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Research article

Genome Characterization for the Antimicrobial Potential of *Streptomyces samsunensis* SA31, a Rhizospheric Actinomycete of *Cymbopogon citratus* (DC) Stapf.

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Abstract

Streptomyces species are promising resources of bioactive compounds that play a vital role in medicinal biotechnology. *Streptomyces* sp. SA31 was isolated from the rhizospheric soil of *Cymbopogon citratus* (DC) Stapf. The strain was identified as *Streptomyces samsunensis* based on genome-based taxonomic analysis. The draft genome of strain SA31 contained 11,850,342 bp with a high percentage of G+C at 71.0% and carried 9,350 predicted protein-encoding sequences (CDSs). Furthermore, the secondary metabolite biosynthesis gene clusters in the genome of strain SA31 were predicted by antiSMASH. *In silico* analysis showed 71 predicted biosynthetic gene clusters (BGCs) responsible for antimicrobial secondary metabolite synthesis. Most gene clusters were involved in the biosynthesis of polyketide synthase. The genome of strain SA31 harbored seven types of polyketide biosynthesis gene clusters that might be associated with antimicrobial activity. Additionally, the genome of strain SA31 contained unexplored secondary metabolite biosynthesis gene clusters. *In vitro* antimicrobial assay showed that an ethyl acetate extract from the culture broth of strain SA31 could inhibit the growth of *Staphylococcus aureus* ATCC 25923, *Kocuria rhizophila* ATCC 9341, *Bacillus subtilis* ATCC 6633, *Candida albicans* ATCC 10231, and *Aspergillus flavus* IMI 242684, implying that the genome of strain SA31 contained potential BGCs for the production of antimicrobial secondary metabolites and led to the isolation of geldanamycin and 17-*O*-demethylgeldanamycin. Therefore, it can be proved that the rhizosphere-associated soil of *C. citratus* (DC) Stapf. is a rich habitat for actinomycetes that are capable of producing promising biologically active compounds.

Keywords: *Streptomyces*; actinomycete; genome analysis; geldanamycin; antimicrobial activity

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1. Introduction

Microorganisms are considered a crucial natural source of bioactive compounds that provide promising biological activities, especially antimicrobial activity (Bérdy, 2005; Supong et al., 2016). Soil is one of the largest reservoirs of microbial diversity (Thawai et al., 2017). It is estimated that one gram of soil regularly contains 10^9 to 10^{10} microorganisms and may include thousands of bacterial species (Armalytė et al., 2019). Antibiotic-producing capabilities of soil microorganisms to inhibit the growth of coexisting microorganisms are influenced by abiotic and biotic factors (Gislin et al., 2018). In addition, sufficient growth factors and nutrients affected antibiotic biosynthesis and influenced other metabolic pathways as well (Karlovsky, 2008). The plant rhizosphere is considered an area with a high complexity of microbiological ecosystems, and the diversity of microbial communities is managed by the mixture of nutrients, including carbon sources and amino acids, released by plant roots (Mendes et al., 2013; Oberhofer et al., 2019). Actinomycetes are saprophytic bacteria and are wildly distributed in the plant rhizosphere. They can produce biological substances that promote plant growth and protect the plant from invading phytopathogens (Khamna et al., 2009; Intra et al., 2011). The genus *Streptomyces* is an outstanding bioactive compound producer, and approximately 60-75% of bioactive compounds have been isolated from this microorganism (Bunbamrung et al., 2020; Sharma & Thakur, 2020). Bioactive compounds with antimicrobial activity from *Streptomyces* species such as nystatin, rapamycin, geldanamycin, streptomycin, neomycin, fosfomycin, platensimycin, and krysinomycin have been used in medical purposes (Quinn et al., 2020; Genilloud, 2017; Boudjeko et al., 2014). It is known that bioactive secondary metabolites are encoded on biosynthetic gene clusters (BGCs) that consist of genes arranged in bacterial genomes. In general, the genomes of *Streptomyces* spp. were found to have non-ribosomal peptide synthetases (NRPSs), polyketide synthases (PKSs), terpenoids, lanthipeptides, and many others that were involved in the production of bioactive compounds (Zheng et al., 2019). To date, the bioinformatic powerful tool antiSMASH has been used to reveal the antimicrobial potential of *Streptomyces* species, where biosynthetic gene clusters (BGCs) could be detected and identified (Weber et al., 2015). Additionally, genome mining generated important genome data that could be used for screening promising *Streptomyces* spp.

In tropical regions, *Cymbopogon citratus* (DC) Stapf. can survive under droughty conditions and is widely used as traditional medicine (Oladeji et al., 2019). This plant plays a role in soil stabilization and erosion control due to its extensive root networks which are able to hold a large volume of soil (Sanusi et al., 2015; Maiti & Kumar, 2016). Our hypothesis is that the rhizospheric soil of *C. citratus* (DC) Stapf. may serve as a potential source of antibiotic-producing *Streptomyces*. In this study, *Streptomyces* sp. SA31 was isolated from the rhizospheric soil of *C. citratus* (DC) Stapf. and genome-based information on its antimicrobial potential and its taxonomic position were reported. Moreover, the separation, structure elucidation, and biological evaluation of the secondary metabolites of strain SA31 were determined as well.

2. Materials and Methods

2.1 Isolation of the actinomycete and primary screening of antimicrobial activity

Soil surrounding the roots of the lemongrass plant (*Cymbopogon citratus* (DC) Stapf.) was collected and dried at room temperature. The dried soil sample was heated at 100°C for 1 h (Nonomura & Ohara, 1969); after that, a ten-fold serial dilution was performed with a basic lauryl sulfate solution (0.01% w/v). The diluted soil solutions (10^{-3}- 10^{-6}) were plated on glycerol yeast extract (GYE) agar supplemented with nystatin (50 µg/mL) and incubated at 30°C for seven days. The colonies of actinomycetes were collected and purified on glycerol yeast extract medium for further experiments. Antibacterial and anticandidal activities were determined by the agar plug diffusion method, according to Messaoudi et al. (2015), with minor modifications. Inoculum of pathogenic bacteria and Candida albicans ATCC 10231 were swabbed on Mueller Hilton Agar (MHA) and Sabouraud Dextrose Agar (SDA), respectively. An agar plug (6 mm in diameter) of the 14 days old actinomycete on GYE medium was transferred onto the surface of inoculated MHA and SDA for screening of antibacterial and anticandidal activities, respectively. The inhibition zone was detected after incubating at 37 °C for 24 h for bacteria and at 30 °C for 48 h for yeast. A dual culture assay was selected to evaluate the antifungal efficacy of the isolated actinomycete as previously described by Sarven et al. (2020) with minor modifications. An agar plug (6 mm in diameter) of 5 days old filamentous fungi was transferred onto Potato Dextrose Agar (PDA) and placed 3 cm away from the periphery of the petri dish. An agar plug (6 mm in diameter) of the 14 days old of actinobacteria was placed 3 cm apart from pathogenic fungi on the opposite side. Each plate was incubated at 30°C for five days. The antifungal activity of actinomycete was expressed as percent of inhibition and was calculated based on the following formula,

$$\text{Percentage of inhibition} = (R1-R2)/R1 \times 100$$

R1 = Radial growth of pathogenic fungi without antagonistic interaction (control)
R2 = Radial growth of pathogenic fungi with antagonistic interaction

2.2 Morphological, physiological, biochemical, and chemotaxonomic characterizations of the actinomycete strain

To assign the taxonomic position of strain SA31 at the genus level, spore morphological analysis and determination of the isomer of diaminopimelic acid (DAP) in the cell wall peptidoglycan were performed. Spore morphology was observed by a scanning electron microscope (SEM) using a culture grown at 30°C for fourteen days on International *Streptomyces* Project medium 2 (ISP2) agar (4 g glucose, 4 g yeast extract, 10 g malt extract, 1 L distilled water, pH 7.2). Samples for the scanning electron microscopy were prepared by the protocol suggested by Duangupama et al. (2022). Growth at different temperatures (10-45°C), and salt levels of 0-10 % (w/v) NaCl (at single unit intervals) was tested on ISP2 agar. The pH range for growth (4.0-12.0 at an increment of 0.5 pH unit) was done by cultivation at 30°C in ISP2 broth for 14 days. Carbon and nitrogen utilization (1%, w/v) were examined using the method recommended by Shirling & Gottlieb (1966), Gordon et al. (1974), and Supong et al. (2013). Nitrate reduction, gelatin liquefaction, and the hydrolysis of casein (1%, w/v) and starch (1%, w/v) were evaluated using the protocols

suggested by Williams & Cross (1971), Gordon et al. (1974), and Arai et al. (1975). The isomer of DAP was prepared and analyzed according to the protocol of Komagata & Suzuki (1988).

