

Antibacterial activity of Decursin from *Streptomyces* sp. GMT-8; an endophyte in *Zingiber officinale* Rosc.

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ARTICLE INFO

Article history:

Received on: 14/08/2013

Revised on: 13/09/2013

Accepted on: 05/10/2013

Available online: 31/10/2013

Key words:

antibacterial activity,
decursin, endophyte,
Streptomyces sp.

ABSTRACT

Strain GMT-8 was isolated from the root tissue of *Zingiber officinale* Rosc. and identified as *Streptomyces* sp. on the basis of morphology, chemotaxonomy and 16SrDNA sequencing. It was an antagonist of Gram positive bacteria; *Staphylococcus aureus* ATCC25932, *Bacillus cereus* ATCC7064 and *Bacillus subtilis* ATCC6633. The inhibitory effect of the crude extract from the strain GMT-8 was examined based on the paper disc diffusion method (5 mg per paper disc) with three replications. It has been shown to have inhibitory activity against Gram positive bacteria. The major active ingredient from the crude extract was purified by silica gel column chromatography, thin-layer chromatography and identified to be decursin by NMR and mass spectral data, respectively. Bioassay studies showed that decursin had antibacterial activities against Gram positive bacteria with the minimum inhibitory concentrations within the range of 32 to 256 µg/ml.

INTRODUCTION

Actinomycetes are prokaryotes which have a hyphal (hence fungal-like) morphology. Most of the actinomycetes described are soil microorganisms and are active in the decomposition of plant tissue, and thereby in the recycling of carbon and nitrogen. Some of actinomycete could be isolated from the tissue of healthy plants which was called endophytic actinomycetes. Several reports refer to endophytic actinomycetes produced secondary metabolites against phytopathogenic fungi (Sardi *et al.*, 1992; Shimizu *et al.*, 2000, Taechowisan *et al.*, 2003), and also produced the novel antibiotics for example: Munumbicins from *Streptomyces* sp. NRRL 30562, an endophyte of *Kennedia nigricans* (Castillo *et al.*, 2002), Kakadumycins from *Streptomyces* sp. NRRL 30566, an endophyte of *Grevillea pteridifolia* (Castillo *et al.*, 2003), Coronamycins from *Streptomyces* sp. MSU-2110, an endophyte of *Monstera* sp. (Ezra *et al.*, 2004). In our previous studies, many endophytic actinomycetes were isolated from plant tissues, some of them produced the secondary metabolites against bacteria and phytopathogenic fungi (Taechowisan *et al.*, 2005, 2008, 2013).

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We report here the isolation of the roots of *Zingiber officinale* Rosc. of another *Streptomyces* sp. GMT-8. Extraction of the culture medium of *Streptomyces* sp. GMT-8 was purified and identified as decursin, which displayed strong antibacterial activity against Gram positive bacteria.

MATERIALS AND METHODS

Organisms and media

Streptomyces sp. GMT-8 was isolated from the root tissues of *Zingiber officinale* Rosc. by the surface-sterilization technique (Taechowisan *et al.*, 2003). Identification of the isolate was based on morphology, chemotaxonomy and also 16S rDNA sequencing as described by Taechowisan and Lumyong (2003). Solid medium for sporulation used in this study was International Streptomyces Project Medium 4 (ISP-4) and the culture medium used for secondary metabolites production was ISP-2 (Shirling and Gottlieb, 1966).

Preparation of the crude extract

A spore suspension of *Streptomyces* sp. GMT-8 was prepared in distilled water from cultures grown on ISP-4 medium at 30 °C for 10 days. The suspension, 10⁸ spores per 100 ml of liquid medium, was added to ISP-2 broth in each 500-ml Erlenmeyer flask.

Cultures were kept on a shaker at 120 rpm at 30 °C for 48 h and used as seed stocks. For large production of culture filtrates, the strain GMT-8 was grown in a modified 3000 ml glass container containing 1500 ml of ISP-2 broth, and incubated in an orbital shaker for 5 days in the same condition. The 5-day-old cultures were filtrated by Whatman paper No. 1 under vacuum. The culture filtrate of the strain GMT-8 was extracted three times with 1/3 volumes of ethyl acetate. This organic solvent was pooled and then taken to dryness under flash evaporation at 40 °C. The yield of dry material per litre was about 2.38 g.

