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Exploring the potency of *Streptomyces koyangensis* strain SHP 9-3 isolated from the soil of Enggano Island (Indonesia) as an antibacterial source

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Abstract. *Pahira DSJ, Damayanti E, Lisdiyanti P, Mustofa, Hertiani T. 2023. Exploring the potency of* Streptomyces koyangensis *strain SHP 9-3 isolated from the soil of Enggano Island (Indonesia) as an antibacterial source. Biodiversitas 24: 1460-1466. Streptomyces* is a Gram-positive bacterium with the potential a produce beneficial antimicrobials. This study aimed to determine the antimicrobial activity, morphological characteristics, cytotoxicity, and chemical profile of *S. koyangensis* SHP 9-3. This bacterium was isolated from Enggano Island, Indonesia, and the morphological characteristics were determined using a *Scanning Electron Microscope* (SEM). Cultivation was conducted in the International Streptomyces Project (ISP-2) media using baffled flasks and a shaker incubator. The secondary metabolites were conducted on the extract against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Candida albicans* by a twofold dilution method. Concurrently, cytotoxicity testing was done on Vero cells. The extract's chemical profile was analyzed using LC-MS/MS. The results showed a prominent antibacterial activity as the extract inhibited the growth of *S. aureus* and *C. albicans* at 7.81 µg/mL. However, a growth inhibition against *E. coli* was observed at 250 µg/mL and *P. aeruginosa* at 31.25 µg/mL. The cytotoxic test showed the IC_{50} value on Vero cells was 5,015 µg/mL, indicating that the extract was nontoxic against Vero cells. The chemical profile of the extract by LC-MS/MS revealed the presence of five major components, i.e., cyclo (Pro-Val) ($C_{10}H_{16}N_2O_2$), kuraramine ($C_{12}H_{18}N_2O_2$), 1,4-diphenyl-1-pentanone ($C_{17}H_{18}O$), (3*S*,4*S*)-4-amino-3-hydroxy-6-methyl heptanoic acid ($C_{21}H_{42}N_2O_7$), and 2-ethyl-8-methyl-2,8-diazaspiro (4,5) decane-1,3-dione ($C_{11}H_{18}N_2O_2$).

Keywords: Antifungal, antimicrobial, LC-MS/MS, secondary metabolites, *Streptomyces*

INTRODUCTION

Antibiotic resistance is still a global concern in the health sector (Jakubiec-Krzesniak et al. 2018; Atikana et al. 2021; Setiawati et al. 2021). Approximately 700,000 people die annually from infectious diseases and drug resistance (Ragheb et al. 2019). In addition, the excessive and irrational use of antibiotics leads to microbial resistance and emerging of multi-drug resistant pathogens. For example, the clinical sites have reported *Staphylococcus aureus* resistant to cefoperazone, rifampicin, ciprofloxacin, cefotaxime, and clindamycin. At the same time, *Escherichia coli* were reportedly resistant to amikacin, cefepime, trimethoprim, erythromycin, gentamicin, imipenem, and netromycin. Therefore, it is necessary to explore new biologically active compounds to overcome the resistance of pathogenic bacteria (Edemekong et al. 2022).

Actinomycetes continue to serve as potential antimicrobial producers from nature (Genilloud 2017; Harir et al. 2018). Actinomycetes show a distinctive morphology compared to common Gram-positive bacteria. These bacteria are filamentous and pleomorphic and grow in colonies with broad branches and have short and narrow basic hyphae and a minor diameter of mycelium (0.05-2 µm) (Sun et al. 2020). The cell structure belongs to that of prokaryotes and is entirely different from fungi. They contain a high guanine+cytosine ranging from 51-70% as Gram-positive bacteria. However, several actinomycetes contain less than 50% GC (Takahashi and Nakashima 2018; Abdelgawad et al. 2020). In addition, actinomycetes are included in the phylum actinobacteria, one of the most extensive descendants of the bacterial domain lineages (Donald et al. 2022).

Actinomycetes are the most beneficial microorganisms from the pharmaceutical and agricultural perspectives. They produce diverse metabolites with outstanding pharmacological values and also produce metabolites that

can potentially restrain the development of plant pathogens (Bhatti et al. 2017; Leetanasaksakul and Thamchaipenet 2018). These bacteria need to be explored further because they have excellent potential for high stability and can be operated at high temperatures.

