Antibacterial compounds derived from marine *Streptomyces aureofaciens* A3 through in-silico molecular docking

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Abstract

Streptomyces aureofaciens widely produces the antibiotic tetracycline and many other compounds during fermentation. The compounds have yet to be known for their antibacterial potential. This work aims to determine new antibiotics or other possible antibacterial compounds produced by marine S.aureofaceiens A3 through an in silico molecular docking method. The ethyl acetate (EA) extracts from fermented marine S. aureofaciens A3 in ISP4 medium enriched with seawater components showed strong antibacterial activity. The antibacterial activity of EA extracts during 6-12 days of fermentation was carried out by the Kirby-Bauer method and the compounds of EA extracts were analyzed by GC/MS. Compounds identified by GC/MS were ligands for an in silico molecular docking study against four target proteins (DNA gyrase, topoisomerase IV, PBP 1a, and DHFR) of pathogenic bacteria. The drug-likeness of selected chemicals as antibacterial agents was assessed using Lipinski's Rule of Five. The results showed the prospective compounds as a narrow-spectrum antibacterial, including 3,5-di-tert-Butyl-4-hydroxyphenylpropionic acid against PBP 1a and Benzenepropanoic acid, and 3,5-bis (1,1-dimethyl ethyl)-4-hydroxy-, methyl esters against DHFR. Substances with broad-spectrum antibacterial activity, such as 3-Acetylphenanthrene and 3-(p-Ethoxyphenyl)-5-(O-tolyloxymethyl)-2-oxazolidone, against multitarget DNA gyrase B and DHFR, 7,9-Di-tert-butyl-1-oxaspiro (4,5) Deca-6,9-diene-2,8-dione against PBP1a and DHFR, and isobenzofuro [5,6-b] benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-, methyl ester against DNA gyrase B, PBP 1a, and DHFR. On the 12th day of fermentation, two compounds were identified: isobenzofuro[5,6-b] benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-, methyl ester, and 3-(p-Ethoxyphenyl)-5-(O-tolyl oxy methyl)-2-oxazolidone. This is the first report that these two compounds, known as potential drugs like antibiotics through in silico molecular docking, were first produced by Streptomyces species.

Keywords*: Antibacterial, Benzofuran, Drug-likeness, Oxazolidone, Streptomyces, molecular-docking*

Introduction

Developing novel compounds with novel chemical structures as antibacterial agents is useful for overcoming antibiotic resistance. Natural sources derived from microorganisms and their analogs and derivatives are the most effective antibiotics for treating infectious diseases and have been widely applied commercially (Stan *et al.*, 2021; Qadri *et al.*, 2022). The prospective source of natural compounds comes from Actinobacteria, which produce a wide range of secondary metabolites such as antibiotics (Zothanpuia *et al.*, 2018; Sharma and Thakur, 2020). Actinobacteria are a group of Gram-positive bacteria with a high G+C content (70-80%) that are widespread in many habitats, both terrestrial and aquatic, and can form symbionts with higher organisms such as plants, animals, and insects (Barka *et al.*, 2016; Cheng *et al.*, 2016). The most important genus of actinobacteria is Streptomyces, a producer of antibiotics. (Alam *et al.*, 2022) stated that many of the secondary metabolites produced by Streptomyces spp. have been successfully applied as an antibiotic in the medical field to treat drug-resistant infections in humans and animals, including aminoglycosides, anthracyclines, chloramphenicol, macrolides, tetracyclines, streptomycin, dactinomycin, erythromycin, chloramphenicol, and rifamycins. A wide range of types and structures of metabolites produced by bacteria or plants can

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quickly be identified for their potential antibacterial agents through in-silico molecular docking.

In silico molecular docking is the first stage of screening metabolite compounds as new drugs. It predicts the binding affinity of all metabolite molecules to a specific high-affinity protein or receptor to select potential compounds as antibacterial agents. It effectively reduces the number of compounds screened and selects multiple compounds with potential antibacterial agents (Agu *et al.*, 2023). Molecular docking has become an essential tool in computer-aided drug design (CADD) to understand the binding interactions between reliable ligands (small molecules) and their receptors (macromolecules), saving cost and time in drug discovery (Agarwal *et al.*, 2016; Singh and Sen, 2016; Agu *et al.*, 2023). It predicts the ligand's conformation in the receptor's active site shown by the docking score (kcal.mol^{-1}) (Fadlan and Nusantoro, 2021; Suwardi *et al.*, 2023). Drug similarity analysis is useful in complementing molecular docking to select candidates with physicochemical properties as drug compounds. In addition, it is useful to complement molecular docking to candidates with physicochemical properties as drug compounds (Agu *et al.*, 2023).

A previous study successfully isolated *Streptomyces aureofaciens* A3 associated with *Amorphinopsis excavans* sponge from Rancabuaya Beach, West Java. *S. aureofaciens* is known to be a tetracycline producer. However, no other compounds of *S. aureofaciens* A3 metabolites were identified with antibacterial potential based on interactions with several antibacterial target proteins such as DNA gyrase, topoisomerase IV, PBP 1a, and DHFR. Therefore, this study of in-silico molecular docking methods aims to predict new antibiotic compounds or other potential antibacterial compounds produced by *S. aureofaciens* A3.

Materials and Methods

Biological material

Streptomyces aureofaciens A3 was isolated from the coastal sponge *Amorphinopsis excavans*. The strain was examined extensively to identify potential compounds as antibacterial agents through molecular docking. The strain was maintained as a working culture on International Streptomyces Project 4 (ISP4) agar slants.

