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Isolation of Sangivamycin from *Streptomyces* sp. A6497 and its Herbicidal Activity

HWANG, EUI IL[1], BONG SIK YUN[2], SUNG WON CHOI[3], JIN SEOG KIM[4], SE JIN LIM[5], JAE SUN MOON[2], SANG HAN LEE[2], AND SUNG UK KIM[2]*

[1]*Bio Research Group, KT&G Central Research Institute, Yusung, Daejon 305-805, Korea*
[2]*Laboratory of Cellular Function Modulator, Korea Research Institute of Bioscience and Biotechnology, Yusung, Daejon 305-333, Korea*
[3]*Research Institute of Biotechnology, Green Biotech Co., Paju 413-830, Korea*
[4]*Biofunction Research Team, Korea Research Institute of Chemical Technology, P. O. Box 107, Yusung, Daejeon 305-600, Korea*
[5]*College of Pharmacy, Dongduk Women's University, Sungbuk, Seoul 136-714, Korea*

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Abstract During the screening for the inhibitors of cellulose biosynthesis as herbicides, we discovered a *Streptomyces* sp. A6497 with a selective antifungal activity against cellulose containing *Phytophthora parasitica*, but not against cellulose lacking *Candida albicans*. The inhibitor was isolated and identified, using a series of chromatographies. Based on structure analyses with UV spectrophotometry, mass and various NMR, the inhibitor was identified as sangivamycin. The compound exhibited strong antifungal activities against *P. parasitica* (MIC; 3.125 μg/ml). In particular, it showed strong herbicidal activities against various weeds in the greenhouse experiment. Taken together, these results suggest that sangivamycin is a useful lead compound for the development of new herbicides.

Key words: Selective screening, chemotaxonomy, sangivamycin, herbicidal activity

The discovery of new herbicidal substances with potent activity is useful to agriculture and horticulture, however, there is also a desire for safer agrochemicals with less environmental and mammalian toxicity. Microbial metabolites attract more attention as potential herbicides than synthetic chemicals, because they are biodegradable and possess a variety of structures and bioactivity. Thus, the search for new types of herbicides of microbial origin has a bright future and is progressing steadily.

The cell walls of fungi, algae, and plants are made of fibrillar polysaccharides embedded in a matrix of amorphous components, including polysaccharides, lipids, and proteins, that maintain the organization of the entire structure [6, 15]. The most characteristic fibrillar polysaccharides are cellulose in plants and chitin in fungi. With the exception of rare examples of bacteria such as *Acetobacter*, which can synthesize cellulose but do not incorporate into their cell walls, prokaryotes do not synthesize microfibrillar polysaccharides [15]. While cellulose is ubiquitous in plant and algae cell walls, cellulose biosynthesis does not occur in human and animal cells [6]. Therefore, cellulose provides a promising target site for novel herbicides that are safe and selective.

Phytophthora parasitica, a phytopathogenic fungus causing serious rot diseases and damaging economically important crops, is known to contain cellulose as one of the cell wall constituents [2], whereas *Candida albicans* lacks cellulose in their cell walls [3]. This difference has been exploited to screen inhibitors from microorganisms [11, 19], having herbicidal activity, and the active compound with a selective activity against *P. parasitica* and herbicidal activity against various weeds was isolated from *Streptomyces* sp. A6497. In this paper, we describe the isolation, structure determination, and herbicidal activity of the active compound.

The *in vitro* bioassay was performed by a slightly modified method reported previously [9, 11, 19]. *Phytophthora parasitica* and *Candida albicans* ATCC 10231 were used as indicator microbial strains. *P. parasitica* was grown at 25°C with shaking for 2–3 days in glucose-V8 medium [0.9% glucose, 14% (v/v) V8 supernatant, 0.2% agar, pH 6.5]. After incubation, the culture was broken by using a waring blender, and diluted 1:5 in glucose-V8 medium (0.75% agar) maintained at 50°C. Plates were prepared by using a base layer of glucose-V8 medium overlaid with inoculated seed medium in a ratio of 2:1 (v/v) and incubated for 2–3 days at 25°C. *C. albicans* was grown in Sabouraud dextrose broth (Difco Detroit, MI, U.S.A.) overnight at

*Corresponding author
Phone: 82-42-860-4554; Fax: 82-42-861-2675;
E-mail: kimsu@kribb.re.kr

30°C until $A_{550\,nm}$ reached to 1.5. An aliquot (5 ml) of the culture was added to 50 ml of overlay medium (0.75% agar in Sabouraud dextrose broth), and then the base medium (Sabouraud dextrose agar, Difco Co.) which was overlaid with the incubated overlay medium at a ratio of 2:1 (v/v) was incubated overnight at 30°C. To identify inhibitors of cellulose biosynthesis, the strains exhibiting growth inhibition against *P. parasitica*, but not against *C. albicans*, were selected.

