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## Antifungal activity of 3-methylcarbazoles from *Streptomyces* sp. LJK109; an endophyte in *Alpinia galanga*

Thongchai Taechowisan, Srisakul Chanaphat, Wanwikar Ruensamran, and Waya S. Phutdhawong

**Thongchai Taechowisan, Srisakul Chanaphat**  
Department of Microbiology,  
Faculty of Science, Silpakorn  
University, Nakorn Pathom 73000,  
Thailand.

**Wanwikar Ruensamran,  
Waya S. Phutdhawong**  
Department of Chemistry,  
Faculty of Science, Silpakorn  
University, Nakorn Pathom 73000,  
Thailand.

**For Correspondence**  
**Thongchai Taechowisan**  
Department of Microbiology,  
Faculty of Science, Silpakorn  
University, Nakorn Pathom 73000,  
Thailand.  
Tel: 66-34-273045,  
Fax: 66-34 255820

### ABSTRACT

Strain LJK109 was isolated from the root tissues of *Alpinia galanga* (L.) Willd. and identified as *Streptomyces* sp. on the basis of morphology, chemotaxonomy and 16SrDNA sequencing. It was an antagonist of phytopathogenic fungi; *Alternaria porri*, *Colletotrichum gloeosporioides*, *Colletotrichum musae*, *Curvularia* sp., *Drechslera* sp., *Exserohilum* sp., *Fusarium oxysporum*, *Verticillium* sp. and *Sclerotium rolfsii*. The culture filtrate and the crude extract from *Streptomyces* sp. LJK109 were all inhibitory to tested phytopathogenic fungi. The major active ingredients from the crude extract were purified by silica gel column chromatography, thin-layer chromatography and identified to be 3-methylcarbazole (compound 1) and 1-methoxy-3-methylcarbazole (compound 2) by NMR and mass spectral data, respectively. Bioassay studies showed that compound 1 and 2 had antifungal activities against tested fungi, and its minimum inhibitory concentrations were found to be within the range of 30 to 240 µg/ml. Conidia germination was also inhibited by these compounds as determined by microscopy.

**Keywords:** alkaloids, antifungal activity, methylcarbazoles, *Streptomyces* sp.

### 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). In a separate study, the strain identified as *Streptomyces aureofaciens* CMUAc130 showed the most effective antifungal activity. The major active ingredients from the culture filtrate were identified as 5,7-dimethoxy-4-p-methoxyphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin (Taechowisan *et al.*, 2005). We report here the isolation from the roots of *Alpinia galanga* (L.) Willd. of another *Streptomyces* sp. LJK109. Extraction of the culture medium of *Streptomyces* sp. LJK109 afforded 3-methylcarbazoles, which displayed strong antifungal activity.

## MATERIALS AND METHODS

### Organisms and media

*Streptomyces* sp. LJK109 was isolated from the root tissues of *Alpinia galanga* (L.) Willd. by the surface-sterilization technique (Taechowisan *et al.*, 2003). Identification of the isolate to species level 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 liquid medium used for fermentation was ISP-2 (Shirling and Gottlieb, 1966). The phytopathogenic fungi *Alternaria porri*, *Colletotrichum gloeosporioides*, *Colletotrichum musae*, *Curvularia* sp., *Drechslera* sp., *Exserohilum* sp., *Fusarium oxysporum*, *Verticillium* sp. and *Sclerotium rolfsii* (provided by Dr. Pongrawee Nimnoi, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand) were used for antifungal assay. They were grown on potato dextrose agar (PDA).

### Preparation of the crude extract

A spore suspension of *Streptomyces* sp. LJK109 was prepared in distilled water from cultures grown on ISP-4 medium at 30 °C for 10 days. The suspension, 10<sup>8</sup> 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 LJK109 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 mycelial mats were washed with distilled water and separated by centrifugation at 5000 rpm for 20 min. The culture filtrate and mycelial mats of the strain LJK109 were 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 523 mg.

### Purification of the compounds

The residue of 785 mg was dissolved in 10 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 (205 mg) were further separated by MPLC (400 x 40 mm column, Merck LiChroprep Si 60, 25-40 µm, UV-detection, 254 nm) to yield 92 mg impure 3-methylcarbazoles. Final purification of 3-methylcarbazoles was achieved by prep TLC (Merck, Si gel 60, 0.5 mm; dichloromethane : diethyl ether = 85 : 15) to give 38 mg and 22 mg of pure compounds **1** and **2**, respectively.

