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Bioactive Secondary Metabolites from Marine *Streptomyces griseorubens* f8: Isolation, Identification and Biological Activity Assay

Wenzhi Yang, Guangjie Liang, Yang Sun and Zhijin Gong *

College of Life Sciences, Qufu Normal University, Qufu 273165, China; yangge@qfnu.edu.cn (W.Y.); 2021400295@buct.edu.cn (G.L.); chechengchuan@qfnu.edu.cn (Y.S.)
* Correspondence: gongzhijin@qfnu.edu.cn

Abstract: Marine actinomycetes are a potential source of a wide variety of bioactive natural products. Herein, four cyclic dipeptides, namely, cyclo(L-Val-L-Pro) (compound 1), cyclo(L-Pro-L-Leu) (compound 2), cyclo(L-Pro-L-Tyr) (compound 3) and cyclo(L-Pro-L-Phe) (compound 5), and an *N*-acetyltyramine (compound 4) were first isolated and identified as products of the marine *Streptomyces griseorubens* f8. Compounds 3 and 5 exhibit antibacterial activity against *Staphylococcus aureus*, *Klebsiella aerogenes* and *Proteus vulgaris*. The minimum inhibitory concentrations (MICs) against *Staphylococcus aureus*, *Klebsiella aerogenes* and *Proteus vulgaris* are 160 μg/mL, 100 μg/mL, 120 μg/mL for the compound 3 and 180 μg/mL, 130 μg/mL 150 μg/mL for the compound 5, respectively. In addition, compounds 1, 2, 3 and 5 was first found to have the ability to inhibit the invasion and migration of A549 cells (lung cancer cells), which exhibited the potentiality for these compounds to be used as novel anticancer drugs. This study provides a novel production strain for compounds 1, 2, 3 and 5, and four potential promising anticancer agents.

Keywords: marine *Streptomyces griseorubens* f8; cyclic dipeptides; antibacterial activity; inhibition of migration and invasion

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1. Introduction

Actinobacteria constitutes one of the largest bacterial phyla and harbors filamentous Gram-positive bacteria with high G + C content [1]. As important microorganisms, many actinomycetes are recognized for their ability to produce secondary metabolites of economic importance and other molecules of pharmaceutical importance [2–4]. In the last few decades, a large number of actinomycete strains have been isolated from terrestrial sources [5–7]. Along with the exploitation of marine resources, increasing attention has been paid to actinobacteria that can potentially produce interesting new bioactive secondary metabolites in marine environments [8–11]. Actinomycetes have been isolated from diverse marine environments, including sea water [12], marine snow and marine sediment [13]. A previous study of marine sediment from the Gulf of California has identified nearly 300 actinomycetes belonging to the genera *Actinomadura*, *Dietzia*, *Gordonia*, *Micromonospora*, *Nonomuraea*, *Rhodococcus*, *Saccharomonospora*, *Saccharopolyspora*, *Salinispora*, *Streptomyces*, *Solwaraspora* and *Verrucosispora* [14]. Marine actinomycetes can not only adapt to extreme habitats but also exhibit unique metabolic and physiological capabilities, and these bacteria have the potential to produce compounds with antitumor properties and other interesting pharmacological activities that are not observed in terrestrial microorganisms [15–18]. Meanwhile, structurally unique natural products with biological activities are being increasingly discovered from marine actinobacteria [19–24].

The Yellow Sea coastline located in the warm temperate zone of China, between the Bo Sea and the Yellow River triangle, constitutes a superior ecological environment due to its unique geographical position. There have been no investigations on marine actinobacteria

and the associated natural products in the environment of the Yellow Sea coastline. In the present study, *S. griseorubens* f8 was isolated from a sample of marine sediment at a depth of 8 m, which was collected from the Yellow Sea, close to Rizhao City, Shandong Province, China. Four cyclic dipeptides and an *N*-acetyltyramine were obtained from *S. griseorubens* f8, and their bioactivities were tested.

2. Materials and Methods

2.1. Isolation of Marine Microorganisms

Microorganisms was isolated from a sample of marine sediment at a depth of 8 m, which was collected from the Yellow Sea, close to Rizhao City, Shandong Province, China. Briefly, the marine sediments (5.0 g) were air-dried for 5 days in a sterile centrifuge tube with sterile air filtered through an air filter (aperture diameter: 0.2 μm, Sartorius) and air pump (model 550W, OUTSTANDING Air Compressor Co., Ltd., Taizhou, China). The dried samples were 1:10 (*m*/*v*) diluted with sterile 25% Ringer's solution. Subsequently, 100 μL of serially diluted sample suspensions (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were spread onto isolation medium (humic acid 1 g, $CaCO_3$ 0.02 g, Na_2HPO_4 0.5 g, $MgSO_4 \bullet 7H_2O$ 0.05 g, KCl 1.7 g, $FeSO_4 \bullet$, $7H_2O$ 0.01 g)]. The isolation medium was prepared with 1 L of natural seawater and 20 g of agar powder supplemented with filtered (0.2-μm pore size) nalidixic acid (25 μg/mL), nystatin (50 μg/mL) and cycloheximide (50 μg/mL) to inhibit the growth of Gram-negative bacteria and fungi [25] and incubated at 28 °C for 14 days. Then one single colony (named strain f8) was transferred into M1 medium (soluble starch 20 g/L, KNO_3 1 g/L, KH_2PO_4 0.5 g/L, NaCl 0.5 g/L, $FeSO_4 \bullet 7H_2O$ 0.01 g/L, $MgSO_4 \bullet 7H_2O$ 0.05 g/L, agar powder 20 g/L, natural seawater 1 L, pH 7.2–7.6). The strain f8 was maintained on M1 medium at 4 °C for short-term storage or in glycerol suspensions (25%, *v*/*v*) at −80 °C for long-term storage. In addition, in this study, general reagents and chemicals are purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). and their purity is analysis pure, unless otherwise stated.

2.2. Genomic DNA Extraction and Taxonomic Identity of Strain f8

The strain f8 was inoculated into M1 medium (50 mL) and incubated at 28 °C in a shaker at 180 rpm for 2 days. Then the cells were harvested by centrifugation at 12,000 rpm for 1 min. Finally, genomic DNA was extracted from the cells using a Gram-positive bacterial DNA extraction kit (model D1130, (Solarbio Science and Technology Co., Ltd., Beijing, China) according to the manufacturer's instructions.

