Identification of Antibiotic Compounds from Thai Mangrove Soil-Derived *Streptomyces iconiensis*

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ABSTRACT

The recent lack of discovery of novel antibiotics and the increase of antibiotic-resistant microorganisms present significant problems in infectious disease therapy. The mangrove forest ecosystem is an important natural source of novel organisms that have high potential to produce bioactive compounds. This study focuses on screening and identification of antimicrobial compound-producing actinobacteria from mangrove sediment samples, determination of some optimal parameters for antimicrobial compound production, as well as characterization of the antimicrobial compounds produced. Among a total of 22 isolates isolated from seven sediment samples, *Streptomyces iconiensis* OUCMDZ-5511, which was identified by colony morphology and 16S rRNA gene sequence, displayed the broadest antimicrobial spectrum against eight target indicator bacteria. An ethyl acetate (EtOAc) extract from culture broth of *S. iconiensis* OUCMDZ-5511 had the highest antibacterial activity against *Bacillus subtilis* ATCC 6051, determined by agar well diffusion method, when this strain was cultured in A_1 broth with initial pH of 9.0 and 0% NaCl. According to bioassay-guided chromatography, three interesting antimicrobial compounds, namely 2(3*H*)-benzothiazolone, indole-3-acetic acid and lumichrome were obtained from culture broth EtOAc extract of *S. iconiensis* OUCMDZ-5511 after purification by HPLC and structure identification by ESI-MS and NMR. However, only lumichrome showed broad-spectrum antibacterial activity against *Salmonella* Weltevreden, *Staphylococcus aureus*, *B. subtilis*, *Micrococcus luteus* and *Escherichia coli*, with MIC and MBC values ranging from 0.125 to 0.5 $mg \cdot mL^{-1}$ and 0.25 to 1.0 $mg \cdot mL^{-1}$, respectively. Notably, this study is the pioneer report on identification of 2(3*H*)-benzothiazolone, indole-3-acetic acid, and lumichrome in EtOAc extract from culture broth of *S. iconiensis*.

Keywords: Antibacterial compound, Lumichrome, Mangrove actinobacteria, Pathogenic bacteria, *Streptomyces iconiensis*

INTRODUCTION

Natural products (NPs) have considerable significance in various industries, including pharmaceuticals, agriculture, cosmetics, and nutritional supplements. The unique pharmacological or biological activity is the result of natural selection and evolution of an organism under a harsh environment or from interspecies defenses (Abozenadah *et al*., 2017). Actinobacteria, particularly *Streptomyces*, have long been recognized as the primary source of pharmaceutically valuable compounds (Watve *et al*., 2001). The majority of antibiotics were derived from NPs such as

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streptomycin, macrolides and fosfomycin from *Streptomyces griseus*, *S. orientalis*, and *S. fradiae*, respectively (Meanwell and Shama, 2008; Das and Patra, 2017). According to their potential for producing antiviral, antifungal, antibacterial and antitumor compounds, actinobacteria have garnered increased attention (Ahmad *et al*., 2017). Antibacterial compounds derived from terrestrial strains have been discovered at a slow rate. On the other hand, the novel antibacterial compounds discovered from microbes living in marine or mangrove habitats are more rapidly increasing (Debbab *et al*., 2010). Mangroves provide food and diverse habitat for a large number of biological species, making them a natural goldmine for novel species and antimicrobial-producing actinobacteria (Abidin *et al*., 2015). In Thailand, there are several reports of actinobacteria strains discovered from mangrove-derived samples such as the novel species *Streptomyces ferrugineus*, (Ruan *et al*., 2015) and hydrolytic enzyme-producing marine actinobacteria (Watanachote *et al*., 2018). Meanwhile, *Streptomyces iranensis* HM35 and *Streptomyces sundarbansensis*, with antimicrobial activity against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231 were also isolated from mangrove sediment samples collected in the central region of Thailand (Phongsopitanun *et al*., 2014). Consequently, isolation and characterization of potential antimicrobial-producing actinobacteria from mangrove-derived samples as well as determination of optimum parameters for growth and the structure of producing antimicrobial compounds are beneficial approaches in the discovery of novel compounds for commercial applications. This research aimed to select and identify antimicrobial-producing actinobacteria isolated from mangrove forest soil samples in Thailand. Furthermore, the effects of NaCl concentration and initial pH in culture media on antimicrobial production as well as characterization of antimicrobial compounds produced by the target strain were also elucidated.

MATERIALS AND METHODS

Indicator bacterial strains

The target indicator strains that are important in fish and fishery products or in medical concerns, including *Bacillus subtilis* ATCC 6051 (BS), *B. thuringiensis* ATCC 10792 (BT), *Escherichia coli* ATCC 11775 (EC), *Pseudomonas aeruginosa* ATCC 10145 (PA), *Salmonella* Paratyphi type A ATCC 9150 (SP), *Staphyloccocus aureus* ATCC 6538 (SA), *S. aureus* ATCC 25923 (capsular polysaccharide production) (SACP), and methicilin-resistant *S. aureus* ATCC 43300 (MRSA) were obtained from Laboratory of Marine Natural Products, Ocean University of China. Meanwhile, *Micrococcus luteus* IFO 12708 (ML) and *Salmonella* Weltevreden DMST 3380 (SW) were obtained from Department of Fishery Products, Faculty of Fisheries, Kasetsart University, Thailand. All indicator bacterial strains were kept in Tryptic soy broth (Bacto™, USA) with 20% (w/v) glycerol below -20 ºC.

Mangrove sediment samples

A total of seven sediment (i.e., mud) samples were collected from two different mangrove forest areas. (1) Sediment samples 1-6 were collected from the area surrounding roots of *Avicenna marina* at two stations of Bang Khun Thian mangrove forest, Bangkok, Thailand. Samples 1 (5 cm depth), 2 (5-10 cm depth) and 3 (>10 cm depth) with pH 5.9 were from station 1 (100°42′50″ E, 13°48′68″ N), while samples 4 (5 cm depth), 5 (5-10 cm depth) and 6 (>10 cm depth) with pH 5.5 were from station 2 (100°42′50″ E, 13°43′68″ N). (2) Sediment sample 7 (5-10 cm depth) with pH 4.8 was collected from the area surrounding roots of *Avicenna marina* from Kung Krabaen Bay (101°54′07″ E, 12°34′24″ N), Chanthaburi, Thailand. All sediment samples were kept in sterile plastic bags and carried to the laboratory by ice box, and then refrigerated below 4 ºC until further use.