Characterization of S. aureofaciens A3

The colony morphology of the isolates was observed in terms of color, aerial, and substrate mycelium. A scanning electron microscope (SEM) with sufficient magnification (5000x) was used to verify the presence of spore chains (Math *et al.*, 2023).

Fermentation and extraction of secondary metabolites

One ose of the strain was grown for three days at 30°C on a shaker rotating at 150 rpm in a 250 mL Erlenmeyer flask containing 100 mL of ISP4 as initial inoculum. Six Erlenmeyer flasks were prepared and each Erlenmeyer was filled with 100 mL of sterile ISP4 medium dissolved in artificial seawater considering that the strain comes from the sea. One percent of the original inoculum was added to each Erlenmeyer flask, incubated at 30°C, and shaken at 150 rpm for zero, three, six, nine, twelve, and fifteen days. The Erlenmeyer-containing culture was taken as a sample from zero days to other days. Cell biomass was then separated from the liquid medium by centrifugation at 13,000 rpm for 10 min. The supernatant was then extracted with ethyl acetate (1:1) and the organic layer was evaporated in a vacuum rotary evaporator at 40°C to obtain the ethyl acetate extract (EA extract). Extracts were prepared for antibacterial analysis (Ambarwati *et al.*, 2020).

Antibacterial test

The antibacterial activity of the extract was determined by the Kirby-Bauer disc diffusion method on Mueller Hinton agar. All test microorganisms (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*) were inoculated into nutrient broth and incubated at 37°C with agitation at 150 rpm for 18-24 h. The pathogenic turbidity of each culture was measured at 530 nm, giving an absorbance of 0.5 McFarland standard, corresponding to 10^8 CFU.ml^{-1} bacteria. Antibiotic paper, 6 mm in diameter, was placed over MHA medium, 50 µl ethyl acetate crude extract was dripped on antibiotic paper, and incubated for 24 h at 30°C. The zone of inhibition was observed and measured with a micrometer (Hudzicki, 2009).

Gas Chromatography-/Mass Spectrometry (GC–/MS) Analysis

The active constituents of the crude extracts soluble in methanol were identified in an Agilent 7890B/ 5977A, a GC coupled with an MS. The separation was performed in an Agilent HP-5ms capillary column (30 m x 250 µm x 0.25 µm). The sample volume was 1 µL and injected in a splitless mode at 280ºC. The separation was performed with helium as the carrier gas at 1 mL.min^{-1}. The oven was initially programmed at 40°C and was held for 1 min. The temperature was then ramped at 10 °C.min^{-1} to 300ºC and was held for 4 min. The total running time is 31 min. The MS source and MS Quad temperature

were set at 230ºC and 150ºC, respectively. The MS was operated in full scan mode. The resulting mass spectra were then compared with the NIST17 Database for similarity.

In silico molecular docking analysis

In silico studies were conducted using the AutoDock 4.2.6 software program, AutoDockTools v.1.5.6, BIOVIA Discovery Studio 2021 (BIOVIA, 2021), MarvinSketch (ChemAxon, 2021), Notepad++ v7.5.9, Pymol 2.4.1, Ligplot+ (The Scripps Research Institute, US), Visual Molecular Dynamics, and Avogadro). The RCSB Protein Data Bank was used to derive 3D structural protein receptors in .pdb format. The 2D structures of the in. mol format test ligands were retrieved from the PubChem database and the NIST Chemistry WebBook, while compounds not in the database were drawn with the MarvinSketch tool. The target protein used in silico was Protein DNA gyrase (PDB ID: 1KZN) from *E. coli* forming compounds with native ligands or Chlorobiocin ligands, having a resolution of 2.3, and using the ATPase domain or the ATP binding site of a protein (Lafitte *et al*., 2002). Topoisomerase IV (PDB ID: 3RAE) from *Streptococcus pneumoniae* formed a complex with the original ligand Levofloxacin, had a resolution of 2.9, and used the BD chain subunit as the binding site for ATP (Veselkov *et al*., 2016). Penicillin-binding protein 1a (PDB ID: 3UDI) from *Acinetobacter baumannii* formed a complex with the ligand from Ceftazidime, had a resolution of 2.6, and used the B chain (Han, 2011). dihydrofolate reductase (PDB ID: 3SRW) from *S. aureus* formed compounds with the native ligand Trimethoprim, has a resolution of 1.7 and utilizes chain A (Li *et al*., 2011). The presence of a negative bond energy value and RMSD <2 indicated that the technique accurately re-docked, allowing the docking parameters (grid box) to be used for the docking stage test ligand against the next target protein. The interaction of the test ligands with the target protein was analyzed, and the percentage of total interactions involving key residues in all interactions was calculated. Using the MarvinSketch tool, the test ligands with the best docking results from each target protein were selected based on their drug-likeness according to Lipinski's Rule (Lipinski *et al*., 2001; Verheij, 2006).

Result and Discussion

Morphological characterization

Colonies of the strain were smooth and gray, had aerial hypa, and did not produce pigment to change the color of the medium (Figure 1A.). The spore measures 0.955 x 1.634 0.955 µm at 5000x magnification and the spore surface was smooth (Figure 1B.).