2.3 Genome-taxonomic and genome analyses for secondary metabolites of the actinomycete strain

To prepare the cells for genomic extraction, the selected actinomycete was cultivated on GYE agar at room temperature for seven days. Then, four single colonies were inoculated in GYE broth for seven days with shaking at 180 rpm, and the gathered cells were subjected to HiPureATM Bacterial Genomic DNA Purification Kit (Himedia, India). The genomic DNA was sequenced using the MiSeq system (Illumina Inc, USA). The raw sequences were assessed for quality using FASTQC software. To remove adapters and poor-quality reads, the sequences were edited using Trim Galore. Then, the filtered reads were submitted to Unicycler, a genome assembly program, and the assembled genome was annotated by Rapid Annotation using Subsystem Technology (RAST) version 2.0 (Aziz et al., 2008; Overbeek et al., 2014) along with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). To analyze the 16S rRNA gene, the 16S rRNA sequence was obtained from the genome of strain SA31 (JABUXT000000000) using the tool in the EzBiocloud web service (http://www.ezbiocloud.net/) (Yoon et al., 2017), and similarity values were determined by the same server. The average nucleotide identity (ANI) values, ANI-blast (ANIb), ANIMUMmer (ANIm), and the correlation indexes of tetra-nucleotide signature (Tetra) were calculated using the JSpecies Web Server (Richter et al., 2016). The average amino acid identity (AAI) value was evaluated using the Kostas Lab ANI calculator (Rodriguez & Konstantinidis, 2014). The digital DNA-DNA hybridization (dDDH) values were calculated using the genome-to-genome distance calculator (GGDC 2.1; blast+method) (Meier-Kolthoff et al., 2013), and the phylogenomic and 16S rRNA gene trees were constructed using the TYGS type strain genome server (https://tygs.dsmz.de/) (Meier-Kolthoff & Göker, 2019). The biosynthetic gene cluster of strain SA31 was determined using antiSMASH (Blin et al., 2021). To determine the genes related to enzyme production, the genome of SA31 was analyzed using blastp on the Uniprot database with matrix; blosum62 (https://www.uniprot.org/blast) (UniProt Consortium, 2019).

2.4 Production and isolation of secondary metabolites from the actinomycete strain

Streptomyces sp. SA31 was cultured in 10 L of ISP2 broth on a rotary shaker (150 rpm) at 30°C for 14 days. After 14 days of cultivation, the culture broth was extracted with an equal volume of ethyl acetate (EtOAc) three times in a separatory funnel. It was evaporated with a rotary evaporator at 40°C to obtain the crude ethyl acetate extract. The antifungal-guided fractionation of the EtOAc extract led to the isolation of geldanamycin **(1)**, together with a known 17-*O*-demethylgeldanamycin **(2)**. The EtOAc crude extract (0.8 g) was firstly separated via a silica gel open-column chromatography with elution by $CHCl_3$:CH_3OH (9:1) to yield 4 fractions. The active EtOAc fraction (fraction F2, 0.38 g) was purified continuously in a Sephadex LH-20 column using an isocratic elution of 100% MeOH to give three subfractions (F2F1-F2F3). The active subfraction F2F2 (100.5 mg) was purified by preparative HPLC. Elution was with a linear gradient system of 30-80% CH_3CN in H_2O over 40 min at the flow rate of 15 mL/min and the following compounds were obtained: **1** (68.4

mg) and **2** (10.2 mg). Geldanamycin **(1)**: Yellow solid; ; $[\alpha]_D^{25}$ +11.32 (*c* 0.100, $CHCl_3$); UV λ_{max} (log ε, MeOH) 305 (3.06), 255 (2.94) and 205 (3.90) nm; IR (ν_{max}, cm^{-1}) 3437, 1700 and 1,653; ^1H (500 MHz) and ^{13}C NMR (125 MHz) data in $CDCl_3$; HRESIMS *m/z* 583.2626 $[M+Na]^+$ (calcd. for $C_{29}H_{40}N_2O_9Na$, 583.2624). 17-*O*-demethylgeldanamycin **(2)**: Brown solid; $[\alpha]_D^{25}$ +5.34 (*c* 0.100, MeOH); UV λ_{max} (log ε, MeOH) 313 (3.99), 244 (3.84) and 204 (4.63) nm; IR (ν_{max}, cm^{-1}) 3393, 1713 and 1648; ^1H (500 MHz) and ^{13}C NMR (125 MHz) data in CD_3OD+$CDCl_3$; HRESIMS m/z 569.2505 [M+Na]+ (calcd. for $C_{28}H_{38}N_2O_9Na$, 569.2503).

2.5 Structure determination of the secondary metabolites of the actinomycete strain

UV spectroscopy was carried out in MeOH on a Spekol 1200 spectrophotometer, Analytik Jena. Optical rotations were measured with a JASCO P-1030 digital polarimeter. FT-IR spectra were measured on a Bruker ALPHA spectrometer. NMR spectra were acquired and calibrated in $CDCl_3$ on a Bruker Avance 500 MHz NMR spectrometer. HRESIMS data were obtained from a Bruker MicrOTOF spectrometer.

2.6 Preparation of pathogenic microorganisms and determination of antimicrobial activities

Staphylococcus aureus ATCC 25923, *Kocuria rhizophila* ATCC 9341, *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, and *Bacillus subtilis* ATCC 6633 were grown on trypticase soy agar (TSA) for 18 h at 37°C. According to Clinical and Laboratory Standards Institute (CLSI, 2008) guidelines, a few single colonies were directly suspended in normal saline solution (0.9% w/v). The optical density of the bacterial suspension was adjusted to 0.1 at 625 nm. *Candida albicans* ATCC 10231 was grown on SDA at 37°C for 24 h, and the tested inoculum was prepared by direct colony suspension as described above. For the dual culture assay, *Aspergillus flavus* IMI 242684, an aflatoxin-producing fungus, was cultivated on PDA at room temperature for five days and the young mycelium on the peripheral edge was cut off for the tests. Antibacterial activity against *B. subtilis*, *E. coli, K. rhizophila, P. Aeruginosa,* and *S. aureus* was tested by resazurin microplate assay (REMA) (Sarker et al., 2007). Minimum inhibitory concentration (MIC) represents the lowest concentration that inhibited 90% growth of the bacterium. Vancomycin was used as a positive control. The maximum concentration for antibacterial activity was 512 µg/mL. Anti-Candidal activity was evaluated using the broth microdilution method recommended in the (CLSI) M27-A3 manual (CLSI, 2008). Fluconazole (FLC) was used as the positive control. The green fluorescent protein microplate assay (GFPMA) was used to evaluate anti-fungal activity against *A. flavus* (Chutrakul et al., 2013). Amphotericin B was used as the positive control. The maximum concentration for anti-*Candida* and anti-*A. flavus* in this study was 25 µg/mL. Each assay was done in triplicate.

