Liberibacter asiaticus*’, ‘*Ca. L. africanus*’, and ‘*Ca. L. americanus*’ associated with Huanglongbing. *Phytopathology* 99, 480-486).

Huanglongbing (HLB) disease (also known as citrus greening or yellow dragon disease) is one such disease associated with the fastidious, Gram-negative, phloem-limited bacterial pathogen, *Candidatus Liberibacter* spp. (Las). It is the most destructive citrus disease worldwide (da Graca, J. V. (1991). Citrus greening disease. *Ann. Rev. Phytopathol.* 29, 109-136; Halbert, S. E., and Manjunath, K. L. (2004). Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: A literature review and assessment of risk in Florida. *Fla. Entomol.* 87, 330-353; and Gottwald, T. R. (2010). Current epidemiological understanding of citrus Huanglongbing. *Ann. Rev. Phytopathol.* 48, 119–139). The current management strategy of HLB is to chemically control psyllids and scout for and remove infected trees. However, current management practices have not been able to stop the spread of HLB disease (Duan, Y., Zhou, L., Hall, D. G., Li, W., Doddapaneni, H., Lin, H., Liu, L., Vahling, C. M., Gabriel, D. W., Williams, K. P., Dickerman, A., Sun, Y. and Gottwald, T. (2009). Complete genome sequence of citrus huanglongbing bacterium, '*Candidatus Liberibacter asiaticus*' obtained through metagenomics. *Mol. Plant Microbe Interact.* 22, 1011-1020).

*Candidatus Liberibacter* species plug the plant's vascular (phloem) tissues to limit nutrient movement. Symptoms of this disease reflect a severe deficiency of essential mineral nutrients (for example copper, manganese, zinc). A temporary masking of symptoms can be achieved by applying high rates of foliar nutrients; however, the bacterial pathogen remains active and infected trees continue to decline in over-all vigor and productivity. The lag time from infection to symptom expression for this disease varies from six months to five years depending on the age of the tree, vigor, and environmental factors (Bove (2006) *J Plant Pathology* 88:7-37). This lag in symptom expression provides ample time for infection before detection and containment in a new area can be accomplished.

The efficacy of current strategies for management of HLB is limited and no conventional measure has shown to provide consistent and effective suppression of the disease. High cost of frequent insect control and tree removal will eventually render citrus groves unprofitable. In addition, large scale application of insecticides will disrupt the ecosystem and pollute the environment (Jun, L. and Xing-Vao. J. (2005). Ecological control of forest pest: a new strategy for forest pest control. *J. Forestry Res.* 16, 339-342). Frequently, insecticides will become non-effective due to the acquisition of resistance. Insecticides could also kill non-target beneficial insects which disrupt the biological control currently in place.

Antibiotics injected into the tree's vascular system are often toxic to the tree, and previously available surface--applied copper compounds are not mobile enough to inhibit bacterial activity within vascular or other plant tissues. Current HLB control strategies of frequent insecticide sprays to limit populations of the psyllid insect vector, removal of infected trees, and nutrient maintenance to keep existing trees as productive as possible until they die provide little confidence for a sustainable citrus industry or incentive to reestablish it (Bove (2006) *J Plant Pathology* 88:7-37; UF/IFAS SWFREC, IMMOKALEE IRREC Seminar, 5 Jun. 2009).

Prokaryotes and eukaryotes have evolved numerous systems for the active export of proteins across membranes. In bacteria, the most common form of secretion of peptides with a signal sequence involves the Sec system. SecA is a protein translocase ATPase subunit that is involved in pre-protein translocation across and integration into the cellular membrane in bacteria. It is one essential component of the Sec machinery which provides a major pathway of protein translocation from the cytosol across or into the cytoplasmic membrane (Manting, E.H., and Driessen, A.J. (2000). *Escherichia coli* translocase: the unravelling of a molecular machine. *Mol. Microbiol.* 37, 226–238). Thus, SecA is a promising antimicrobial agent because it is a protein conserved and essential in all bacteria and is absent in humans (Chen, W., Huang, Y.J., Gundala, S.R., Yang, H., Li, M., Tai, P.C. and Wang, B. (2010). The first low microM SecA inhibitors. *Bioorg. Med. Chem.* 18, 1617-1625; Li, M., Tai, P.C., and Wang, B. (2008). Discovery of the first SecA inhibitors using structure-based virtual screening. *Biochem. Biophys. Res. Commun.* 368, 839-845; and Jang, M. Y., De Jonghe, S., Segers, K., Anné, J., Herdewijn, P. (2011). Synthesis of novel 5-amino-thiazolo[4,5-d]pyrimidines as *E. coli* and *S. aureus* SecA inhibitors. *Bioorg. Med. Chem.* 19, 702).

SecA cooperates with the SecB chaperone to target pre-proteins to SecYEG as an active ATPase gene to drive protein translocation across the bacterial membrane when it is bound to the SecYEG complex (Economou, A., and Wickner, W. (1994). SecA promotes preprotein translocation by undergoing ATP-driven cycles of membrane insertion and deinsertion. *Cell.* 78, 835–843). SecA is the peripheral membrane ATPase, which couples the hydrolysis of ATP to the stepwise translocation of pre-proteins (Van den Berg, B., Clemons, W. M. J., Collinson, I., Modis, Y., Hartmann, E., Harrison, S.C., and Rapoport, T. A. (2004). X-ray structure of a protein-conducting channel. *Nature.* 427, 36–44). The crystal structures of SecA are available for other bacteria such as *Escherichia coli* (Papanikolau, Y.,

Papadovasilaki, M., Ravelli, R. B., McCarthy, A. A., Cusack, S., Economou, A., Petratos, K. (2007). Structure of dimeric SecA, the *Escherichia coli* preprotein translocase motor. J. Mol. Biol. 366, 1545-1557) and the ATPase active site has been clearly defined. This structural information had been utilized for structure based design to identify antimicrobial compounds with IC<sub>50</sub> value up to 2.5  $\mu$ M against SecA of *Ca. L. asiaticus* (Akula, N., Zheng, H., Han, F. Q., Wang, N. (2011). Discovery of novel SecA inhibitors of *Candidatus Liberibacter asiaticus* by structure based design. Bioorg. Med. Chem. Lett. 15, 4183-4188).

Development of alternative or complementary approaches for effective management of the disease is highly desirable and will greatly help the citrus industry due to the difficulty to control the HLB disease. Considering the highly destructive nature of HLB disease and the lack of control measures, there is a huge potential to develop antimicrobial small molecules against the causal agent thus to suppress the population of *Ca. L. asiaticus* in plants and to reduce the inoculum for psyllid transmission. Development of antimicrobial small molecules may provide economic and ecological benefits by reducing producing costs, decreasing insecticide application, preserving the natural habitat and populations of beneficial insects, and enhancing productivity of citrus in the presence of HLB.

There is thus a need for antimicrobials that (i) are not subject to the types of antibiotic resistance currently hampering antibiotic treatment of bacteria, (ii) can be developed rapidly and with some reasonable degree of predictability as to target-bacteria specificity, (iii) are effective at low doses, meaning, in part, that they are efficiently taken up by wild-type bacteria or even bacteria that have reduced permeability for antibiotics, and (iv) show few side effects. In particular, there is currently a need in the art for an effective antimicrobial compound to target *Ca. L. asiaticus*.

#### BRIEF SUMMARY

The present invention provides novel compositions and methods for improving plant health and controlling phytopathogenic bacteria and endophytic microorganisms on or within plant tissue.