Despite its abundant presence in terrestrial and water environments (Barka et al. 2016). However, approximately 80-90% of actinomycetes are isolated from the soil (Sheik et al. 2017; Budhathoki and Shrestha 2020) and produce around 10,000 bioactive compounds (Sebak et al. 2019). Several factors affect the number and types of actinomycetes in the soil: geographic location, pH, temperature, soil type, organic matter content, cultivation, aeration, and air (Devanshi et al. 2022; Edemekong et al. 2022). The largest actinomycetes group is the *Streptomyces* group (Khadayat et al. 2020).

Streptomyces produces a broad spectrum of biologically active compounds. During the growth phase, *Streptomyces* produces the compounds required to maintain the exponential growth of bacterial cells. In addition, these secondary metabolites from *Streptomyces* can absorb metals, protect against ultraviolet light, inhibit competing organisms (antibiotics), and promote communication with other species (Quinn et al. 2020). The active compounds from *Streptomyces* are active against several phytopathogenic bacteria and serve as potential antituberculosis, antibiofilm, antifungal, antiplasmodial, antioxidant, anticancer, antitumor, antiparasitic, and inhibitor of bacterial toxin production (Promnuan et al. 2020; Gebily et al. 2021; Rakhmawatie et al. 2021). Two hundred seventy-nine new secondary metabolites from 121 species of *Streptomyces* showed diverse biological activities (Donald et al. 2022).

This follow-up research was based on a preliminary screening of actinomycetes isolated from Enggano Island's soil (Pahira et al. 2020). This study focused on the most promising isolate SHP 9-3. It was aimed to determine the morphological characteristics of the isolate while determining antimicrobial potential, assessing the cytotoxicity against Vero cells analyzing the chemical profile of the ethyl acetate extract resulting from its fermentation.

MATERIALS AND METHODS

Preparation of *Streptomyces koyangensis* strain SHP 9-3

The *S. koyangensis* strain SHP 9-3 was isolated from the soil of Enggano Island, Bengkulu, Indonesia (Pahira et al. 2020). Cultivation of *S. koyangensis* SHP 9-3 was carried out on the International Streptomyces Project-2 (ISP-2) media (consisting of 4 g yeast extract, 10 g malt extract, 4 g glucose in 1 L water, pH 7.0) for 7-14 days at 37°C (Ratnakomala et al. 2016).

Morphological characterizations

Morphological characteristics of the isolate were performed using the Scanning Electron Microscope (SEM) (Hitachi SU3500, Japan) (Nirwati et al. 2022). First, bacterial biomass was applied to the outer cover. Next, the samples were covered with Au by the Au ion sputter (Hitachi MC1000). Then, it was dried before being examined by SEM with a voltage speed of 5 kV, 30% intensity in high mode, and 5000-10,000x magnification (Damayanti et al. 2021).

Production of secondary metabolites

Streptomyces koyangensis SHP 9-3 from the solid medium was inoculated into 100 mL of ISP-2 and incubated for 72 hours at 30°C and 130 rpm in a shaker incubator (Setiawati et al. 2021). Afterward, 90 mL of production medium and 10 mL of pre-culture stock were added to a 500 mL baffled flask (Susanti et al. 2019). The mixture was incubated further at 30°C in a shaker incubator at 130 rpm. After incubation, ethyl acetate was added to *S. koyangensis* SHP 9-3 culture and left in a shaker incubator at a speed of 130 rpm for 12 hours (overnight) to extract the secondary metabolites (Bakheit and Saadabi 2014; Retnowati et al. 2018). The mixture was then placed in a separatory funnel to gain the ethyl acetate extract.

Antimicrobial assay

After being adjusted to 0.1 (equivalent to 0.5 Mc Farland standard, or 1.5x108 CFU/mL), the microbial culture density was diluted in fresh medium to obtain OD600 values of 0.01 for each bacterial species 0.38 for *Candida albicans* (Hamzah et al. 2020). The antimicrobial potency was determined using a microdilution method on 96-well microtiter plates (Iwaki, Japan). In the first step, 150 µL of the microbial suspension was put into 1-3 wells, added with 20 µL of extract (2 mg/mL), and 130 µL of Brain Heart Infusion (BHI) broth media (Oxoid, Germany) for bacteria (Wang et al. 2017) and RPMI 1640 media (Sigma, USA) for fungi (Hamzah et al. 2020). Then, two-fold serial dilutions were carried out to obtain a series of sample dilutions (Charousová et al. 2016; Atikana et al. 2021) with a concentration range of 1000 to 7.81 µg/mL. Chloramphenicol (50 µL/mL) was applied as a control for antibacterial, and 1% nystatin as a control for antifungal. Then, the plate was incubated at 37°C for 24 hours for antibacterial assay and 72 hours for antifungal assay. Finally, the absorbance was measured at a wavelength of 595 nm.