3. Results and Discussion

3.1 Isolation of actinomycete and primary screening of antimicrobial activity

It is known that the rhizome of *C. citratus* (DC) Stapf. produces a variety of volatile oils that show antimicrobial activity against several pathogenic microorganisms (Oladeji et al., 2019). Furthermore, the microorganisms living around the root of *C. citratus* (DC) Stapf.

may differ from soil microorganisms. A *Streptomyces*-like microorganism, designated strain SA31, was isolated from the rhizospheric soil of *C. citratus* (DC) Stapf. To our knowledge, we found that the strain SA31 exhibited antimicrobial activity against *B. subtilis*, *K. rhizophila*, *S. aureus*, and *C. albicans* with inhibition zones at 20.3, 22.1, 18.2, and 16.5 mm, respectively. Moreover, strain SA31 inhibited the mycelial growth of *A. flavus* with a percentage of inhibition at 61.1. This corresponded to the study of Anansiriwattana et al. (2006) and Campos-Avelar et al. (2021), who reported that many *Streptomyces* spp. were able to inhibit the growth of several Gram-positive bacteria and fungi. Moreover, our results were in agreement with others who found that several members of the genus *Streptomyces* produced a variety of secondary metabolites that exhibited antimicrobial activity (Tohyama et al., 2004; Phongsopitanun et al., 2014; Buatong et al., 2019). Thus, it can be deduced that strain SA31 may produce a range of interesting bioactive secondary metabolites since it could inhibit the growth of many pathogens.

3.2 Identification of the actinomycete strain SA31

Based on morphology, strain SA31 produced a dark brown substrate mycelium and dark brownish grey aerial spore mass with brown diffusible pigment on ISP2 medium. Spiral chain spores with rugose surfaces developed on the aerial mycelium (Figure 1). In the case of the chemotaxonomic analysis, strain SA31 presented LL-diaminopimelic acid (DAP) in the peptidoglycan. This chemotaxonomic characteristic is commonly found in the cell walls of members of the genus *Streptomyces* (Kämpfer, 2012). The 16S rRNA gene sequence (1,520 nt) of strain SA31 was deposited at DDBJ/ENA/GenBank under the accession OM527181. The EzBioCloud analysis demonstrated that the complete 16S rRNA gene sequence of strain SA31 had the highest percentage of similarity with the 16S rRNA gene sequence of *Streptomyces samsunensis* M1463^T at 99.93% followed by *S. malaysiensis* NBRC 16446^T (99.44%) and *S. rhizosphaericus* NBRC 100778^T (98.96%). According to the evolutionary pattern of strain SA31, it could be confirmed that strain SA31 was a member of the genus of *Streptomyces*. It shared a common ancestor with *S. samsunensis* M1463^T in the same cluster with a high bootstrap value of 88% (Figure 2).

Furthermore, we also analyzed the genome-based taxonomy of strain SA31 to clarify its taxonomic position at the species level. The position of strain SA31 in the phylogenomic tree indicated that it formed a tight cluster with *Streptomyces solisilvae* HNM 0141^T and *S. samsunensis* DSM 42010^T (Figure 3). Strain SA31 shared ANIb, ANIm, and AAI values with *S. samsunensis* DSM 42010^T (96.5, 97.7, and 96.4%) and *S. solisilvae* HNM 0141^T (98.6, 98.7, and 98.7%), which were significantly above the 95-96% cut-off point for species demarcation (Richter & Rossello-Mora, 2009; Konstantinidis et al., 2017).

In addition, strain SA31 showed dDDH values with *S. solisilvae* HNM 0141^T (88.3%) and *S. samsunensis* DSM 42010^T (75.8%), which were higher than 70%, which was the cut-off values for strain SA31 to be assigned to the same species (Wayne et al., 1987) (Table 1). In our study, we found that *S. solisilvae* HNM 0141^T exhibited high similarity levels of ANIb (96.5%), ANIm (97.7%), AAI (96.8%), and dDDH (75.6%) values with *S. samsunensis* DSM 42010^T, indicating that *S. solisilvae* HNM 0141^T may be the same species as *S. samsunensis* DSM 42010^T.

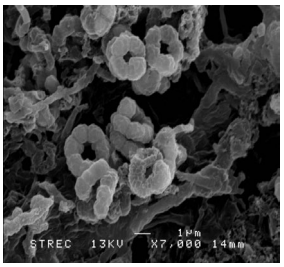


Figure 1. Scanning electron micrograph of *Streptomyces* sp. SA31 grown on ISP2 medium at 30°C for 14 days. Bar, 1 μm

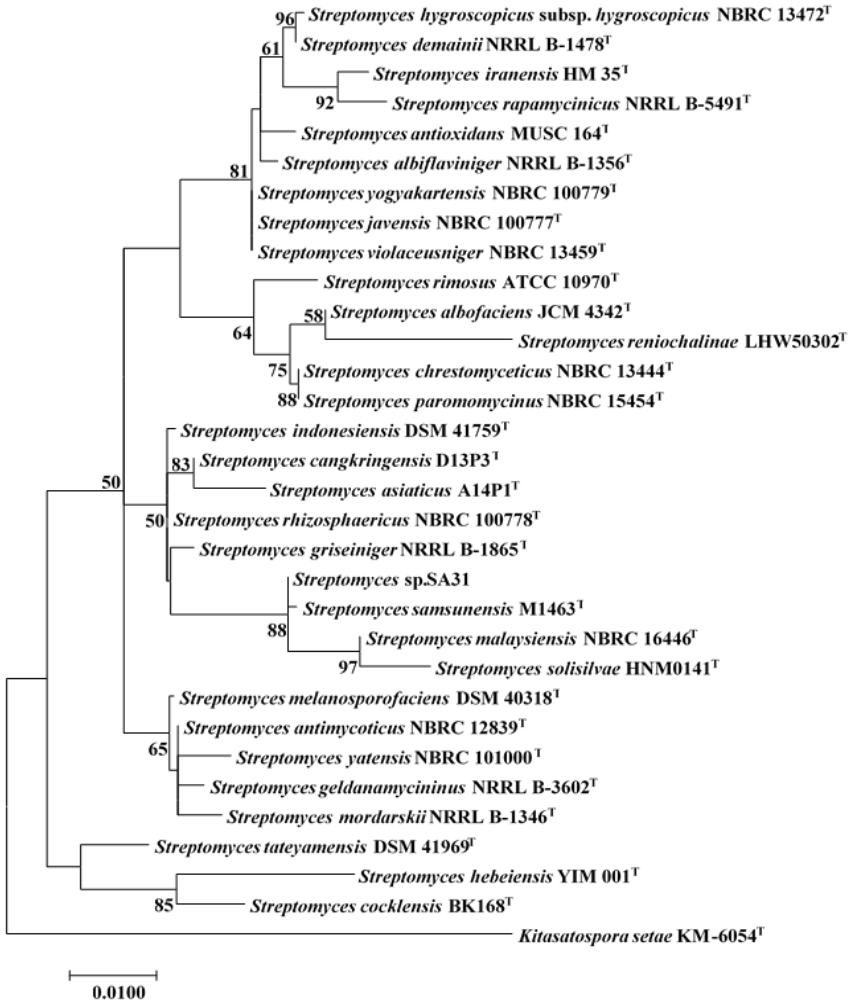


Figure 2. A maximum likelihood phylogenetic tree based on 16S rRNA gene sequence of *Streptomyces* sp. SA31, along with closely related members in the genus *Streptomyces*. The percentage of bootstrap value (1,000 replicates) is presented on each internal node; only values higher than 50% are presented. Scale bar, 0.0100 substitutions per nucleotide position.