The subject invention provides antimicrobial compounds that interrupt, in bacteria, the ATP-hydrolysis process in a Sec system and disrupt pre-protein translocation. In one embodiment, SecA inhibitors are provided that interfere with the ATP binding pocket of SecA, thus affecting protein translocation, including potential signal peptide dependent

virulence factors. In a specific embodiment, the SecA inhibitors affect protein translocation and potential signal peptide dependent virulence factors of *Ca. L. asiaticus*.

In accordance with the subject invention, SecA inhibitors are administered to plants to contact bacteria. In one embodiment, an SecA inhibitor is directly injected into the plant vascular system.

The compounds of the invention can be used against bacterial diseases of annual as well as perennial crops and ornamental plants. In preferred embodiments, the SecA inhibitors are particularly effective against *Ca. L. asiaticus* in citrus plants.

### BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawings(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

**Figure 1** illustrates a homology model of *Ca. L. asiaticus* SecA used for structure based design. (A) Superimposed three-dimensional monomeric structures of SecA ATPase of *Ca. L. asiaticus* and *Escherichia coli* SecA (2FSG.pdb). The RMS deviation between these two proteins is 0.8Å. Various domains of the protein structure were represented in different colors and defined as: NBD (Nucleotide Binding Domain), IRA (1 or 2) Intramolecular Regulator of ATPase, VAR (Variable region of IRA 2), CTD (Carboxy-Terminal Domain also called C-domain), SD (scaffold sub-domain) and WD (wing sub-domain). ATP binding site is in between NDB and IRA2-VAR domains in blue and green colors. (B) Intermolecular interactions of ligand-protein (ATP - SecA of *Las*) complex after molecular minimization. H-bond interactions are observed between ATP and SecA active site residues including R344, G79, K82, T83, L84, Q61 & R56 and  $\pi$ - $\pi$  interactions with F58 are also present. ATP is represented as stick model with dot surface and the remaining active site residues are in sticks.

**Figure 2** provides the structures of 20 inhibitory compounds (C1–C20) against SecA of *Las*.

**Figure 3** shows inhibitory activities of C1-C20 compounds against SecA of *Ca. L. asiaticus*. (A) Compounds 1–15 at 20µM, 2µM & 1µM concentrations; (B) Compounds C16–C20 at 1 µM, 750 nM, 500 nM, 250 nM, 200 nM, 100 nM & 50 nM concentrations.

**Figure 4** illustrates the molecular docking interactions of SecA with different ligands including ATP, high & low activity structures. Intermolecular H-Bond interactions, between ligand – protein complexes. (A) ATP-SecA active site interactions with R344, R112, T78, T83, Q64 & P57 (Dock Score: -8.7412 k.cal/mol); (B) C16-SecA active site interactions with R344, T83, K82 & Q64 (Docking Score: -7.2140 k.cal/mol); (C) C4-SecA active site interactions with T83 & Q64 (Docking Score: -5.6561 k.cal/mol).

**Figure 5** illustrates the intermolecular interactions of SecA with ligand after molecular minimization. Intermolecular (H-bond, Hydrophobic &  $\pi$ - $\pi$ ) interactions between ligand – Protein complexes. (A) C16-SecA active site interactions with R344, D347, T78, G79, K82, T83, R112 & F58 (Binding Energy: -73878 k.cal/mol); (B) C4-SecA active site interactions with D347, T83, L84 & F58 (Binding Energy: -73039 k.cal/mol).

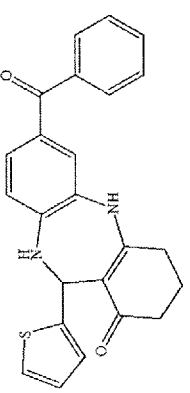
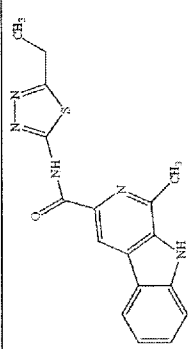
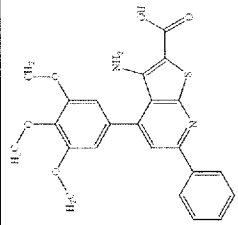
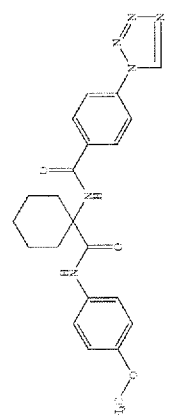
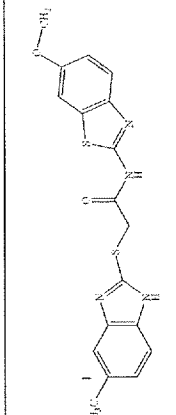
#### DETAILED DISCLOSURE

The invention provides SecA inhibiting compounds that have antimicrobial activities. The term “inhibiting” indicates a reduction in the rate or amount of a measurable interaction. An SecA inhibiting compound of the present invention inhibits SecA activity in a Sec system, preferably in a bacterial Sec system, as measured by a block or delay in the ATP-hydrolysis process in a Sec system.

The SecA inhibiting compounds of this invention can be used in treating bacterial diseases of annual as well as perennial crops and ornamental plants. Preferably, the SecA inhibiting compounds are used with citrus plants; however, in other embodiments, the compounds of the invention can be used with a variety of plant species including, for example, trees, vines, forage, and annual plants.

The SecA inhibiting compounds of the invention are active against a variety of bacteria, including *Ca. L. asiaticus*. According to the subject invention, the SecA inhibiting compound interferes with the ATP binding pocket of SecA, thus affecting pre-protein translocation, including potential signal peptide dependent virulence factors of *Ca. L. asiaticus* and other bacteria.

Examples of SecA inhibiting compounds of the invention are as follows:

| Structure                                                                             | ID  | Mol Weight | Mol Formula                                                                  | Mol Name                                                                             | cLogP | LogSW  | hDon | hAcc |
|---------------------------------------------------------------------------------------|-----|------------|------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|-------|--------|------|------|
|    | C20 | 400.5      | C <sub>24</sub> H <sub>20</sub> N <sub>2</sub> O <sub>2</sub> S              | 7-benzoyl-11-(2-thienyl)-2,3,4,5,10,11-hexahydro-1H-diabenzo[b,e][1,4]diazepin-1-one | 3.95  | -5.493 | 2    | 2    |
|    | C16 | 337.4      | C <sub>17</sub> H <sub>15</sub> N <sub>5</sub> OS                            | N-(5-ethyl-1,3,4-thiadiazol-2-yl)-1-methyl-9H-beta-carboline-3-carboxamide           | 3.47  | -5.734 | 2    | 4    |
|   | C17 | 436.5      | C <sub>23</sub> H <sub>20</sub> N <sub>2</sub> O <sub>5</sub> S              | 3-amino-6-phenyl-4-(3,4,5-trimethoxyphenyl)thieno[2,3-b]pyridine-2-carboxylic acid   | 5.03  | -6.677 | 2    | 6    |
|  | C18 | 420.5      | C <sub>22</sub> H <sub>24</sub> N <sub>6</sub> O <sub>3</sub>                | N-(1-[(4-methoxyphenyl)amino]carbonyl)cyclohexyl-4-(1H-tetrazol-1-yl)benzamide       | 3.494 | -5.294 | 2    | 6    |
|  | C19 | 384.5      | C <sub>18</sub> H <sub>16</sub> N <sub>4</sub> O <sub>2</sub> S <sub>2</sub> | N-(6-methoxy-1,3-benzothiazol-2-yl)-2-[(5-methyl-1H-benzimidazol-2-yl)thio]acetamide | 4.67  | -7.094 | 2    | 4    |

Their potent antimicrobial action is evident from their antimicrobial activities against *Agrobacterium tumefaciens* with MBC ranging from 128 µg/ml to 256 µg/ml

In view of the aforementioned antimicrobial activities, the subject invention provides compositions comprising SecA inhibiting compounds, and salts thereof, as the active ingredient in anti-microbial compositions. The subject invention further provides methods of combating microbial growth through the use of an effective amount of the SecA inhibiting compounds, or salts thereof.