The producing microorganism, a strain of *Streptomyces* sp. A6497, was isolated from soil collected on Oct. 28, 1997 at mountain Odae, Gangwon Province, Korea. This strain was grown on YM medium (0.4% yeast extract, 1.0% malt extract, 0.4% glucose, 2.0% agar, pH 7.2) and stored in 20% glycerol at 70°C.

One ml of spore suspension of strain A6497 in 20% glycerol was cultivated on YM medium at 25°C. Well-grown spores on YM medium were inoculated into a 500-ml baffled flask, containing fermentation medium (0.1% yeast extract, 2.0% starch, 0.4% soytone, 0.2% polypeptone, 0.3% phamamedia, 0.2% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% K_2HPO_4, 0.3% $CaCO_3$, 0.002% $FeSO_4$, 0.001% $MnCl_2$, 0.001% $ZnSO_4$, 0.0005% $CoCl_2$), and the spores were incubated for 3 days at 26°C on a rotary shaker at 150 rpm (radius 7 cm). An aliquot (20 ml) of culture was transferred into a 5-l baffled flask, containing 1 l of the same medium. The fermentation was carried out for 4 days at 26°C on a rotary shaker at 150 rpm.

The structure analyses were performed by using ESI-MS (Hewlett Packard 5989A, U.S.A), NMR (Varian UNITY 300, U.S.A.), and UV (Shimazu UV265, Japan).

Minimum inhibitory concentrations (MICs) were determined by the two-fold serial agar dilution method [7]. The plant pathogens, including *Alternaria kikuchiana*, *Botrytis cinerea*, *Colletotrichum lagenarium*, *Fusarium oxysporum*, *Magnaporthe grisea*, and *Rhizoctonia solani*, were grown on potato dextrose agar (Difco. Co.) medium. *Phytophthora capsici* and *P. parasitica* were grown on the glucose-V8 agar medium. All plant pathogens were incubated for 7 days at 25°C. Antifungal activities were observed after 2–5 days of incubation at 25°C.

The post-emergence herbicidal activity of bioactive compound, sangivamycin, was determined in the greenhouse. The plastic pots (350 cm^2) stuffed with soil were planted with seeds of plants, including *Sorghum bicolor* Moench, *Echinochloa crusgalli* P. Beauv, *Agropyron smithii* Rydb., *Digitaria sanguinalis* (L.) Scop., *Panicum dichotomiflorum* Michx. *Solanum nigrum* L., *Abutilon avicennae* Gaertn, *Xanthium strumarium* L., and *Calystegia japonica* Choisy, and covered with soil to a depth of 5 or 10 mm. The pots were placed in a greenhouse for 10 days to allow the plant to grow. Sample solutions were directly sprayed over the leaves and stems of the plants. Herbicidal effect was examined on the 14th day after application of the sample and assigned the grade of effectiveness in a manner as follows: The score is 0 when there is no damage of the stems or leaves, while 100 is given for a complete kill in comparison with that of untreated control. For overall herbicidal potency of the test compound, the score was determined at four concentrations of 23.3, 70, 210, and 630 g ha^{-1}.

To identify the genus of the actinomycete strain A6497, the cultural and morphological characteristics and chemotaxonomy were performed by the methods of International Streptomyces Project (ISP) and *Bergey's Manual of Systematic Bacteriology* [20]. The color of the aerial mycelium was white to grayish yellow green, while the reverse side color was moderate yellow to pale yellow, depending on the medium used. The spore surface of the isolate strain was shown to be smooth, and the long chain spore was connected as retinaculum-apertum. No soluble pigment was produced in the media. The type of diaminopimelic (DAP) acid in the cell wall of this strain was found to be a LL-type, and the strain did not contain mannose, ribose, xylose, galactose, or arabinose in the cell wall. From these results, this strain was identified as the genus *Streptomyces* and designated as *Streptomyces* sp. A6497.