Vero cells proliferation assay

Cells were harvested after reaching 80% confluency. The liquid culture medium was discarded and added with 5 mL PBS followed by trypsin-EDTA 0.25% solution was added into the media to release the cells. The culture flask was incubated for 3 minutes in a CO_2 incubator at 37°C. After incubation, 5 mL of DMEM was put into the 96-well plate. The cell suspension was transferred into a new sterile conical tube to attach the cells. Afterward, 100 µL of Vero cell culture suspension and 100 µL of test fractions or compounds at various concentrations were put into a 96-well plate and incubated for 24 hours at 37°C. After incubation, the culture medium was discarded and substituted with 100 µL of the new complete one and added with 10 µL of MTT solution. Then, the 96-well plate was incubated again for 3 hours at 37°C. Following

incubation, 100 µL of 10% SDS solution in 0.01 M HCl was added and incubated for 24 hours. The absorbance was measured at 595 nm. The half-maximal inhibitory concentration (IC_{50}) value determines its toxicity to the traditional cell culture (To'Bungan et al. 2022).

Identification of secondary metabolite of the extract by LC-MS/MS

Liquid Chromatography-tandem Mass Spectrometry (LC-MS/MS, waters MS technologies, Milford, USA) was used to analyze and identify the chemical compound in the extract. Electrospray ionization ranges from 100-1,200 m/z. The positive electron spray mode was used in the analysis. The capillary and cone voltages were set at 0.8 and 30 kV, respectively. Desolvation gas is determined at 1,000 µL/h and 500°C. The source temperature is 120°C, and the cone gas flow rate is 50 µL/hr. The water acquity ultra-performance LC system supports Ultra Performance Liquid Chromatography (UPLC) performance. LC analysis using a 100 mmx2.1 mm, 1.7 m ACQUITY UPLC HSS T3 column at 40°C with a gradient polarity (A: B) of 95:5 to 5:95. The mobile phase was composed of a solvent. A (0.1 percent methanoic acid in H_2O, v/v) and solvent B (0.1 percent methanoic acid in acetonitrile, v/v) with a 0.3 mL/min flow rate. The autosampler was stored at 20°C, while the column was stored at 40°C. The sample was injected at 1 µL with a 0.002 g/mL concentration. UNIFI software is used to analyze data. The observed Retention Time (RT) was from 1 to 16 minutes (modified method from Damayanti et al. (2021).

RESULTS AND DISCUSSION

Characteristics of *Streptomyces koyangensis* strain SHP 9-3

Our preliminary research supports further investigation of the isolate SHP 9-3, which showed prominent broad-spectrum antimicrobial activity (Pahira et al. 2020). Based on the 16S rRNA analysis, the isolate was identified as *S. koyangensis* SHP 9-3. Macroscopic observations show the characteristics of dry colonies: mycelium, rounded colonies, raised elevation, and wrinkled, white to dark gray (Figure 1). The characteristics are similar to previously reported actinomycetes from Mangrove (Katili and Retnowati 2017; Fatimah et al. 2022; Quintero et al. 2022) belonging to the genus *Streptomyces*.

In general, actinomycetes grow in various soil areas (Guo et al. 2015; Barka et al. 2016), produce spores, and grow slower than other bacteria (Abidin et al. 2016). The colony's growth is initiated by single spores or fragments of hyphae (Chater 2016). Colonies grow from a compact mass, although some show additional expansion. Colony size depends on the species, growing condition, and age. The colony can be raised or flat, sometimes covered with a velvety or powdery rough surface. The consistency of the colony itself varies from very soft to very hard (Devanshi et al. 2022).