Culture media

Five selective agar media including A_1 medium (A_1M) (Fu and MacMillan, 2015), modified actinomyces 2 medium (A_2M) (Jiang *et al*., 2015), Gause's medium (GM) (Zhou *et al*., 2017), modified rare actinomyces medium (RAM) (Cho *et al*., 2013) and modified yeast extract medium (YEM) (Muzyed *et al*., 2021) were used to isolate and purify actinobacteria strains from mangrove sediment samples. ISP-2 (International *Streptomyces* Project-2) medium was used for studying *Streptomyces* colony morphology. Luria-Bertani (LB) agar (Bacto™, USA) was used for screening antimicrobial-producing actinobacteria. Muller-Hinton broth (MHB) (Bacto™, USA) and Muller-Hinton agar (MHA) (Bacto™, USA) were used for determining minimum inhibition concentration (MIC) and minimum bactericidal concentration determination (MBC), respectively.

Chromatographic material

Silica gel 60 (Mesh size 0.036-0.200 mm) (Millipore Sigma, USA) was used in vacuum liquid chromatography (VLC). Thin layer chromatography (TLC) plates (Merck, KGaA, Germany) were used for preliminary detection of the purity of each fraction from VLC. A high-performance liquid chromatography (HPLC) system (Agilent Technologies, Agilent 1200 Infinity Series, USA) fitted with a reversed-phase analytical column (Agilent 1200 Infinity Series C_{18}, 4.6×250 mm, 5 µm) and a semi-preparative high-performance liquid chromatography system (semi-preparative HPLC) (Waters™, Water 600, USA) fitted with a reversed-phase C_{18} column (ACE C_{18} Analytical column, 2.1×50 mm, 3.0 µm) were used to purify the antibacterial compounds.

Standard compounds

The standards 2(3*H*)-benzothiazolone and lumichrome were purchased from Tokyo Chemical Industry, Japan. Idole-3-acetic acid was purchased from Sigma-Aldrich, USA.

Structure identification

Bruker AVANCE NEO 400 spectrometer (Bruker AVANCE NEO 400, USA) was used to record NMR spectra, and chemical shifts were referenced to the corresponding residual solvent signals ($\delta_{H/C}$ 2.50/39.52 for DMSO-d_6). ESI-MS data were obtained on a Waters ACQUITY SQD 2 UPLC/MS system with a reversed-phase C_{18} column (ACE C_{18}-AR, 2.1×50 mm, 3.0 µm). UV spectra were recorded on a UV-visible spectrophotometer (Shimadzu UV-1700 Spectrophotometer, Japan) with measuring absorbance of 200-400 nm.

Isolation of actinobacteria strains

The sediment samples were dried for seven days on an aluminum tray at room temperature. Consequently, samples were heated at 35 °C for 24 h to reduce undesirable bacteria (Jiang *et al*., 2016). A 10-mg sample of dried sediment was added to 10 mL of sterile distilled water and mortared until homogenized. After that, the suspension was centrifuged at 2,500 rpm under 4 °C for 5 min, and the supernatant was centrifuged at 18,000 rpm under 4 °C for 25 min to obtain the spore pellet of actinobacteria. The spore pellet was diluted from 10^{-1} to 10^{-4} by sterile distilled water via serial ten-fold dilution and spread on five selective agar media plates. The plates were incubated at 28 ºC for two weeks. The obtained single colony of actinobacteria was purified twice on the same media.

Identification of actinobacteria strains

Pure cultures of all isolated actinobacteria strains growing on ISP-2 medium were preliminarily characterized by using identical *Streptomyces* colony morphology (cell morphology and spore surface) according to the method of Shirling and Gottlieb (1966) under light microscopy (OLYMPUS CX31). Genomic DNA extraction and PCR amplification of the 16S rRNA gene were carried out according to the methods of Hozzein and Goodfellow (2007). The target PCR product was confirmed by gel

electrophoresis (2.0% agarose/EtBr gel) and 100 bp DNA ladder from 100 to 2,000 bp (Takara Biomedical Technology, China) was used as a marker. Then, the PCR product was purified and nucleotide sequences were determined by accredited private laboratory (Peseno Biotech Co., Ltd). The obtained target DNA sequence was compared to 16S rRNA gene sequences of validly published species by using the Basic Local Alignment Search Tool (Blast) of National Center for Biotechnology Information (NCBI) in the GenBank database (Madden, 2003).

Screening of antimicrobial-producing actinobacteria

All actinobacteria strains were cultured in A_1 broth containing 3% NaCl, initial pH at 7.0, and with shaking at 180 rpm at 28 °C for 7 days. Then, culture medium was added with an equal volume of ethyl acetate (EtOAc) and extracted by sonicating at 40 kHz for 30 min in a sonicator bath (Bransonic model 3510-MT); the extraction process was then repeated with the same procedure. All EtOAc extracts were evaporated to remove EtOAc under reduced pressure (30 °C, 120 bar) in a rotary evaporator. The remaining crude antimicrobial extract was dissolved in 5 mL of 1 M methanol and centrifuged at 4° C and 12,000 rpm for 30 min. After centrifugation, the supernatant was collected and dried by flushing with nitrogen gas.

The antibacterial activity of dried EtOAc crude extract was determined by using agar disk diffusion method proposed by Hudzicki (2009). Each targeted indicator bacteria suspension was spread on the surface of LB agar by a sterile cotton swab and was allowed to dry at room temperature for 5 min. Then, 10 µL of EtOAc crude extract dissolved in methanol at a final concentration of 1 $mg{\cdot}mL^{-1}$ was dropped onto a paper disk (6 mm in diameter) and kept under ambient environment to remove methanol. Three prepared paper disks containing EtOAc crude extract of each actinobacteria strain were placed on the LB agar spread with each target indicator bacteria and incubated at 37 °C for 24 h. An antibiotic disk of ciprofloxacin hydrochloride (5 $\mu g{\cdot}mL^{-1}$) was used as positive control together with paper disks (without antibiotic) as negative control. After incubation, paper disks with a clear inhibition zone indicated antimicrobial activity against target indicator bacteria, and diameters of inhibition zones were measured.

Effect of NaCl concentration and initial pH in culture media on antimicrobial compound production of strain OUCMDZ-5511

Streptomyces iconiensis OUCMDZ-5511 was cultured in A_1 broth containing different NaCl concentrations (0%, 3%, 5%, 7%) and under different pH (3.0, 5.0, 7.0, 9.0), which was adjusted by 1 M of HCl and NaOH. All treatments were incubated by shaking at 180 rpm at 28 °C for 7 days, then EtOAc extract of culture media was prepared as previously described. Each obtained sample was determined for antibacterial activity in dry EtOAc extract against *Bacillus subtilis* ATCC 6051 by agar well diffusion method as described by Sylvia *et al*. (2004). Briefly, a suspension of log-phase *B. subtilis* ATCC 6051 was spread on MHA plates with a sterile cotton swab, and a well of 8 mm diameter was punched into the prepared MHA plates. Agar wells were filled with 20 µL (1 $mg{\cdot}mL^{-1}$) of crude extract in methanol and the suspension was allowed to diffuse at room temperature for 30 min; then the plates were incubated at 37 °C for 24 h. Wells containing the same volume of distilled water and methanol were used as negative control.