The subject compounds can be used in suitable solvents or diluents, in the form of emulsions, suspensions, dispersions, on suitable solid or semi-solid carrier substances, if desired, together with other compounds having antimicrobial activity.

Solid carrier substances that are suitable for the preparation of compositions in powder form include various inert, porous and pulverous distributing agents of inorganic or organic nature, such as, for example, tricalcium phosphate, calcium carbonate, in the form of prepared chalk or ground limestone, kaolin, bole, bentonite, talcium, kieselguhr and boric acid; powdered cork, sawdust, and other fine pulverous materials of vegetable origin are also suitable carrier substances.

The active ingredient is mixed with these carrier substances by, for example, by being ground therewith. Alternatively, the inert carrier substance can be impregnated with a solution of an SecA inhibiting compound of the invention in a volatile solvent and the solvent is thereafter eliminated by heating or by filtering with suction at reduced pressure. By adding wetting and/ or dispersing agents, such pulverous preparations can also be made readily wettable with water, so that suspensions are obtained.

Inert solvents used for the production of liquid preparations should preferably not be flammable and should be as far as possible odorless and as far as possible non-toxic to warm-blooded animals or plants in the relevant surroundings. Solvents suitable for this purpose are high-boiling oils, for example, of vegetable origin, and lower-boiling solvents with a flash point of at least 30°C, such as, for example, polyethylene glycol isopropanol, dimethylsulfoxide, hydrogenated naphthalenes and alkylated naphthalenes. It is, of course, also possible to use mixtures of solvents. Solutions can be prepared in the usual way, if necessary, with assistance of solution promoters.

Other liquid forms that can be used include emulsions or suspensions of the active compound in water or suitable inert solvents, or also concentrates for preparing such emulsions, which can be directly adjusted to the required concentration. For this purpose, an SecA inhibiting compound of the invention can be, for example, mixed with a dispersing or emulsifying agent. The active component can also be dissolved or dispersed in a suitable inert solvent and mixed simultaneously or subsequently with a dispersing or emulsifying agent.

Furthermore, it is possible for the SecA inhibiting compounds of the invention to be used in the form of aerosols. For this purpose, the active component is dissolved or dispersed, if necessary, with the aid of suitable inert solvents as carrier liquids, such as difluorodichloromethane, which at atmospheric pressure boils at a temperature lower than room temperature, or in other volatile solvents. In this way, solutions under pressure are obtained which, when sprayed, yield aerosols which are particularly suitable for controlling or combatting fungi and bacteria, *e.g.*, in closed chambers and storage rooms, and for application to vegetation for eradicating or for preventing infections by bacteria.

The subject compounds and compositions thereof can be applied by conventional methods. For example, a plant to be treated or to be protected against attack by bacterium can be treated with the subject compounds and the compositions thereof by dusting, sprinkling, spraying, brushing, dipping, smearing, impregnating, injection into the vascular system, application to root system or other suitable means. In a preferred embodiment, the SecA inhibiting compounds of the invention are directly injected in the vascular system of a plant.

When the subject SecA inhibiting compounds are employed in combination with suitable carriers, *e.g.*, in solution, suspension, dust, powder, ointment, emulsion, and the like forms, a high activity over a very high range of dilution is observed. For example, concentrations of the SecA inhibiting compounds can range from 100 $\mu$ M, 500 $\mu$ M and 1mM. Of course, higher or lower concentrations may also be employed as warranted by the particular situation. Moreover, the subject SecA inhibiting compounds can be employed with other programs, such as commercial fertility programs.

Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

## EXAMPLE 1—Activity of SecA inhibiting compounds

**Materials & Methods***Computational methodology*

Homology model of SecA was built with Prime structure prediction of Schrodinger software (Schrödinger, LLC (2005) Schrödinger Suite 2006. Induced Fit Protocol, Prime version 1.5, Schrödinger, LLC, New York). Reported X-ray crystal structures in Protein Data Bank (PDB ID: 2VDA, 2FSF & 2FSG) (Papanikolaou, Y., Papadovasilaki, M., Ravelli, R. B., McCarthy, A. A., Cusack, S., Economou, A., Petratos, K. (2007). Structure of dimeric SecA, the *Escherichia coli* preprotein translocase motor. J. Mol. Biol. 366, 1545-1557; and Gelis, I., Bonvin, M. A., Keramisanou, D., Koukaki, M., Gouridis, G., Karamanou, S., Economou, A., Kalodimos, C. G. (2007). Structural Basis for Signal-Sequence Recognition by the Translocase Motor SecA as Determined by NMR. Cell. 131, 756-769) of *E. coli* SecA homodimer bound with ATP was used to build SecA homology model of Las. The coordinates for all reported proteins were obtained from the PDB.

Structures were prepared using the Maestro (Schrödinger, LLC (2005). Maestro, Version 7.5, Schrödinger, LLC, New York) software package and aligned using the Protein Structure Alignment module in Prime. If a PDB structure was missing side-chain atoms, Prime was used to predict their locations. Water molecules in all structures were removed. A brief relaxation was performed on each starting structure using the *Protein Preparation* module in Maestro with the “Refinement Only” option. The modeled protein structure was prepared with appropriate bond orders and formal charges by protein preparation wizard of Maestro module. Then ATP ligand was manually docked as reported (Akula, N., Zheng, H., Han, F. Q., Wang, N. (2011). Discovery of novel SecA inhibitors of *Candidatus Liberibacter asiaticus* by structure based design. Bioorg. Med. Chem. Lett. 15, 4183-4188) against the homology model and ligand-protein complexes were energy minimized. The receptor grid file was generated by excluding ATP and defining 8Å radius from ATP without any constraints.

Structure-based virtual screening was used to screen putative SecA inhibitors from approximately 11 million small molecule compounds available from ZINC<sup>11</sup> database (Irwin and Shoichet, (2005). ZINC – A Free Database of Commercially Available Compounds for Virtual Screening. J. Chem. Inf. Model. 45, 177-182). To reduce the workload into the pipeline, twenty thousand structures were selected based on the physicochemical properties

e.g. Net charge (-1 to +1), H-Bond donor/acceptor (2-6), and molecular weight (300-600 Da). Glide program (Friesner, R. A., Banks, J. L., Murphy, R. B., Halgren, T. A., Klicic, J. J., Mainz, D. T., Repasky, M. P., Knoll, E. H., Shelley, M., Perry, J. K., Shaw, D. E., Francis, P., Shenkin, P. (2004). Glide: A New Approach for Rapid, Accurate Docking and Scoring. 1. Method and Assessment of Docking Accuracy. J. Med. Chem. 47, 1739– 1749) was used to build receptor grid file and for the docking studies.