Marine *S. aureofaciens* A3 was a filamentous Gram-positive aerobic bacterium with simple spore chains. Despite its use as a tetracycline antibiotic, *S. aureofaciens* has been the subject of extensive biochemical research (Gradnigo *et al.*, 2016).

Antibacterial activity

The growth curve presented in Figure 2A shows the specific growth rate (µ) in the logarithmic phase is 0.064 h with a generation time (GT) of 10.81 h. There was a decrease in pH indicating organic acid production during fermentation. The EA extract depicts antibacterial activity against all pathogens, as shown in Figure 2B. The EA extracts performed moderate to strong activity and were produced from day 6 to day 15. Between day 6 and day 15, there was a 4.15 mm and 3.83 mm daily increase in antibacterial activity against *E. Coli* and *B. subtilis*, respectively. On the other hand, the antibacterial metabolites' activity rates against *S. aureus* and *P. aeruginosa* were 4.86 $mm.d^{-1}$ and 5.85 $mm.d^{-1}$, each, from day 6 through day 12.

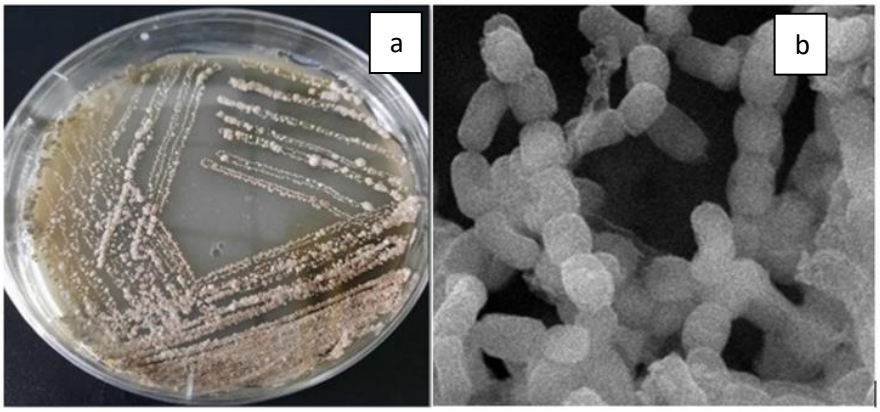

Figure 1. Macroscopic characteristics (a) and spore chain morphology of *S. aureofaciens* A3 (b).

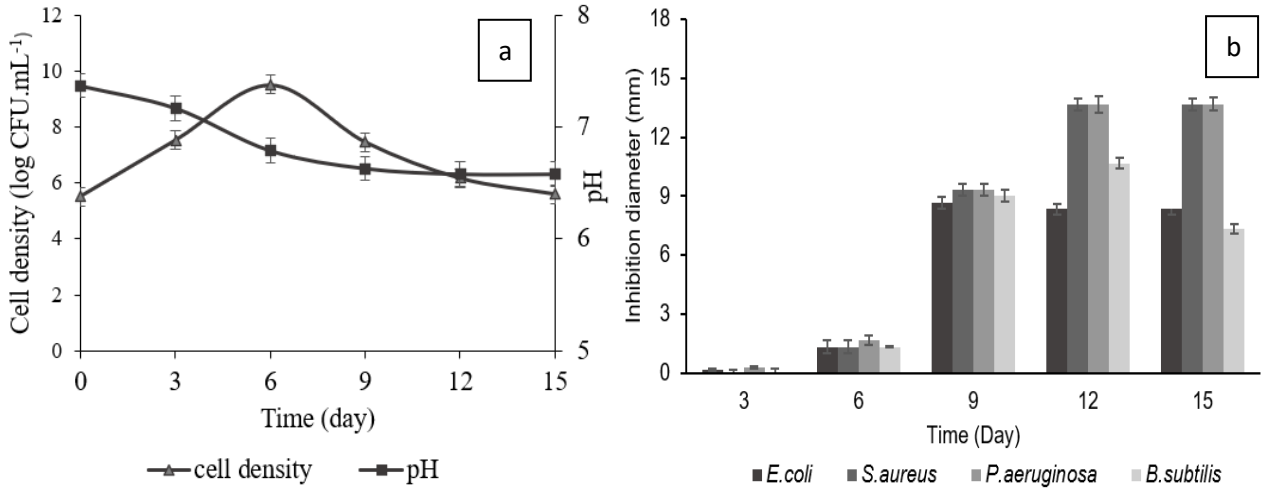

Figure 2. Growth profile *of S. aureofaciens* A3 on ISP4A with 1% inoculum (2.2 X 10^5 CFU.mL^{-1}), 150 rpm agitation, and 30°C temperature (a). Antibacterial activity of crude extract ethyl acetate from *S. aureofaciens* A3 (b).

From day 6 (stationary phase) until day 15 (death phase), the strain produced antibacterial secondary metabolites containing different types and amounts of molecules. The diameters of the zone of inhibition reached the maximum during the 12th and 15th days of fermentation when the supposed antibacterial components were produced. Antibacterial agents from the Actinobacteria group can exist in exponential, stationary, and death phases (Cheng *et al.*, 2016). In addition, secondary metabolites appear to be produced during environmental stress due to nutrient depletion-limiting growth conditions (Law *et al.*, 2019; Krysenko, 2023).

GC/MS analysis

A total of 85 annotated compounds consisting of 35 compound classes are responsible for the formation of bioactivity. The compounds annotated in the EA extract using GCMS were ligands in molecular docking analysis. Metabolite compounds from ethyl acetate extract during antibacterial production are shown in Table 1.