Purification of bioactive compounds produced by Streptomyces iconiensis OUCMDZ-5511

The EtOAc extract (2.0 g) was mixed with 10 g Silica gel 60 (mesh size 0.063-0.200) mm and subsequently subjected to the upper part of a vacuum liquid chromatography (VLC) column (1.5 cm in diameter×120 cm height) which was already packed with 160 g of Silica gel 60. Then, 300 mL of each gradient elution solvent, namely (1) a mixture of dichloromethane (DCM) and petroleum ether (PE) at 1:1 (v/v), (2) dichloromethane (DCM), and (3) a mixture of dichloromethane and methanol (MeOH) at 99:1, 95:5, 9:1, 8:2 and 1:1 (v/v) were serially used for eluting absorbed antimicrobial compounds from silica gel. A TLC plate coated with silica gel and solvent mixture of DCM and MeOH at 95:5 (v/v) was used for preliminary observation

of the chemodiversity in each eluted fraction. Migrated bands on TLC plates were observed under ultra-violet light at 254 nm. Fractions showing the same chemodiversity were combined. After that, 100 µL of each fraction was determined for antimicrobial activity against *B. subtilis* ATCC 6051 and *S. aureus* ATCC 6538 by agar well diffusion method as previously described; PE, DCM, MeOH were used as negative controls. The eluting fraction with the widest inhibition zone against *B. subtilis* ATCC 6051 and *S. aureus* ATCC 6538 was selected for further purification.

The eluting fraction from VLC with the widest inhibition zone against *B. subtilis* ATCC 6051 and *S. aureus* ATCC 6538 was further purified by an HPLC system fitted with a reversed-phase column (Agilent 1200 Infinity Series C_{18}, 4.6×250 mm, 5 µm). Antimicrobial compound absorbed in reversed-phase column was eluted by gradient program of acetonitrile (MeCN) aqueous solution, 10% MeCN (0-2 min), the gradient 10-90% MeCN (2-14 min), and 100% MeCN (14-20 min), at flow rate of 1.0 mL·min^{-1}. Subsequently, eluted fractions related to well-separated symmetrical peaks in the chromatogram and corresponding to antimicrobial activity against target indicator bacteria when determined by agar well diffusion method were selected. The selected fractions were determined for dry weight and further purified by semi-preparative HPLC fitted with a reversed-phase analytical column (ACE C_{18} Analytical column, 2.1×50 mm, 3.0 µm). Antimicrobial compound absorbed in ACE C_{18} Analytical column was eluted by gradient program of MeCN-H_2O (0.1% formic acid), 10% MeCN (0-0.5 min), the gradient 10-100% MeCN (0.5-3.5 min), 100% MeCN (3.5-4.0 min), and the gradient of 100-10% MeCN (4.0-4.5 min) at a flow rate 0.4 mL·min^{-1}.

Structure elucidation of the purified antimicrobial compounds produced by Streptomyces iconiensis OUCMDZ-5511

Structural elucidation of purified antimicrobial compounds produced by *Streptomyces iconiensis* OUCMDZ-5511 was carried out by ESI-MS and NMR (Chen *et al*., 2013). ESI-MS data were obtained on a Waters ACQUITY SQD 2 UPLC/MS system with a reversed-phase C_{18} column (ACE C_{18}-AR, 2.1×50 mm, 3.0 µm). The pure compound absorbed in ACE C_{18} Analytical column was eluted by the gradient program of MeCN-H_2O (0.1% formic acid), the 10% MeCN (0-0.5 min), the gradient 10-100% MeCN (0.5-3.5 min), 100% MeCN (3.5-4.0 min), and the gradient 100-10% MeCN (4.0-4.5 min) at a flow rate of 0.4 mL·min^{-1}. Meanwhile, Bruker AVANCE NEO 400 spectrometer (Bruker AVANCE NEO 400, USA) was used to record NMR spectra, and chemical shifts were referenced to the corresponding residual solvent signals ($\delta_{H/C}$ 2.50/39.52 for DMSO-d_6).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of target antimicrobial compounds

The MIC and MBC values of target antimicrobial compounds against indicator strains that are important fish and fishery products or in medical settings including *Bacillus subtilis* ATCC 6051, *Escherichia coli* ATCC 11775, *Micrococcus luteus* IFO 12708, *Salmonella* Weltevreden DMST 3380 and *Staphylococcus aureus* ATCC 25923 were carried out according to methods of Plangsom and Kanjabavas (2015). Briefly, 2(3*H*)-benzothiazolone, indole-3-acetic acid and lumichrome were dissolved in 0.1 mL of 1 N MeOH, distilled water, and 1 N KOH, respectively. Each antimicrobial compound solution was diluted with MHB medium to the final concentrations from 62.5 to 1,000 µg·mL^{-1} by a series of two-fold dilutions. Indicator strains were cultured in 10 mL of MHB at 37 °C for 24 h, and the turbidity of each indicator strain was adjusted until it reached 0.5 McFarland. The target indicator strain (1 mL) was inoculated into each MHB tube containing target antimicrobial compounds at designed final concentration and incubated at 37 °C for 24 h. The minimum inhibitory concentration (MIC) was evaluated by the lowest concentration of each target antimicrobial compounds in the dilution series that did not cause the target indicator strain to proliferate compared to the negative control when determined by spectrophotometer at 600 nm (OD_{600}). Amoxicillin and MHB were used as positive and negative controls, respectively. Culture broth (0.1 mL) in each tube from the MIC determination step that did not show any turbidity after incubation

was spread on the dry surface of Muller Hinton Broth (MHA) with sterile glass spreader, and the MHA plate was incubated at 37 °C for 24 h. The minimum bactericidal concentration (MBC) value was calculated based on the lowest concentration of target antimicrobial compound that did not show the indicator strain growing on MHA.

Statistical analysis

All experiments were done in triplicate and the data are presented as mean±SD. Data were subjected to analysis of variance, and means were compared using Duncan's multiple range tests. The tests were considered significant at $p<0.05$. Statistical analysis was run by the SPSS package (SPSS for Windows, Inc., USA).

RESULTS AND DISCUSSION

Isolation and identification of actinobacteria strains

In total, 22 isolates of actinobacteria were isolated from the seven sediment samples collected in Thai mangrove forests. Based on colony morphology and 16S rRNA gene sequences, only 16 of the isolates were selected; these showed more than 98 % similarity to sequences of published species available from the NCBI platform (Table 1). All of the 16 isolates belonged to one of five genera: *Streptomyces, Norcardiopsis, Brevibacterium Agrococcus* and *Streptomonospora*. Several studies have reported on actinobacteria diversity derived from mangrove forests in Southeast Asia. The genera *Gordonia, Leifsonia, Microbacterium,*

Table 1. Actinobacteria strains isolated from sediment samples from Thai mangrove forests.

