PHTHOXAZOLIN A, A SPECIFIC INHIBITOR OF CELLULOSE BIOSYNTHESIS FROM MICROBIAL ORIGIN

I. DISCOVERY, TAXONOMY OF PRODUCING MICROORGANISM, FERMENTATION, AND BIOLOGICAL ACTIVITY

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A new inhibitor of cellulose biosynthesis named phthoxazolin A was discovered as a metabolite of *Streptomyces* sp. OM-5714. A newly established screening method, which utilized a cellulose-containing fungus, *Phytophthora* sp. as a test organism, was successfully empolyed for discovery of the compound. Phthoxazolin A, $C_{16}H_{22}N_2O_3$ (MW of 290), is a lipophilic triene compound with an oxazole moiety. It has moderate antifungal activity against *Phytophthora* spp. only and has potent herbicidal activity. Phosphate-limited fermentation conditions favored production of the active compound.

Herbicides are useful in agriculture and horticulture. Herbicides today are required to have potent activity and be safe to animals, humans and to ecosystems. Microbial metabolites attract attention as potential herbicides, because they are biodegradable, cause less pollution, and are diverse in variety of structure and bioactivity. Cellulose is contained in all the plant cell walls, but is not in human and animal cells. Therefore, cellulose biosynthesis provides a promising target site for safe herbicides and plant growth regulators.

We have developed a new method of screening for cellulose biosynthesis inhibitors from microorganisms. This method utilizes two microorganisms as test organism: *Phytophthora*, a plant-pathogenic fungus containing cellulose as one of the cell wall polysaccharide components,[1] and *Candida* containing no cellulose in the cells. Our screening efforts using this method led to the discovery of a new compound, named phthoxazolin A (Fig. 1).

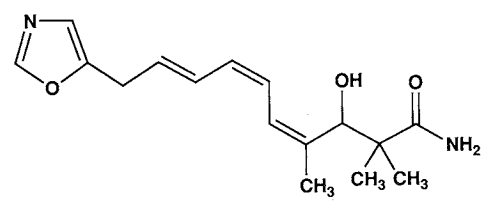
This paper describes the taxonomy of the producing microorganism, fermentation, and biological activity of phthoxazolin A. A preliminary account of this compound has appeared,[2] in which this compound was called phthoxazolin. It is now renamed phthoxazolin A, because new phthoxazolin homologs were recently discovered (K. SHIOMI, N. ARAI, H. YOSHIDA, Y. TANAKA and S. ŌMURA, in preparation).

Materials and Methods

Microorganisms

Strain OM-5714 was isolated from a soil sample collected at Fukuyama-city, Hiroshima Prefecture, Japan. This original isolate and strain OM-5714-S-3 were used. Strain OM-5714-S-3 was obtained from the original strain by the conventional single colony

Fig. 1. Structure of phthoxazolin A.



isolation technique, and produces a higher phthoxazolin A titer than strain OM-5714 does. Test organisms used in discovery screens were: *Phytophthora parasitica* subsp. *nicotianae* IFO 4783, grown on V8 agar[3] at 27°C; *Candida albicans* KF-1, grown on glucose-yeast extract agar (pH 6.0) at 27°C; and *Acetobactor aceti* subsp. *xylinum* IFO 3288, grown on an ethanol-nutrient agar[4] (nutrient agar supplemented with filter-sterilized ethanol at 2% (v/v) at 27°C).

Taxonomic Studies

The type of diaminoplimlic acid (DAP) was determined by the method of TAKAHASHI *et al.*[5] Cultural and physiological characteristics were studied using International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB,[6] and those by WAKSMAN.[7] Observations were done after two weeks of incubation at 27°C. Color names and hue numbers indicated in Table 1 are those of Color Harmony Manual (4th ed.).[8] The carbon source utilization was examined by growth at 27°C in Pridham and Gottlieb medium containing 1% of individual carbon sources.

Fermentation

Strain OM-5714-S-3 was used. The seed medium consisted of glucose 0.1%, starch 2.4%, peptone 0.3%, meat extract 0.3% yeast extract 0.5%, $CaCO_3$ 0.4%, pH 7.0 before autoclaving. The inoculum for fermentation was prepared by growth strain OM-5714-S-3 at 27°C for two days in 500-ml conical flasks containing 100 ml of seed medium. Vegetative growth of the seed culture was transferred, at 2% (v/v), to 500 ml conical flasks containing 100 ml of a production medium. The production medium consisted of soluble starch 2%, glycerol 0.5%, wheat germ 1%, meat extract 0.3%, dry yeast 0.3%, $CaCO_3$ 0.4%, allophane (Shinagawa Chemicals Co., Tokyo), 0.5%, presterile pH of 7.2. The culture was grown at 27°C for 4 days at 27°C on a rotary shaker (110 rpm).