Molecular minimization of ATP, highly active ligands with protein complexes were performed by MacroModel suite program (Mohamadi, F., Richards, N. G. J., Guida, W.C., Liskamp, R., and Lipton, M. (1990). MacroModel - an Integrated Software System for Modeling Organic and Bioorganic Molecules Using Molecular Mechanics. J. Comput. Chem. 11, 440–467). OPLS (Jorgensen, W. L., Maxwell, D. S., and Tirado-Rives, J. (1996). Development and Testing of the OPLS All-Atom Force Field on Conformational Energetics and Properties of Organic Liquids. J. Am. Chem. Soc. 118, 11225-11236) force field and “distance-dependent” dielectric constant were used during minimization. All the minimizations were carried out by means of 5000 iterations of Polak–Ribiere conjugate gradient method and followed by converge on gradient until a convergence threshold of 0.05 kJ/mol.Å. All the molecular modeling studies have been performed on HP ProLiant, RedHat Linux operating system and docking postures were taken by PYMOL program (DeLano, L. L. (2002).

#### *Inhibition assay against SecA of Las*

Cloning, expression, and purification of SecA were conducted as described previously (Akula, N., Zheng, H., Han, F. Q., Wang, N. (2011). Discovery of novel SecA inhibitors of *Candidatus Liberibacter asiaticus* by structure based design. Bioorg. Med. Chem. Lett. 15, 4183-4188). Quantichrom ATPase/GTPase kit (Bioassay Systems, Hayward, CA) was used to test the inhibitory effect of different compounds on SecA of *Ca. L. asiaticus* as described previously (Denis, L., Guy, G.P., and Andrien, R. B. (1978). A convenient method for the ATPase assay. Anal. Biochem. 85, 86-89; and Richard, D.H., John, L.V., and Richard, A. W. (1998). A microassay for ATPase. Anal. Biochem. 169, 312-318). All these assays were done in triplicate and repeated three times with similar results. The OD values are measured from Benchmark plus ELISA micro plate spectrophotometer (Bio-Rad Hercules, CA). ATPase activities were determined by the release of phosphate ion (Pi) detected

spectrophotometrically, using malachite green and inhibition was calculated by showing the percentage (%) of the remaining ATPase activities (Akula *ibid.*).

#### *Antimicrobial activity against A. tumefaciens*

*A. tumefaciens* was obtained from the culture collection of citrus associated bacteria initially isolated from the roots and rhizosphere of citrus and maintained in our laboratory as glycerol stock stored at -80°C. Minimum bactericidal concentrations (MBC) of the selected compounds against *A. tumefaciens* were determined by the broth microdilution method. Streptomycin was used as a positive control. Each small molecule compound was dissolved in DMSO and two-fold serially diluted with LB medium to give final concentrations of 8 µg/ml to 512 µg/ml. Aliquots of 100 µl were poured into 96-well microplates. *A. tumefaciens* was grown overnight and diluted with LB medium to give final concentrations  $5 \times 10^5$  colony-forming unit (CFU) ml<sup>-1</sup>. Samples of 100 µl were inoculated onto LB microdilution plates containing the tested compounds and incubated at 28 °C for 24 h. After 24 h of incubation at 28°C, 100 µl samples from different dilutions were transferred onto antibiotic-free LB agar plates, which were then incubated at 28°C. After incubation for 2 days, the number of colonies recovered was counted. Survival rate was determined as the percentage of recovered cells compared to viable cells in the initial inoculum. MBC was defined as the lowest drug concentration producing a survival rate of <0.1%. Experiments were carried out in triplicate and repeated three times.

## **Results & Discussion**

### *Homology modeling & optimization of ATP binding site*

Previously, only one PDB structure (2FSG) was used as a template to build the SecA homology model (Akula, N., Zheng, H., Han, F. Q., Wang, N. (2011). Discovery of novel SecA inhibitors of *Candidatus Liberibacter asiaticus* by structure based design. Bioorg. Med.

Chem. Lett. 15, 4183-4188). In this study, three PDB structures (2VDA, 2FSF and 2FSG) were used as templates to further optimize the homology model of SecA. The precise positions, orientations/conformations of the helices in the homology modeled SecA are slightly different from the reported templates. The homology model of SecA was used to superimpose on the reported PDB (2FSG) structure to identify the ATP binding site on SecA protein of *Ca. L. asiaticus* (Fig. 1). The ATP- binding site is located in between NBD and IRA2-VAR. ATP was extracted from 2FSG and was manually placed on SecA at the similar receptor site of the model and merged with the ligand protein complex (Fig. 1A). Then the ligand protein complex was minimized using OPLS5 force field with 5000 minimization cycles until the minimization was terminated once the RMSD reached a maximum value of 0.05Å. The intermolecular interactions were also observed. In the optimized complex, ATP retained its orientation without losing the H-bond interactions between ATP and SecA active site residues (R344, G79, K82, T83, L84, Q61 and R56). The observed  $\pi$ - $\pi$  interactions with F58 were similar to the reported crystal structure (Fig. 1B).

*Molecular docking: High throughput virtual screening/SP/XP*

High throughput screening (HTVS) by molecular docking was performed and calculated docking scores to filter the structures, and the filtrates were subjected to standard and extra precision docking to evaluate the scoring functions. Approximately, 20,000 structures identified from the physicochemical properties were subjected to glide HTVS to filter the low scoring ones. The initial step to screen the small molecule databases is grid generation. Receptor grid file was generated that enabled Glide (Friesner, R. A., Banks, J. L., Murphy, R. B., Halgren, T. A., Klicic, J. J., Mainz, D. T., Repasky, M. P., Knoll, E. H., Shelley, M., Perry, J. K., Shaw, D. E., Francis, P., Shenkin, P. (2004). Glide: A New Approach for Rapid, Accurate Docking and Scoring. 1. Method and Assessment of Docking Accuracy. *J. Med. Chem.* 47, 1739– 1749) to search for favorable interactions between the ligand and ATP binding-site region. The shape and properties of the receptor are represented on a set of grids for positioning and scoring ligand poses. From HTVS, approximately 5000 structures were selected based on the docking scores against SecA. The resulting poses of the docked compounds were ranked according to their Glide standard and extra precision docking scores. Compounds detected by 2D and 3D ligand-based searches were analyzed

with the binding modes, intermolecular interactions, and with the chemical intuition twenty compounds selected for biological activity studies (Fig. 2).

*Inhibitory assay against SecA ATPase activity of Ca. L. asiaticus and antimicrobial assay against A. tumefaciens*

The selected twenty compounds were tested for their inhibition against SecA ATPase activity. Among the twenty compounds, only five of them showed greater than 50% inhibition at 1  $\mu$ M (Fig. 3A). The IC<sub>50</sub> values of the five compounds C16, C17, C18, C19, and C20 were 0.25, 0.92, 0.48, 0.64, and 0.44  $\mu$ M respectively. These values are calculated based on the 50% remaining ATPase activity (Fig. 3B).

The minimal bactericidal concentrations (MBCs) of the five identified compounds and streptomycin were determined by broth microdilution method. The MBC values of five compounds C16, C17, C18, C19, C20 and streptomycin are 256, 256, 256, 128, 256 and 64  $\mu$ g/ml, respectively. The MBC values of all compounds are 2-4 fold higher than streptomycin.