The 16S rRNA gene was amplified by DNA amplification kit (2×Pfu PCR MasterMix, Solarbio Science and Technology Co., Ltd., Beijing, China) using primer A and primer B (primer A: 5′-AGAGTTTGATCC TGGCTCAG-3′, stock concentration: 10 μmol/L; primer B: 5′-AAGGAGGTGATCCAGCCGCA-3′, stock concentration: 10 μmol/L) [26]. Amplification was performed in a 50 μL reaction volume consisting of 2 μL genomic DNA, 25 μL 2× MasterMix, 2 μL primer A, 2 μL primer B and 19 μL ddH_2O on an PCR instrument (Eppendorf Mastercycler Gradient 5331, Hamburg, Germany). Briefly, after an initial denaturation at 95 °C for 5 min, amplification was carried out with 35 cycles at a melting temperature of 95 °C for 40 s, an annealing temperature of 55 °C for 45 s and an extension temperature of 72 °C for 75 s, followed by an additional extension step at 72 °C for 10 min. PCR products were sequenced by using an ABI PRISM 3730 XL sequencer (Thermo Fisher Scientific, Massachusetts, America). The sequences were compared with those in the National Center of Biotechnology Information (NCBI) database [25] using the basic local alignment search tool for nucleic acids (BLASTN) algorithm. A phylogenetic tree was constructed using the neighbor-joining method, which was implemented in the Molecular Evolutionary Genetics Analysis (MEGA), version 5.0 program [27]. The resultant tree topologies were evaluated by bootstrap analysis based on 1000 replicates. Only values greater than 50% are shown.

2.3. Morphological Observation of S. griseorubens f8

The *S. griseorubens* f8 was inoculated onto an M1 medium (soluble starch 20 g/L, KNO_3 1 g/L, KH_2PO_4 0.5 g/L, NaCl 0.5 g/L, $FeSO_4 \bullet 7H_2O$ 0.01 g/L, $MgSO_4 \bullet 7H_2O$ 0.05 g/L, agar 20 g/L, natural seawater 1 L, pH 7.2–7.6) agar plate and cultured for 14 days to observe the morphology of the strain. Samples for scanning electron microscopy (SEM) were prepared by the insertion method. Briefly, *S. griseorubens* f8 was cultured on solid medium, and sterilized coverslips were inserted into the M1 agar plate at a 45° angle. After 14 days of cultivation, the coverslips were removed, frozen at −80 °C for 24 h and then lyophilized for 48 h to remove water. Finally, the coverslips were sprayed with gold, and then strain morphology was assessed by SEM (Sigma 500 VP, Carl Zeiss AG, Jena, Germany).

2.4. Fermentation and Compound Extraction and Isolation

S. griseorubens f8 was inoculated onto M1 agar plates (diameter: 90 mm) from a glycerol stock under aseptic conditions and incubated at 28 °C for 14 days. The mycelium was then inoculated onto new M1 agar plates and incubated at 28 °C for 14 days. A total of about 1200 agar plates (a total of 30 L M1 medium) were inoculated and cultured. After cultured, these agar medium were chopped and extracted three times with ethyl acetate-methanol-acetic acid (EAMAC, 80:15:5; *v*/*v*/*v*) to obtain the extract. Then the extract was repeatedly partitioned with H_2O and ethyl acetate (EA) until the EA was colorless. Subsequently, the EA solution was dried over Na_2SO_4, and the solvent was removed under vacuum at 45 °C. The EA extract was partitioned with petroleum ether (PE) and methanol (MeOH) until the PE layer was colorless. The MeOH solution was concentrated under vacuum at 38 °C to obtain the MeOH extract (4.2 g).

The MeOH extract (4.2 g) was subjected to column chromatography over Sephadex LH-20 and eluted with MeOH to obtain 10 fractions (Fr1-10). Fr4 (550.7 mg) was subjected to Medium Pressure Liquid Chromatography (MPLC, sepacore ×50, Buchi Laboratory Equipment Trading(Shanghai)Ltd, Shanghai, China) over RP-18 silica gel, and 35 subfractions were obtained from the elution with ddH_2O, 10%, 20%, 30%, 50%, 70% and 100% MeOH, after combing the same subfractions and evaporating to dryness, five subfractions were obtained: Fr4-1 (171.8 mg), Fr4-2 (70.9 mg), Fr4-3 (68.3 mg), Fr4-4 (58.3 mg) and Fr4-5 (47 mg). Fr4-2 (70.9 mg) was eluted by isocratic High Performance Liquid Chromatography (HPLC, 1260 Infinity II, Agilent Technologies Inc., California, America) with 13% acetonitrile at a flow rate of 4 mL/min to obtain compound 1 (5.9 mg), with a retention time of 6.5 min. Fr4-3 (68.3 mg) was eluted by isocratic HPLC with 13% acetonitrile at a flow rate of 4 mL/min to obtain compound 2 (4.4 mg), with a retention time of 16.5 min. Fr5 (535.3 mg) was subjected to MPLC over RP-18 silica gel, and 40 200 mL subfractions were obtained for each gradient from the elution with ddH_2O, 10%, 20%, 30%, 40%, 50%, 70% and 100% MeOH and five fractions were obtained as follows: Fr5-1 (262.7 mg), Fr5-2 (126.7 mg), Fr5-3 (29.7 mg), Fr5-4 (23.6 mg) and Fr5-5 (17.3 mg). Fr5-2 (126.7 mg) was subjected to column chromatography over Sephadex LH-20 and eluted with MeOH to obtain 50 subfractions and five fractions were obtained: Fr5-2-a (10 mg), Fr5-2-b (6.3 mg), Fr5-2-c (20.4 mg), Fr5-2-d (92.3 mg) and Fr5-2-e (2.4 mg). Fr5-2-d (92.3 mg) was eluted by isocratic HPLC with 20% acetonitrile at a flow rate of 4 mL/min to obtain compound 3 (2.4 mg) with a retention time of 3.5 min and compound 4 (1.4 mg) with a retention time of 4.4 min. Fr5-3 (29.7 mg) was eluted by isocratic HPLC with 35% acetonitrile at a flow rate of 4 mL/min to obtain compound 5 (5.4 mg) with a retention time of 3.5 min. The process of compound extraction and isolation is shown in Figure 1.