For larger scale fermentations, a 100-liter fermentor was charged with 70 liters of production medium followed by a supplement of 0.03% (v/v) adecanol LG-109 (Asahi Denka, Tokyo). After steam-sterilization at 121°C for 30 minutes, a vegetative seed culture (2%, v/v) was transferred. The fermentation was run at 27°C for 4 days with an agitation of 200 rpm, and aeration of 0.5 v/v/m. Other production media employed are described in Table 4.

Antimicrobial Activity

The antimicrobial spectrum of phthoxazolin A shown in Table 5 was determined by the conventional two-fold serial agar dilution method. The test organisms, growth media, and incubation conditions employed are described in Table 5.

Herbicidal Activity

An aliquot (0.5 ml) of a test sample was dropped onto a defatted cotton piece (1 cm × 1 cm), dried overnight at room temperature. The cotton piece was put into the bottom of a test tube (2 cm × 10 cm), into which 0.5 ml of water was added. Five seeds of radish (*Phaphanus sativus* L.) were placed onto the wet cotton, and incubated at 27°C for 3 to 4 days under lightening. The hight of plant growth was scored against no durg control.

Other Analyses

Microbial growth was estimated as volume (ml) of packed mycelial mass obtained after centrifugation (3,000 rpm, 10 minutes) of 10 ml of cultured broths. Antibiotic activity was monitored microbiologically by anti-*Phytophthora* activity with *P. parasitica* IFO 4783 as test organism and with V8 agar[3] for its growth at 27°C. Scanning electron micrographs were obtained with a Hitachi electron microscope (model S-430).

Results and Discussion

Discovery

The phthoxazolin A-producing culture OM-5714 was chosen from about 25,000 soil isolates for its selective growth inhibition against *P. parasitica* and no growth inhibition against *C. albicans* and *Bacillus*

subtilis. This was followed by the observation of its herbicidal activity. Concentrates of the ethyl acetate extract of a whole cultured broth of strain OM-5714 inhibited the incorporation of ^{14}C-labeled glucose into an alkali-insoluble cellulose fraction of *A. aceti* subsp. *xylinum*, an acetic acid bacterium known to produce cellulose extracellularly.[9] The rationale and details of this screening system will be described in a forthcoming paper.

Taxonomy of the Producing Strain OM-5714

Vegetative mycelia grow abundantly on both chemically defined and complex agar media, and do not show fragmentation into coccoid or bacillary elements. The aerial mycelia grow abundantly on most of the agar media tested. The matured sporophores formed spiral spore chains, which contained more than 20 spores per chain. The spores were cylindrical in shape, $1.1 \times 0.8\ \mu m$ in size, and had a smooth to somewhat wrinkled surface (Fig. 2). No screlotic granules, sporangia nor flagellated spores were observed.

Fig. 2. Scanning electron micrograph of spore chains of strain OM-5714 grown on yeast extract - malt extract agar for 14 days.

Bar represents $1.0\ \mu m$.

Table 1. Cultural characteristics of strain OM-5714.

Medium	Growth	Reverse	Aerial mycelium	Soluble pigment
Yeast extract - malt extract agar[a]	Good, melon yellow (3ga)	Amber (3pe)	Good, pussywillow gray (5dc)	None
Oatmeal agar[a]	Good, light cherry red (7la)	Cherry rose (7ia)	Good, silver gray (3fe)	None
Inorganic salts - starch agar[a]	Good, light coral red (6la)	Light coral rose (6ga)	Good, silver gray (3fe)	None
Glycerol - asparagine agar[a]	Good, bamboo-shrimp pink (2fb~6½ga)	Bamboo-dusty cedar (2fb~6½ie)	Good, natural-white (3dc~a)	None
Glucose - asparagine agar[b]	Good, bisque (3ec)	Bamboo (2gc)	Good, silver gray (3fe)	None
Peptone - yeast extract - iron agar[a]	Good, shrimp pink (6½ia)	Amber (3pc)	Good, natural-white (3dc~a)	None
Tyrosine agar[a]	Good, brite red (7½pa)	Wine (7½pg)	Good, silver gray (3fe)	None
Sucrose - nitrate agar[b]	Good, biscuit (2ec)	Biscuit (2ec)	Moderate Covert tan (2ge)	None
Glucose - nitrate agar[b]	Poor, bamboo (2gc)	Bamboo (2gc)	None	None
Glycerol - calcium malate agar[b]	Good, light ivory (2ca)	Light ivory (2ca)	Poor, bamboo (2fb)	None
Glucose - peptone agar[b]	Good, bright orange (4na)	Amber (3pc)	Good, natural-white (2dc~a)	None
Nutrient agar[b]	Good, light lacquer red (6nc)	Orange (4la)	Good, covert gray-white (2fe~a)	None

[a] Medium recommended by International Streptomyces Project.[6]
[b] Medium recommended by S. A. WAKSMAN.[7]

Table 2. Physiological properties of strain OM-5714.