Scanning Electron Microscopy (SEM) observation was performed on the isolate after 14 days of incubation at 30°C (Ratnakomala et al. 2016; Pahira et al. 2020). The bacterium shows a spherical shape, a long spiral chain colony, and fine hyphae (Figure 2). These characteristics confirmed that the strain SHP 9-3 belongs to the *Streptomyces genus.*

Antimicrobial activity against selected pathogenic microbes

The isolated was fermented in an ISP-2 liquid medium using 500 mL baffled flasks and then incubated in an orbital shaker for five days at room temperature. Incubation using an orbital shaker helps maintain the homogeneity of the nutrient distribution in the media and facilitates absorption into the cells. After five days of incubation, ethyl acetate was added in a 1:1 v/v (Rakhmawatie et al. 2022) to extract the extracelullar secondary metabolites of actinomycetes (Selim et al. 2021).

The antimicrobial activity was carried out using the microdilution broth against four pathogenic microbes, i.e., *C. albicans, E. coli, Pseudomonas aeruginosa,* and *S. aureus*. The antimicrobial test results showed that *S. aureus* and *C. albicans* growth was inhibited at 7.81 µg/mL. On the other hand, against *E. coli* and *P. aeruginosa*, a higher concentration was required to inhibit the growth (Figure 3).

Vero cells proliferation assay

The toxicity of the extract was determined by proliferation assay of the extract against normal cells (Vero cells), and the result showed that the IC_{50} value observed was 5.015 µg/mL, indicating that the ethyl acetate extract of *S. koyangensis* SHP 9-3 was relatively nontoxic (Figure 4). Cytotoxicity assay on normal cells (non-malignant or non-cancerous cells) was performed to determine the selection of the extract. As reported elsewhere, Vero cells were used to evaluate the toxicity of antimalarial, antimycobacterial, and antibacterial from *Streptomyces* sp. BCC 27095 (Intaraudom et al. 2015). The IC_{50} values of the antiproliferative assay in this study were higher than other research, which indicates that SHP 9-3 extract was nontoxic than active compounds of *Streptomyces* BCC26924, which had IC_{50} values between 26.8 and >50 µg/mL on normal cells (Intaraudom et al. 2015). Another study by (Isaka et al. 2002) showed that IC_{50} values of the metacyclic-prodigiosin compound, bafilomycin A, and spectinabilin from *Streptomyces spectabilis* BCC 4785 ranged from 1.14-20 µg/mL on Vero cells, so these compounds are more toxic than the SHP 9-3 extract.

The LC-MS/MS analysis of the ethyl acetate extract of *S. koyangensis* showed the presence of Five major compounds with Retention Time (RT) of 3.48, 3.64, 4.70, 5.10, and 9.36 min (Figure 5, Table 1).

The electrospray ionization method was used in the analyses as a soft ionization technique which causes little, or no molecular fragmentation occurs (Banerjee and Mazumdar 2012). Based on LCMS/MS database, peaks on the chromatogram were identified as cyclo (Pro-Val) ($C_{10}H_{16}N_2O_2$), kuraramine ($C_{12}H_{18}N_2O_2$), 1,4-diphenyl-1-pentanone ($C_{17}H_{18}O$), (3*S*,4*S*)-4-amino-3-hydroxy-6-methyl heptanoic acid ($C_{21}H_{42}N_2O_7$), and 2-ethyl-8-2,8-diazaspiro (4,5) decane-1,3-dione ($C_{11}H_{18}N_2O_2$) (https://pubchem.ncbi.nlm.nih.gov) (Figures 6-10).

The cyclo (Pro-Val) has been reported previously to exhibit antimicrobial activity (Song et al. 2021), while no reports on biological activities of 1,4-diphenyl-1-*pentanone*, (3*S*,4*S*)-4-amino-3-hydroxy-6-methyl heptanoic acid, 2-ethyl-8-methyl-2,8-diazaspiro (4,5) decane-1,3-dione, and kuraramine (Damayanti et al. 2021).