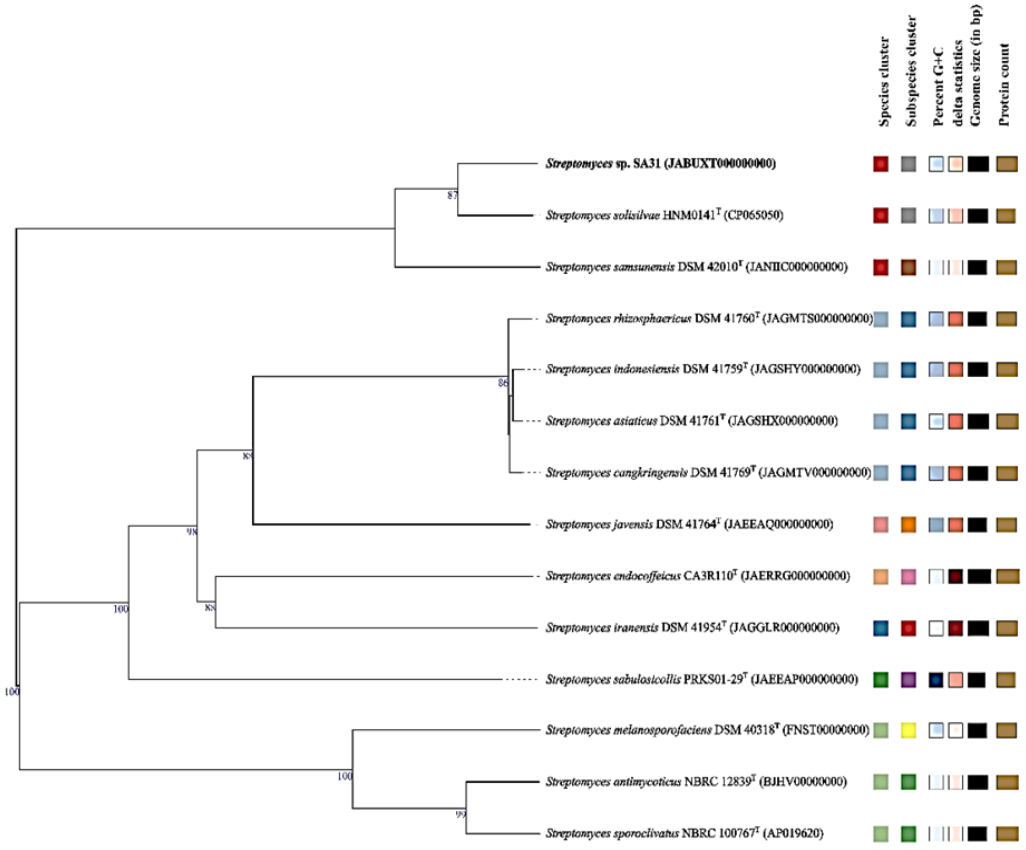


Figure 3. The phylogenomic tree of *Streptomyces* sp. SA31 and its related type strains of the genus *Streptomyces* obtained from TYGS. The branch lengths are scaled in terms of GBDP distance formula d_5. The numbers above branches are GBDP pseudo-bootstrap support values > 60% from 100 replications, with an average branch support of 83.1%.

Table 1. ANIb, ANIm, tetra values, AAI, and dDDH of *Streptomyces* sp. SA31 and related type strains

Query Genome	Reference Genome	ANIb (%)	ANIm (%)	Tetra Values	AAI (%)	Digital DNA-DNA Hybridization Relatedness Formula 2*				G+C Difference
						% dDDH	Model C.I. (%)	Distance	Prob. DDH ≥ 70 (same species)	
Streptomyces sp. SA31	*S. samsunensis* DSM 42010^T	96.5	97.7	0.99959	96.4	75.80	72.8 - 78.5	0.0285	86.63	0.1
	S. solisilvae HNM0141^T	98.6	98.7	0.99980	98.7	88.30	85.9 - 90.4	0.0139	95.2	0.0

* A formula (identities/HSP length) that is liberated of genome length and is thus prosperous against the use of incomplete draft genomes.

Thus, we suggested that *S. solisilvae* HNM 0141^T should be reclassified as *S. samsunensis* after further polyphasic taxonomic study. *Streptomyces samsunensis* M1463^T was first described by Sazak et al. (2011); it was isolated from the rhizospheric soil of *Robina pesudoacacia*. In the case of strain SA31, it formed a spiral spore chain with a rugose surface. Moreover, it could utilize D-cellobiose, D-fructose, D-galactose, D-glucose, D-ribose, L-methionine, L-phenylalanine, DL-2-aminobutyric acid, 4-hydroxyproline, L-arginine. It could also perform gelatin liquefaction, starch hydrolysis, milk peptonization and nitrate reduction. The strain grew in media containing 0 to 3% NaCl (w/v) at pH 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0, and at 15 to 40°C but no growth at 45°C. These phenotypic characteristics were consistent with the phenotypic details found for *S. samsunensis* (Sazak *et al*. 2011). It is evident from the genotypic and phenotypic results that the actinomycete strain SA31 could be identified as *S. samsunensis*.

3.3 Genome analysis for secondary metabolites of *Streptomyces* sp. SA31

Streptomyces sp. SA31 was stated for draft genome sequence and the sequence was deposited to GenBank under bioproject PRJNA638085 and accession number JABUXT000000000. The genome sequences were assembled with an average coverage at 200X, resulting in 242 contigs and N50 of 177 kb. The draft genome covered 11,850,342 bp with a high G+C content at 71.0%. The number of protein-encoding sequences (CDSs) was 9,350. The annotation result from the RAST server showed a total of CDSs classified into 357 subsystems. More than 50% of the predicted CDSs were related to metabolic and biosynthesis pathways and could be subdivided into sequences related to amino acids and derivatives at 20.34%, carbohydrates at 16.42%, fatty acids at 10.45%, cofactors, and vitamins at 9.42%, and proteins at 8.03%. The other genes present were involved with the necessity for surviving and thriving in the environment as well as cellular processes and information storage (Figure 4).

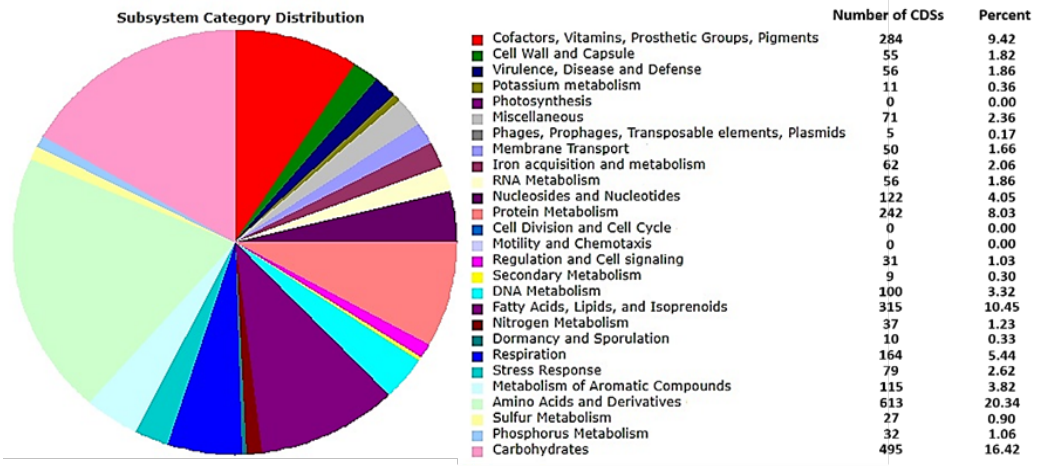


Figure 4. Subsystem categories and features distribution of the *Streptomyces* sp. SA31 genome predicted by RAST annotation server. The percentage of CDSs involved in metabolic and biosynthesis pathways is indicated.