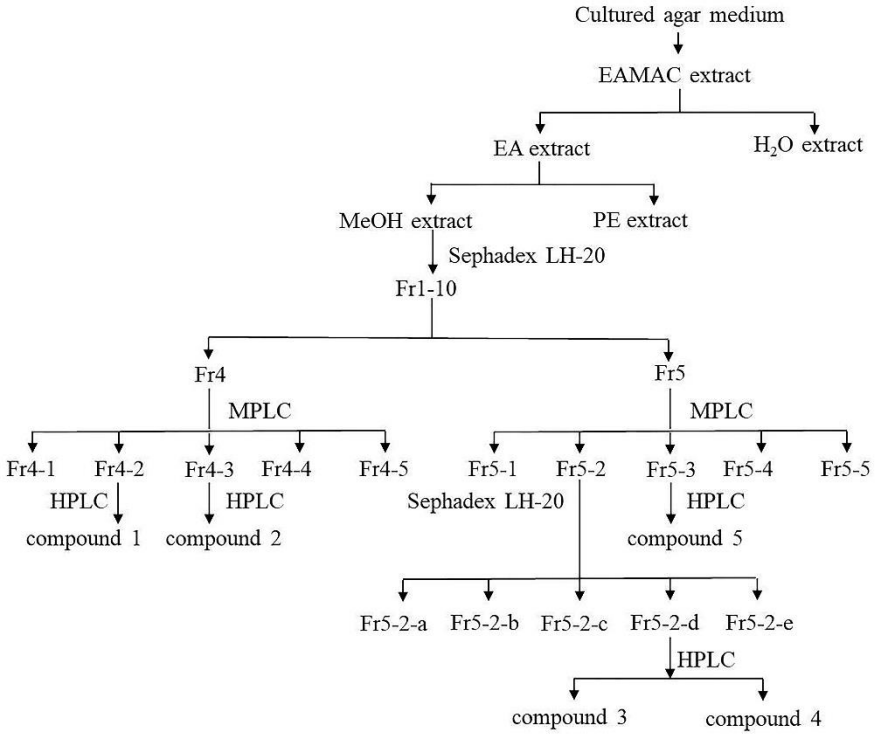

Figure 1. Isolation process of compounds 1 to 5.

2.5. Structural Analysis of the Compounds

^{1}H, ^{13}C and 2D Nuclear Magnetic Resonance (NMR) spectra were recorded on a NMR spectrometer with tetramethylsilane as an internal standard (500 MHz, Bruker BioSpin, Karlsruhe, Germany). Mass Spectrometry (MS) identification of compounds 1 to 5 was performed using an Agilent 1100 series HPLC INSTRUMENT (Agilent Technologies Inc., California, America). The HPLC system consisted of an online vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A) and a thermostated column compartment (model G1316A). The mass spectrometer (MSD Trap SL, model G2445D) from Bruker Daltonik (Bremen, Germany) was equipped with an electrospray ionization (ESI) source (model G1947A). ESI was performed in positive ion detection mode; the nebulizer pressure was 30 psi; the dry gas temperature was 350 °C; the dry gas flow rate was 9.0 L/min; and the capillary voltage was 3500 V. The ampl value was set at 1.00; the trap drive level was 80%; and the cutoff value was 30%. The full-scan mass spectrum of the quasimolecular ions was recorded.

2.6. Antibacterial Activity Assay

To determine the MICs of compounds 1 to 5 against the *Staphylococcus aureus* (ATCC25923), *Klebsiella aerogenes* (CGMCC 1.183) and *Proteus vulgaris* (CGMCC 1.491), antibacterial susceptibility test was carried out according to the guidelines of the Clinical and Laboratory Standards Institute (Volume 32 Number 2, 2009) [28]. Briefly, the bacteria were cultured at 37 °C overnight with constant shaking and then diluted with Mueller-Hinton (MH, beef powder 2 g/L, soluble starch 1.5 g/L, casein acid hydrolysate 17.5 g/L, pH 7.2–7.4) broth to obtain a final concentration of 10^5 colony forming units (CFU)/mL (turbidity standard). Compounds 1 to 5 were prepared in MeOH to obtain different sub-inhibitory concentrations (10–200 μg/mL) by serial microdilution. Then 10 μL aliquots of the sub-inhibitory dilutions of compounds 1 to 5 and 90 μL of the bacterial suspension were added into individual wells of a 96-well microtiter plate. Wells treated with MeOH and Ampicillin (AMP) served as the negative control groups and positive control groups, respectively (three parallel experiments were performed for each group). Next, the plates were incubated at 37 °C for 16 to 24 h. The MICs was defined as the lowest concentration of drug that inhibited 90% of

the growth of the microorganisms, which was determined by measurement of the optical density (OD) at a wavelength of 600 nm using a microplate reader [29]. The inhibition zone diameter of compounds against the indicator bacteria at the MICs was determined by the filter paper disk method. Briefly, the indicator bacteria cultured overnight were diluted to 10^5 CFU/mL in MH broth. Next, 200 µL of the bacterial suspension was uniformly spread on the MH broth agar plates by confluent swabbing of the surface. Sterile filter paper disks (6 mm in diameter) impregnated with 5 µL of the compound solution were placed on the surfaces of seeded agar plates. Filter paper disks impregnated with AMP and MeOH served as the positive control groups and negative control groups, respectively. After incubation at 37 °C for 16 h to 24 h, the diameter of the inhibitory zones surrounding the disks (disk diameter included) was used to evaluate the bacterial inhibitions (three parallel experiments were performed for each group).

2.7. Cytotoxicity Assays

Compounds 1 to 5 were evaluated for cytotoxic activity using A549 cells (A549 cell line human, are human lung carcinoma derived and growth as monolayer and presented by professor Hongbin Ji of Shanghai Institute of Biochemistry and Cell Biology). A549 cells were maintained in high-glucose Dulbecco's modified Eagle medium (DMEM) supplemented with 10% bovine calf serum, 100 U/mL penicillin and 100 U/mL streptomycin at 37 °C in a humidified atmosphere containing 5.0% CO_2, and 0.02% Trypsin-EDTA and 0.25% trypsin were mixed at a ratio of 1:9 for cells subculturing. Cells in logarithmic growth phase were used for cell experiments. The viability of the A549 cells was evaluated using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, A549 cells (1×10^5 cells/96-well plate) were pre-incubated in drug-free medium overnight before addition of the compounds at different concentrations (0.39, 0.78, 1.56, 3.13, 6.25, 12.5, 25, 50, 75 and 100 µg/mL; the stock solutions of the compounds were dissolved in methanol). Cells treated with MeOH and cisplatin served as the negative control groups and positive control groups, respectively. After exposure to compounds 1 to 5 at different concentrations for 24 h, the absorbance of peer well at 570 nm was measured by microplate reader (BioTek Mini-Subcell, Vermont, America). The absorbance of peer well at 570 nm was measured by microplate reader. Three parallel experiments were performed for each group.