Previous studies by Lee and Hwang (2002) and Lee et al. (2005) reported the antimicrobial activity of *S. koyangensis* isolated from Koyang, Korea. The isolate was fermented on Yeast-Malt-extract-Agar seclusion media (YMA) for 5-7 days at 30°C. Antifungal activities of *S. koyangensis* have been tested against groups of molds, i.e., *Alternaria mali*, *Cladosporium cucumerinum*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum sp. cucumerinum*, and *Colletotrichum orbiculare* (Lee and Hwang 2002). Lee and Hwang (2002) reported that *S. koyangensis* contains 4-phenyl-3-butenoic acid, an active antifungal compound. However, our result showed that *S. koyangensis* isolated from Enggano Island produced a different chemical profile from that reported by Lee and Hwang (2002) and Lee et al. (2005). We did not identify the presence of 4-phenyl-3-butenoic acid in the extract, which may be due to the differences in the cultivation method (Fahrurrozi et al. 2017). The media and baffled flasks used in our study tend to support the growth of the actinomycetes better but are less beneficial in supporting the production of bioactive secondary metabolites. Further research on optimization of the *S. koyangensis* SHP 9-3 fermentation condition should be conducted to support higher production of active antimicrobials from the isolate.

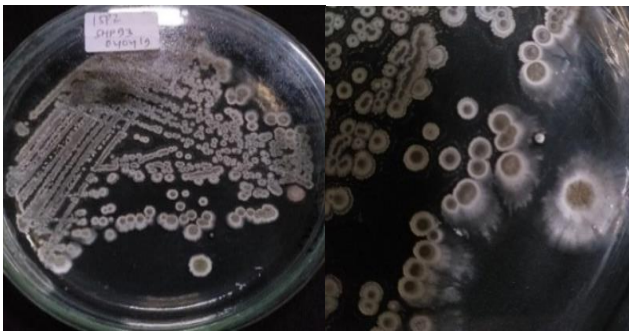

Figure 1. Colony appearance of *Streptomyces koyangensis* strain SHP 9-3 following rejuvenation in ISP-2 media observed on day 14

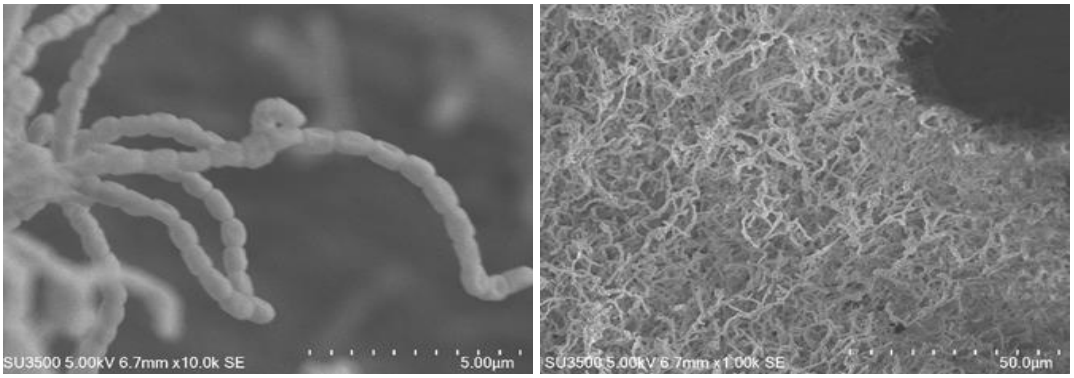

Figure 2. The morphological characteristic of *Streptomyces koyangensis* SHP 9-3 observed 5000x using a *Scanning Electron Microscope* (SEM)