Sediment sample	Isolated strain	Closest species in NCBI GenBank	Similarity (%)	Accession No. in NCBI GenBank	Isolation Media
7	OUCMDZ-5500	*Streptomyces lividans*	98.80	KC898819.1	GM
2	OUCMDZ-5502	*Norcardiopsis* sp.	98.09	KU382693.1	GM
3	OUCMDZ-5505	*Norcardiopsis* sp.	98.25	EU214943.1	A_1M
3	OUCMDZ-5506	*Streptomyces lividans.*	98.94	KC898819.1	GM
4	OUCMDZ-5507	*Bravibacterium* sp.	98.87	KY492051.1	A_1M
5	OUCMDZ-5508	*Norcardiopsis dassonvillei*	99.63	KF146896.1	GM
7	OUCMDZ-5509	*Agrococcus casei*	99.56	LN906630.1	YEM
6	OUCMDZ-5511	*Streptomyces iconiensis*	99.36	MK878389.1	GM
2	OUCMDZ-5512	*Streptomyces griseoplanus*	99.13	MG755810.1	GM
4	OUCMDZ-5513	*Streptomonospora* sp.	98.64	JX007947.1	GM
7	OUCMDZ-5514	*Streptomyces colicolor*	99.11	CP050522.1	A_1M
7	OUCMDZ-5515	*Streptomyces* sp.	98.39	MN629139.1	GM
1	OUCMDZ-5516	*Streptomyces graminearus*	99.90	MT071574.1	GM
7	OUCMDZ-5517	*Streptomyces coelicoflavus*	99.40	MN052663.1	RAM
7	OUCMDZ-5518	*Norcardiopsis* sp.	98.67	MN486497.1	A_2M
7	OUCMDZ-5519	*Brevebacterium linens*	99.32	CP030797.1	YEM

Note: GM = Gause's medium; A_1M = A_1 medium; YEM = yeast extract medium; RAM = rare actinomyces medium; A_2M = actinomyces 2 medium

Micromonospora, *Mycobacterium, Nocardia, Nocardioides, Sinomonas, Streptacidiphilus, Strepomyces* and *Terrabacter* were identified from soil samples in Malaysia (Lee *et al*., 2014), while *Amycolotopsis, Norcardiopsis, Streptomyces* and *Saccharomonospora* were found in soil samples in Indonesia (Retnowati *et al*., 2017). Additionally, *Streptomyces parvulus*, with antimicrobial activity against *Bacillus subtilis, Escherichia coli,* and *Staphylococcus aureus*, was isolated from mangrove soils in Chumphon Province of Thailand (Watanachote *et al*., 2017). These discoveries indicate that mangrove forests support high diversity of actinobacteria. However, a more detailed description of the characteristics of each actinobacteria species is needed, such as the isomer of diaminopimelic acid in the cell wall and sugar components; this work requires the use of a scanning electron microscope.

Screening of antimicrobial-producing actinobacteria

Antimicrobial activity in EtOAc extracts from A_1 broth of 16 actinobacterial strains against eight target indicator strains was determined by disk diffusion method. The results revealed that only eight actinobacteria isolates were able to inhibit at least one strain of target indicator bacteria. Strain OUCMDZ-5511, which was identified as *Streptomyces iconiensis* by colony morphology and 16S rRNA gene sequence, showed the broadest antimicrobial activity against all eight indicator strains (Table 2). Similar to our finding, *S. iconiensis* isolated from soil samples collected from a salt lake in Turkey also exhibited broad-spectrum antimicrobial activity against gram-positive and gram-negative pathogenic bacteria (Demet *et al*., 2014). Furthermore, other antimicrobial-producing *Streptomyces* have

Table 2. Antimicrobial activity in EtOAc extracts of 16 actinobacteria isolates against target indicator strains when determined by agar disk diffusion method.

EtOAc extract	Inhibition zone diameter (mm)							
	SA	**EC**	**BS**	**PA**	**MR**	**CP**	**BT**	**SP**
OUCMDZ-5500	-ve	-ve	-ve	-ve	-ve	15	-ve	-ve
OUCMDZ-5502	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
OUCMDZ-5505	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
OUCMDZ-5506	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
OUCMDZ-5507	-ve	-ve	13	-ve	-ve	-ve	-ve	-ve
OUCMDZ-5508	-ve	-ve	-ve	-ve	-ve	-ve	10	-ve
OUCMDZ-5509	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
OUCMDZ-5511	10.5	12	10	11	11	10	11	11.5
OUCMDZ-5512	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
OUCMDZ-5513	20	-ve	-ve	-ve	-ve	-ve	14	-ve
OUCMDZ-5514	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
OUCMDZ-5515	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
OUCMDZ 5516	-ve	-ve	-ve	-ve	19	-ve	-ve	-ve
OUCMDZ 5517	-ve	-ve	-ve	-ve	-ve	-ve	16	-ve
OUCMDZ-5518	-ve	-ve	-ve	-ve	-ve	-ve	14	-ve
OUCMDZ-5519	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Note: -ve = no inhibition zone against target indicator strain was observed; SA = *Staphylococcus aureus* ATCC 6538; EC = *Escherichia coli* ATCC 11775; BS = *Bacillus subtilis* ATCC 6051; PA = *Pseudomonas aeruginosa* ATCC 10145; MR = Methicillin-resistant *Staphylococcus aureus* ATCC 43300; CP = *Staphylococcus aureus* (capsular polysaccharide) ATCC 25923; BT = *Bacillus thuringiensis* ATCC 10792; SP = *Salmonella* Paratyphi type A ATCC 9150

been isolated from sediment and soil samples, such as *S. plicatus* from soil samples in Iran (Kariminik and Baniasadi, 2010) and *S. euryhalinus* from sediment samples in Lothian Island of the Indian Sundarbans (Biswas *et al*., 2022). However, few scientific studies concerning antimicrobial-producing *S. iconiensis* have been published.

Streptomyces can be found in almost any environment, from deep seas to high mountains, and has been recognized as the most significant microorganism in terms of bioactive compound production (Manteca and Yagüe, 2019; Quinn *et al*., 2020). Moreover, *Streptomyces* sp. can either produce specific or broad-spectrum antimicrobial activity. For example, *Streptomyces triostinicus* CKM7 exhibited narrow-spectrum antimicrobial activity against gram-positive bacteria (Chanthasena and Nantapong, 2016). Meanwhile, *Streptomyces* strain with broad-spectrum antimicrobial activity against pathogenic bacteria was isolated from a soil sample collected in southern Thailand (Sripreechasak *et al*., 2013).