*Molecular Docking and Minimization: Comparative study of ATP binding of high and low activity compounds)*

The optimized homology model of SecA was used for molecular docking study. To validate the generated grid model and molecular docking methodology, the ATP structure was docked to the active site of SecA of Las. Glide Xtra-Precision software was used to rigidly dock the ligand at the binding site of the Ca. L. asiaticus SecA without any constraints and water molecules. The obtained results indicate that the binding mode of ATP inside the receptor is similar and in agreement with the experimental mode of the crystal structure (Fig. 4A). This result indicates that the receptor grid file can be used to dock rigidly with the selected twenty structures. The observed dock results of the identified twenty structures showed similar binding orientations and  $\pi$ - $\pi$  interactions with Phe58 as ATP. The structural difference and binding mode between high & low activity structures were analyzed and compared with ATP in order to understand the differences between the activities among the compounds. Specifically ATP is involved in several H-Bond interactions in the active site

due to presence of more nucleophilic phosphate groups. Also the adenine moiety has  $\pi$ - $\pi$  stacking with the aromatic ring of residues Phe58 and forms H-Bond with Gln64 (Fig. 4A). The conformational orientation of rigidly docked ATP at *Ca. L. asiaticus* SecA binding sites is slightly different from its original minimized ATP-SecA complex. Nevertheless, the critical interactions with Arg344, Arg112, Thr83, Gly79 and Phe58 remained the same in both models (Fig. 1B & 4A). The high activity structure C16 displayed similar interactions as ATP, particularly in H-Bond interactions (involving residues Gln64, Lys82, Thr83 & Arg344) and  $\pi$ - $\pi$  stacking with Phe58 (Fig. 4B). The low activity structure C4 lacked of interactions with the inside binding site residues Gly81, Lys83 & Arg344 which are more important to keep the ligand inside the binding site to competitively inhibit the ATP. Although the low activity structure has  $\pi$ - $\pi$  stacking with its aromatic ring and Phe58 (Fig. 4C), the lack of internal interactions might be responsible for the lower activity of C4 compound. In addition, the docking scores also correlated well with the in vitro activities of C16 & C4. These experimental and theoretical results indicated the importance to study residue interactions for selecting compounds with higher activity against selected proteins.

To further understand the difference between high and low activity structures, energy minimization studies were performed after the molecular docking analysis. While the docking methodology facilitates studying the binding of the *Ca. L. asiaticus* SecA inhibitors with flexible and rigid binding modes, it does not allow the protein mobility. In order to give free molecular motion of SecA protein along with ligands, the ligand protein complexes were minimized and the binding energies calculated (Fig. 5). Interestingly, it was observed that the high activity structure C16 has hydrophobic interactions with active site residues (Thr78, Gly79 & Arg344) along with other contacts after energy minimization (Fig. 6A), whereas the low activity structure C4 is missing many of these interactions in energy minimized complex (Fig. 6B). This demonstrates that the electronic effects of these substituents have an impact toward higher affinity. The need for a strong H-bond donor/acceptor in the ligand is critical for the binding interactions between the ligand and active site residues. The lack of these interactions in low activity compounds results in minor competitive inhibition of ATP binding compared with high activity compounds.

**Conclusions**

Structure based virtual screening was performed to identify the novel SecA inhibitors of *Las*. Five compounds were found to inhibit the ATPase activity of SecA of *Ca. L. asiaticus* in nano molar concentrations. These compounds showed antimicrobial activities against *A. tumefaciens* with with MBC ranging from 128 µg/ml to 256 µg/ml. Molecular docking and minimizations studies showed the correlation between the experimental and theoretical studies.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

## CLAIMS

We claim:

1. A method of treating or preventing plant disease comprising administering to a plant in need thereof a composition comprising an effective amount of a SecA inhibiting compound selected from the group consisting of: N-(5-ethyl-1,3,4-thiadiazol-2-yl)-1-methyl-9H-beta-carboline-3-carboxamide (C16); 3-amino-6-phenyl-4-(3,4,5-trimethoxyphenyl)thieno[2,3-b]pyridine-2-carboxylic acid (C17); N-(1-[(4-methoxyphenyl)amino]carbonyl)cyclohexyl-4-(1H-tetrazol-1-yl)benzamide (C18); N-(6-methoxy-1,3-benzothiazol-2-yl)-2-[(5-methyl-1H-benzimidazol-2-yl)thio]acetamide (C19); and 7-benzoyl-11-(2-thienyl)-2,3,4,5,10,11-hexahydro-1H-diabenzo[b,e][1,4]diazepin-1-one (C20).

2. The method of claim 1, wherein the composition is administered to the plant by a method selected from the group consisting of dusting, sprinkling, spraying, brushing, dipping, smearing, impregnating, injection of the composition into plant vasculature, and application to a root system.

3. The method of claim 2, wherein the composition is administered to the plant by injecting the composition into plant vasculature or by application to a root system.

4. The method of claim 1, wherein the method is practiced in conjunction with application of a regular commercial fertilizer program.

5. The method of claim 1, wherein the plant is an annual or perennial ornamental plant.

6. The method of claim 1, wherein the plant is an annual or perennial crop plant.

7. The method of claim 6, wherein said plant is tree.

8. The method of claim 7, wherein said tree is a citrus tree.

9. The method of claim 1, wherein the plant disease is caused by *Candidatus liberibacter asiaticus*.

10. The method of claim 1, wherein the concentration of the SecA inhibiting compound in the composition is between about 100 $\mu$ M and 1 mM.

11. The method of claim 1, wherein the concentration of the SecA inhibiting compound in the composition is about 500 $\mu$ M.

12. The method of 1, wherein the composition comprises the SecA inhibiting compound in solution, suspension, dust, powder, ointment, or emulsion form.

13. An agricultural composition, formulated for administration to a plant, wherein said composition comprises a SecA inhibiting compound selected from the group consisting of: C16, C17, C18, C19, and C20; and a carrier.

14. The agricultural composition of claim 13, further comprising an antimicrobial.

15. The agricultural composition of claim 13, wherein the carrier is solid.

16. The agricultural composition of claim 13, wherein the carrier is liquid.

17. The agricultural composition of claim 13, further comprising a plant fertilizer.

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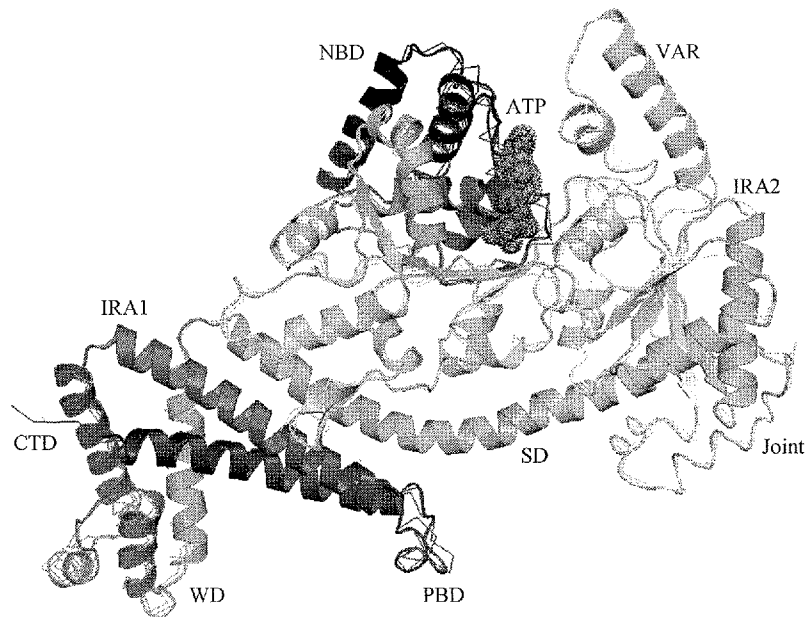