Purification of the compounds

The residue of 2.38 g was dissolved in 23.8 ml of chloroform and fractionated on column chromatography (Merck silica gel 60, 35-70 mesh) with hexane, diethyl ether and methanol. The combined fractions eluted with 50% diethyl ether in hexane, 100% diethyl ether, and 5% methanol in diethyl ether (315 mg) were further separated by MPLC (400 x 40 mm column, Merck LiChroprep Si 60, 25-40 µm, UV-detection, 254 nm) to yield 104 mg impure decursin. Final purification of decursin was achieved by prep TLC (Merck, Si gel 60, 0.5 mm; dichloromethane : diethyl ether = 75 : 25) to give 78 mg of purified decursin.

Structure elucidation of the compounds

The structures of the active compounds have been identified using NMR and mass spectral data. The melting point of the compounds was determined on a Buchi-540 melting point apparatus. Optical rotations were measured on a Perkin-Elmer 241 polarimeter, IR spectra on a Perkin-Elmer 1 spectrometer, ¹H and ¹³C NMR spectra on a Bruker DRX 500 spectrometer, and EI-MS and FAB-MS respectively on a Hewlett-Packard 5989 B and a Finnigan/Thermo Quest Mat 95 XL mass spectrometer.

Antagonism and antibacterial activity assay

An *in vitro* plate assay technique was used to test the inhibitory effects of *Streptomyces* sp. GMT-8 on the tested bacteria as described in the previous report (Taechowisan *et al.*, 2008). For screening of antibacterial activity of the endophytic actinomycetes, we used the solid media bioassay test against *Staphylococcus aureus* ATCC25932, *Bacillus cereus* ATCC7064, *Bacillus subtilis* ATCC6633, *Escherichia coli* ATCC10536 and *Pseudomonas aeruginosa* ATCC27853, the bacteria was cultured in ISP-2 broth at 37°C for 24 h; the cells were diluted to 10⁵ cells/ml in soft agar and then were overlaid on pre-grown (7-day-old culture at 30°C) colony of endophytic actinomycetes on ISP-2 plates. For antibacterial activity assay, the crude extract and purified compound were tested for antibiosis against the tested bacteria using the paper disk method (Taechowisan *et al.*, 2005). Two pieces of 8-mm sterile paper disks (Advantec, Toyo Roshi Kaisha, LTD., Japan) were respectively soaked in crude extract (5 mg/disc) and purified compound (50 µg/disc). The air-dried discs were placed on ISP-2 plates. Each plate was then overlaid with top agar containing 10⁵ cells/ml of bacteria test strains. The plates

were incubated at 37°C for 24 h. The width of inhibition zones was measured. Each treatment consisted of three replicates. The experiment was repeated twice.

Minimum inhibitory concentrations (MICs)

MICs of crude extract and purified decursin were determined by NCCLS microbroth dilution methods (National Committee for Clinical Laboratory Standards, 1997). The crude extract and purified decursin were dissolved in DMSO. A dilution suspension of bacteria was inoculated into each well of a 96-well microplate, each containing a different concentration of the test agents. We performed doubling dilutions of the test agents. The range of sample dilutions was 512 to 1 µg/ml in nutrient broth supplement with 10% glucose (NBG) and a final concentration of test agent that inhibited bacterial growth, as indicated by the absence of turbidity. Test agent-free broth containing 5% DMSO was incubated as growth control. Minimum bactericidal concentration (MBC) was determined by inoculating on to nutrient agar plates, a 10 µl of medium from each of the well from the MIC test which showed no turbidity. MBCs were defined as the lowest concentration of test agent where was no microbial growth on the plates.

RESULTS AND DISCUSSION

Identification of microorganism

An endophyte designated actinomycete GMT-8 was isolated from the root tissue of *Zingiber officinale* Rosc. This strain was of great interest, because of its potent antibacterial activity. Morphological observation of 7-day-old cultures of GMT-8 grown on ISP-2 medium revealed that sporophores to be monopodially branched, producing open spirals of oval-shaped spores (1x1.5 µm) with smooth surfaces (Figure 1).

The substrate mycelium was extensively branched with non-fragmenting hyphae. The aerial mycelium was orange changing to brown with yellow soluble pigment occasionally discernible. Based on results in morphological observation as well as on the presence of LL-type diaminopimelic acid in the whole-cell extracts, endophytic actinomycetes GMT-8 was identified as belonging to the genus *Streptomyces*. Almost the complete 16S rDNA sequence was determined for the endophytic *Streptomyces* sp. GMT-8 from position 25 to position 1470. BLAST search results for strain GMT-8 came from GenBank; when reference sequences were chosen.