However, it was noted that the antimicrobial spectrum against target microorganisms produced by the same strain of actinobacteria was related to culture media and culture conditions. In the case of *Streptomyces* isolated from sponge, sand, and mangrove sediment samples, six of ten broad-spectrum antimicrobial-producing isolates did not exhibit any antimicrobial activity against target indicator bacteria when cultured in four other trial culture media (Phongsopitanun *et al*., 2019). Furthermore, minor elements, NaCl and initial pH in the culture medium played significant roles in the production of antimicrobial compounds (Actinomycin-$X_{0\beta}$, X_2 and D) by *Streptomyces heliomycini* (Wang *et al*., 2017). Meanwhile, Grasso *et al*. (2016) suggested that a lack of some nutrients in culture media such as phosphate and nitrogen compounds, which are perceived by microorganisms through complex signaling mechanisms, were involved in inducing the production of antimicrobial compounds and other secondary metabolites by actinobacteria.

Effect of NaCl concentration and initial pH in culture media on antimicrobial compound production of strain OUCMDZ-5511

Determination of the culture conditions that enhance antimicrobial compound production of a target strain is needed for further large-scale production. *Streptomyces iconiensis* OUCMDZ-5511 was cultured in A_1 broth under different NaCl concentrations (0%, 3%, 5%, 7%) and initial pH (3.0, 5.0, 7.0, 9.0). The results in Table 3 indicate that *S. iconiensis* OUCMDZ-5511 was able to grow and produce antimicrobial compound in A_1 broth with 0-7% NaCl and initial pH of 5.0-9.0, whereas no growth was observed at pH 3.0. The growth of *S. iconiensis* OUCMDZ-5511 under 0-7% NaCl environments suggested that this strain was halotolerant. Similar to our finding, *S. iconiensis* isolated from Tuz (Salt) Lake in Turkey were described as halotolerant actinobacteria (Demet *et al*., 2014). Generally, actinobacteria growth and survival are also significantly affected by the pH of the environment (Akond *et al*., 2016), and the optimum pH for cell growth of genus *Streptomyces* was reported to be 6.5-8.0 (Li *et al*., 2021). Normally, antimicrobial and other bioactive compounds produced by *Streptomyces* are secondary metabolites that are produced in response to cell stress in the stationary growth phase (Brock, 1961; Higgens and Kastner, 1971; Lam, 2006; Kumar and Naraian, 2019). In this study, production of antimicrobial compounds by *S. iconiensis* OUCMDZ-5511 was induced by the pH-stress environment of A_1 broth at pH 9.0, which exceeds the optimal pH for cell growth. Meanwhile under an alkaline environment (pH 9.0), as NaCl concentration was increased, the antimicrobial activity decreased. This might due to NaCl creating a significant osmotic pressure difference between actinobacteria cells and their environment, which results in extracellular polymeric substance disruption and cell lysis (Pang *et al*., 2020).

In a similar case, the marine-dwelling *Salinispora* showed higher rifamycin production in environments with 3% or less NaCl compared to environments with greater than 3% NaCl. It was

Table 3. Inhibition zone diameter against *Bacillus subtilis* ATCC 6051 of EtOAc extracts produced by *Streptomyces iconiensis* OUCMDZ-5511 grown in A_1 broth under different initial pH and NaCl concentrations.

pH	NaCl (%)	Inhibition zone diameter (mm)
3	0	0
	3	0
	5	0
	7	0
5	0	9.75 ± 0.35^{ef}
	3	10.25 ± 0.35^{e}
	5	13.75 ± 0.35^{b}
	7	13.00 ± 1.41^{bcd}
7	0	9.75 ± 0.35^{ef}
	3	12.00 ± 0.00^{cd}
	5	12.50 ± 0.70^{bcd}
	7	13.50 ± 0.70^{b}
9	0	16.00 ± 0.00^{a}
	3	13.25 ± 0.35^{bc}
	5	11.75 ± 0.35^{d}
	7	8.75 ± 0.35^{f}

Note: Mean±SD superscripted with different lowercase letters indicate significant ($p<0.05$) difference.

suggested that this was because the cells had to go through a long lag phase under the high osmotic pressure created by low salinity before entering the logarithmic and stationary growth phases (Rolfe *et al*., 2012; Ng *et al*., 2014). Meanwhile, the pH-stress-inducing interaction may account for the highest antimicrobial activity against target indicator strain in EtOAc extract of *S. iconiensis* OUCMDZ-5511 cultured in A_1 broth under an alkaline environment (pH 9.0 and 0% NaCl). In *Streptomyces*, bioactive secondary metabolites are mainly produced by the activation of cryptic gene clusters that are not active under optimum conditions for cell growth (Pettit, 2011). Furthermore, secondary metabolite production in some actinobacteria can be induced by using a variety of techniques including genetic manipulation and expression of biosynthetic gene clusters (BGCs) (Richard, 2015) as well as modification of cultivation parameters such as pH, temperature, and aeration (Joachim *et al*., 2018; Stefano *et al*., 2018). As such, in further experiments, *S. iconiensis* OUCMDZ-5511 was cultured in 10 L of A_1 broth under initial pH at 9.0 and 0% NaCl (based on the highest antimicrobial activity found for these conditions) and extracted by EtOAc to obtain at least 2.0 g of dry EtOAc extracts for subsequent purification of bioactive compounds.

Purification of bioactive compounds produced by Streptomyces iconiensis OUCMDZ-5511

The EtOAc extract from culture broth of *Streptomyces iconiensis* OUCMDZ-5511 was partially purified by VLC with different eluents. Results in Table 4 indicate that fractions 12 and 13 eluted from VLC with the mixture of DCM-MeOH at 9:1 (v/v) had the widest inhibition zone against *Bacillus subtilis* ATCC 6051 (14 and 12 mm diameter) and *Staphylococcus aureus* ATCC 6538 (16 and 13 mm diameter) when determined by agar well diffusion method. Therefore, these two fractions were selected for the further purification process by HPLC fitted with a reversed-phase column. According to the HPLC chromatogram in Figure 1,

antimicrobial compound 1 (6.7 mg, t_R 16.4 min) was obtained from fraction 12 eluted with mixture gradient consisting of 100% MeCN. Meanwhile, antimicrobial compound 2 (3.9 mg, t_R 9.25 min) and antimicrobial compound 3 (1.8 mg, t_R 11.86 min) were obtained from fraction 13 eluted with mixture gradient consisting of 58.26% and 75.50% MeCN, respectively. The three eluted fractions with antimicrobial compound from HPLC in the previous purification step were further purified by semi-preparative HPLC, and the chromatogram of each purified antimicrobial compound is shown in Figure 2.

Structure elucidation of the purified antimicrobial compounds produced by Streptomyces iconiensis OUCMDZ-5511

Structure elucidation and identification of purified antimicrobial compounds are significant steps for expanding an in-depth study and for further application in related fields. The three purified antimicrobial compounds produced by *Streptomyces iconiensis* OUCMDZ-5511 derived from semi-preparative HPLC purification were elucidated and identified based on data obtained from ESI-MS and NMR, as well as by comparison with analytical results of each commercial target compound.

Table 4. Bioassay-guided isolation of compounds from *Streptomyces iconiensis* OUCMDZ-5511.