**Fig. 1A**

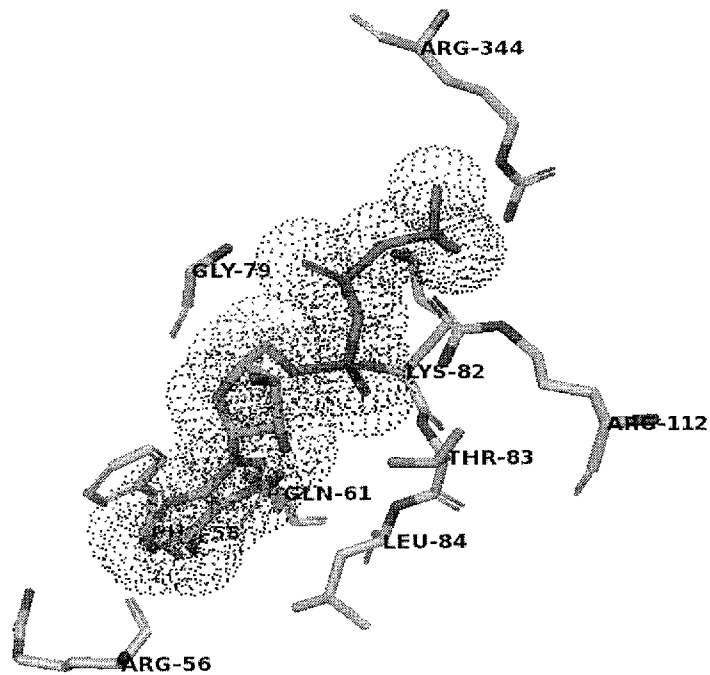


**Fig. 1B**

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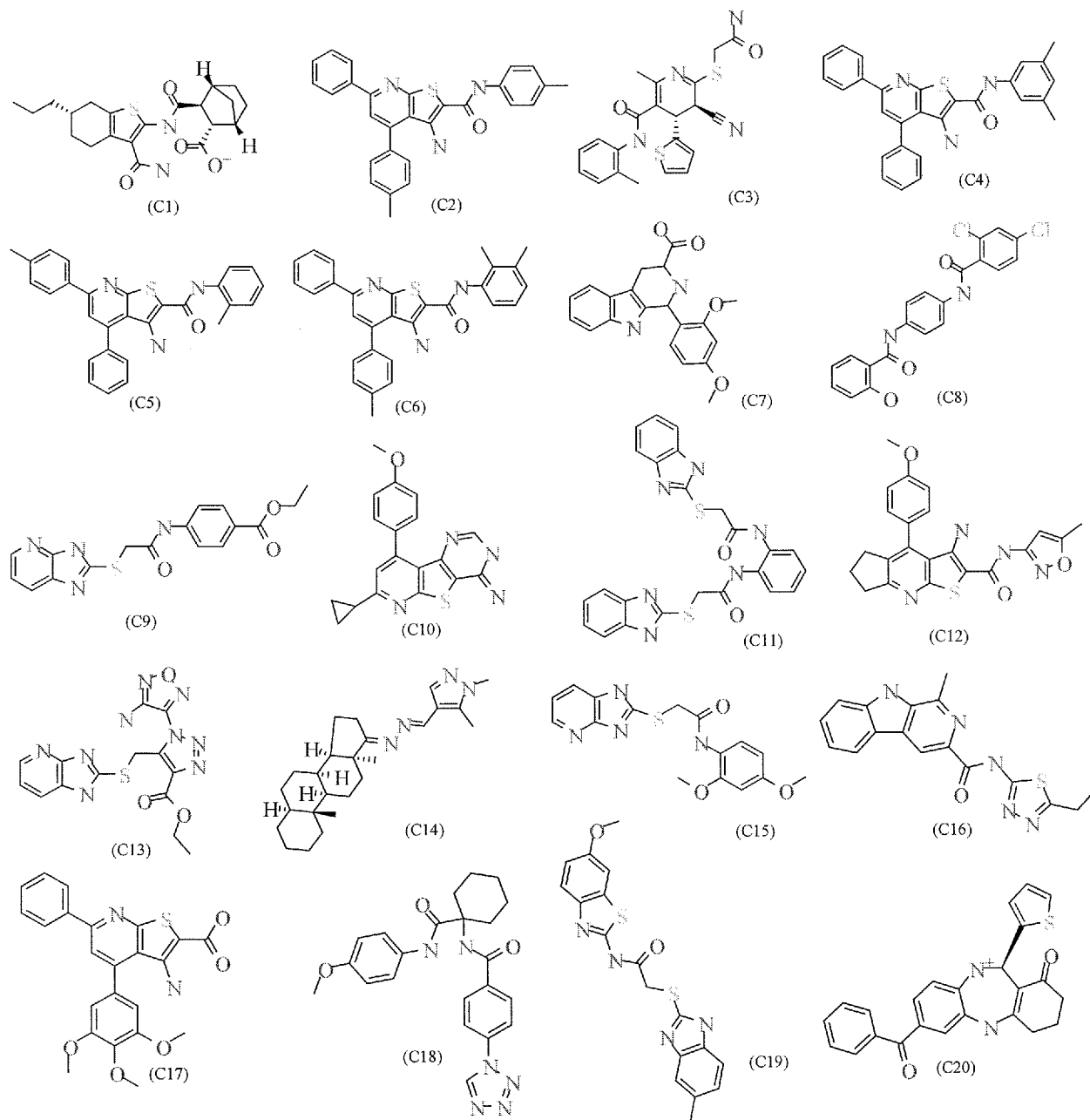


Fig. 2

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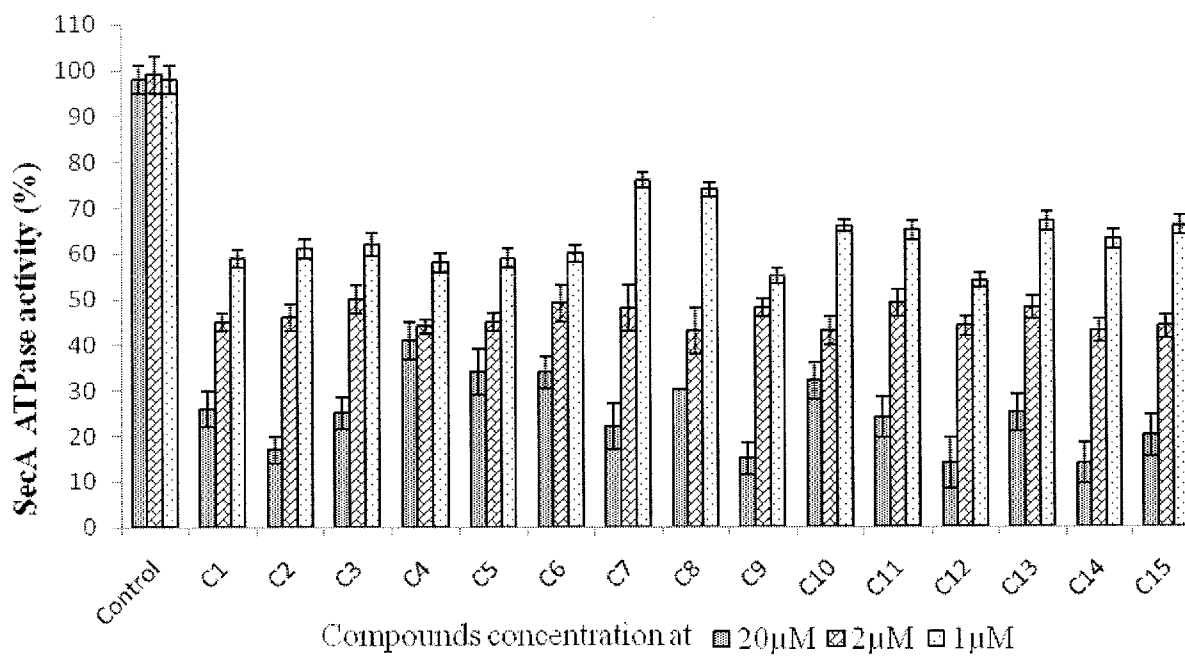