The BLAST search results and the phylogenetic tree generated from representative strains of the related genera showed that strain GMT-8 had high levels of sequence similarity to species of *Streptomyces nodosus* ATCC 14899 (accession number: AF114035) (Figure 3). 16S rDNA analysis revealed that strain GMT-8 is phylogenetically closely related to *Streptomyces nodosus* (the sequence similarity levels were 98%). The nucleotide sequence data reported in this paper appeared in the GenBank, EMBL and DDBJ databases with accession number AB845421.

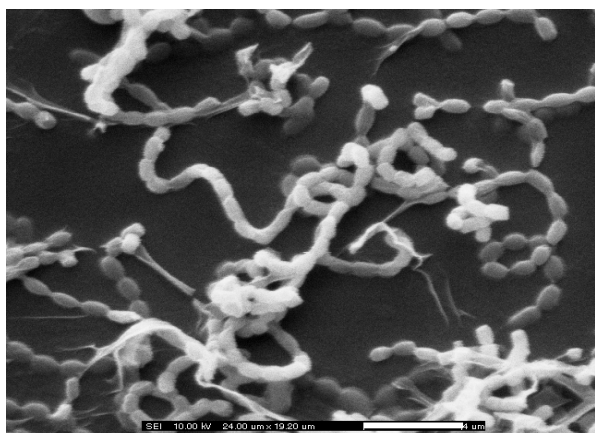


Fig. 1: Spores and hyphae of *Streptomyces* sp. GMT-8. BAR = 4 μ m.

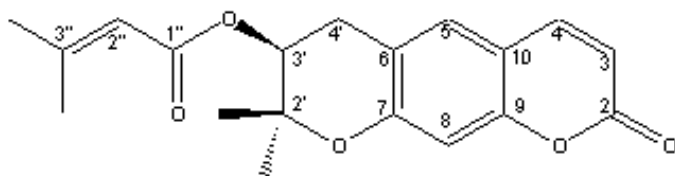


Fig. 2 Chemical structure of decursin.

Isolation and structure elucidation of the compound

The purified compound was isolated from the crude extract by column chromatography and TLC technique. This compound was identified by comparison with previous reports as decursin (Hata and Sano, 1966, Ryu *et al.*, 1990). Properties of purified decursin: white crystal from MeOH; m.p. 111-112 °C; $[\alpha]_D^{15}$: 172.9° (CHCl₃); UV λ_{max} (MeOH)(nm): 220, 328; IR ν_{max} (KBr)(cm⁻¹): 2960 (CH), 1725 (C=O), 1630, 1653, 1495 (aromatic ring), 1390, 1380 ((CH₃)₂C=); MS: *m/e* (%) = 328 (M⁺, 0.7), 228 (21.4), 213 (100.0), 83 (24.7), 55 (20.1); ¹H-NMR (300 MHz, CDCl₃): δ (ppm) = 1.36 (3H, s, Me-2''), 1.39 (3H, s, Me-2'), 1.88 (3H, d, *J* = 1.2 Hz, Me-4''), 2.15 (3H, d, *J* = 1.2 Hz, Me-3''), 2.87 (2H, dd, *J* = 17.6, 4.8 Hz, H-4'), 3.20 (2H, dd, *J* = 17.6, 4.8 Hz, H-4''), 5.09 (1H, t, *J* = 4.8 Hz, H-3'), 5.67 (1H, m, H-2''), 6.23 (1H, d, *J* = 9.5 Hz, H-3), 6.80 (1H, s, H-5), 7.16 (1H, s, H-8), 7.59 (1H, d, *J* = 9.5 Hz, H-4); ¹³C-NMR (75.5 MHz, CDCl₃): δ (ppm) = 20.3 (3''-Me), 23.2 (2'-Me), 25.0 (2'-Me), 27.5 (C-4''), 27.6 (C-2''), 69.0 (C-4'), 76.6 (C-3'); 104.6 (C-8), 112.8 (C-10), 113.2 (C-3), 115.5 (C-2''), 115.9 (C-6), 128.7 (C-5), 143.2 (C-4), 154.1 (C-9), 156.4 (C-7), 158.5 (C-3''), 161.3 (C-2), 165.7 (C-1'').

