Postprint: Investigation of Secondary Metabolites from Marine Streptomyces sporoverrucosus 33510

Authors: Liu Ying, Zhu Zhenxin, Wang Cong, Liu Hongcun, Jiang Mingguo, Zhu Yuzhang, Lin Kun, Lu Xiaomei, Liang Haina, Yang Lifang, Yang Lifang

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Abstract

To obtain bioactive substances from marine actinomycete secondary metabolites, the marine streptomycete *Streptomyces sporoverrucosus* 33510 was investigated. Antimicrobial activity assays of the *Streptomyces sporoverrucosus* 33510 strain demonstrated that the strain *Streptomyces sporoverrucosu* 33510 exhibited inhibitory activity against various plant pathogens. The fermentation products were isolated and purified using analytical and separation techniques including High Performance Liquid Chromatography (HPLC), semi-pre HPLC (Semi-pre HPLC), Colume Chromatography (CC), Thin Layer Chromatography (TLC), and recrystallization. The obtained monomeric compounds were identified via Nuclear Megnetic Resonance (NMR). A total of ten monomeric compounds were isolated from its secondary metabolites: bisphenol A (1), 2-(4-methoxyphenyl)acetic acid (2), N-phenethylacetamide (3), methyl 2-(1H-indol-3-yl)acetate (4), dibutyl phthalate (5), cyclo(D)-pro-(D)-Leu (6), cyclo(D-Pro-L-Leu) (7), cyclo-(D-ProL-Ile) (8), cyclo(L-Pro-L-Phe) (9), and cyclo-(L-Leu-L-Val) (10). Except for compounds 5 and 9, the remaining eight compounds were isolated from this bacterium for the first time. Antimicrobial assays of the monomeric compounds revealed that compounds 3, 4, 5, and 6 exhibited significant inhibitory effects against *Rhizoctonia solani*; compounds 7, 8, and 9 showed significant inhibitory effects against *Cryphonectria parasitica*; and compounds 3 and 7 demonstrated significant inhibitory effects against *Fusarium pseudograminearum*. The strain *Streptomyces sporoverrucosus* 33510 possesses potential for development as an antimicrobial agent.

Full Text

Study on Secondary Metabolites of Marine Streptomyces sporoverrucosus 33510

Abstract

To investigate the bioactive secondary metabolites of marine actinobacteria *Streptomyces sporoverrucosus* 33510 and evaluate the antibacterial activity of purified compounds, the strain was tested for antimicrobial activity against various plant pathogenic bacteria. The compounds were isolated and purified by high performance liquid chromatography (HPLC), semi-preparative HPLC, thin-layer chromatography (TLC), and recrystallization techniques, and their chemical structures were characterized by nuclear magnetic resonance (NMR) analysis. Ten compounds were identified: bisphenol A (1), 2-(4-methoxyphenyl)acetic acid (2), *N*-phenethylacetamide (3), methyl 2-(1*H*-indol-3-yl)acetate (4), dibutyl phthalate (5), cyclo(D)-Pro-(D)-Leu (6), cyclo(D-Pro-L-Leu) (7), cyclo-(D-Pro-L-Ile) (8), cyclo(L-Pro-L-Phe) (9), and cyclo-(L-Leu-L-Val) (10). Except for compounds 5 and 9, the remaining eight compounds were isolated from *Streptomyces sporoverrucosus* for the first time. Antibacterial activity was evaluated by disk diffusion assay. Compounds 3, 4, 5, and 6 showed inhibitory activity against *Rhizoctonia solani*; compounds 7, 8, and 9 showed activity against *Cryphonectria parasitica*; and compounds 3 and 7 showed activity against *Fusarium pseudograminearum*. Marine actinomycetes *Streptomyces sporoverrucosus* 33510 represents a potential resource for antibacterial agents.

Keywords: *Streptomyces sporoverrucosus* 33510; secondary metabolites; antibacterial activity

Introduction

The discovery of microorganisms capable of producing bioactive compounds from terrestrial environments has become increasingly limited, prompting researchers to explore marine ecosystems as important sources of natural medicinal chemistry. Since the late 1960s, with the expansion of research into marine flora and fauna, novel secondary metabolites have emerged as a new direction in drug discovery. Today, marine natural products represent a significant component of bioactive compound research, with over 20,000 active metabolites identified to date (Jensen et al. 1994). Actinomycetes remain the primary source of new compounds, contributing more than half of the marine-derived metabolites, followed by *Bacillus*, *Nocardia*, and *Pseudomonas* species (Blunt et al. 2021). Currently, approximately two-thirds of clinically used antibiotics originate from actinomycetes (Jiang et al. 2007). The genus *Streptomyces* continues to yield new compounds with antibacterial activity (Bi et al. 2016). This study investigates the secondary metabolites of *Streptomyces sporoverrucosus* 33510, isolated from mangrove soil in the Maowei Sea of Guangxi, to obtain compounds with

promising antibacterial activity.