On the 12th day of fermentation, 2 compounds were detected for the first time produced by Streptomyces, namely isobenzofuro[5,6-b] benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-, methyl ester, and 3-(p-Ethoxyphenyl)-5-(O-tolyl oxy methyl)-2-oxazolidinone as shown in Figure 3.

Isobenzofuro[5,6-b] benzofuran-8-carboxylic acid 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-methyl ester is a heterocyclic aromatic compound consisting of benzene and a furan ring, the carbon compound has a carboxylic group (- COOH). Tetrasiloxane, decamethyl is a non-cyclic silicon oligomer and compound bioactivity is unknown. This compound has been identified as a minor compound in several plant extracts such as *Hybanthus enneaspermus*, *Clinacanthus nutans*, *Amomum subulatum,* and bacteria *Lysinibacillus sphaericus* (Saleh *et al.,* 2018; Toha *et al.*, 2020; Alam and Singh, 2021). The oxazolidone class is the only new class of synthetic antibiotics developed for clinical use in the last 50 years. Oxazolidone compounds have a broad spectrum of properties, especially against multidrug-resistant pathogens such as *Enterococcus* spp. (VRE) resistant to vancomycin, MRSA, and *Mycobacterium tuberculosis* (Foti *et al.*, 2021). Lipoxazolidinones A-C (2-alkydene-5-alkyl-4-oxazolidinones) are new compounds isolated from the new genus *Marinispora*. and *Nocardia brasiliensis*. Lipoxazolidinones A-C are broad-spectrum antibiotics and have similar activity to the commercial antibiotic linezolid (Zyvox) (Macherla *et al.*, 2007; Espinoza-González *et al.*, 2008; Subramani and Aalbersberg, 2013).

Molecular docking analysis

The compounds were successfully docked onto the active site of target proteins (DNA gyrase, Topoisomerase type IV subunit B, Penicillin-binding protein 1a, and Dihydrofolate reductase). Table 2 shows the binding energy, amino acid residues in H-Bond, and Hydrophobic Interactions in docking experiments. Due to their binding energies being lower than or equal to the positive control antibiotic of each target protein, eleven compounds had antibacterial potential in silico. The amino acid residues were essential components of protein active sites and played a role in hydrophobic interactions and hydrogen bond formation.

Table 1. Annotated Compounds of crude extract ethyl acetate from *S. aureofaciens* A3

Compound Class	Annotated compounds
Dicarboxylic acid ester	Oxalic acid, isohexyl neopentyl ester
	Phthalic acid, di (2-propyl pentyl) ester
	1,2-Benzenedicarboxylic acid, bis (4-methyl pentyl) ester
	Phthalic acid, hexadecyl 3-methyl butyl ester
	1,2-Benzenedicarboxylic acid, dinonyl ester
Dicarboxylic acids	2,2,4-Trimethyl-1,3-pentanediol di-isobutyrate
Alkanes	Pentacosane
	Docosane, 1-iodo-
	Hexacosane
	Decane, 4-ethyl-
	Dodecane, 5-methyl-
	Nonane, 5-methyl-5-propyl-
	Hexane, 3,3-dimethyl-
	Eicosane
	Heneicosane
	Heptacosane
	Hentriacontane
Naphthopyrans	2-Bromo dodecane
Phenylpropanes	2,4-Di-tert-butyl phenol
	Phenol, 4-(1,1,3,3-tetramethyl butyl)-
	Phenol, 2-methyl-4-(1,1,3,3-tetramethyl butyl)-
	Phenol, 4-(1,1-dimethyl propyl)-
	Benzenepropanoic acid, 3,5-bis (1,1-dimethyl ethyl)-4-hydroxy-, methyl ester
	phenol, 2-(1,1,3,3-tetramethyl butyl)-
	Phenol, p-tert-butyl-
	Phenol, 2-(1,1-dimethyl ethyl)-4-methyl-
	Benzene, 1-(1,1-dimethyl ethyl)-4-methoxy-
Sulfonamide	Acetamide, N-(2,4-dimethyl phenyl)-
Steroid ester	Succinic acid, (adamant-1-yl) methyl 2,2,3,3,4,4,5,5-octafluoropentyl ester
Benzene	Phenol, m-tert-butyl-
	4-(1,1-Dimethylpropyl) phenyl acetate
	Ethylbenzene
	1-(Isocyanatomethyl)-4-(methyloxy)benzene
	Benzeneacetic acid
Phenylpropanes ketone	2-tert-Butyl-6-methyl phenol, 2-methyl propyl ether
Ketones	2-Methyl-4-hydroxy benzoxazole
	1,3-Cyclopentadiene, 5,5-dimethyl-1,2-Dipropyl-
	Decane, 3,6-dimethyl-
Gamma butyrolactones	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione
Halogen	Octadecane, 1-iodo-
	Triacontane, 1-iodo-
	Octacosane, 1-iodo-
	Dodecane, 1-chloro-
	Tetradecane, 1-chloro-
	Tetracosane, 1-iodo-
Quinoline	5-Methylmercapto-6-methoxy-8-aminoquinoline
Fatty acid methyl esters	Hexadecanoic acid, methyl ester
	Methyl stearate
Fatty acids and terpenoids	Cyclotrisiloxane, hexamethyl-
Long-chain fatty acids	n-Hexadecanoic acid
	Octadecanoic acid
	Palmitic Acid, TMS derivative
Phenylpropanoid acids	3,5-di-tert-Butyl-4-hydroxyphenyl propionic acid
Organosilicon	2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene
	Tetrasiloxane, decamethyl-
Dithioketals heterols	7,7,9,9,11,11-Hexamethyl-3,6,8,10,12,15-hexaoxa-7,9,11-trisilaheptadecane