Antibacterial activity of the crude extract and purified compound

Results of the antibacterial screening indicated that, the crude extract showed strong activity against Gram positive bacteria at the amount of 5 mg/disc, while decursin exhibited moderate activity against Gram positive bacteria at the amount of 50 μ g/disc. At these amount of substances, crude extract showed weak activity against Gram negative bacteria, while decursin showed no activity (Table 1).

The crude extract and purified decursin showed both antibacterial activity at tested MIC and MBC limit of 512 μ g/ml (Table 2). The MIC values obtained with the crude extract varied from 32-128 μ g/ml for Gram positive bacteria and 512 μ g/ml for Gram negative bacteria. For decursin isolated from the crude extract, the MIC values also varied from 32-256 μ g/ml for Gram positive bacteria and >512 μ g/ml for Gram negative bacteria. Regarding the degree of activity of decursin, the lowest MIC value (32 μ g/ml) was noted on *Staphylococcus aureus* ATCC25932. The results of the MBC determinations indicated that the MBC values obtained with the crude extract and decursin varied from 128->512 μ g/ml on Gram positive bacteria. The results of the MBC determinations indicated that cidal effect of the tested sample could be expected. However, a keen look of the results of MIC and MBC, showed that the MIC values obtained are two-three times lesser than MBCs on corresponding microorganisms, confirming the microbicidal effects of the concerned samples (Taechowisan *et al.*, 2008). Previous reports indicated that decursin was a pyranocoumarin isolated from the root of *Angelica gigas* Nakai (Umbelliferae) (Konoshima *et al.*, 1968). It has been reported to have variable pharmacological activities, such as neuroprotection (Kang and Kim, 2007), anticancer activities (Ahn *et al.*, 1996, Lee *et al.*, 2003a, Jiang *et al.* 2007) and anti-inflammatory properties (Kim *et al.*, 2006). In the course of our research regarding antimicrobial agents from actinomycetes, we found decursin from *Streptomyces* sp. GMT-8 to be a potent antibacterial activity against Gram positive bacteria with MIC values in the range 32-256 μ g/ml, according to the previous report by Lee *et al.* (2003b), that decursin isolated from *Angelica gigas* roots exhibited significant antibacterial activity against *Bacillus subtilis* with MIC of 12.5 μ g/ml. Our data added decursin had been isolated from endophytic *Streptomyces* sp. GMT-8 and their antimicrobial activity was observed. These results indicated that some endophytic actinomycetes were potent for investigation of antimicrobial agents.

Table 1: Antimicrobial activity of crude extract and purified decursin from *Streptomyces* sp. GMT-8.

Test agents	Potential of growth inhibition on tested bacteria				
	S.a. ^a	B.c.	B.s.	E.c.	P.a.
Crude extract	4+ ^b	3+	4+	1+	1+
Decursin	3+	2+	3+	NA ^c	NA

The potential of antibacterial activity was evaluated by the zone of growth inhibition on ISP-2 medium after incubation at 37°C for 24 h. ^aS.a.; *Staphylococcus aureus* ATCC25932, B.c.; *Bacillus cereus* ATCC7064, B.s.; *Bacillus subtilis* ATCC6633, E.c.; *Escherichia coli* ATCC10536, P.a.; *Pseudomonas aeruginosa* ATCC27853. ^b4+: Width of growth inhibition zone > 20 mm, 3+: >10-20 mm, 2+: >1-10 mm, 1+: \leq 1 mm. ^cNA: Not active.

Table 2: Minimum inhibitory concentration of the crude extract and purified compounds against tested bacteria.

Test agents	MIC (μ g/ml)					MBC (μ g/ml)		
	S.a. ^a	B.c.	B.s.	E.c.	P.a.	S.a.	B.c.	B.s.
Crude extract	32	128	32	512	512	128	512	256
Decursin	32	256	64	>512	>512	128	>512	256

^aS.a.; *Staphylococcus aureus* ATCC25932, B.c.; *Bacillus cereus* ATCC7064, B.s.; *Bacillus subtilis* ATCC6633, E.c.; *Escherichia coli* ATCC10536, P.a.; *Pseudomonas aeruginosa* ATCC27853.