1. Materials and Methods

1.1 Instruments Low-temperature cooling liquid circulation pump (LC-LTC-10/20, Shanghai Lichen Bangxi Instrument Technology); rotary evaporator (N-1300, Shanghai Ailang Instrument); circulating water vacuum pump (SHZ-III, Shanghai); water bath (HH-S6, Jintan City Jingda Instrument Manufacturing); large-capacity floor-standing shaker (MQZ-632, Shanghai Minquan Instrument); electronic balance (ME204T, Mettler-Toledo); semi-preparative HPLC system (SCL-10AVP, Shimadzu, Japan); analytical HPLC (LC-10AT, Shimadzu, Japan); NMR spectrometer (Bruker 400 MHz); biochemical incubator (ZXSD-B1160, Shanghai Zhicheng Analysis Instrument Manufacturing); ultra-clean workbench (ZHJH-C1112B, Shanghai Zhicheng Analysis Instrument Manufacturing).

1.2 Reagents Petroleum ether, ethyl acetate, methanol, dichloromethane, and other organic solvents were analytical grade. HPLC-grade organic solvents were purchased from Chengdu Kelong Chemical. Column chromatography silica gel (200-300, 300-400 mesh) was obtained from Qingdao Marine Chemical. ODS column chromatography packing material: YMC Gel ODS-A. Analytical column: YMC HPLC column (250 × 4.6 mm I.D., S-5 m, 12 nm, AA12S05-2510WT). Semi-preparative column: YMC-Pack ODS-A (250 × 10 mm I.D., S-5 m, 12 nm, AA12S05-2510WT).

1.3 Materials Potato dextrose agar medium. The *Streptomyces sporoverrucosus* 33510 strain was obtained from the Marine Biotechnology Research Institute of Guangxi Minzu University and Guangxi Haibo Biotechnology Co., Ltd. Pathogenic fungal strains were sourced from the Yunnan Microbial Institute of Yunnan University.

2. Experimental Procedures

2.1 Antibacterial Activity Testing Sample preparation: Methanol solutions of test samples (10 L total) were applied to filter paper disks prepared with a puncher. Control disks were treated with methanol only. For testing against plant pathogens, compounds were dissolved in methanol/dichloromethane to prepare 0.1 $mg \cdot mL^{-1}$ solutions. Five microliters of each sample were applied to filter disks and allowed to dry completely. Positive control disks contained ketoconazole (0.1 $mg \cdot mL^{-1}$). Disks were placed on PDA agar plates inoculated with test organisms. An 8 mm agar plug containing the indicator fungus was placed at the center of each plate using sterile bamboo, positioned 2 mm from the filter disks. Plates were incubated at 28°C for 3 days, after which inhibition zones were examined.

2.2 Crude Extract Preparation One hundred eighty liters of fermentation broth from large-scale fermentation was extracted three times with an equal volume of ethyl acetate. The combined extracts were concentrated under reduced pressure to yield 24.9 g of crude extract.

2.3 Isolation and Purification of Secondary Metabolites The crude extract was subjected to normal-phase silica gel column chromatography using dry packing and dry loading methods. Elution was performed with petroleum ether/ethyl acetate mixtures at ratios of 1:0, 10:1, 8:1, 6:1, 4:1, 2:1, 1:1, 1:2, and 0:1, yielding 27 fractions. Compound 1 (11 mg) was obtained from fraction 20 by recrystallization. HPLC analysis was used to combine fractions containing identical compounds, resulting in 11 major fractions (Fr.1-Fr.11).

Fraction Fr.5: ODS reversed-phase column chromatography with methanol/water (20%-80%) yielded 21 subfractions (Fr.5.1-Fr.5.21). Subfractions Fr.5.10 and Fr.5.11 were combined and purified by semi-preparative HPLC using 70% methanol/water to afford compound 2 (1.8 mg).

Fraction Fr.7: ODS reversed-phase chromatography with methanol/water (10%-80%) gave 24 subfractions (Fr.7.1-Fr.7.25). Subfraction Fr.7.8 was analyzed by HPLC and purified by semi-preparative HPLC with 30% methanol/water to obtain compounds 3 (4.6 mg) and 4 (18.7 mg). Subfractions Fr.7.11 and Fr.7.20 were prepared under conditions of 50% methanol at 264 nm and 45% methanol at 267 nm, respectively, yielding compound 5 (2.2 mg) and compound 6 (11 mg).