Fraction	Inhibition zone diameter (mm)		Eluent
	Bacillus subtilis **ATCC 6051**	***Staphylococcus aureus*** **ATCC 6538**	
1	-ve	-ve	1:1 (300 mL) PE-DCM
2	-ve	-ve	1:1 (300 mL) PE-DCM
3	-ve	-ve	1 (300 mL) DCM
4	-ve	-ve	1 (300 mL) DCM
5	-ve	-ve	1 (300 mL) DCM
6	-ve	-ve	99:1 (300 mL) DCM-MeOH
7	-ve	-ve	99:1 (300 mL) DCM-MeOH
8	11	9	99:1 (300 mL) DCM-MeOH
9	9	9	99:5 (300 mL) DCM-MeOH
10	9	9	99:5 (300 mL) DCM-MeOH
11	4	4	99:5 (300 mL) DCM-MeOH
12	14	16	9:1 (300 mL) DCM-MeOH
13	12	13	9:1 (300 mL) DCM-MeOH
14	11	11	9:1 (300 mL) DCM-MeOH
15	-ve	-ve	8:2 (300 mL) DCM-MeOH
16	10	9	8:2 (300 mL) DCM-MeOH
17	10	10	8:2 (300 mL) DCM-MeOH
18	9	10	7:3 (300 mL) DCM-MeOH
19	9	9	7:3 (300 mL) DCM-MeOH
20	9	9	7:3 (300 mL) DCM-MeOH
21	-ve	-ve	1:1 (300 mL) DCM-MeOH

Note: -ve = no inhibition zone against target indicator strain was observed.

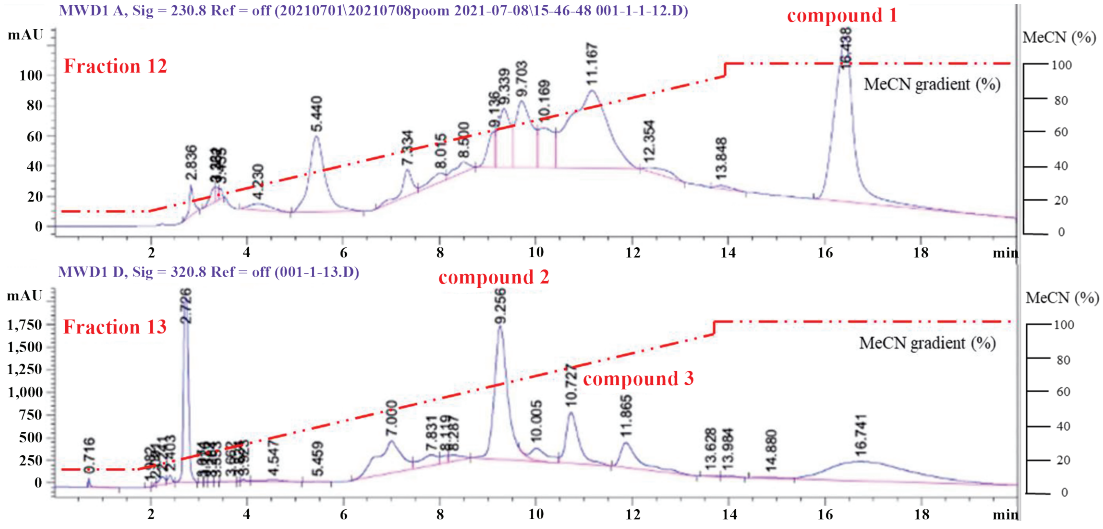

Figure 1. HPLC chromatograms of fractions 12 and 13 of *Streptomyces iconiensis* OUCMDZ-5511 after eluted from VLC.

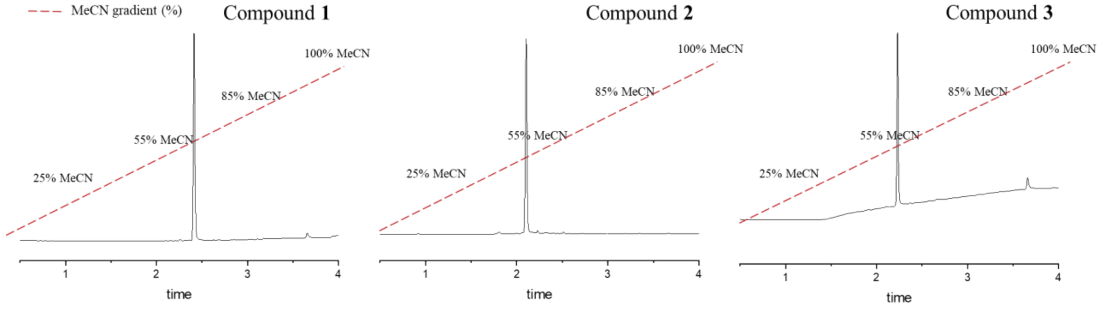

Figure 2. Chromatograms of purified antimicrobial compound 1, 2 and 3 eluted from semi-preparative HPLC.

Antimicrobial compound 1 was obtained as a white solid. UV (MeOH) λmax (log ε) 218, 280 nm (Figure 3). The ^{1}H NMR (600 MHz, DMSO-d_6) only displayed four aromatic proton signals, at δ_H 7.50 (1H, d, J = 7.7 Hz, H-7), 7.21 (1H, ddd ("dt" like), J = 7.7, 8.0, 1.3 Hz, H-5), 7.04 (1H, t, J = 7.7 Hz, H-6) and 7.07 (1H, dd, J = 8.0, 1.2 Hz, H-4) (Figure 4). These data indicate an *ortho* disubstituted benzene ring in antimicrobial compound 1. The molecular weight of 151 Da from the ESI-MS peak at *m/z* 152 $[M+H]^+$ implies a nitrogen atom in the molecule of antimicrobial compound 1, with the molecular formula of C_7H_5NOS. The NMR data are very similar to those of 2(3*H*)-benzothiazolone (Itoh and Mase, 2007). Furthermore, antimicrobial compound 1 showed the same retention time (17.6 min, 100% MeCN) to the standard 2(3*H*)-benzothiazolone. Thus, antimicrobial compound 1 was identified as 2(3*H*)-benzothiazolone (Figure 6). In a previous report, 2(3*H*)-benzothiazolone derivative was also produced by *Streptomyces* sp. FORM5 (Groenhagen *et al.*, 2014). However, there is no report describing its biological effects on target microorganisms.

Antimicrobial compound 2 was a white solid. UV (MeOH) λmax (log ε) 214, 240, 300 nm (Figure 3). ESI-MS at *m/z* 176 $[M+H]^+$ corresponded to 175 Da of molecular weight, indicating a nitrogen atom in the molecule of antimicrobial compound 2.