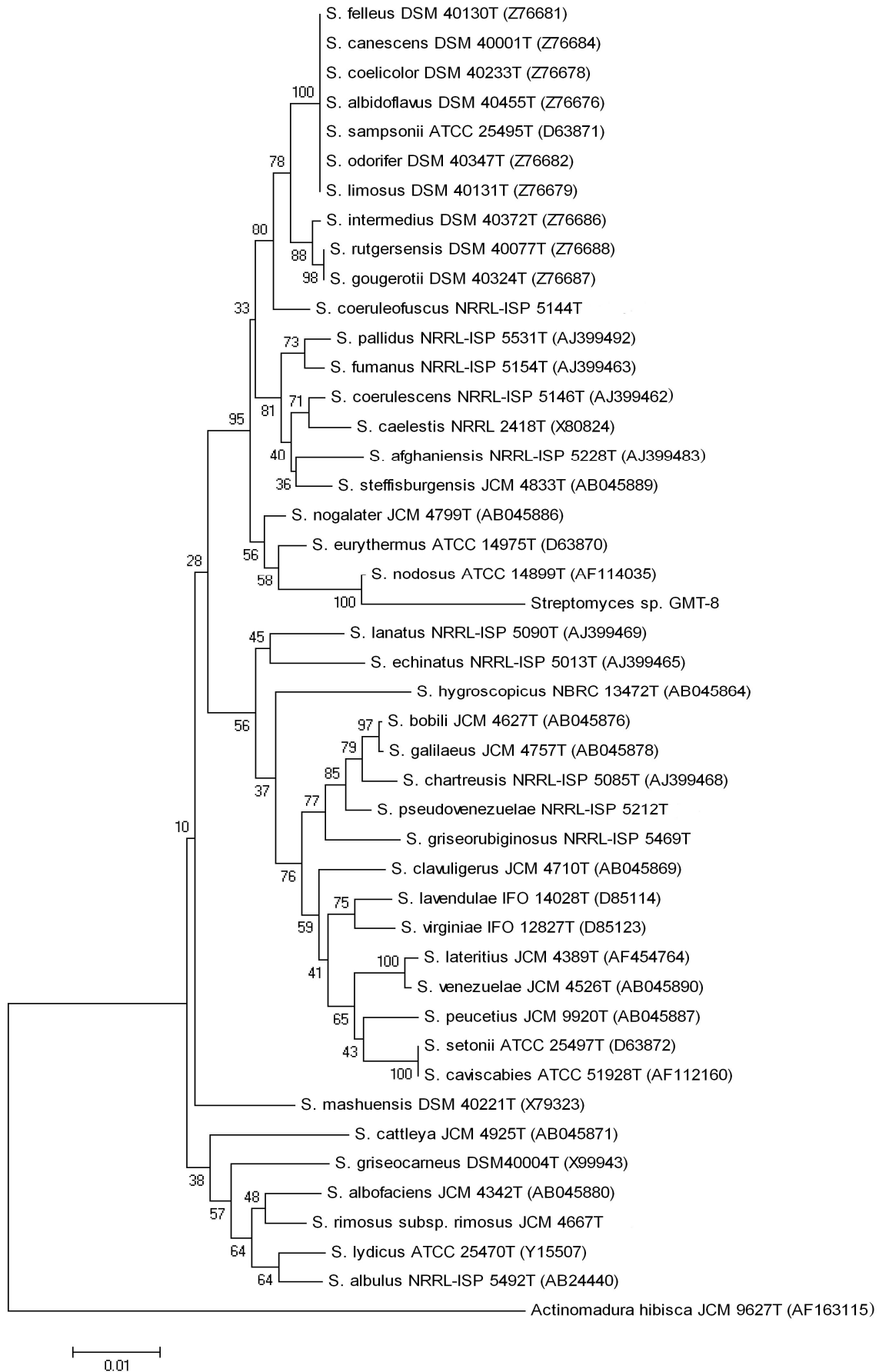


Fig. 3: Neighbor-joining phylogenetic tree of *Streptomyces* sp. strain GMT-8, including representatives of the most closely-related type strains which were retrieved from GenBank, and accession numbers appear in parentheses. Bootstrap (1,000 replicates) values are given in percentage. Bar, 0.01 substitutions per nucleotide.

ACKNOWLEDGEMENT

This work was supported by Thailand Research Fund (Research grant No. RMU5480005) and Faculty of Science, Silpakorn University, Thailand.

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How to cite this article:

Thongchai Taechowisan, Srisakul Chanaphat, Wanwikar Ruensamran, and Waya S. Phutdhawong. Antibacterial activity of Decursin from *Streptomyces* sp. GMT-8; an endophyte in *Zingiber officinale* Rosc. *J App Pharm Sci*, 2013; 3 (10): 074-078.