Fraction Fr.11: Semi-preparative HPLC with 30% methanol/water afforded four pure compounds: compound 7 (36.2 mg), compound 8 (26.2 mg), compound 9 (13.4 mg), and compound 10 (55.2 mg).

All semi-preparative HPLC runs were performed at a flow rate of 3.5 mL/min. Elution procedures are detailed in Table 1 .

3. Results and Discussion

3.1 Antibacterial Activity of S.33510 The antibacterial assays demonstrated that *S. sporoverrucosus* 33510 exhibited significant inhibitory effects against multiple plant pathogens. Representative results are shown in Figure 1 [Figure 1: see original paper], with detailed data presented in Table 2 .

Figure 1 shows the antibacterial efficacy of S.33510 against (from left to right) *Botryosphaeria dothidea*, *Bipolaris sorokiniana*, and *Colletotrichum musae*.

Table 2 summarizes the inhibition results of S.33510 strain against various pathogenic bacteria including *Fusarium oxysporum*, *Colletotrichum musae*, *Bacillus subtilis*, and *Fusarium pseudograminearum*. Note: "√" indicates obvious antibacterial effect, "×" indicates no obvious effect. *Plectosphaer cucumerina* and *Cryphonectria parasitica* were also tested.

3.2 Structural Identification of Pure Compounds Compound 1: Transparent flaky crystals. ^{1}H NMR (400 MHz, Methanol-d$_4$) δ 4.26 (ddd, J = 9.5, 7.2, 1.8 Hz, 1H), 4.17–4.09 (m, 1H), 3.55–3.47 (m, 2H), 2.31 (dddd, J = 11.3, 6.9, 4.6, 2.9 Hz, 1H), 2.10–1.82 (m, 5H), 1.57–1.47 (m, 1H), 0.96 (dd, J = 6.4, 2.6 Hz, 6H). ^{13}C NMR (101 MHz, MeOD) δ 172.80, 168.92, 60.28, 54.62, 46.44, 39.39, 29.07, 25.76, 23.66, 23.30, 22.20. The data matched literature values (Liu et al. 2012), identifying it as the known compound cyclo-(S-Pro-S-Leu).

Compound 2: Dark oily substance, soluble in methanol. ^{1}H NMR (400 MHz, Methanol-d$_4$) δ 7.02 (d, J = 8.7 Hz, 1H), 6.66 (d, J = 8.7 Hz, 1H), 1.58 (s, 2H). ^{13}C NMR (101 MHz, MeOD) δ 155.97, 143.46, 128.73, 115.52, 42.49, 31.67. The data matched literature values (Xu et al. 2009), identifying it as bisphenol A.

Compound 3: Colorless oily substance. ^{1}H NMR (400 MHz, Methanol-d$_4$) δ 7.07 (d, J = 8.5 Hz, 1H), 6.72 (d, J = 8.5 Hz, 1H), 3.66 (s, 2H), 3.52 (s, 1H). ^{13}C NMR (101 MHz, CD$_3$OD_{SPE}) δ 174.58, 157.57, 131.30, 126.32, 116.26, 52.37, 40.89. The data matched literature values (Shin et al. 2003), identifying it as methyl *p*-hydroxyphenylacetate.

Compound 4: Dark oily substance. ^{1}H NMR (400 MHz, Methanol-d$_4$) δ 7.32–7.25 (m, 3H), 2.78 (dd, J = 8.1, 6.7 Hz, 3H), 1.90 (s, 4H), 1.89 (s, 1H). ^{13}C NMR (101 MHz, CD$_3$OD_{SPE}) δ 173.24, 140.50, 129.77, 129.48, 127.34, 42.10, 36.48, 22.50. The data matched literature values (Wang et al. 2018), identifying it as *N*-phenethylacetamide.

Compound 5: Colorless oily substance. ^{1}H NMR (400 MHz, Methanol-d$_4$) δ 7.51 (dt, J = 7.9, 1.0 Hz, 1H), 7.34 (dt, J = 8.2, 1.0 Hz, 1H), 7.16 (d, J = 1.0 Hz, 1H), 7.10 (ddd, J = 8.2, 7.0, 1.2 Hz, 1H), 7.01 (ddd, J = 8.0, 7.0, 1.1 Hz, 1H), 3.77 (d, J = 0.9 Hz, 2H), 3.68 (s, 3H). ^{13}C NMR (101 MHz, MeOD) δ 174.85, 138.02, 128.57, 124.64, 122.48, 119.88, 119.34, 112.26, 108.51, 52.35, 31.86. The data matched literature values (Liu et al. 2018), identifying it as methyl 3-indole-ethanoate.