Table 1 (continue). Annotated Compounds of crude extract ethyl acetate from *S.aureofaciens* A3

Compound Class	Annotated compounds
Secondary alcohols	2-Hexanol
Pyrimidine derivatives	4-Pyrimidinamine, 2,6-dimethyl-
Propanoic acids-ester	2- Chloropropionic acid, octadecyl ester
Dibutylhydroxytoluene	2-tert-Butyl-6-methyl phenol, n-butyl ether
Amida aromatic	m-Anisoyl amide, N-(2-phenyl ethyl)-N-pentyl-
Hydroxybenzaldehydes	3,5-di-tert-Butyl-4-hydroxybenzaldehyde
Unsaturated aliphatic hydrocarbons	5-Eicosene, (E)-
Benzoic acid esters	Bis(2-ethylhexyl) phthalate
	Dibutyl phthalate
	Di-isononyl phthalate
Fatty acid esters	n-Propyl 11-octadecenoate
	Hexadecanoic acid, propyl ester
	Octadecanoic acid, propyl ester
	Hexanedioic acid, bis(2-ethylhexyl) ester
	Phthalic acid, hept-4-yl isohexyl ester
	Phthalic acid, isohexyl 3-methyl butyl ester
	Phthalic acid, hept-2-yl isohexyl ester
	Phthalic acid, 2-ethyl butyl nonyl ester
	Phthalic acid, 2,2-dimethyl pent-3-yl nonyl ester
	Phthalic acid, 2-ethyl butyl heptyl ester
	Phthalic acid, 3-methyl butyl octadecyl ester
	Hexadecanoic acid, ethyl ester
Alkene	1-Octadecene
Phenanthrene	3-Acetylphenanthrene
Dialkyl ethers	Methane, chloromethoxy-
Aldehydes	Nonanal
Cinnamaldehydes oxazolines	3-(p-Ethoxyphenyl)-5-(O-tolyloxymethyl)-2-oxazolidone
Perfluoro carboxylic acids ester	Pentadecafluorooctanoic acid, octadecyl ester
Benzofuranones	Isobenzofuro[5,6-b] benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-, methyl ester

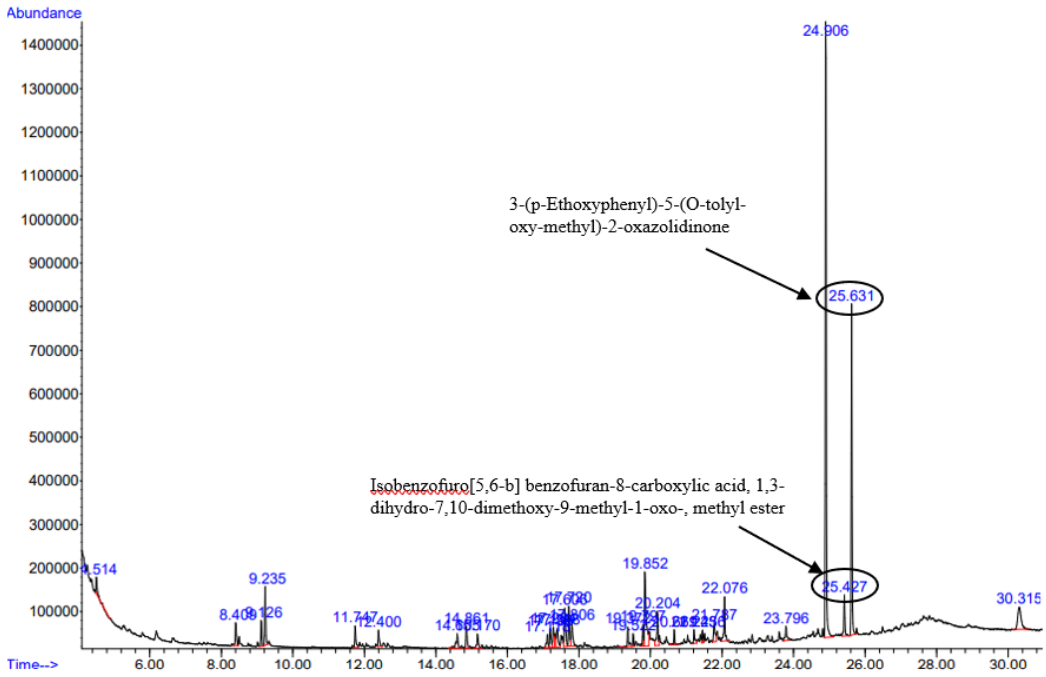

Figure 3. GC/MS chromatogram of two first annotated compounds from marine *S.aureofaciens* A3.

Compounds isobenzofuro[5,6-b] benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-, methyl ester showed lower bond energy values or the same for positive control antibiotics from 3 target proteins (DNA gyrase, Penicillin-binding protein 1a, and Dihydrofolate reductase). Compound 3-(p-Ethoxyphenyl)-5-(O-tolyloxymethyl)-2-oxazolidone was a chemical that binds to DNA gyrase effectively since it had a little higher binding energy than ciprofloxacin.