### 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, <sup>1</sup>H- and <sup>13</sup>C-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 antifungal activity assay

An *in vitro* plate assay technique was used to test the inhibitory effects of *Streptomyces* sp. LJK109 on the phytopathogenic fungi as described in the previous report (Taechowisan *et al.*, 2005). For antifungal activity assay, the culture filtrate, crude extract, or purified compounds were tested for antibiosis against the phytopathogenic fungi 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 culture filtrate, crude extract and each of two purified compounds (50 µg/disc). The air-dried discs were placed on PDA plates. Each plate was then inoculated with an agar block (8 mm diameter) containing mycelial mats of the fungi in the centre of the plate. The paper disks were 2.2 cm from the fungi. Inhibition percentage was obtained 4 days after treatment at 30 °C from the equation as follows:

$$\text{Inhibition (\%)} = \frac{[(\text{growth diameter in untreated control} - \text{growth diameter in treatment}) \times 100]}{\text{growth diameter in untreated control}}$$

Each treatment consisted of three replicates. The experiment was repeated twice.

### Minimum inhibitory concentration

The purified compound **1** and **2** were assayed on PDA in Petri dishes to determine the minimum inhibitory concentration (MIC) of this compound against phytopathogenic fungi. The purified compound was dissolved in DMSO, then serially diluted to obtain final concentration 480, 240, 120, 60, 30 and 15 µg/ml in PDA. An 8 mm diameter plug of the fungi, removed from the margin of a 5 day-old colony on PDA, was placed on the PDA containing purified compounds. The plates were incubated at 30 °C for 5 days. The MIC was the concentration of the compound inhibited the mycelium growth. Each treatment consisted of three replicates.

### Fungal spore germination assays

A stock suspension of *Colletotrichum gloeosporioides* (the most sensitive fungi in MIC assay), 10<sup>6</sup> conidia/ml of the fungus in 20% glycerol was prepared and kept at -20 °C. Equal volumes (400 µl) of spore suspension, 3X potato dextrose broth and the purified compound (1440, 720, 360, 180 and 90 µg/ml in 10% (v/v) DMSO) were mixed in sterile microcentrifuge tube. The test solutions were replaced with sterile water in control samples. Tubes were incubated at 30 °C for 24 h. A drop of the mixture from each was placed on microscope slide and the percentage of conidial germination was determined from the first 100 spores chosen at random. Data were transformed into values representing the treatment as a percentage of the control (in which % inhibition of control = 0) by the following equation:

Inhibition (%) = [1- (the percentage of spores germinating in the treatment / the percentage of spores germinating in the control)] x100

The results of each experiment are reported as the average of three replicates.

## RESULTS

Results of the dual cultures showed that *Streptomyces* sp. LJK109 was inhibitory to the growth of phytopathogenic fungi; *Alternaria porri*, *Colletotrichum gloeosporioides*, *Colletotrichum musae*, *Curvularia* sp., *Drechslera* sp., *Exserohilum* sp., *Fusarium oxysporum*, *Verticillium* sp. and *Sclerotium rolfsii*. The inhibition zone of these tested fungi was over 18 mm when they were dually cultured with *Streptomyces* sp. LJK109 for 4 days at 30 °C on PDA. Both ethyl acetate extracts of liquid filtrate and mycelial mats showed antifungal activity.