This compound displayed the same retention time (9.0 min, 52% MeCN/H_2O) as the standard indole-3-acetic acid. These data indicate the structure of antimicrobial compound 2 as indole-3-acetic acid (Figure 6). Similarly, indole-3-acetic acid was also produced by *Streptomyces artovirens* and was reported to have a key function as a signaling molecule crucial for plant organ development and growth (Petroski and Deshaies, 2005; Abd-Alla *et al*., 2013).

Antimicrobial compound 3 was obtained as a yellow amorphous powder. UV (MeOH) λmax (log ε) 220, 260, 360 nm (Figure 3). ESI-MS at *m/z* 243 $[M+H]^+$ indicated an even number of nitrogen atoms in the molecule of antimicrobial compound 3.

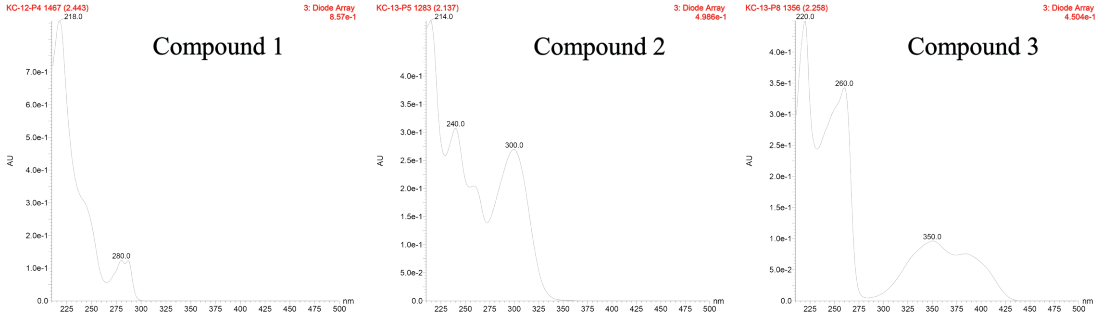

Figure 3. UV spectra of antimicrobial compound 1, antimicrobial compound 2, and antimicrobial compound 3.

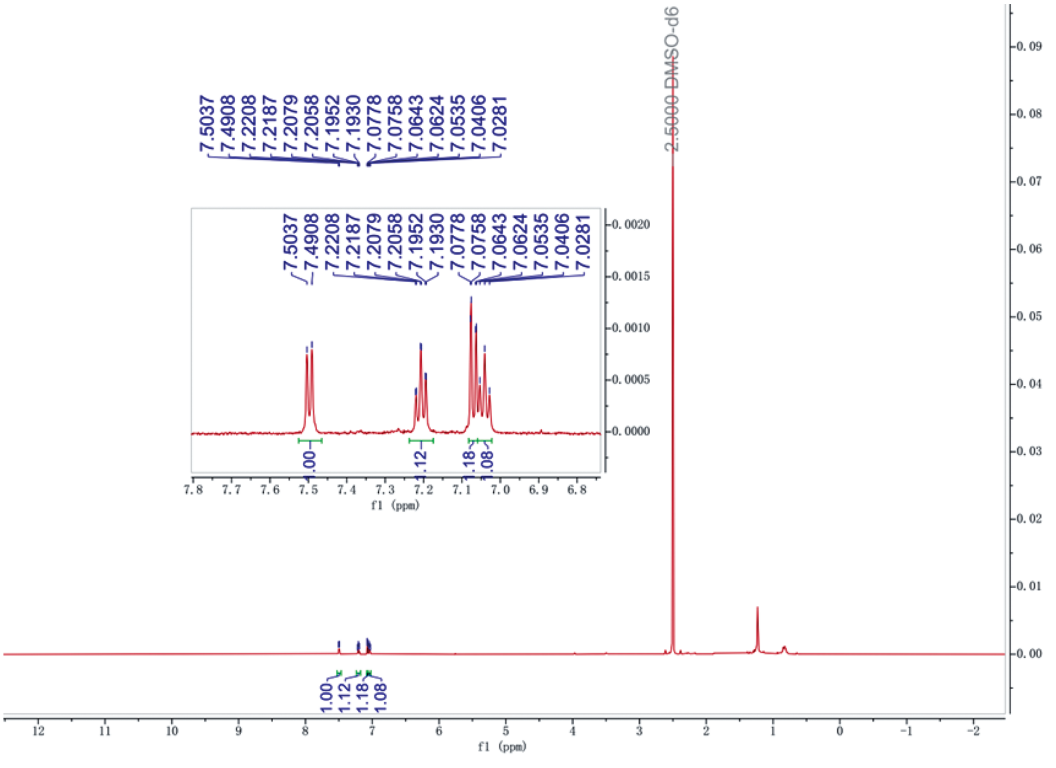

Figure 4. ^1H NMR spectrum of antimicrobial compound 1.

The ^{1}H NMR (400 MHz, DMSO-d_6) of antimicrobial compound 3 displayed two aromatic proton signals at δ_H 7.90 (1H, s, H-9) and 7.70 (1H, s, H-6), two methyl proton signals at δ_H 2.48 (3H, s, H3- 12) and 2.45 (3H, s, H3-11), as well as two exchangeable proton signals at δ_H 11.74 (2H, brs, NH-1 & NH-3) (Figure 5). These data are consistent with recordings for lumichrome in a previous report (Ding *et al*., 2009). In addition, antimicrobial compound 3 showed the same retention time (11.9 min, 52% MeCN/H_2O) as the standard lumichrome. Therefore, antimicrobial compound 3 was identified as lumichrome (Figure 6). Lumichrome was also produced by *Streptomyces* sp. MBTH32 and was capable of inhibiting sortase A, which is responsible for the covalent binding of protein to the cell wall (Beomkoo *et al*., 2019), as well as inhibiting lung cancer cell proliferation by upregulating p53 and downregulating its target BCL-2 (Wipa *et al*., 2019).