Fig. 3A

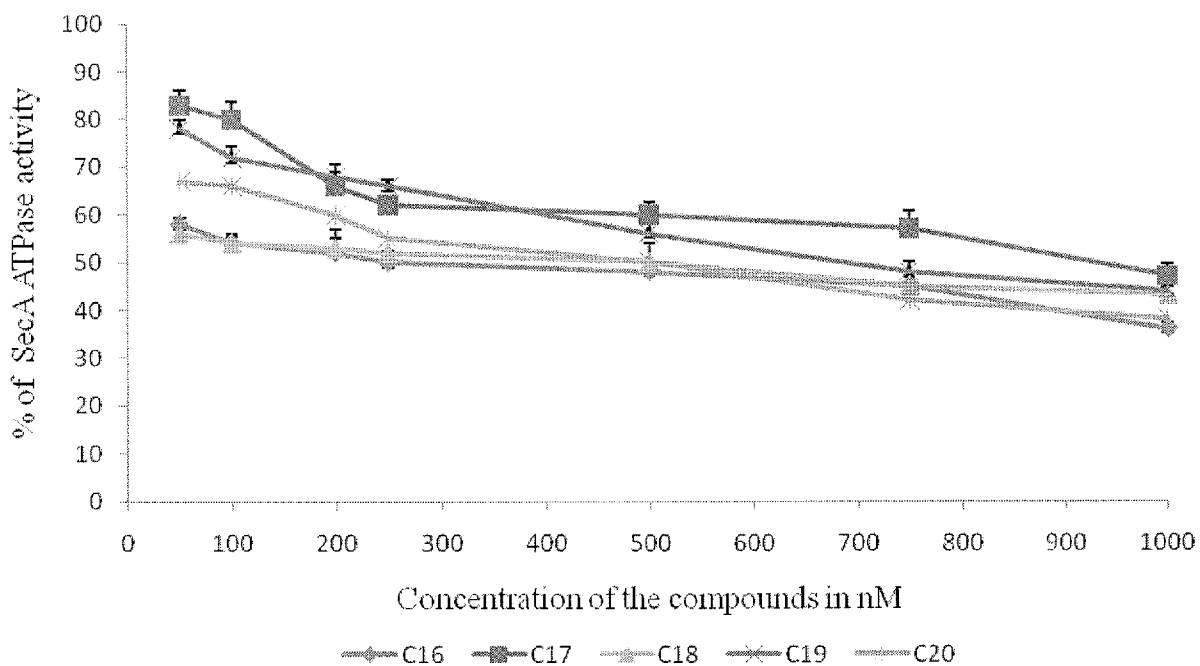
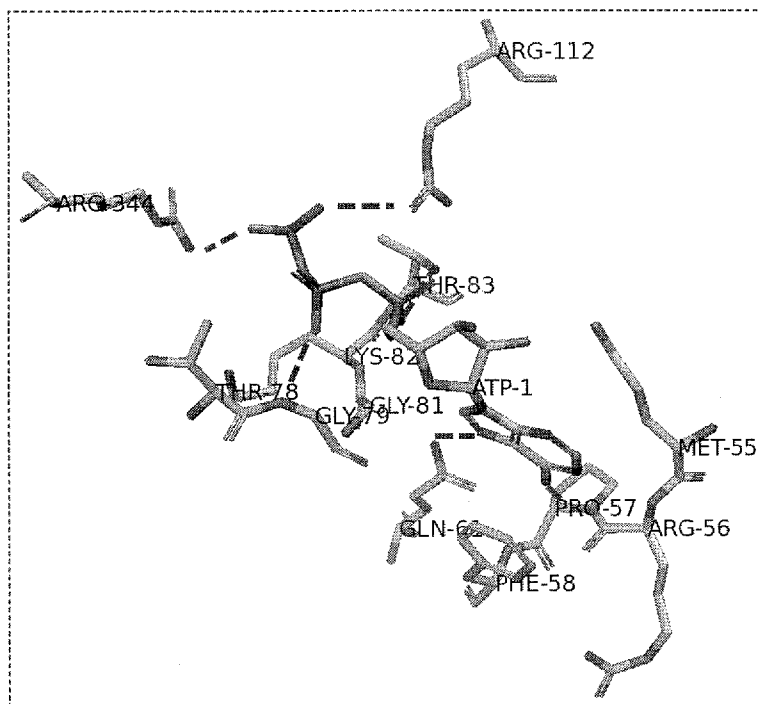
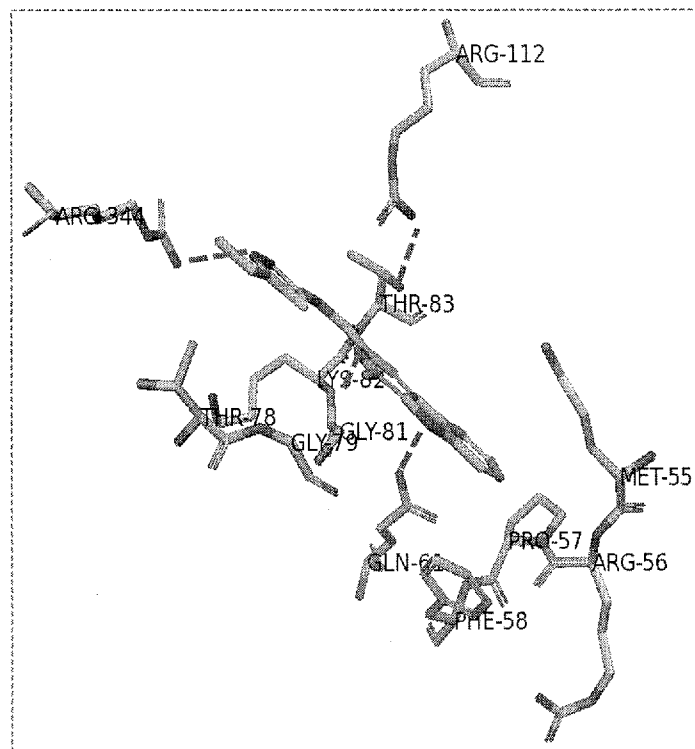
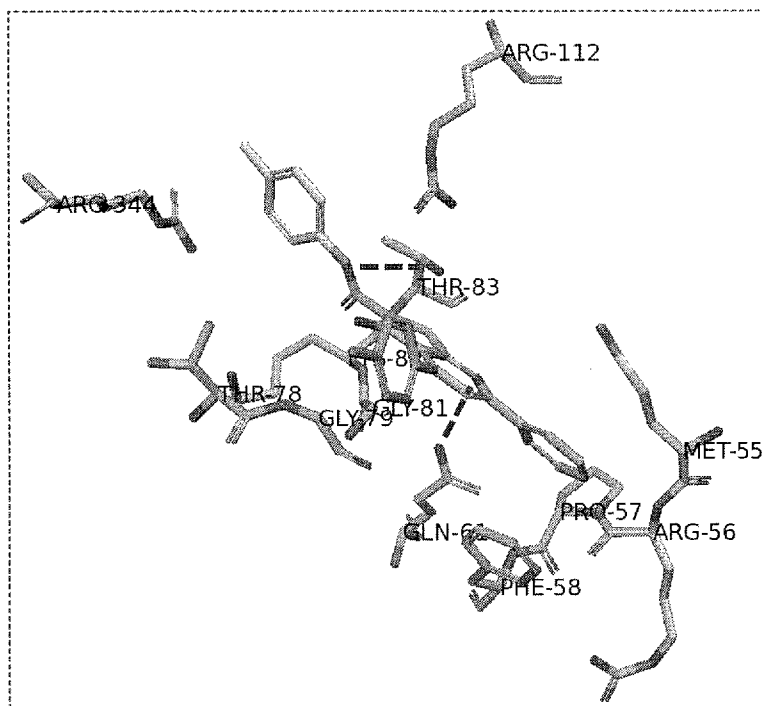