Compound **1**, identified by NMR and mass spectral data as 3-methylcarbazole (C<sub>13</sub>H<sub>11</sub>N), was a colorless prisms having: mp 207-208 °C (from acetone). UV λ<sub>max</sub>nm: 216 sh, 230 sh, 236, 246 sh, 260, 290 sh, 296, 328, 342. IR ν<sub>max</sub> cm<sup>-1</sup>: 3380, 1600, 1490, 1450. MS m/z: 181 (M<sup>+</sup>, 100%), 180, 152. <sup>1</sup>H-NMR (acetone-d<sub>6</sub>) Δ: 2.45 (3H, s, aryl CH<sub>3</sub>), 6.96-7.50 (5H, m, 1-, 2-, 6-, 7-, 8-H), 7.82 (1H, s, 4-H), 7.99 (1H, d, J=8 Hz, 5-H), 10.10 (1H, br s, NH). Compound **2**, identified by NMR and mass spectral data as 1-methoxy-3-methylcarbazole (C<sub>14</sub>H<sub>13</sub>NO), was a colorless plates having: mp 53-55 °C (from hexane). Picrate: brown needles, mp 189-191 °C (from benzene). UV λ<sub>max</sub>nm (log ε): 225 (4.47), 243 (4.58), 251 sh (4.44), 283 sh (3.83), 292 (4.01), 330 (3.53), 344inf (3.49). IR ν<sub>max</sub> cm<sup>-1</sup>: 3480, 1640, 1610, 1590, 1505. MS m/z : 211 (M<sup>+</sup>, 100%), 196 (89), 180 (10), 168 (54), 167 (33). <sup>13</sup>C-NMR δ: 145.2 (s), 139.5 (s), 129.2 (s), 128.0 (s), 125.4 (d), 124.3 (s), 120.3 (d), 119.1 (d), 112.5 (d), 110.9 (d), 107.7 (d), 55.1 (q), 21.8 (q). As the compound **1** and **2** were identified as 3-methylcarbazole (**1**) and 1-methoxy-3-methylcarbazole (**2**), respectively. Their <sup>1</sup>H- and <sup>13</sup>C-NMR spectral data were identical with those of 3-methylcarbazoles previously reported (Chakraborty *et al.*, 1968; Furukawa *et al.*, 1985; Chakraborty and Batabyal, 1992). These compounds also showed growth inhibition of all tested fungi at 50 μg/disc using the paper disk method, the results were presented in Table 1.

The results of MIC determination showed that the crude extract, compound **1** and **2** were able to prevent the growth of the nine tested fungi. The lowest MIC value of 480 μg/ml for the crude extract was recorded on *C. musae* and *F. oxysporum* (Table 2). The MIC of the compound **1** against *C. gloeosporioides* was 30 μg/ml, it was the most potent MIC in this experiment. The spore germination inhibition of *C. gloeosporioides* by the crude extract and purified compounds was reduced when increasing the concentration of the crude extract and purified compounds. The percentage of spore germination inhibition was observed 100% in a final concentration of 480 μg/ml of compound **1** and **2** in all tested fungi (Figure 2). In contrast, a high degree of germination occurred in both experiments when spores were mixed with either distilled water or with 10% (v/v) DMSO. The presence of solvent in the crude extract sample did not have any negative effect on spore germination when compared with distilled water.

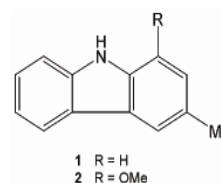


Fig. 1: Chemical structures of 3-methylcarbazole (**1**), and 1-methoxy-3-methylcarbazole (**2**).

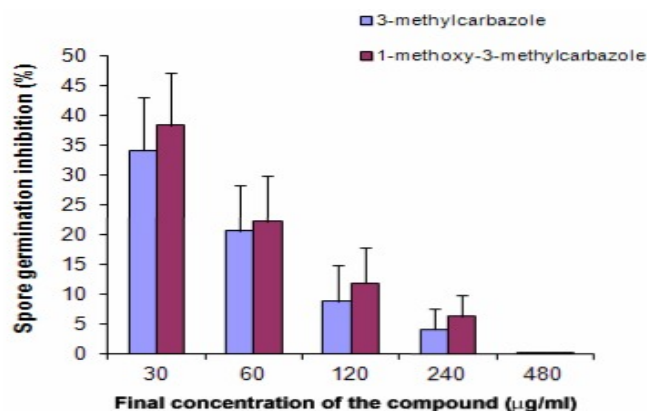


Fig. 2: Spore germination inhibition of tested fungi by the crude extract and purified compounds against tested fungi. Apo.; *Alternaria porri*, Cgl.; *Colletotrichum gloeosporioides*, Cmu.; *Colletotrichum musae*, Cur.; *Curvularia* sp., Dre.; *Drechslera* sp., Exs.; *Exserohilum* sp., Fox.; *Fusarium oxysporum*, Ver.; *Verticillium* sp. and Sro.; *Sclerotium rolfsii*.

Table 1: Growth inhibition of tested fungi by the crude extract and purified compounds.

| Test agents   | Growth inhibition of tested fungi (%) <sup>a</sup> |      |      |      |      |      |      |      |      |
|---------------|--|------|------|------|------|------|------|------|------|
|               | Apo.   | Cgl. | Cmu  | Cur. | Dre. | Exs. | Fox. | Ver. | Sro. |
| Crude extract | 64.2   | 52.0 | 72.1 | 68.0 | 59.4 | 51.4 | 76.5 | 57.3 | 62.4 |
| Com-pound 1   | 20.5   | 16.4 | 35.6 | 28.9 | 27.2 | 22.9 | 40.0 | 30.6 | 28.6 |
| Com-pound 2   | 24.1   | 20.2 | 33.8 | 31.2 | 36.1 | 34.3 | 36.2 | 29.4 | 26.7 |

<sup>a</sup> Growth inhibition was carried out by using the paper disk method at 50 μg/disc.