Compound 6: Colorless oily substance. ^{1}H NMR (400 MHz, Methanol-d$_4$) δ 7.72 (dd, J = 7.9, 1.0 Hz, 2H), 1.72 (ddt, J = 9.0, 8.0, 6.4 Hz, 2H), 1.52–1.38 (m, 2H), 0.98 (t, J = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) δ 169.31, 133.58, 132.34, 129.87, 66.65, 31.71, 20.25, 14.05. The data matched literature values (Liu et al. 2018), identifying it as dibutyl phthalate.

Compound 7: Colorless oily substance. ^{1}H NMR (400 MHz, Methanol-d$_4$) δ 4.26 (dd, J = 9.6, 6.6 Hz, 1H), 1.97 (m, 1H), 2.01–1.86 (m, 2H), 1.78 (dtd, J = 8.6, 6.6, 4.0 Hz, 1H), 1.68 (ddd, J = 13.5, 9.5, 5.5 Hz, 1H), 1.57 (ddd, J = 13.8, 8.5, 5.5 Hz, 1H). ^{13}C NMR (101 MHz, MeOD) δ 171.62, 169.07, 59.33, 57.09, 46.71, 43.63, 29.91, 25.52, 23.33, 23.06, 21.93. The data matched literature values (Peng et al. 2015), identifying it as cyclo(D-Pro-L-Leu).

Compound 8: Light-colored flaky crystals. ^{1}H NMR (400 MHz, Methanol-d$_4$) δ 4.84 (s, 3H), 4.24 (dd, J = 9.9, 6.4 Hz, 1H), 3.34–3.28 (m, 1H), 2.39–2.31

(m, 1H), 1.91 (s, 2H), 1.89 (ddd, J = 11.5, 8.7, 4.4 Hz, 2H), 1.65–1.55 (m, 1H), 1.03–0.91 (m, 7H). ^{13}C NMR (101 MHz, MeOD) δ 171.58, 167.91, 63.44, 59.73, 46.76, 40.95, 30.29, 26.03, 22.87, 15.65, 11.60. The data matched literature values (Hwang et al. 2017), identifying it as cyclo-(D-Pro-L-Ile).

Compound 9: Light-colored oily crystals. ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.31–7.21 (m, 4H), 7.23 (s, 1H), 4.06 (ddd, J = 10.9, 6.4, 2.0 Hz, 1H), 3.31 (t, J = 1.7 Hz, 1H), 3.17 (dd, J = 5.0, 1.8 Hz, 2H), 1.85–1.74 (m, 2H). ^{13}C NMR (101 MHz, MeOD) δ 170.92, 166.90, 137.28, 131.05, 129.46, 128.09, 60.06, 57.68, 45.96, 38.24, 29.37, 22.74. The data matched literature values (Yu et al. 2019), identifying it as cyclo-(L-Pro-L-Phe).

Compound 10: Light-colored substance. ^{1}H NMR (400 MHz, Methanol-d_4) δ 1.05 (d, J = 7.0 Hz, 1H), 1.00–0.93 (m, 3H). ^{13}C NMR (101 MHz, MeOD) δ 171.28, 169.67, 61.54, 54.37, 46.00, 33.67, 25.29, 23.61, 21.84, 19.31, 17.79. The data matched literature values (Wu et al. 2020), identifying it as cyclo-(L-Leu-L-Val).

The chemical structures of compounds 1–10 are shown in Figure 2 [Figure 2: see original paper].

Antibacterial Activity of Pure Compounds

At a concentration of 0.1 mg·mL^{-1}, compounds 3, 4, 5, and 6 showed significant inhibitory effects against *Rhizoctonia solani*. Compounds 3 and 7 exhibited clear inhibition against *Fusarium pseudograminearum*. Compounds 7, 8, and 9 demonstrated significant activity against *Cryphonectria parasitica*. None of the compounds showed inhibitory activity against human pathogenic bacteria. Representative results are shown in Figure 3 [Figure 3: see original paper].

Figure 3 shows the antibacterial activity of compounds against (from left to right) *Rhizoctonia solani*, *Cryphonectria parasitica*, and *Fusarium pseudograminearum*. Numbers 1, 10, and 18 correspond to compounds 2, 6, and 7, respectively.

4. Conclusion

This study isolated ten compounds from the marine actinomycete *Streptomyces sporoverrucosus* 33510. Eight of these compounds were reported for the first time from this species. Antibacterial activity testing confirmed that the crude extract significantly inhibited multiple plant pathogens. At a concentration of 0.1 mg·mL^{-1}, several purified compounds demonstrated clear inhibitory effects against plant pathogenic fungi. These results confirm that *Streptomyces sporoverrucosus* 33510 has potential as a source of antibacterial agents and enrich the diversity of secondary metabolites from marine actinomycetes.

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Note: Figure translations are in progress. See original paper for figures.