Secondary metabolite genes were analyzed using antiSMASH server 6.0, and the results of the distribution of biosynthetic gene clusters are shown in Figure 5. The genome of strain SA31 consisted of 71 gene clusters from 19 types of secondary metabolite-related gene clusters. Polyketide synthase biosynthesis was found as the majority (>50%) of gene clusters including 24 gene clusters for Type 1 polyketide synthase (T1PKS), and 2 gene clusters for Type 2 polyketide synthase (T2PKS) and Type 3 polyketide synthase (T3PKS). The rest of the clusters were involved with biosynthetic pathways of NRPS (15.18%), NRPS-like fragment (7.14%), terpenes (7.14%), betalactone (3.57%), siderophores (2.68%), ladderane (2.68%), arylpolyene (1.79%), bacteriocin (1.79%), and butyrolactone (1.79%). As similarity result from antiSMASH, 15 gene clusters of the strain SA31 genome exhibited high similarities (>50%) to the following known secondary metabolite gene clusters: enterocin biosynthetic gene cluster (100%), coelichelin biosynthetic gene cluster (100%), geosmin biosynthetic gene cluster (100%), pristinol biosynthetic gene cluster (100%), ectoine biosynthetic gene cluster (100%), 2-methylisoborneol biosynthetic gene cluster (100%), nigericin biosynthetic gene cluster (88%), spore pigment biosynthetic gene cluster (83%), ochronotic pigment biosynthetic gene cluster (75%), elaiophylin biosynthetic gene cluster (66%), mediomycin A biosynthetic gene cluster (65%), niphimycins C-E biosynthetic gene cluster (64%), belactosin A biosynthetic gene cluster (62%), 5-isoprenylindole-3-carboxylate β-D-glycosyl ester (61%), and hopene biosynthetic gene cluster (53%). The genome of *Streptomyces* sp. SA31 was assumed for the putative secondary metabolite biosynthesis gene clusters (smBGCs) responsible for the synthesis of antimicrobial secondary metabolites, including enterocin, nigericin, mediomycin A, niphimycins C-E, and elaiophylin. The enterocin biosynthesis gene cluster of strain SA31 is shown to be closely related to the enterocin biosynthetic gene cluster from *Streptomyces maritimus* (MIBiG accession BGC0000220, GenBank accession AF254925.1, positions 69,507 - 142,049). Enterocin is a bacteriostatic polyketide antibiotic that was first isolated and characterized from two strains of soil actinomycetes, *Streptomyces candidus* var. *enterostaticus* WS-8096 and *Streptomyces viridcochromogenes* M-127 and this compound demonstrated an antibacterial spectrum against *E. coil*, *Proteus vulgaris*, *Sarcina lutea*, *S. aureus*, and *Corynebacterium xerosis* (Miyairi et al., 1976). Another interesting compound was nigericin. Nigericin is a polyether ionophore with various biological activities such as antibacterial, antifungal, antiviral, antimalarial, and anticancer. This compound was first isolated from *Streptomyces hygroscopicus* (Faul & Huff, 2000). The nigericin synthesis gene clusters of strain SA31 were related to known clusters from *Streptomyces violaceusniger* DSM 4137 (MIBiG accession BGC0000114, GenBank accession DQ 354110.1, positions 1–28,126) at 80%. Mediomycin A, which is a natural linear polyene polyketide antibiotic with strong antifungal activity, was first found in *Streptomyces mediocidicus* ATCC 23936 and *S. hygroscopicus* TP-A0623 (Sun et al., 2018). The mediomycin A biosynthesis gene cluster in the genome of strain SA31 showed 65% similarity to the corresponding gene cluster of *Kitasatospora mediocidica* (MIBiG accession BGC0001662, GenBank accession MF139773.1, positions 222,800–405,501).

Finally, the elaiophylin and niphimycins C-E biosynthetic gene clusters of strain SA31 exhibited correspondence with the nucleotide sequences of the elaiophylin biosynthetic gene cluster of an unknown organism (MIBiG accession BGC0000053, GenBank accession GP697151.1, position 1–49,127), and the niphimycins C-E biosynthetic genes cluster of *Streptomyces* sp. IMB7-145 (MIBiG accession BGC0001700, GenBank accession MF671979.1, positions 1–58,225) with the percentages of similarity at 66% and 64%, respectively. Both elaiophylin, and niphimycins are members of the macrolide antibiotic group. Elaiophylin exhibited an extensive range of biological activities that included anthelmintic, anticancer, immunosuppressive, anti-inflammatory, and antiviral

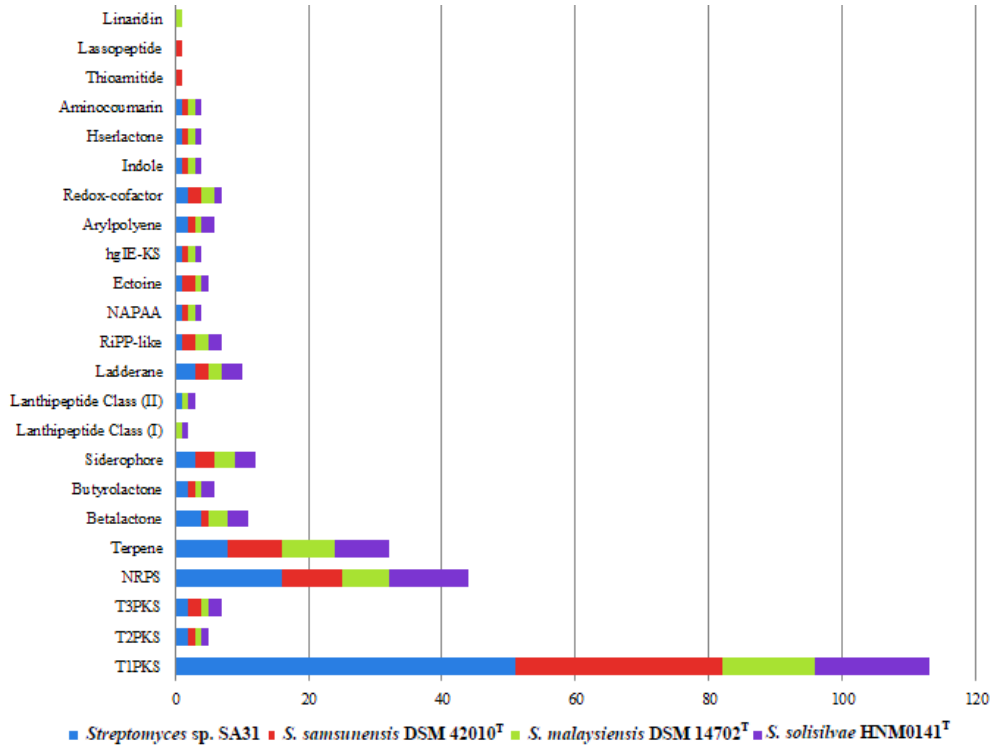


Figure 5. Biosynthetic gene clusters presented in the genomes of *Streptomyces* sp. SA31 and its close relatives using antiSMASH 6.0. Highly variable profiles were found between the strains. The genomes of the *Streptomyces* strains in the study were found to be particularly rich in clusters of PKS, terpene, and NRPS.