2.8. Cell Invasion and Migration Assays

Compounds 1 to 5 were dissolved in MeOH to concentrations of 20 µg/mL, 30 µg/mL and 40 µg/mL. Subsequently, the cell migration and invasion experiments were performed with MeOH as the negative control groups, cisplatin as positive control groups as follow: (1) Cell migration assays: transwell migration assays were performed by using transwell chamber in 24-well cell culture plate with 8 µm pores. Chambers were washed with PBS for three times. Then the 600 µL medium was placed in the lower chamber, and A549 cells (2×10^5/well) in 200 µL serum-free medium were seeded in the top chamber. Cells were treated with concentrations of 20 µg/mL, 30 µg/mL, 40 µg/mL of the drug at 37 °C in a humidified atmosphere of 5% CO_2. After incubation for 16 h, non-migrated cells on the top surface of the membrane were gently scraped away with cotton swab, and migrated cells were fixed with 4% paraformaldehyde for 20 min and stained with 0.1% crystal violet for 15 min. The cells that migrated to the lower side of the membranes were imaged and counted using an Olympus light microscope (BX51/61, Olympus Corporation, Tokyo, Japan). Cells were counted using Image J_v1.8.0 (17/08/2017) and manually verified. The experimental results were all repeated three times. (2) Cell invasion assays: the BioCoat Matrige (24 Wells, 8.0 µm pore size) was removed from the −20 °C freezer to encase the invaded compartment. In the upper chamber, 200 µL diluted cell suspension was added, and in the lower chamber, 600 µL fresh medium and corresponding concentration of drugs were added. The cells were cultured in an incubator at 37 °C and 5% CO_2 for 16 h. After the incubation period, the medium was blotted out and the membranes were rinsed twice with phosphate buffered saline (PBS) buffer. 1 mL 4% paraformaldehyde fixing solution

was added to all wells and fixed at room temperature for 20 min. Then 1 mL iced MeOH was added to all wells and fixed at room temperature for 15 min. The residual liquid on the chamber was cleaned twice with PBS, and the residual liquid was dried up. Washing the PBS buffer twice. Using a cotton swab to gently scrape off the cells on top of the compartment and place the compartment on a slide. Photographs were taken and counted with an Olympus light microscope (BX51/61, Olympus Corporation), and the results were analyzed. Cells were counted using Image J _v1.8.0 (17/08/2017) and manually verified. The experimental results were all repeated three times.

2.9. Method of Statistical Calculations

The inhibition zone diameter in the mothed of "2.6. antibacterial activity assay" is the average value of three parallel experiments performed for each group. The cell viability in the mothed of "2.7. Cytotoxicity Assays" is calculated via the following formula (1) (*Y*: Cell viability; A1: average value of absorbance at 570 nm of culture system added compounds or cisplatin; A2: average value of absorbance at 570 nm of culture system added MeOH); A3: average value of absorbance at 570 nm of culture system without culture. The number of migrated cells and invaded cells in "2.8. Cell Invasion and Migration Assays" is counted by using Image J _v1.8.0 (17/08/2017) and manually verified, and the significant difference is calculated by *t*-test calculation program of graphpad prism 7.

$$\boldsymbol{Y} = \frac{\mathbf{A1} - \mathbf{A3}}{\mathbf{A2} - \mathbf{A3}} \times \mathbf{100}\% \quad (1)$$

3. Results

3.1. Isolation and Taxonomy of Strain f8

Strain f8 was isolated from a sample of marine sediment at a depth of 8 m, which was collected from the Yellow Sea (119°25′ E, 35°13′ N), close to Rizhao City, Shandong Province, China. The 16S rRNA sequence of strain f8 was deposited in GenBank (https://www.ncbi.nlm.nih.gov/nuccore/MG515747; sequence size: 1428 bp; accession number: MG515747; accessed date: 25 November 2017). A BLASTN search employing the PCR-amplified 16S rRNA sequence indicated that the strain was related to *Streptomyces griseorubens* strain G19 (KU535562.1) (99% similarity). The phylogenetic tree (Figure 2) was constructed using the neighbor-joining method corrected with the Jukes-Cantor algorithm showing the relatedness with *S. griseorubens* strain G19 (KU535562.1). Therefore, the strain f8 was named *S. griseorubens* f8.

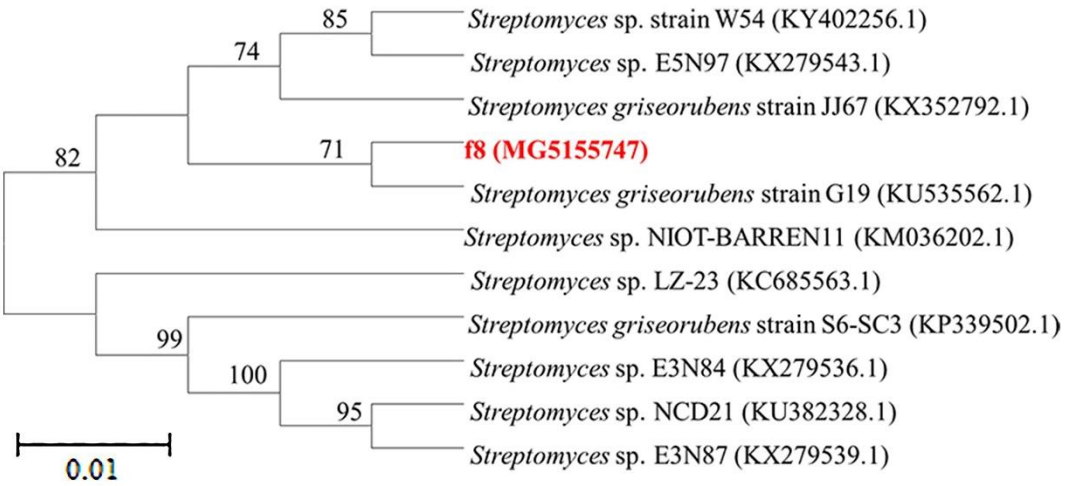

Figure 2. The phylogenetic relationship of the *S. griseorubens* f8 based on 16S rRNA gene homology.

3.2. Morphology of Strain S. griseorubens f8

After strain *S. griseorubens* f8 was grown at 28 °C for 14 days on M1 medium, the colonies were found to be gray and in irregularly round shape (with diameters of approximately 2 mm) and had rough surfaces and central bulges. SEM showed that the spores were in sphere, and the diameters were approximately 0.8–1.0 μm (Figure 3).

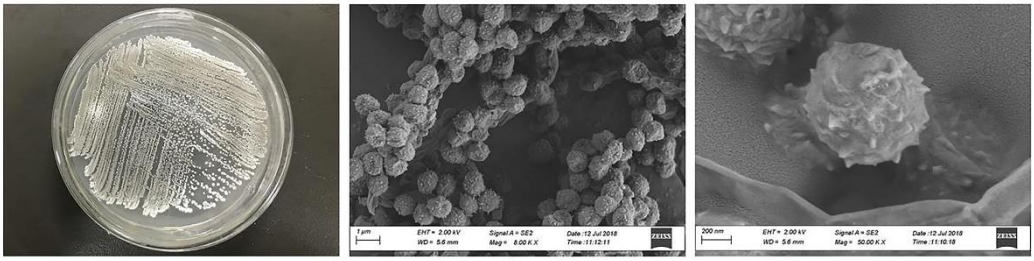

Figure 3. The morphology of strain *S. griseorubens* f8. (**a**) shows the morphology of strain *S. griseorubens* f8 on M1 medium. (**b**) shows the morphology of the strain *S. griseorubens* f8 spores under SEM. (**c**) shows the morphology of a single spore under SEM.