Melanin formation	−
Tyrosine reaction	−
H_2S production	−
Nitrate reduction	+
Liquefaction of gelatin	−
Peptonization of milk	+
Coagulation of milk	−
Cellulolytic activity	−
Hydrolysis of starch	+
Temperature range for growth	15~38°C

+: Active.
−: Not active.

Table 3. Utilization of carbon sources by strain OM-5714.

D-Glucose	++
D-Fructose	++
L-Rahmnose	++
D-Mannitol	++
L-Arabinose	++
i-Inositol	+
Raffinose	+
D-Xylose	++
Sucrose	+
Melibiose	++

+: Weakly utilized.
++: Utilized.

The DAP in cell walls of strain OM-5714 was determined to be LL-type. The cultural and physiological characteristics and the utilization of carbon sources are shown in Tables 1, 2, and 3, respectively.

Based on the taxonomic properties described above, strain OM-5714 is considered to belong to the genus *Streptomyces*. This strain has been deposited in Fermentation Research Institute, Agency of Industrial Sciences and Technology, Japan, under the accession No. FERM P-10775.

Fermentation

The antifungal activity of phthoxazolin A was first detected in the culture grown in an allophane-supplemented production medium (medium C in Table 4). Allophane is a non-chrystalline clay, which was reported to act as a phosphate ion-trapping agent in other fermentation systems, thereby increasing antibiotic titers with concomitant decreases in phosphate ion concentration in the medium.[10] Thus, allophane, added to a fermentation medium, increases antibiotic titers, when the biosynthesis of an antibiotic is susceptible to inorganic phosphate ions. In order to examine if this occurred in phthoxazolin A production, potassium phosphate (0.2%) was added to a production medium, and strain OM-5714-S-3 was grown as described. Phthoxazolin A production was almost nil in this medium, indicating that phthoxazolin A biosynthesis was inhibited by a high concentration of phosphate ions. Thirteen kinds of complex media varying in contents of organic nutrients, such as yeast extract, peptone, and glycerol, starch *etc.*, were used for phthoxazolin A production, into which allophane (0.5%) was added, and phthoxazolin A titers were compared with those in non-supplemented media. Table 4 shows that phthoxazolin A was produced in three of the 13 media when they contained allophane. The rest 10 media did not supported phthoxazolin A production even when allophane was supplemented. It is obvious that the addition of allophane to production media is essential for phthoxazolin A production by OM-5714-S-3. This is an another example showing that phosphate ion-trapping agents (allophane, magnesium carbonate *etc.*) and ammonium ion-trapping agents (zeolite, magnesium phosphate, *etc.*) are

Table 4. Phthoxazolin A production in media with an without added allophane.

Production medium	Phthoxazolin A (μg/ml)	
	None	+Allophane (0.5%)
A	<5	95
B	<5	79
C	<5	90

Medium A: Soluble starch 2.0%, glycerol 0.5%, wheat germ 1.0%, meat extract 0.3%, dry yeast 0.3%, $CaCO_3$ 0.3%, pH 7.5.

Medium B: Lactose 1.0%, glycerol 0.5%, pectin 0.5%, NZ-amine 0.5%, dry yeast 1.0%, $CaCO_3$ 0.3%, pH 6.7.

Medium C: Starch 2.4%, glucose 0.1%, peptone 0.3%, meat extract 0.3%, yeast extract 0.5%, $CaCO_3$ 0.4%, trace salt solution 0.5% (v/v), pH 7.0. Trace salt solution contained (each at 1 g/liter): $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and $CoCl_2 \cdot 6H_2O$, pH 6.7.

Fig. 3. A typical time-course of phthoxazolin A production by *Streptomyces* sp. OM-5714 in the presence of allophane, a phosphate ion-trapping agent.

●, Phthoxazolin A (μg/ml); ▲, packed mycelial volume (ml/10 ml of cultured broth); ○, pH.