Apo.; *Alternaria porri*, Cgl.; *Colletotrichum gloeosporioides*, Cmu.; *Colletotrichum musae*, Cur.; *Curvularia* sp., Dre.; *Drechslera* sp., Exs.; *Exserohilum* sp., Fox.; *Fusarium oxysporum*, Ver.; *Verticillium* sp. and Sro.; *Sclerotium rolfsii*.

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

| Test agents   | MIC against tested fungi (μg/ml) |      |      |      |      |      |      |      |      |
|---------------|----------------------------------|------|------|------|------|------|------|------|------|
|               | Apo.                             | Cgl. | Cmu. | Cur. | Dre. | Exs. | Fox. | Ver. | Sro. |
| Crude extract | 120                              | 120  | >480 | 120  | 240  | 480  | >480 | 480  | 240  |
| Compound 1    | 60                               | 30   | 120  | 60   | 60   | 120  | 240  | 60   | 120  |
| Compound 2    | 60                               | 60   | 120  | 60   | 60   | 120  | 240  | 60   | 60   |

Apo.; *Alternaria porri*, Cgl.; *Colletotrichum gloeosporioides*, Cmu.; *Colletotrichum musae*, Cur.; *Curvularia* sp., Dre.; *Drechslera* sp., Exs.; *Exserohilum* sp., Fox.; *Fusarium oxysporum*, Ver.; *Verticillium* sp. and Sro.; *Sclerotium rolfsii*.

## DISCUSSION

3-methylcarbazoles and their carbazole alkaloids constitute an important group of natural products that are of particular interest owing to their wide ranging biological activities for example; scavenge reactive oxygen species-free radicals, such as hydroxyl radicals, superoxide radicals, or hypochlorous acid, and to influence processes involving free-radical injury (Tembhurne and Sakarkar, 2010; Pandey *et al.*, 2010), they have also been found to inhibit lipid peroxidation and to possess vasorelaxant (Rawat and Wulff, 2004; Sathaye *et al.*, 2011; Ahmad *et al.*, 2010) and anti-inflammatory/antioxidant activity (Ramsewak *et al.*, 1999). Moreover they have been reported for their other pharmacological activities such as anticancer (Muthumani *et al.*, 2009), anti-diarrhoeal (Mandal *et al.*, 2010), anti-diabetic (Prasad *et al.*, 2009), anti-asthmatic (Parmar *et al.*, 2010), antiplasmodial (Bringmann *et al.*, 1998), antibacterial, antifungal and anthelmintic activities (Khuntia and Panda, 2011). Previous reports indicated that 3-methylcarbazole was produced by numerous species of plants including *Murraya euchrestifolia* (Furukawa *et al.*, 1985), *Murraya koenigii* (Chakrabarty *et al.*, 1997), *Clausena dunniana* (Cui *et al.*, 2002), *Micromelum hirsutum* (Ma *et al.*, 2005). The formation of 3-methylcarbazole alkaloids in plants restricted to the four Rutaceae genera *Clausena*, *Glycosmis*, *Micromelum*, and *Bergera* (formerly section Bergara of the genus *Murraya*) (Samuel *et al.*, 2001). In our study, 3-methylcarbazoles was obtained from the culture of *Streptomyces* sp. LJK109, isolated from the root tissue of *Alpinia galanga* (L.) Willd. It proved that 3-methylcarbazoles were the major antifungal component, as evidenced by the fact that dual culture, crude extracts, and purified 3-methylcarbazoles from *Streptomyces* sp. LJK109 were all inhibitory to phytopathogenic fungi. Since 3-methylcarbazoles had antifungal activities in inhibition of mycelium extension and conidia germination of phytopathogenic fungi, other human pathogenic fungi for example *Candida albicans* and *Cryptococcus neoformans* should be studied further.

## CONCLUSIONS

This work shows the antifungal activity of 3-methylcarbazoles isolated from the culture of *Streptomyces* sp. LJK109, an endophyte in *Alpinia galanga* (L.) Willd. Since 3-methylcarbazoles have antagonism against phytopathogenic fungi. They should be further studied in application of horticulture biocontrol and other biological activities of 3-methylcarbazoles should be studied as well.

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