3.3. Structure Elucidation

Compound 1: cyclo(L-Val-L-Pro); white crystal; molecular formula: $C_{10}H_{16}N_2O_2$; ESI-MS (*m/z*): 219.1 $[M+Na]^+$. Compound 1 is consistent with a previous report [30] (shown in Supplementary Table S1). The structural formula and mass spectrum of compound 1 are shown in Figure 4a.

Compound 2: cyclo(L-Pro-L-Leu); white powder; molecular formula: $C_{11}H_{18}N_2O_2$; ESI-MS (*m/z*): 233.0 $[M+Na]^+$. Compound 2 is consistent with a previous report [31] (shown in Supplementary Table S2). The structural formula and mass spectrum of compound 2 are shown in Figure 4b.

Compound 3: cyclo(L-Pro-L-Tyr); white crystals; molecular formula: $C_{14}H_{16}N_2O_3$; ESI-MS (*m/z*): 261.3 $[M+H]^+$, 283.1 $[M+Na]^+$. Compound 3 is consistent with a previous report [30] (shown in Supplementary Table S3). The structural formula and mass spectrum of compound 3 are shown in Figure 4c.

Compound 4: *N*-acetyltyramine; yellow oil; molecular formula: $C_{10}H_{13}NO_2$; ESI-MS (*m/z*): 180.0 $[M+H]^+$, 202.0 $[M+Na]^+$. Compound 4 is consistent with a previous report [32] (shown in Supplementary Table S4). The structural formula and mass spectrum of compound 4 are shown in Figure 4d.

Compound 5: cyclo(L-Pro-L-Phe); white crystals; molecular formula: $C_{14}H_{16}N_2O_2$; ESI-MS (*m/z*): 245.1 $[M+H]^+$, 267.1 $[M+Na]^+$. Compound 5 is consistent with a previous report [33] (shown in Supplementary Table S5). The structural formula and mass spectrum of compound 5 are shown in Figure 4e.

3.4. Antimicrobial Activity

Table 1 shows the MICs of compounds 1 to 5 and AMP. Figure 5a shows the inhibitory zone diameter of compound 3 and compound 5 against *Staphylococcus aureus*, *Proteus vulgaris* and *Klebsiella aerogenes* at the MICs. As shown in Figure 5a, the the inhibitory zone diameter of *Proteus vulgaris* is the biggest, which indicated that compound 3 and compound 5 had the best inhibitory effect on *Proteus vulgaris*. On the contrary, it has the least inhibitory effect on *Staphylococcus aureus*. Figure 5b shows the antibacterial activity of the AMP, compound 3 and compound 5 on the three indicator bacteria. It could be concluded from Table 1 and Figure 5 that compounds 3 and 5 had weak antibacterial activity compared with the AMP. Unfortunately, compounds 1, 2 and 4 did not exhibit antibacterial activity against any of the tested bacteria at concentrations up to 200 μg/mL (datas is not shown).

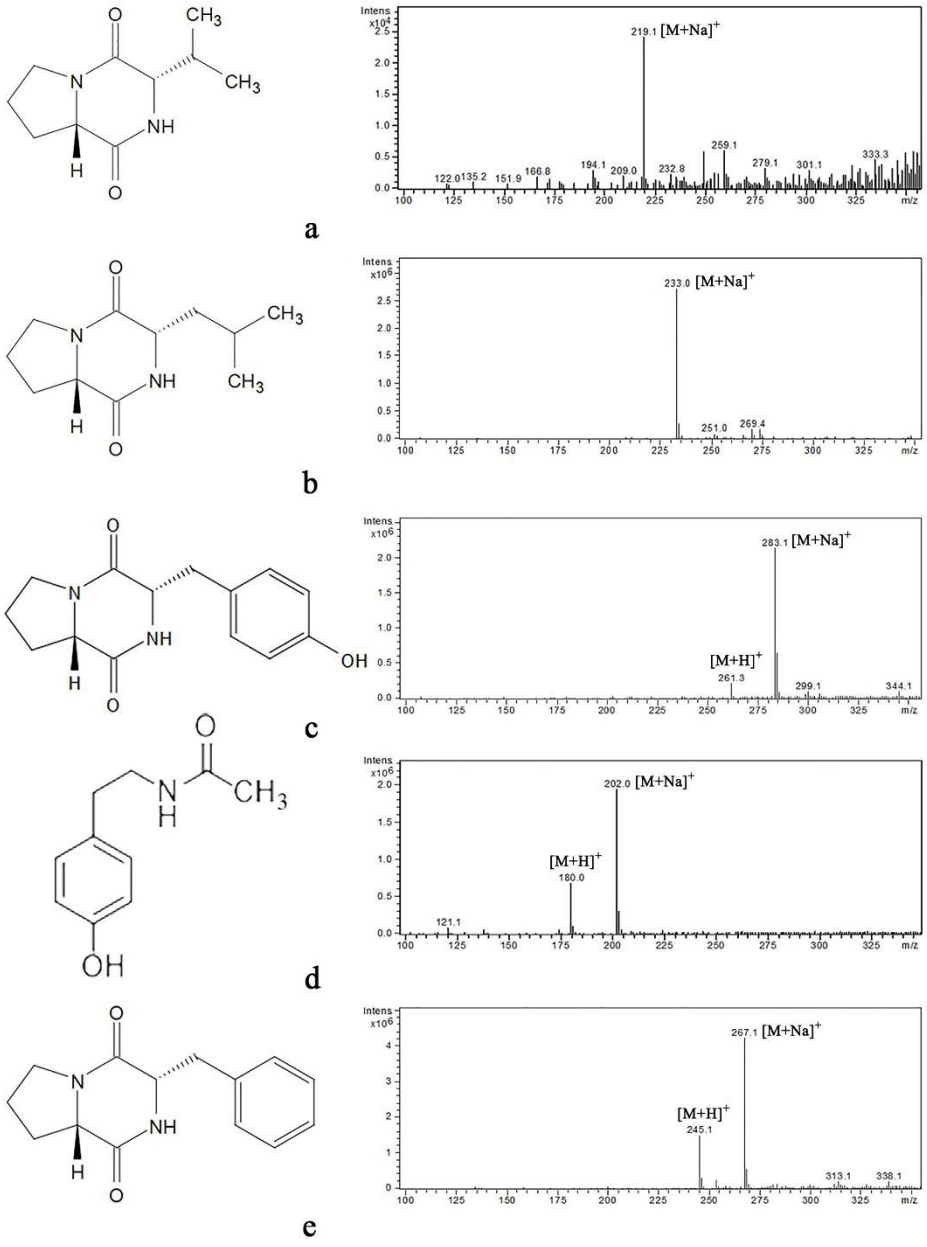

Figure 4. Structural formula and mass spectrum of compounds. (**a**–**e**) show the structures and mass spectra of compounds 1 to 5, respectively. (M: molecular weight of the compound).