Table 3 reveals the results of the drug-likeness study performed using Lipinski's rules on the best-docked compounds. Phthalic acid di(2-propylpentyl) ester had one violation of the Lipinski rule out of the 11 drug-likeness compounds, with a log P value greater than 5. This indicates the compound may still be useful as a medication. Lipinski's rule states that in general, oral active drugs must not have more than one violation of the following criteria Molecular weight <500, Number of hydrogen bond donors (nHBD) ≤5, Number of hydrogen bond acceptors (nHBA) ≤10, Calculated Log p ≤5 and Polar surface area (PSA) < 140 Å 2). Phthalic acid di(2-propyl pentyl-ester (compound 9) violated one log with a P-value greater than 5. This is of concern because further in vivo testing of this chemical may cause severe toxicity and be dangerous. According to Lipinski's rules, this substance can still be a drug because it meets other criteria. Docking results are the docking score or binding energy value of the affinity of the ligand for the receptor's active site (negative value) to confirm that the tested ligand can interact with the antibacterial receptors.

Among the above 11 docking components, there are six main antibacterial candidates for each target protein as shown in Figure 4, namely 3,5-di-tert-butyl-4-hydroxyphenyl propionic acid, benzene propanoic acid, 3,5-bis(1,1-dimethyl ethyl-4-hydroxy-, methyl ester, 3-acetyl phenanthrene, 3-(p-ethoxy phenyl)-5-(O-tolyloxymethyl)-2-oxazolidone,7,9-di-tert -butyl-1-oxaspiro (4,5) Deca-6,9-diene-2,8-dione and isobenzofuro[5,6-b] benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo -, methyl ester. The compounds obtained in the best docking can be grouped into (1) compounds that can be narrow-spectrum antibacterial agents, namely compounds with high affinity for only one target protein, such as 3,5-di-tert-butyl. -4-Hydroxyphenylpropionic acid and benzene propanoic acid, 3,5-bis (1,1-dimethyl ethyl)-4-hydroxy-, methyl ester and (2) compounds that can act as broad-spectrum antibacterial agents targeting multiple proteins, namely compound 3. - acetyl phenanthrene, benzene propanoic acid, 3,5-bis (1,1-dimethyl ethyl)-4-hydroxy-, methyl ester, 3-(p-ethoxy phenyl)-5-(O-tolyloxymethyl)-2-oxazolidone and isobenzofuro [5,6-b] Benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-methyl ester.

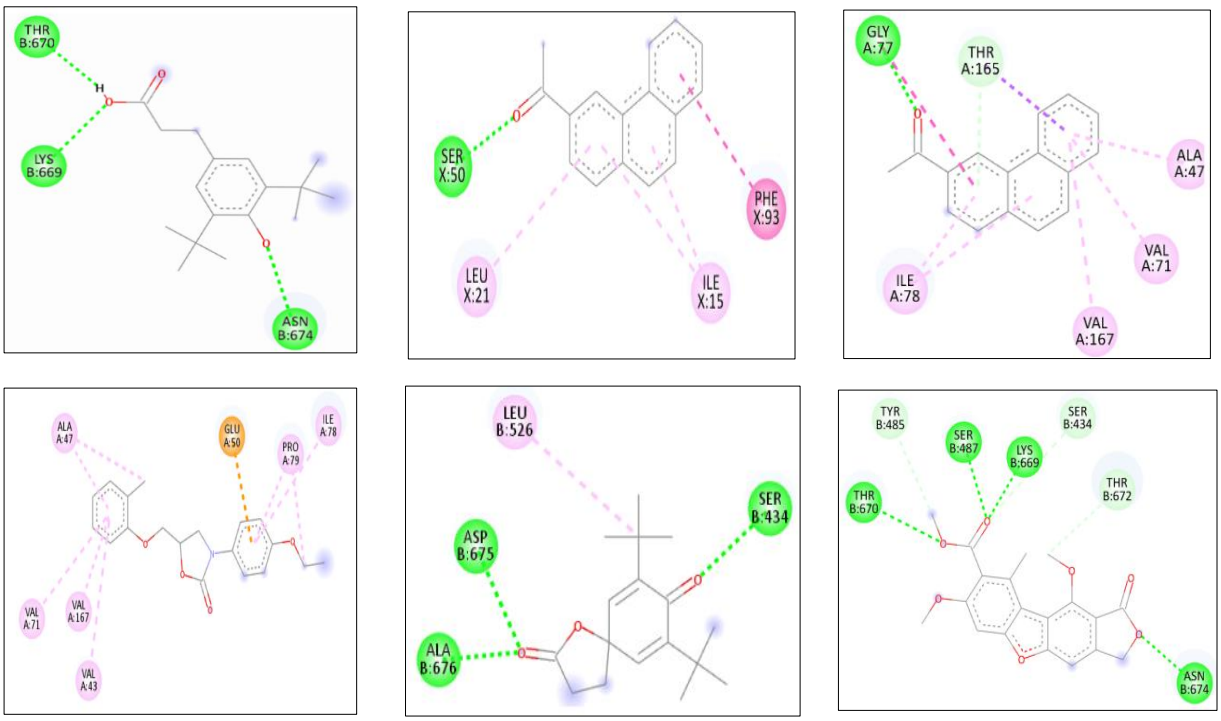

Figure 4. The 2D visualization results of ligands: (a) 3,5-di-tert-butyl-4-hydroxyphenylpropionic acid, (b) benzene propanoic acid, 3,5-bis(1,1-dimethylethyl-4-hydroxy-, methyl ester, (c) 3-acetyl phenanthrene, (d) 3-(p-ethoxyphenyl)-5-(O-tolyloxymethyl)-2-oxazolidone, (e) 7,9-di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione, (f) isobenzofuro[5,6-b] benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo -, methyl ester as the best docking results with target proteins.