(Usuki et al., 2006; Gui et al., 2019). In the case of terpene biosynthetic gene clusters, the genome of strain SA31 harbored 2 terpene biosynthesis gene clusters that exhibited 100% identity with geosmin and pristinol biosynthetic gene clusters in *Streptomyces coelicolor* A3(2) (MIBiG accession BGC0001181, GenBank accession AL645882.2, positions 321447–343810) and *Streptomyces pristinaespiralis* ATCC 25486 (MIBiG accession BGC0001746, GenBank accession NZ_CM000950.1, positions 1–19686), respectively (Jiang et al., 2007; Klapschinski et al., 2016). The remaining terpene biosynthesis genes of strain SA31 exhibited 61% similarity to the hopene biosynthetic gene cluster of *S. coelicolor* A3(2) (MIBiG accession BGC0000663, GenBank accession AL645882.2, positions 1–18,423). For NRPS biosynthetic gene clusters, the coelichelin and ochronotic pigment biosynthetic gene clusters were found in the genome of strain SA31 with 100% similarity to the coelichelin biosynthetic gene cluster in *S. coelicolor* A3(2) (MIBiG accession BGC0000325, GenBank accession AL645882.2, positions 64,847–115,742) and 75% similarity to the ochronotic pigment biosynthetic gene cluster of *Streptomyces avermitilis* (MIBiG accession BGC0000918, GenBank accession AB070935.1, positions 89,586–133,596), respectively (Redenbach et al., 1996; Ōmura et al., 2001; Bentley et al., 2002;). Furthermore, strain SA31 contained gene clusters exhibiting relatedness to clusters known to synthesize ectoine (100%) in *Streptomyces anulatus* (MIBiG accession BGC0000853, GenBank accession AY524544.1, positions 64,847–115,742), betalactosin

A/C (62%) in *Streptomyces* sp. (MIBiG accession BGC0001441, GenBank accession KY249118.1, positions 53,684–82,918) (Asai et al., 2000; Asai et al., 2004; Prabhu et al., 2004; Wolf et al., 2017), together with 5-isoprenylindole-3-carboxylate β-D-glycosyl ester (61%) in *Streptomyces* sp. RM-5-8 (MIBiG accession BGC0001483, GenBank accession KT895008.1, positions 104,988–126,136) (Wang et al., 2015; Elshahawi et al., 2017). In our analysis of the genome of strain SA 31, there were several type I polyketide (T1PKS) gene clusters with low similarity scores (<50%) compared with known clusters according to antiSMASH analysis. Importantly, this suggested that strain SA31 might be a potential source for producing new valuable compounds with potent biological activity, especially antimicrobial activity. Moreover, the geldanamycin biosynthetic gene cluster in the genome of strain SA31 showed only 34% similarity to *S. hygroscopicus* NRRL 3602 (Figure 6) (Rascher et al., 2003).

The work of Rascher et al. (2003) provides evidence that a group of genes, including *gdmH*, *gdmI*, *gdmJ*, *gdmK*, *gdmG*, *gdmO,* were involved in geldanamycin biosynthesis. Previous studies reported by Shin et al. (2008) and He et al. (2008) proved that the genes *gdmL, gdmM, gdmRI, gdmRII*, and *gdmRIII* were required to synthesize geldanamycin and its derivative, 17-*O*-demethylgeldanamycin. Similarly, the genes involved in geldanamycin production, including *gdmF*, *gdmL*, *gdmM*, *gdmN*, *gdmH*, *gdmI*, *gdmJ*, *gdmK*, *gdmG*, *gdmO*, *gdmP*, *gdmRI*, *gdmRII*, *gdmRIII*, were retrieved in the genome of strain SA31 as well (Table 2). In this study, we found that *S. samsunensis* DSM 42010^{T} and *S. solisilvae* HNM 0141^{T}, the closely related *Streptomyces* species to strain SA31, also contained the geldanamycin biosynthetic gene cluster in their genomes. This result indicated that the geldanamycin biosynthetic gene clusters are related to the species within this phylogenetic cluster. Therefore, it can be implied that strain SA31 has a high possibility to produce geldanamycin. Our assumption based on the genome data led to the isolation of geldanamycin in the culture broth of strain SA31.

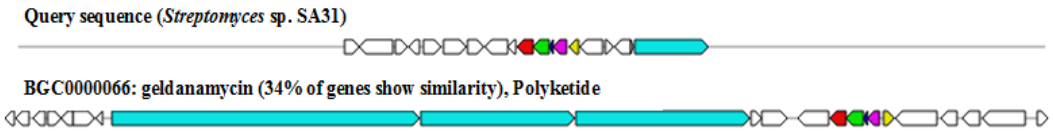


Figure 6. The BLAST result using antiSMASH 6.0 shows the type 1 polyketide synthase (T1PKS) in the genome of *Streptomyces* sp. SA31, which is 34% similar to the known biosynthetic gene clusters of geldanamycin of *S. hygroscopicus* (BGC0000066).

3.4 Isolation and structure elucidation of secondary metabolites of strain SA31

The culture broth of strain SA31 was extracted using ethyl acetate and evaporated under vacuum to yield 0.8 g of the crude extract. Then, the crude EtOAc extract of strain SA31 was purified by silica gel and Sephadex LH-20 columns. This was followed by preparative HPLC to give two compounds **1-2**. The chemical structures of the isolated compounds (Figure 7) were elucidated by several spectroscopic techniques including NMR, MS, UV, and IR spectroscopy. Compound **1** was obtained as a yellow solid showing optical rotation $[\alpha]_D^{25}$ +11.32 (c 0.100, $CHCl_3$). The UV spectrum exhibited UV λmax (log ε, MeOH) at 305, 255, and 205 nm. The IR absorption spectrum displayed characteristic bands at 3,437 cm^{-1} (O-H stretching), 1,700 cm^{-1} (C=O stretching), and 1,653 cm^{-1} (C=O stretching, amide band).

Table 2. *In silico* genes encoding proteins relating to the geldanamycin production of *Streptomyces* sp. SA31

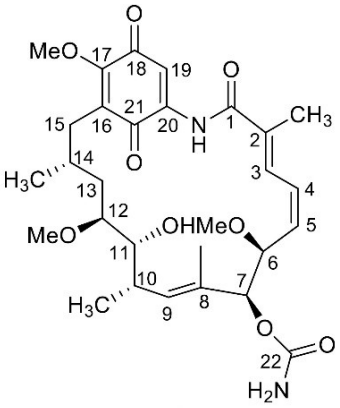
Product	Span (nt)	Score	Percent Similarity	Closest Match
Geldanamycin production				
*Rht*B family transporters	1-214	1,048	98.6	*S. autolyticus*
Alpha/beta hydrolase	17-101	492	100.0	*S. autolyticus*
*Ara*C family transcriptional regulator	4-294	1,475	98.6	*S. autolyticus*
Flavin-dependent monooxygenase (*gdm*L)	1-480	2,071	83.7	*Streptomyces* sp. NRRL F-5122
Type I polyketide synthase	3282-4868	12,052	88.0	*S. solisilvae*
		12,046		*S. malaysiensis*
Amide synthase (*gdm*F)	1-256	1,326	98.4	*S. autolyticus*
Flavin-dependent oxidase (*gdm*M)	1-547	2,838	98.9	*S. autolyticus*
Carbamoyltransferase (*gdm*N)	4-687	3,524	99.6	*S. autolyticus*
*gdm*H	1-370	1,901	98.9	*S. autolyticus*
*gdm*I	1-359	1,818	99.2	*S. autolyticus*
*gdm*J	6-85	420	98.8	*S. autolyticus*
*gdm*K	1-288	1,440	99.3	*S. autolyticus*
*gdm*G	1-218	1,085	98.6	*S. autolyticus*
*Lux*R-type domain-containing protein (*gdm*RI)	1-948	4,879	99.6	*S. autolyticus*
*Lux*R-type domain-containing protein (*gdm*RII)	1-910	4,531	98.5	*S. autolyticus*
*Tet*R family transcriptional regulator (*gdm*RIII)	1-212	922	90.8	*Streptomyces spongiae*
3-dehydroquinate synthase (*gdm*O)	17-353	1,711	99.1	*S. autolyticus*
Cytochrome P450 (*gdm*P)	5-400	2,006	99.2	*S. autolyticus*
Putative hydrolase	1-235	1,227	100.0	*S. autolyticus*