Table 1. The MIC of compound 3, compound 5 and AMP.

Indicator	Compound 3	Compound 5	AMP
Staphylococcus aureus	160 μg/mL	180 μg/mL	30 μg/mL
Klebsiella aerogenes	100 μg/mL	130 μg/mL	20 μg/mL
Proteus vulgaris	120 μg/mL	150 μg/mL	20 μg/mL

3.5. Cytotoxic Activity

The effects of different concentrations of compounds 1 to 5 and cisplatin on the viability of A549 cells were evaluated (Supplementary Figure S1). The results show that the cell viability against compounds 1 to 5 were all more than 90%, which was almost the same as that of the negative control group (MeOH) but opposite to the positive control groups (cisplatin) that shown remarkable dose-dependent toxicity to A549 cells. In addition, the effects of the same concentrations of compounds 1 to 5 and cisplatin on the viability of

A549 cells were analyzed (Supplementary Figure S2) and the result shows that the cell viability of the same concentrations of compounds were not statistically significant, even at high concentrations. These results indicated that compounds 1 to 5 did not exhibit the potential to inhibit A549 cells.

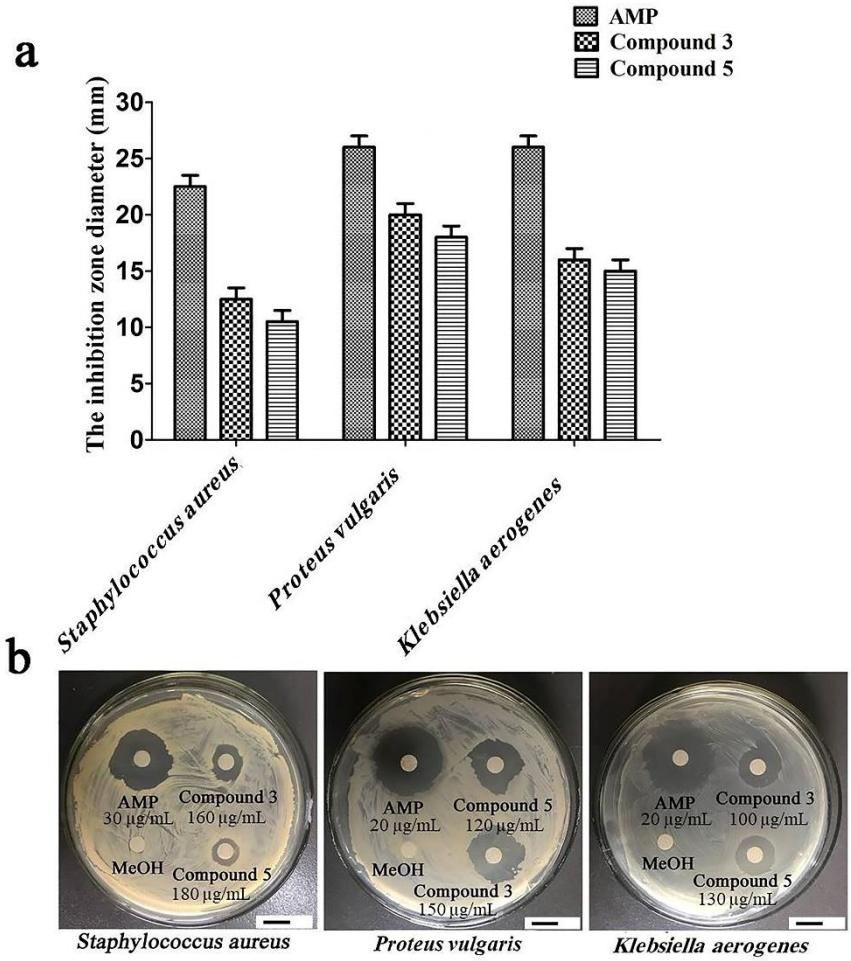

Figure 5. The inhibition zone diameter. (**a**) shows the inhibitory zone diameter of AMP, compound 3 and compound 5 against *Staphylococcus aureus*, *Proteus vulgaris* and *Klebsiella aerogenes* at the MICs (the MICs of AMP, compound 3 and compound 5 for *Staphylococcus aureus* are 30 μg/mL, 160 μg/mL and 180 μg/mL, for *Proteus vulgaris* are 20 μg/mL, 120 μg/mL and 150 μg/mL, and for *Klebsiella aerogenes* are 20 μg/mL, 100 μg/mL and 130 μg/mL, respectively). (**b**) shows the antibacterial activity of the AMP, compound 3 and compound 5 on the three indicator bacteria. Scale bar = 10 mm.

3.6. Effects of the Compounds on Cell Invasion and Migration

Inhibition of the invasion and migration of tumor cells is an important approach for the treatment of cancer. Therefore, we conducted experiments to determine the effects of compounds 1 to 5 on cells invasion and migration. Figures 6 and 7 show the effects of different concentrations compounds 1 to 5 and cisplatin on the invasion and migration of A549 cells. Based on the results shown in Figures 6 and 7, we concluded that compounds 1, 2, 3 and 5 could inhibit the invasion and migration of A549 cells. Unfortunately, compound 4 did not exhibit the potential to inhibit the invasion and migration of A549 cells. The effects of different concentrations of compounds 1 to 5 and cisplatin on the invasion ability and migration ability of A549 cells were evaluated. As shown in Figure 8, the number of invaded and migrated cells was gradually decreased with increasing concentrations of compounds 1, 2, 3 and 5. In addition, the number of invaded and migrated cells at the same concentration of different compounds were analyzed (Supplementary Figures S3 and S4). In the invasion

experiment, compound 5 have better inhibitory ability than other compounds used in the experimental concentrations (Supplementary Figure S3). In the migration experiment, compound 1 have the most significant inhibition on cell migration at the concentrations of 20 and 30 μg/mL, while compound 5 inhibits cell migration most significantly at the concentration of 40 μg/mL (Supplementary Figure S3). From the analysis results of invasion and migration, we concluded that compounds 1, 2, 3 and 5 could significantly inhibit the invasion and migration of A549 cells. Among them, compound 5 may be a better potential compound that can inhibit invasion and migration of A549 cells simultaneously, which suggests that it may be a more promising potential drug for the treatment of cancer.