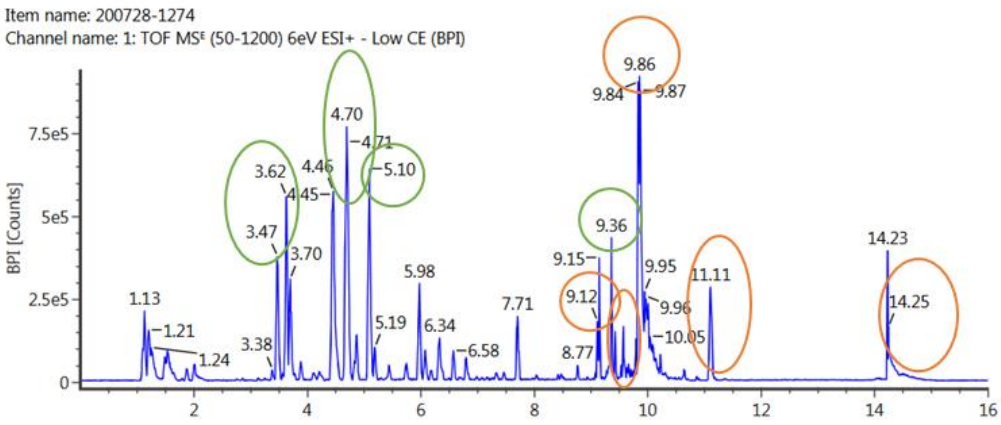

Figure 5. LC-MS/MS chromatogram of the ethyl acetate extract of *S. koyangensis* SHP 9-3 from Enggano. Spectrometer Xevo G2-XS instruments. ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.7 µm). The A solvent (0.1% methanoic acid in H_2O, v/v) and the B solvent (0.1% methanoic acid in acetonitrile (v/v)), with gradient polarity (A: B) of 95:5 to 5:95. The set of the flow rate was set at 0.3 mL/min. Note: The orange highlighted sample was a major blank sample; the green marked was a major detected compound

Table 1. Identified chemical compounds of the ethyl acetate of *Streptomyces koyangensis* SHP 9-3 from Enggano based on the LC-MS/MS analysis

Component name	Identification status	Observed m/z	Neutral mass (Da)	Observed RT (min)	Detector counts
Cyclo(Pro-Val) $C_{10}H_{16}N_2O_2$	Identified	197.1280	196.12118	3.64	316247
Kuraramine $C_{12}H_{18}N_2O_2$	Identified	245.1283	222.13683	5.10	650498
Candidate Mass $C_{17}H_{18}O$	Identified	261.1234	238.13577	3.48	36.7510
Candidate Mass $C_{21}H_{42}N_2O_7$	Identified	457.2866	434.29920	9.36	184749
Candidate Mass $C_{11}H_{18}N_2O_2$	Identified	211.1438	210.13683	4.70	850426

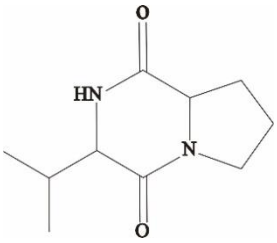

Figure 6. Cyclo (Pro-Val) ($C_{10}H_{16}N_2O_2$, exact mass 196.12118)

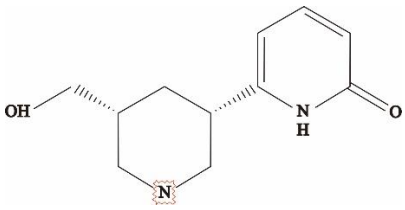

Figure 7. Kuraramine ($C_{12}H_{18}N_2O_2$, exact mass 222.13683)

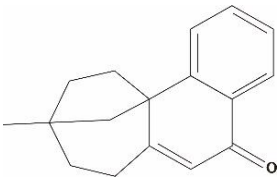

Figure 8. 1,4-*diphenyl*-1-*pentanone* ($C_{17}H_{18}O$, exact mass 238.13577)

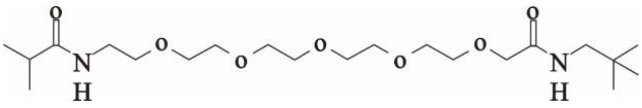

Figure 9. (3*S*,4*S*)-4-amino-3-hydroxy-6-methyl heptanoic acid ($C_{21}H_{42}N_2O_7$, exact mas 434.29920)

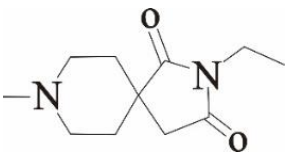

Figure 10. 2-Ethyl-8-methyl-2,8-diazaspiro (4,5) decane-1,3-dione ($C_{11}H_{18}N_2O_2$, exact mass 210.13682783)

In conclusion, *S. koyangensis* SHP 9-3 from Enggano Island, Indonesia, is a potential source of antimicrobial. The LC-MS/MS analyses detected the presence of cyclo (Pro-Val) ($C_{10}H_{16}N_2O_2$), kuraramine ($C_{12}H_{18}N_2O_2$), 1,4-diphenyl-1-pentanone ($C_{17}H_{18}O$), (3*S*,4*S*)-4-amino-3-hydroxy-6-methyl heptanoic acid ($C_{21}H_{42}N_2O_7$), and 2-ethyl-8-2,8-diazaspiro (4,5) decane-1,3-dione ($C_{11}H_{18}N_2O_2$). Those compounds have never been reported before to be produced by *Streptomyces*. Further research to optimize fermentation should be conducted to produce active compounds in higher concentrations.

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