Table 2. Interaction of compounds with ligands

Target protein code	Compounds	Binding energy (kcal.mol^{-1})	Residues Involved in H-Bond Interactions	Residues Involved in Hydrophobic Interactions
DNA gyrase (PDB ID: 1KZN)	Ciprofloxacin	-6,8	Gly, Arg, Val, Asp	Ala, Ile, Pro
	3-Acetylphenanthrene	-7,71	Gly, Thr	Thr, Gly, Ala, Val, Ile
	Cyclotrisiloxane, hexamethyl-	-7,61		
	2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene	-7,31	Asn, Pro	Thr, Val, Ala, Ile
	Isobenzofuro[5,6-b] benzofuran -8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-, methyl ester	-7,25	Thr, Glu, Gly, Pro	Thr, Asn, Gly, Ala, Arg, Ile, Val120
	Tetrasiloxane, decamethyl-	-6,79		
	3-(p-Ethoxyphenyl)-5-(0-tolyloxymethyl)-2-oxazolidone	-6,79		Val, Ala, Ile, Pro
Topoisomerase type IV subunit B	Levofloxacin	-9,37	Glu, Arg	Arg
	3,5-di-tert-Butyl-4-hydroxyphenyl propionic acid	-9,08	Gly, Lys	
Penicillin-binding protein 1a	Penicillin G	-6,25	Asn	Pro
	3,5-di-tert-Butyl-4-hydroxyphenyl propionic acid	-6,58	Lys, Thr, Asn	
	7,9-Di-tert-butyl-1- oxaspiro (4,5) deca-6,9-diene-2,8-dione	-6,5	Ser, Asp, Ala, Tyr, Thr	Leu
	Cyclotrisiloxane, hexamethyl-	-6,34	Asn, Phe, Thr	
	Isobenzofuro[5,6-b] benzofuran -8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-, methyl ester	-6,34	Ser, Lys, Thr, Asn	
Dihydrofolate reductase (PDB ID: 3SRW)	7-(2-ethoxynaphthalen-1-yl)-6- methyl quinazoline-2,4-diamine (Q27)	-6,8	Ala, Ile, Asp	Leu
	7,9-Di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione	-8,56	Ala, Ser, Val	Leu
	Tetrasiloxane, decamethyl-	-8,39	Ala	
	Isobenzofuro[5,6-b] benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-, methyl ester	-8,13	Ser, Asn, Thr	Phe, Ile, Lys
	Cyclotrisiloxane, hexamethyl-	-7,76	Ala	Phe
	Benzenepropanoic acid, 3,5-bis(1,1-dimethyl ethyl)-4-hydroxy-, methyl ester	-7,64	Ala, Ser	Ala, Leu, Ile
	3-Acetylphenanthrene	-7,55	Ser	Ile, Leu, Phe
	3-(p-Ethoxyphenyl)-5-(0-tolyloxymethyl)-2-oxazolidone	-7,51	Ala, Val	Ile, Leu, Val, Phe
	2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene	-7,07	Ala, Val	Ile
	m-Anisoyl amide, N-(2-phenyl ethyl)-N-pentyl-	-7,02	Ala	Leu, Ala, His, Val
	Phthalic acid, di (2-propyl pentyl) ester	-6,83	Thr, Gly94	Leu, Ala, Ile, Leu, Val, Phe

Analysis of the production time of antibacterial candidate compounds

The best docking compounds are created during the fermentation process. The production time of the best-candidate antibacterial compound is shown in Figure 5. The following is the preparation time of the best antibacterial candidates: 3,5-di-tert-butyl-4-hydroxyphenylpropionic acid was prepared on days 0 and 6. Benzenepropanoic acid, 3,5-bis (1,1-dimethyl ethyl) -4-hydroxy-, methyl ester) produced on days 0 and 6. 3-acetyl phenanthrene) was produced only on the 9th day. 3-(p-ethoxy phenyl)-5-(0-tolyloxymethyl)-2-oxazolidone) was produced only on day 12. 7,9-di-tert-butyl-1-oxaspiro (4,5) deca-6, 9-diene-2,8-dione) was prepared on days 0 and 6. Isobenzofuro[5,6-b] benzofuran- 8- carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl- 1-oxo-, methyl ester) was produced only on the 12th day. These docking results correspond to the results of the Kirby-Bauer test shown in Figure 2b. Most secondary metabolites were the main candidates for antibacterial compounds and began to form on the sixth day (initial stationary phase).