The fermentation broth (10 l) of *Streptomyces* sp. A6497 was extracted twice with 10 liters each of EtOAc and partitioned between EtOAc and H_2O. The EtOAc layer (4.7 g) was evaporated and applied to a silica gel column (Merck, Kieselgel 60, 230–400 mesh), and the column was eluted with a gradient of $CHCl_3$-MeOH to give active fractions (MeOH fraction). The active fractions were combined and concentrated *in vacuo*. The residue on an ODS column (Merck, Lichroprep RP-18, 40–63 μm) was isolated by eluting with a gradient of MeOH-H_2O to yield active fractions (50 to 60% MeOH fractions), and the active fraction was then subjected to Sephadex LH-20 (Sigma, Lipophilic LH-20, 25–100 μm) column with MeOH. To further isolate the active fraction, it was subjected to ODS Sep-Pak column chromatography with a gradient of

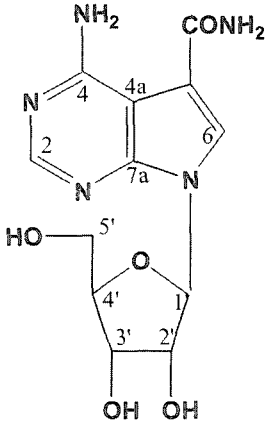

Fig. 1. Structure of sangivamycin isolated from *Streptomyces* sp. A6497.

Table 1. The antifungal activities of sangivamycin isolated from *Streptomyces* sp. A6497 against various phytopathogenic fungi.[a]

Phytopathogenic fungi	MICs (μg/ml)
Alternaria kikuchiana	100
Botrytis cinerea	100
Colletotrichum lagenarium	100
Fusarium oxysporum	>100
Magnaporthe grisea	>100
Phytophthora capsici	50
Phytophthora parasitica	3.125
Rhizoctonia solani	50

[a]Each experiment was repeated three times with essentially the same results.

Table 2. Morphological characteristics and chemotaxonomy of the isolate A6497.

Characteristics	A6497
Morphology	
Spore chain	Retinaculum-apertum
Spore size	0.8-1.0×1.0-1.6 μm
Spore surface	Smooth
Chemotaxonomy	
Cell wall composition	LL-DAP[a]
Phospholipid fatty acids	Anteiso-$C_{15:0}$(28.36%), iso-$C_{16:0}$(19.79%) Iso-$C_{15:0}$(16.62%), Anteiso-$C_{17:0}$(11.74%)
Whole cell sugars	-[b]

[a]DAP: Diaminopimelic acid.
[b]Xylose, arabinose, galactose, ribose, mannose, or rhamnose were not detected.

CH_3CN-H_2O to give a crude compound (10% CH_3CN). Finally, the crude compound was further purified by precipitation with 50% MeOH at 4°C. The active fraction was filtered to give a white powder of pure active compound (6 mg).

The bioactive compound was obtained as white powder with an R_f value of 0.46 in 50% MeOH on a Merck C_{18} plate. The UV absorption spectrum (λmax) showed maxima at 230 and 280 nm in MeOH. The molecular formula was determined to be $C_{12}H_{15}N_5O_5$ on the basis of ESI mass spectrum (*m/z* 309.8 $[M+H]^+$) in combination with ^1H-, ^{13}C-NMR, and DEPT spectral data. In addition, the melting point and optical rotation of the compound were determined as 260° and $[\alpha]_D^{25}$; -46.0 (*c* 1.0, 0.1 N HCl), respectively. Therefore, this compound was determined to be sangivamycin (Fig. 1), a pyrrolopyrimidine nucleoside, and the spectral data were in good agreement with those previously published for sangivamycin [12, 16].

In bioassay, sangivamycin isolated from *Streptomyces* sp. A6497 selectively inhibited the growth of *P. parasitica* at 100 μg $disk^{-1}$ (inhibition zone; >35 mm), whereas it exhibited no inhibitory activity against *C. albicans* (data not shown). Sangivamycin showed antifungal activities against cellulose containing *P. parasitica* and *P. capsici* with MICs of 3.125 and 50 μg/ml, respectively. Except for the case of *Rhizoctonia solani*, the compound was inactive or weakly active against phytopathogenic fungi which contain no cellulose, such as *A. kikuchiana*, *B. cinerea*, *C. lagenarium*, *F. oxysporum*, and *M. grisea* (Table 1).