Fig. 3B

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**Fig. 4A**

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**Fig. 4B****Fig. 4C**

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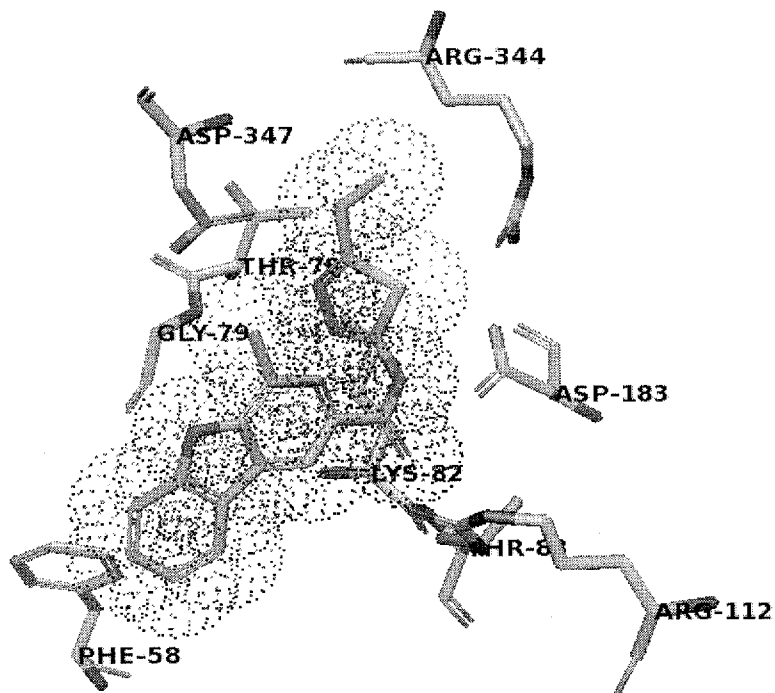


Fig. 5A

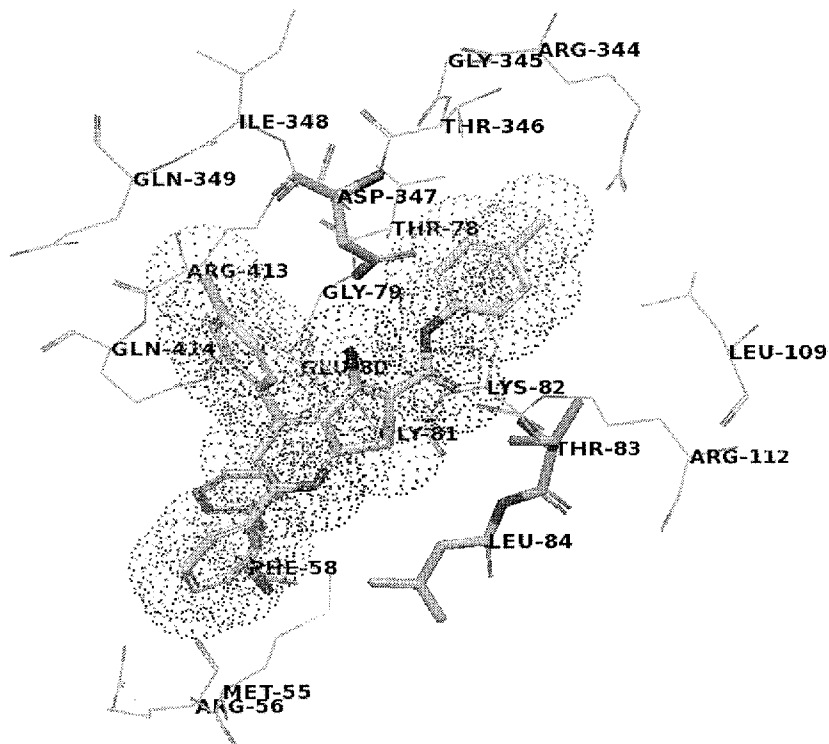


Fig. 5B

**A. CLASSIFICATION OF SUBJECT MATTER****A01N 43/48(2006.01)i, A01N 43/64(2006.01)i, A01N 43/72(2006.01)i, A01N 31/08(2006.01)i, A01P 1/00(2006.01)i**

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A01N 43/48

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

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) &amp; keywords: SecA, membrane, antimicrobial, inhibitor, plant, disease

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                    | Relevant to claim No. |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| A         | CHEN, WEIXUAN et al. 'The First Low $\mu$ M SecA Inhibitors.' Bioorganic & Medicinal Chemistry, 15 February 2010, Vol. 18, No. 4, pp.1617-1625.<br>See abstract; page 1617 right column, page 1618 right column, page 1619 and page 1620 left column; Scheme 3; figs. 3-5.            | 1-17                  |
| A         | LI, MINYONG et al. 'Discovery of the first SecA inhibitors using structure-based virtual screening.' Biochemical and Biophysical Research Communications, 18 April 2008, Vol. 368, No. 4, pp.839-845.<br>See abstract; page 840 right column and page 844 left column; figs. 1 and 2. | 1-17                  |
| A         | AKULA, NAGARAJU et al. 'Discovery of novel SecA inhibitors of Candidatus Liberibacter asiaticus by structure based design.' Bioorganic & Medicinal Chemistry Letters, 15 July 2011, Vol. 21, No. 14, pp.4183-4188.<br>See abstract; page 4186-4188; fig. 2.                           | 1-17                  |



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents:

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

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

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

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

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

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

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

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

"&amp;" document member of the same patent family

Date of the actual completion of the international search

24 June 2013 (24.06.2013)

Date of mailing of the international search report

**28 June 2013 (28.06.2013)**

Name and mailing address of the ISA/KR

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189 Cheongsa-ro, Seo-gu, Daejeon Metropolitan City,  
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Facsimile No. 82-42-472-7140

Authorized officer

HONG, Sung Ran

Telephone No. 82-42-481-5405



**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/US2013/033871**Patent document  
cited in search reportPublication  
datePatent family  
member(s)Publication  
date

None