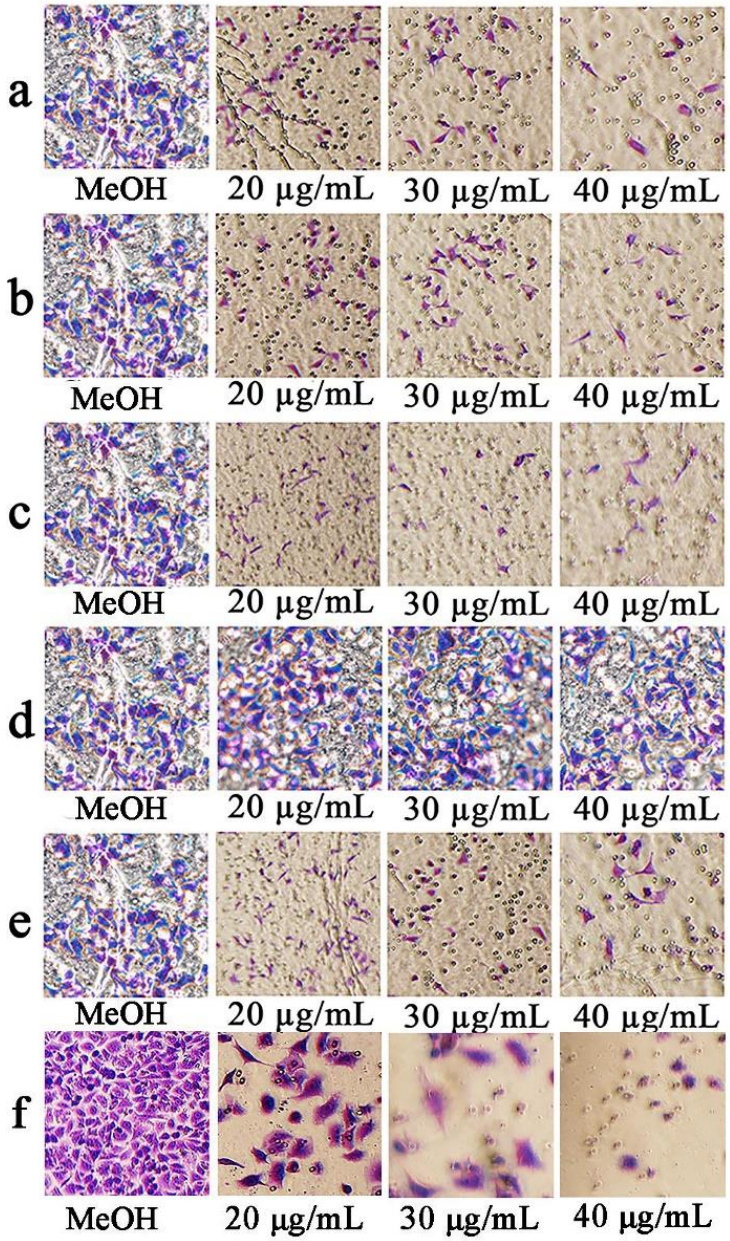

Figure 6. Cell invasion assays. (**a**–**f**) show the effects of different concentrations (20 μg/mL, 30 μg/mL and 40 μg/mL) of compounds 1 to 5 and cisplatin on the invasion of A549 cells, respectively.

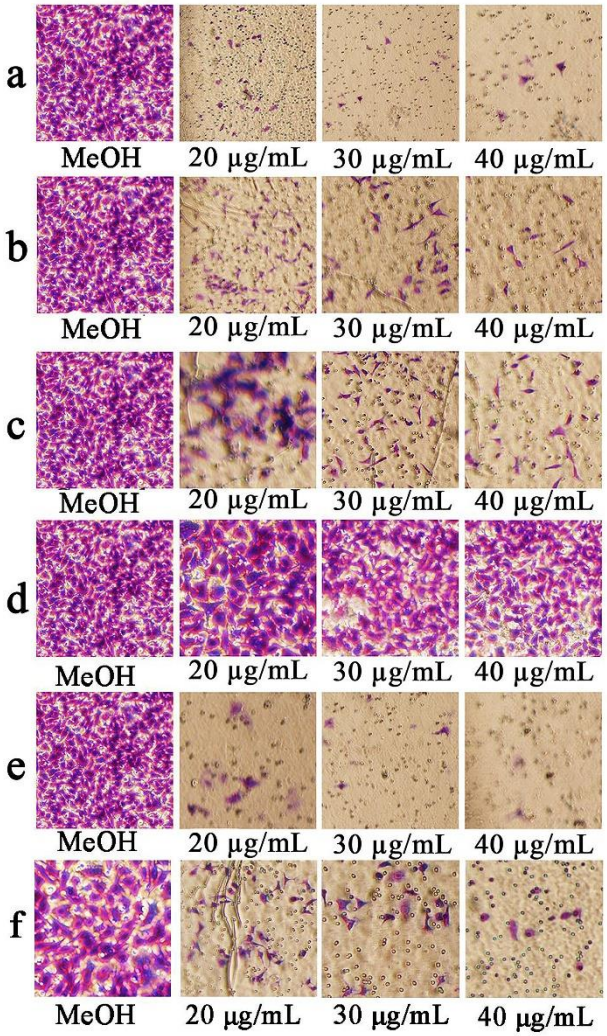

Figure 7. Cell migration assay. (**a**–**f**) show the effects of different concentrations (20 μg/mL, 30 μg/mL and 40 μg/mL) of compounds 1 to 5 and cisplatin on the migration of A549 cells, respectively.

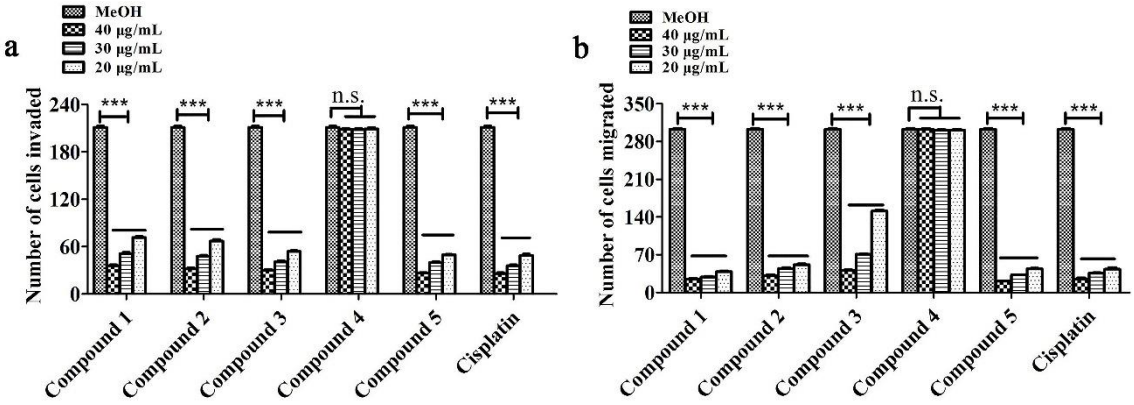

Figure 8. Number of invaded and migrated cells. (**a**) shows the number of cells invaded using different concentrations of the compounds and cisplatin for treatment. (**b**) shows the number of cells migrated using different concentrations of the compounds and cisplatin for treatment. ***: $p < 0.001$; n.s.: no significance compared to the corresponding control; the p-value is determined by t-test.