HRESIMS data revealed a molecular formula of $C_{29}H_{40}N_2O_9Na$, showing a mass ion peak at m/z 583.2626 $[M+Na]^+$. Therefore, the molecular formula of this compound was determined to be $C_{29}H_{40}N_2O_9$ with MW of about 560.

The spectroscopic information including ^{1}D (^{1}H NMR, ^{13}C NMR) spectral data led to the elucidation of the chemical structure of compound **1** as follows: ^{1}H-NMR ($CDCl_3$, 500

MHz) δ: 0.97 (3H, d, *J* =6), 1.00 (3H, d, *J* =6.8), 1.8 (2H, m), 2.02 (3H, s), 3.30 (3H, s), 3.36 (3H, s), 4.12 (3H, s), 1.79 (3H, s), 2.37 (2H, m), 1.60 (brs), 2.76 (m), 3.34 (m), 3.53 (d, *J* =7.2), 4.31 (d, *J* =9.4), 5.18 (s), 5.81 (d, *J* =9.37), 5.89 (dd, *J* =10.2, 10.3), 6.57 (dd, *J* =11.3, 11.5), 6.94 (d, *J* =11.6), 7.28 (s), 4.73 (NH_2, brs), 8.75 (NH, s), ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: 183.3 (C-18), 185.0 (C-21), 168.2 (C-1), 156.0 (7-$OCONH_2$) (Figures 8 & 9). The ^{1}H NMR and ^{13}C NMR spectral data corresponded to those previously reported for geldanamycin (Rinehart & Shield, 1976; Omura et al., 1979), which was isolated from the culture broth of *S. hygroscopicus* (DeBoer et al., 1970).

Compound 1



Compound 2

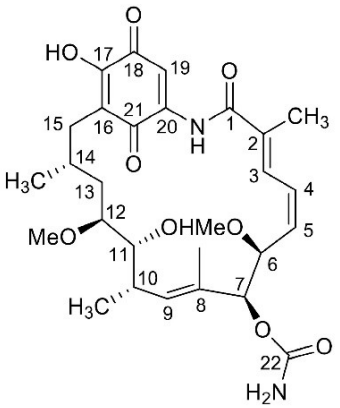


Figure 7. Structures of compound **1** (geldanamycin) and compound **2** (17-*O*-demethylgel- danamycin)

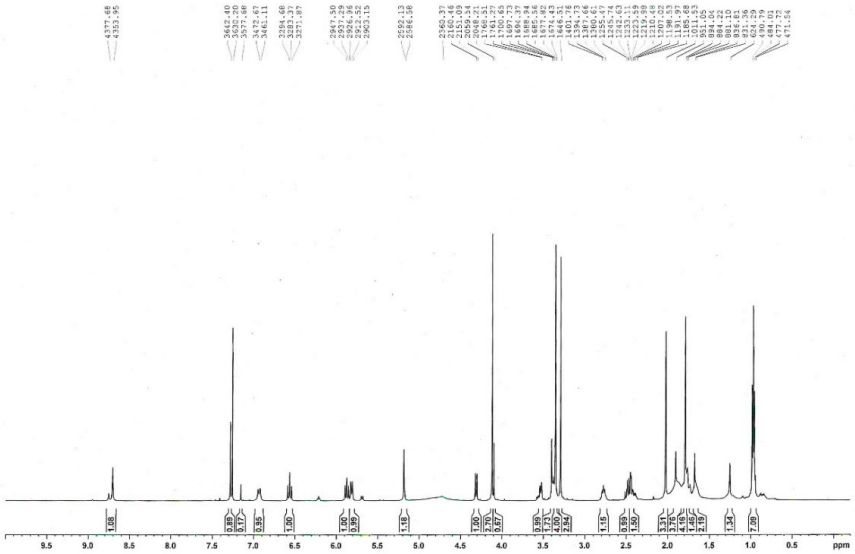


Figure 8. ^{1}H NMR spectrum of compound **1** in $CDCl_3$ (500 MHz)

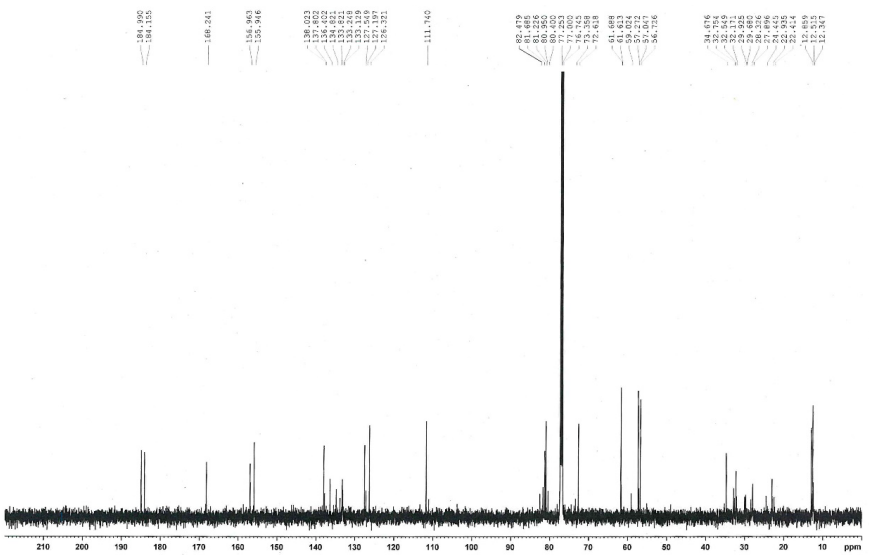


Figure 9. ^{13}C NMR spectrum of compound **1** in $CDCl_3$ (125 MHz)

Compound **2** was obtained as a brown solid. The molecular formula $C_{28}H_{38}N_2O_9$ was deduced by the analysis of HRESIMS spectrum, giving a mass ion peak at m/z 569.2505 $[M+Na]^+$. Partial spectroscopic information from the ^1H and ^{13}C NMR spectra data were identical to those reported for 17-*O*-demethylgeldanamycin (Clermont et al., 2010): ^1H-NMR ($CD_3OD+CDCl_3$, 500 MHz) δ: 0.92 (3H, d, *J* =6.9), 0.98 (3H, d, *J* =6.3), 1.62 (3H, s), 2.00 (3H, s), 3.34 (3H, s), 3.35 (3H, s), 1.63 (2H, m), 2.30 (2H, m), 1.73 (s), 2.71 (m), 3.36 (s), 3.44 (s), 4.43 (d, *J* =7.7), 5.12 (s), 5.65 (d, *J* =8.7), 5.87 (t, *J* =9.4, 10.1), 6.60 (t, *J* =11.4, 11.5), 7.01 (d, *J* =9.6), 7.70 (s), 8.13 (NH, s). ^{13}C-NMR (MeOD+$CDCl_3$, 125 MHz) δ: 183.9 (C-18), 185.3 (C-21), 170.1 (C-1) (Figures 10 and 11). A comparison of the spectral data of this compound to those reported (Clermont et al., 2010) confirmed that compound **2** was the known compound, 17-*O*-demethylgeldanamycin, which was isolated from the culture broth of *Streptomyces autolyticus* CGMCC 0516 (Yin et al., 2011).