Table 3. Characterization of drug-likeness molecular docking results

Target protein code	Annotated compounds	Lipinski rule parameter		
		Molar mass ($g.mol^{-1}$)	Hydrogen bonds donor	Number of hydrogen bond acceptors
		<500	<5	<10
Penicillin-binding protein 1a	3,5-di-tert-Butyl-4-hydroxyphenyl propionic acid	278,40	2	3
Dihydrofolate reductase	Benzenepropanoic acid, 3,5-bis (1,1-dimethyl ethyl)-4-hydroxy-, methyl ester	292,41	1	2
	Phthalic acid, di (2-propyl pentyl) ester	390,56	0	2
	m-Anisoyl amide, N-(2-phenyl ethyl)-N-pentyl-	325,45	0	2
DNA gyrase subunit B, Dihydrofolate reductase	3-Acetylphenanthrene	220,26	0	1
	3-(p-Ethoxyphenyl)-5-(O-tolyloxymethyl)-2-oxazolidone	327,38	0	4
	Tetrasiloxane, decamethyl-	310,69	0	3
	2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene	282,29	0	2
Penicillin-binding protein 1a, Dihydrofolate reductase	7,9-Di-tert-butyl-1- oxaspiro(4,5)Deca-6,9-diene-2,8-dione	276,40	0	2
DNA gyrase subunit B,	Cyclotrisiloxane, hexamethyl-	222,46	0	3
Penicillin-binding protein 1a, Dihydrofolate reductase	Isobenzofuro[5,6-b] benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-, methyl ester	356,30	0	4

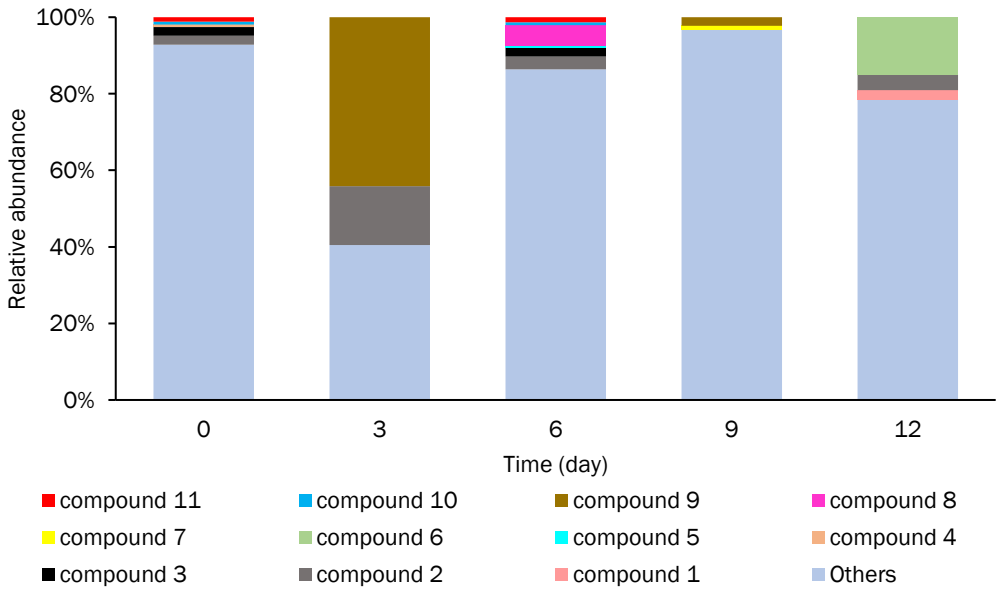

Figure 5. Relative GC-MS area peak dynamic curve of production of the antibacterial candidate. Compound 1: Isobenzofuro[5,6-b]benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy-9-methyl-1-oxo-, methyl ester; Compound 2: Cyclotrisiloxane, hexamethyl-; Compound 3: 7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione; Compound 4: 2-(Acetoxymethyl)-3-(methoxycarbonyl)biphenylene; Compound 5: Tetrasiloxane, decamethyl-; Compound 6: 3-(p-Ethoxyphenyl)-5-(O-tolyloxymethyl)-2-oxazolidone; Compound 7: 3-Acetylphenanthrene; Compound 8: m-Anisoyl amide, N-(2-phenyl ethyl)-N-pentyl-; Compound 9: Phthalic acid, di(2-propyl pentyl) ester; Compound 10: Benzenepropanoic acid, 3,5-bis(1,1-dimethyl ethyl)-4-hydroxy-, methyl ester; Compound 11: 3,5-di-tert-Butyl-4-hydroxyphenyl propionic acid.

Antibacterial candidate compounds were produced from the beginning to the end of the growth phase starting on days 0, 3, 6, 9, and 12, but most of these compounds were produced in the stationary phase (day 6) and death phase (day 12). The antibacterial candidate compounds had a low relative

abundance compared to other compounds produced on the 6th and 12th days. Therefore, the production of antibacterial metabolites from *S. aureofaciens* A3 can be done on day 6 and day 12 of fermentation. However, fermentation methods must also be optimized to improve the abundance of antibacterial compounds needed. Furthermore, antibacterial compounds of crude extracts also can be purified and characterized.

Conclusion

Secondary metabolites of the sponge symbiont *Streptomyces aureofaciens* A3 have been identified as useful bioactive compounds against pathogens that cause infectious diseases. Through study, new bioactive compounds have also been discovered. Isobenzofuro[5,6-b] benzofuran-8-carboxylic acid, 1,3-dihydro-7,10-dimethoxy benzofuran-8-carboxylic acid3-(p-Ethoxyphenyl) and -9-methyl-1-oxo-, methyl ester-O-tolyoxymethyl-5-(O-tolyoxymethyl)-2-oxazolidone demonstrated the efficiency of pharmaceuticals targeting bacterial infections, providing it a promising ingredient for pharmaceutical applications with antibacterial properties that deserve further investigation. Further research will focus on the purification of potential antibacterial substances compounds in ethyl acetate extract produced by *S. aureofaciens* A3.

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