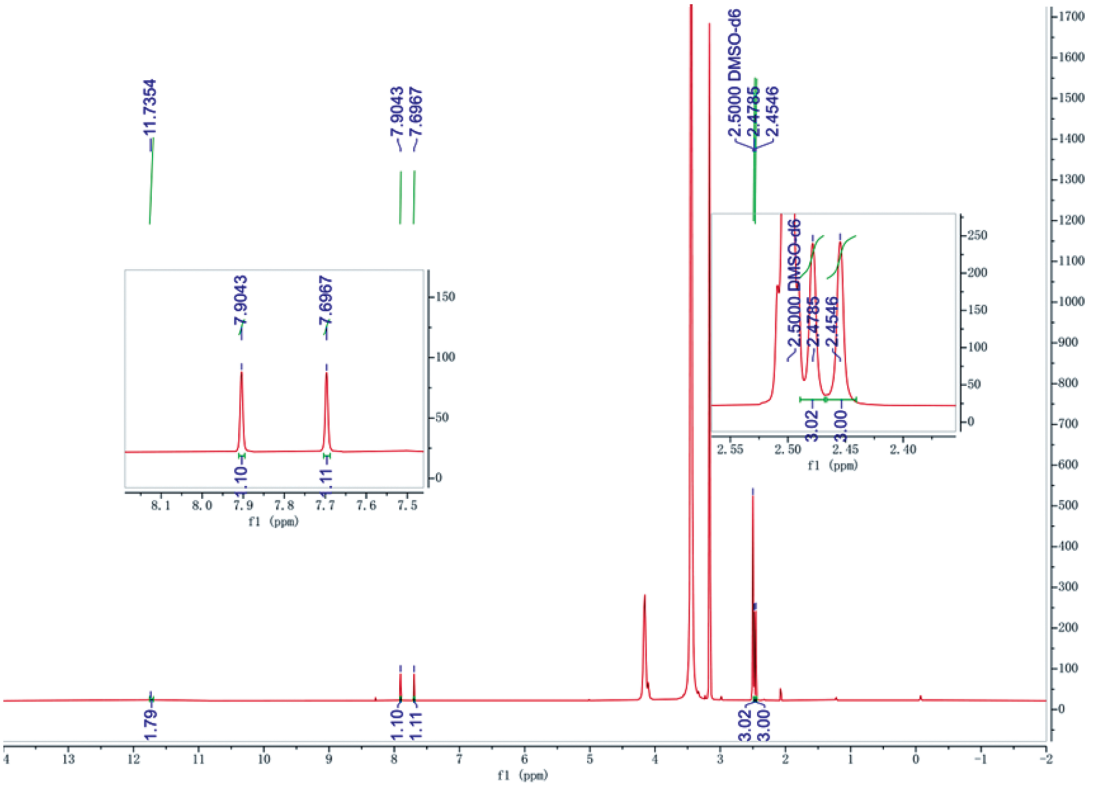

Figure 5. ^{1}H NMR spectrum of antimicrobial compound 3.

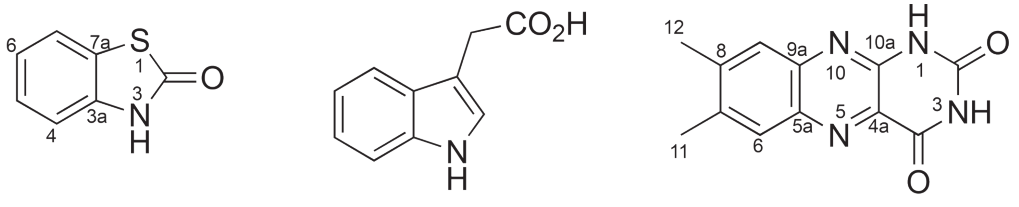

Figure 6. Chemical structures of compounds 1-3 produced by *Streptomyces iconiensis* OUCMDZ-5511.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of target antimicrobial compounds

The inhibitory and bactericidal activities of 2(3*H*)-benzothiazolone (antimicrobial compound 1), indole-3-acetic acid (antimicrobial compound 2), and lumichrome (antimicrobial compound 3) against five target indicator strains that play an important role in fish and fishery products or in medical concerns were evaluated. The results indicated that lumichrome (antimicrobial compound 3) exhibited antibacterial activity against all five target strains, with MIC and MBC values of 0.125-0.5 mg·mL^{-1} and 0.25-1.0 mg·mL^{-1}, respectively (Table 5). Similarly, lumichrome was also produced by *Streptomyces* sp. MBTH32 and was capable of inhibiting sortase A, which is responsible for the covalent binding of protein to the cell wall (Beomkoo *et al*., 2019). Meanwhile, indole-3-acetic acid (antimicrobial compound 2) had antimicrobial activity against only *Staphylococcus aureus* ATCC 25923, with MIC and MBC values of 0.25 mg·mL^{-1} and 0.5 mg·mL^{-1}, respectively. However, 2(3*H*)-benzothiazolone (antimicrobial compound 1) did not show antimicrobial activity against any of the tested bacterial strains, even though this substance, which has a benzene ring fused with a thiazole ring, and its derivative are used worldwide in a variety of antimicrobial therapeutic applications (Jamkhandi and Disouza, 2012; Sharma *et al*., 2012). The inability of 2(3*H*)-benzothiazolone to inhibit target indicator strains in this study may due to an inadequate concentration used, or perhaps the target indicator strains were resistant to this compound. Likewise, in this study, some benzothiazole derivatives did not possess any antimicrobial activity against *E. coli* ATCC 25922, *S. aureus* ATCC 29213, or *S. aureus* ATCC 25923 (Saeed *et al*., 2010).

Table 5. MIC and MBC values (mg·mL^{-1}) of 2(3*H*)-benzothiazolone (compound 1), indole-3-acetic acid (compound 2) and lumichrome (compound 3) against target pathogenic bacteria.

Indicator strain	2(3*H*)-benzothiazolone		indole-3-acetic acid		lumichrome	
	MIC (mg•mL^{-1})	MBC (mg•mL^{-1})	MIC (mg•mL^{-1})	MBC (mg•mL^{-1})	MIC (mg•mL^{-1})	MBC (mg•mL^{-1})
Salmonella Weltevreden DMST 3380	NA	NA	NA	NA	0.5	1
Staphylococcus aureus ATCC 25923	NA	NA	0.25	0.5	0.25	0.5
Bacillus subtilis ATCC 6051	NA	NA	NA	NA	0.25	1
Micrococcus luteus IFO 12708	NA	NA	NA	NA	0.25	0.5
Escherichia coli ATCC 11775	NA	NA	NA	NA	0.125	0.25

Note: NA = could not inhibit target bacteria

CONCLUSION

Eight antimicrobial-producing actinobacteria were isolated from sediment samples collected in mangrove forests of Thailand. Among the eight isolates, *Streptomyces iconiensis* OUCMDZ-5511 exhibited the broadest antimicrobial spectrum against target indicator bacteria that are important for fish, fishery products and medical concerns. The most suitable culture medium for obtaining

high antimicrobial activity production of this strain was A_1 broth without NaCl and initial pH at 9.0. After serial purification by VLC, HPLC and semi-preparative HPLC, three purified antimicrobial compounds were obtained. According to structural elucidation by ESI-MS and NMR, these three purified antimicrobial compounds were identified as 2(3*H*)-benzothiazolone, indole-3-acetic acid, and lumichrome. However, only lumichrome exhibited antibacterial activity against all five selected target strains, with MIC and MBC values of 0.125-0.5 $mg \cdot mL^{-1}$ and 0.25-1.0 $mg \cdot mL^{-1}$, respectively. Meanwhile, indole-3-acetic acid had antimicrobial activity against only *S. aureus* ATCC 25923, with MIC and MBC values of 0.25 and 0.5 $mg \cdot mL^{-1}$, respectively. Finally, the data we have presented here can serve as a basis for more in-depth study of *S. iconiensis* OUCMDZ-5511, and for its further application in related fields.

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