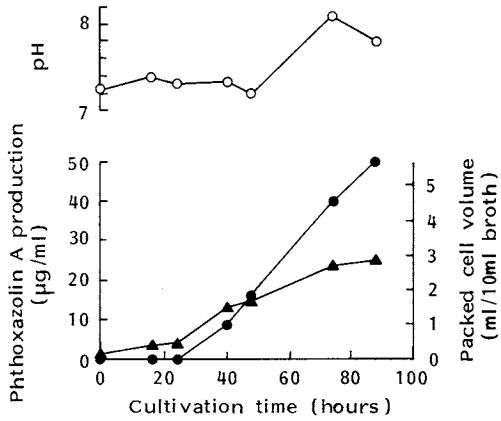


Fig. 4. Herbicidal activity of phthoxazolin A against radish seedlings.

Radish seeds were grown in small test tubes (2 cm × 10 cm) at 27°C for 4 days under lightening in the presence of indicated amounts of phthoxazolin A (μg/tube).

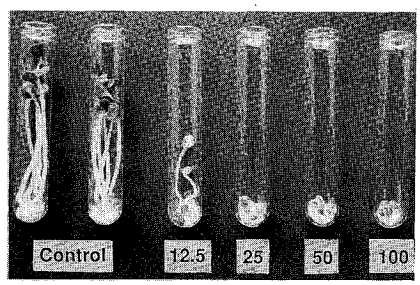


Table 5. Antimicrobial spectrum of phthoxazolin A.

Test organism	Medium	MIC (μg/ml)
Staphylococcus aureus FDA 209	A	>200
Micrococcus luteus ATCC 9341	A	>200
Bacillus subtilis PCI 219	A	>200
Escherichia coli NIHJ	A	>200
Xanthomonas oryzae KB-88	A	>200
Pseudomonas aeruginosa P-3	A	>200
Acetobacter aceti subsp. *xylinum* IFO 3288	B	>200
Phytophthora parasitica IFO 4873	C	125
P. capsici KF-278	C	31.3
Aspergillus niger ATCC 6275	D	>200
Pyricularia oryzae KF-180	D	>200
Mucor racemosus IFO-4581	D	>200
Candida albicans KF-1	D	>200
Saccharomyces sake KF-26	D	>200
Trichophyton interdigitale KF-62	D	>200
Microsporum gypseum KF-65	D	>200
Penicillium chrysogenum KF-97	D	>200
Fusarium oxysporum lycopersici KF-166	D	>200
Botrytis cinerea KF-184	D	>200

A: Sensitivity disc agar (Nissui), 37°C, 24 hours.
B: Composition: glucose 2.5%, yeast extract 0.5%, peptone 0.3%, agar 15% (pH 4), 27°C, 96 hours.
C: V8 glucose agar (pH 7)[3], 27°C, 72 hours.
D: Potato glucose agar (pH 6), 27°C, 72 hours.

useful in fermentation for new drug discovery.[11] Fig. 3 shows a typical time-course of phthoxazolin A production in a 50-liter fermentor in the presence of allophane.

Phthoxazolin A was isolated from an allophane-supplemented culture by solvent extraction and column chromatography. The analyses of the physico-chemical and spectroscopic properties of a purified preparation revealed the structure of phthoxazolin A, as shown in Fig. 1. Details of isolation and structure determination are described in an accompanying paper.[12]

Biological Activity

The minimum inhibitory concentrations (MICs) of phthoxazolin A against bacterial and fungal strains are shown in Table 5. Phthoxazolin A is moderately active only against cellulose-containing *Phytophthora* spp., the test organism used in the screening steps. The compound was inactive against all the fungal strains tested which contain no cellulose in the cells, such as *Aspergillus niger*, *Pyricularia oryzae*, *Mucor racemosus*, and *Saccharomyces cerevisieae*, nor bacteria including *Staphylococcus*, *Bacillus*, and *Escherichia*. This is in good agreement with the preliminary results that phthoxazolin A inhibits cellulose biosynthesis in growing culture of *A. aceti* subsp. *xylinum* (data not shown).

No mice died after oral administration of phthoxazolin A at 100 mg/kg.

A loboratory test was undertaken for herbicidal activity of phthoxazolin A with radish seedlings as test plant grown in small test tubes. Phthoxazolin A inhibited the growth of radish seedlings (Fig. 4). It is noteworthy that the inhibitory effect of phthoxazolin A was evident for plant growth, and not for the germination of radish seeds. The ID_{90}, the amount of phthoxazolin A added in a test tube that causes 90% reduction in the hight of radish growth, was around 25 μg/test tube under the assay conditions employed.

The results presented in this paper show that phthoxazolin A is a new inhibitor of cellulose biosynthesis, isolated from a microorganism for the first time. It provides a novel class of non-selective herbicides.

As suggested by many experiences, the discovery of new compounds is often favored by three factors: sensitive and selective assay methods, effective fermentation techniques, and unique microorganisms. It is obvious that the discovery of phthoxazolin A was realized by the combination of a newly established screening method and phosphate ion-depressed fermentation.

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