3.5 Biological activity of compounds 1 and 2

Compounds **1** and **2** exhibited antifungal activity against *C. albicans* ATCC 10231 and *A. flavus* IMI 242684, with IC_{50} values ranging from 6.4±2.21 to >25 µg/mL. Both compounds exhibited weak antibacterial activity against *S. aureus* ATCC 25923, *K. rhizophila* ATCC 9341, and *B. subtilis* ATCC 6633 with MIC values of more than 512 µg/mL, and showed no activity against *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922 (Table 3). These findings opened up exciting possibilities for further research and potential applications. Compounds **1** and **2**, for instance, displayed mild antimicrobial activity, but they possessed moderate antifungal activity against *C. albicans* ATCC 10231 and *A. flavus*, mirroring the results of the geldanamycin derivatives reported in previous studies (Taechowisan et al., 2019; Wu et al., 2012).

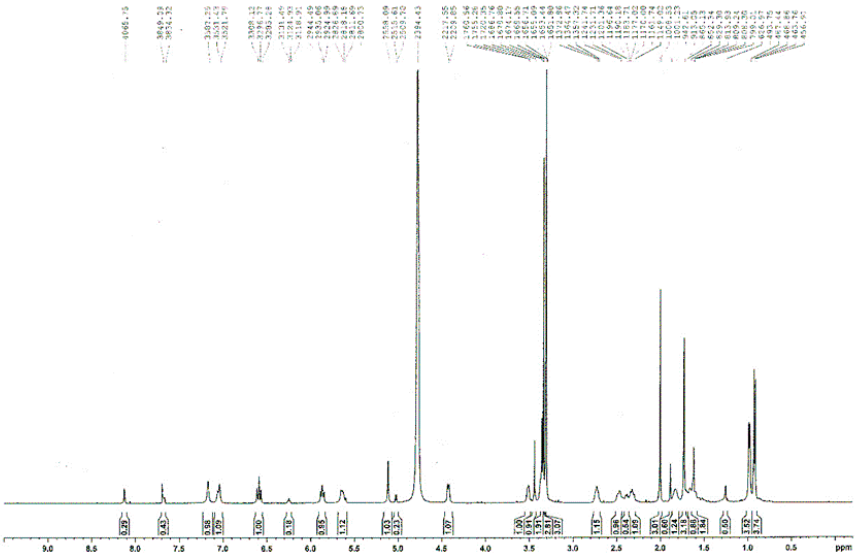


Figure 10. ^{1}H NMR spectrum of compound **2** in CD3OD+CDCl3 (500 MHz)

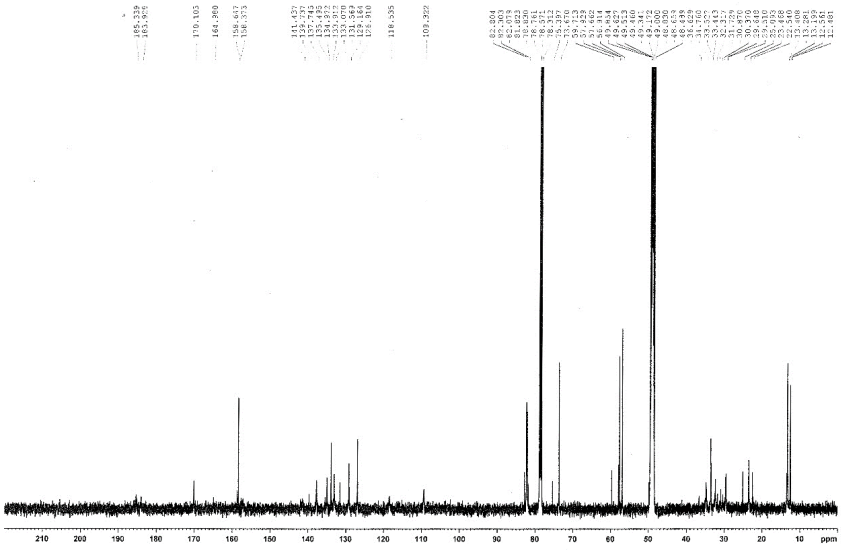


Figure 11. ^{13}C NMR spectrum of compound **2** in CD_3OD+$CDCl_3$ (125 MHz)

Table 3. Biological activity of secondary metabolites isolated from *Streptomyces* sp. SA31

Compound	Anti-*B. subtilis* MIC (µg/mL)	Anti-*K. rhizophila* MIC (µg/mL)	Anti-*S. aureus* MIC (µg/mL)	Anti-*C. albicans* MIC (µg/mL)	Anti-*A. flavus* MIC (µg/mL)
1	>512	>512	>512	6.4 ± 2.2	16.0 ± 1.8
2	>512	>512	>512	>25.0	>25.0
Vancomycin	2.0 ± 0.8	<2.00	<2.00	-	-
Amphotericin B	-	-	-	-	3.1 ± 1.9
Fluconazole	-	-	-	0.1 ± 0.04	-

Geldanamycin (**1**) was originally isolated from *S. hygroscopicus* var. *geldanus* var. *nova*. It was reported as a broad spectrum of antimicrobial agents against phytopathogenic fungi such as *Alternaria*, *Pythium*, *Botrytis*, and *Penicillium*. Furthermore, it also possessed intense anticancer activity (Clermon et al., 2010). To date, there were several *Streptomyces* species, including *Streptomyces melanosporofaciens* EF-76, *S. hygroscopicus* JCM 4427^T, *S. autolyticus* CGMCC 0516^T, *Streptomyces cameroonensis* JJY4^T, *Streptomyces zerumbet* W14, and *S. malaysiensis* SCSIO 41397, have been reported as geldanamycin producers (Wu et al., 2012; Boudjeko et al., 2017; Yin et al., 2017; Taechowisan et al., 2019; Xie et al., 2021). 17-*O*-demethylgeldanamycin **(2)** was first reported from *S. autolyticus* CGMCC 0516. It showed excellent antimalarial activity against *Plasmodium falciparum* and anticancer activity against NCI-H187 cell lines (Yin et al., 2011).

4. Conclusions

In conclusion, information on the identification and genome properties of *Streptomyces* sp. SA31 was reported in this study. Based on phenotypic and genome taxonomic characterization, strain SA31 was identified as *S. samsunensis*. Draft genome analysis of strain SA31 showed the presence of abundant secondary metabolite biosynthesis gene clusters such as terpene, PKS, LAP, NRPS, NRPS-like fragments, and beta lactone. The ethyl acetate extract of extracellular substances in the culture broth of strain SA31 showed the potential to inhibit the growth of Gram-positive bacteria, yeast, and filamentous fungi. This indicated that strain SA31 might be a potential source of the production of bioactive secondary metabolites. Interestingly, the BGCs predicted using antiSMASH indicated strain SA31 contained a geldanamycin biosynthetic gene cluster in its genome and this led to the isolation of two compounds, geldanamycin, and 17-*O*-demethylgeldanamycin. It can be concluded that the genomic information of strain SA31 provides important data that will contribute to the further study and understanding of metabolomic and transcriptomic that underly mechanisms for synthesizing bioactive compounds.

5. Acknowledgements

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6. Conflicts of Interest

The authors declare no conflict of interest.

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