4. Discussion

Marine *actinomycetes* are a prolific source of secondary metabolites and about 80% of the actinomycete natural products reported from marine *actinomycetes* to date. Peptides are short polymers of amino acids and is important bioactive secondary metabolites of marine *Streptomyces* [34]. For example, piperazimycins A–C are cytotoxic hexadepsipeptides isolated from *Streptomyces* sp, among them, piperazimycin A exhibited potent in vitro cytotoxicity against multiple tumor cell lines, such as leukemia cell lines, prostate cell lines and the melanoma cell lines [35]; salinamides A and B are bicyclic depsipeptides produced by a *Streptomyces* sp. CNB-091 isolated from the jelly fish *Cassiopea xamachana* and can be used as potential antibiotics and anti-inflammatory agents [36]; allophycocyanin is one of the most important active peptides from marine *Streptomyces* that could remarkably inhibit the S-180 carcinoma in mice [34]. *S. griseorubens* are a genus of Gam-positive bacteria that form filamentous mycelium-like eukaryote fungi [37]. Many *S. griseorubens* strains were isolated from the terrestrial environment, but few were isolated from the oceans. In this study, a marine *S. griseorubens* was isolated and its five secondary metabolites (compounds 1 to 5) were purified and analyzed. Among them, compounds 1, 2, 3 and 5 are cyclic dipeptides that were first isolated from *S. griseorubens*.

The diketopiperazines cyclo(*L*-Val-*L*-Pro) (compound 1) has been isolated from the different strains, such as *Pseudomonas aurantiaca* [30], *Streptomyces albospinus* [38] and *Aspergillus fumigatus* [39], and is weakly antibacterial inhibiting the growth of *Staphylococcus aureus* and *Micrococcus luteus* at the concentration up to 2.9 mmol/L [40]. In this study, compound 1 did not exhibit antibacterial activity against any of the tested bacteria at the concentration of 200 μg/mL (1 mmol/L), which indicated that compound 1 is indeed a weakly antibacterial for many bacteria. Compound 2 has been isolated from *Rhodococcus rhodochrous* [40] and the marine sponge *Callyspongia* [31], and does not exhibit the potential to cytotoxic activity against SGC-7901 (human stomach cancer), HepG2 (human liver cancer) and HeLa (human ovarian cancer) cell lines using the MTT method [31]. However, it has not been verified whether compound 2 has the potential to inhibit the invasion and migration of tumor cells. In our study, compound 2 did not also exhibit the potential to cytotoxic activity against A549 cells (dates is not shown), but was first discovered that it had the ability to inhibit the invasion and migration of A549 cells, indicating compound 2 may be used as a potential drug to inhibit the invasion and migration rather than to inhibit the growth of cancer cells. Compound 3 [30,38,41] and compound 4 [32] have been reported but no biological activity was assayed. In this study, we found that compound 3 may be a potential antimicrobial agent due to the significant antibacterial activity against the different tested bacteria, which is conducive to promoting the further study of the biological activity of compound 3. Compound 4 did not show any ability to inhibit the growth of bacteria tested and the invasion and migration of A549 cells, and the biological activity of compound 4 need to be further investigated. According to previous studies, compound 5 has high antimicrobial activity against *Ralstonia solanacearum*, a causative agent of bacterial wilt in many important crops throughout the world, and also interfered with the expression levels of some pathogenicity-contributors of *R. solanacearum* [33]. Furthermore, compound 5 effectively inhibited spore formation of *Magnaporthe grisea*, which is a vital pathogenesis process of the fungal pathogen [33]. Meanwhile, our found that compound 5 had antibacterial activity against *Staphylococcus aureus*, *Klebsiella aerogenes* and *Proteus vulgarisin*, suggesting compound 5 is a promising potential antimicrobial agent with broad-spectrum activity. In addition, compounds 1, 3 and 5 were also first discovered to have the potential to inhibit the invasion and migration of A549 cells, indicating that these cyclic dipeptides isolated from *Streptomycetes griseorubens* have the promising potential pharmaceutical use in cancer treatment. This study shown that marine *Streptomycetes griseorubens* are a promising resource for the discovery of potential natural antimicrobial and antitumor peptides agents5.

5. Conclusions

In the present study, five compounds (1 to 5) were isolated and identified from the secondary metabolites of strain *S. griseorubens* f8 that isolated from the Yellow Sea, close to Rizhao City, Shandong Province, China. Among them, compounds 1, 2, 3 and 5 are first isolated from the secondary metabolites of marine *S. griseorubens*. Compounds 3 and 5 exhibit significant antibacterial activity against *Staphylococcus aureus*, *Klebsiella aerogenes* and *Proteus vulgaris*. Compounds 1, 2, 3 and 5 are first found to inhibit the invasion and migration of A549 cells. Our results provided a novel production strain for compounds 1, 2, 3 and 5. These extracted secondary metabolites from *S. griseorubens* f8 may be potential antimicrobials against *Staphylococcus aureus*, *Klebsiella aerogenes* and *Proteus vulgaris*, and novel anti-lung cancer agents clinically.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jmse9090978/s1, Figure S1: The viability of A549 cells (%) between the same compounds and cisplatin of the different concentration., Figure S2: The viability of A549 cells (%) between the same concentration of the different compounds and cisplatin., Figure S3: The number of migrated cells using different compounds and cisplatin at the same concentration., Figure S4: The number of invaded cells using different compounds and cisplatin at the same concentration., Table S1: Comparison of 1H and 13C NMR data of compound 1 with those found in the literature (δ in ppm, J in Hz)., Table S2: Comparison of 1H and 13C NMR data of compound 2 with those found in the literature (δ in ppm, J in Hz)., Table S3: Comparison of 1H and 13C NMR data of compound 3 with those found in the literature (δ in ppm, J in Hz)., Table S4: Comparison of 1H and 13C NMR data of compound 4 with those found in the literature (δ in ppm, J in Hz)., Table S5:, Comparison of 1H and 13C NMR data of compound 5 with those found in the literature (δ in ppm, J in Hz).

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