Sangivamycin belongs to a group of pyrrolopyrimidine nucleosides which includes toyocamycin and tubercidin. In earlier reports [14, 17, 18], the action mechanism of sangivamycin was considered to be identical with that of toyocamycin and tubercidin, which have the common nucleoside skeleton, 7-deazaadenosine. However, toyocamycin, possessing the CN instead of $CONH_2$ of sangivamycin, inhibited the germination of various plant seeds and the growth of *C. albicans* [8, 16], whereas tubercidin [1], which possesses the H at the C-5 position of sangivamycin, inhibited both *P. parasitica* and *C. albicans* at 100 μg $disk^{-1}$ (data not shown). On the other hand, sangivamycin selectively inhibited *P. parasitica* and exhibited a strong antifungal activity against *P. parasitica* (MIC; 3.125 μg/ml). Thus, the antifungal activity of sangivamycin was found to be distinct from those of tubercidin and toyocamycin. These

Table 3. Effect of sangivamycin on the herbicidal activities against several weeds at foliar treatment.

Days	Rate (g ha^{-1})	Grasses				Broadleaves					
		SOR	ECH	AGR	DIG	PAN	SOL	AES	ABU	XAN	CAL
2	23.3	0	0	0	0	0	0	0	0	0	0
	70	10	0	0	20	20	0	20	0	20	15
	210	40	70	15	70	60	30	100	10	60	30
	630	70	80	60	90	60	60	100	10	60	90
6	23.3	0	0	0	0	0	0	0	0	0	0
	70	20	20	0	20	30	0	20	0	10	0
	210	80	100	25	85	60	85	100	20	90	60
	630	100	100	90	100	90	100	100	70	100	100
11	23.3	0	0	0	0	0	0	0	0	0	0
	70	0	0	0	0	15	0	10	0	0	0
	210	60	100	20	70	50	65	100	20	90	60
	630	100	100	60	100	90	100	100	80	100	100

The experiment was designed to examine post-emergence herbicidal activity of sangivamycin. Herbicidal activity was assigned on a 0 to 100 scale, where 0 represents 0% damage and 100% a complete kill. Test plants were SOR, *Sorghum bicolor* Moench; ECH, *Echinochloa crusgalli* P. Beauv; AGR, *Agropyron smithii* Rydb.; DIG, *Digitaria sanguinalis* (L.) Scop.; PAN, *Panicum dichotomiflorum* Michx.; SOL, *Solanum nigrum* L.; AES, *Aeschynomene indica* L.; ABU, *Abutilon avicennae* Gaertn; XAN, *Xanthium strumarium* L.; and CAL, *Calystegia japonica* Choisy.

results led us to suggest that the selective anti-*P. parasitica* activities of pyrrolopyrimidine nucleosides are dependent on the position of the carbonyl group.

The *in vivo* herbicidal activity of sangivamycin against various weeds was examined by using the two classes of weeds; grasses and broadleaves. Sangivamycin caused 100% damage only against *Echinochloa crusgalli* P. Beauv and *Aeschynomene indica* L. within 6 days after treatment with the compound at the rate of 210 g ha^{-1}, whereas almost all the weeds used were completely killed within 6 days at the rate of 610 g ha^{-1}. The visual observation revealed that the necrosis and a burning effect on the leaves were much more severe in broadleaves than in grasses.

Several cellulose biosynthesis inhibitors have previously been reported [4, 5, 10, 11, 19], however, only a few, including phthoxazolin A [11, 19] and phthoramyzin [10], appear to be inhibitors of cellulose biosynthesis of microbial origin. Sangivamycin has a structure that is entirely different from that of other cellulose biosynthesis inhibitors which have so far been reported.

Considering the fact that sangivamycin has diverse biological activities, such as antiviral [14], antitumor [12], and antimicrobial activities [13], this compound may have multiple target sites. The mode of action for the herbicidal activity of this compound, including the inhibition of cellulose biosynthesis, remains to be investigated. Although this compound shows weaker herbicidal activity than organic synthetic herbicides, it may serve as a useful lead compound for the development of new herbicides.

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