Communications to the Editor

PHTHOXAZOLIN, A SPECIFIC INHIBITOR OF CELLULOSE BIOSYNTHESIS, PRODUCED BY A STRAIN OF *STREPTOMYCES* SP.

Sir:

Cellulose is ubiquitous among plant and algae cell walls.[1,2] Because cellulose biosynthesis is not involved in human and animal cells, it provides a promising target site for safe and non-selective herbicides of a novel class. Inhibitors of cellulose biosynthesis of microbial origin have not been documented, although a recent article[3] from our laboratories suggested phthoramycin as a non-specific inhibitor of polysaccharide biogenesis, probably acting on cellulose synthesis as well. We described here the discovery of a specific inhibitor of cellulose biosynthesis produced by *Streptomyces* sp. OM-5714.

Screening for inhibitors of cellulose biosynthesis was based on the selective antimicrobial activity of actinomycete cultures against *Phytophthora parasitica*, a phytopathogenic fungus known to contain cellulose as one of the cell wall constituents,[4] but no activity against common fungi such as *Candida albicans* and *Piricularia oryzae*, which do not contain cellulose in their cells. Among *ca.* twenty thousand soil isolates, strain OM-5714, for which the genus *Streptomyces* was assigned by the taxonomical studies reported elsewhere, was chosen as the best candidate producer. This strain was thought to produce a cellulose biosynthesis inhibitor, because the culture, when grown in suitable nutrient-rich but phosphate-limited media, exhibited selective antifungal activity against *P. parasitica*, as well as herbicidal activity against radish seedlings, and inhibited the incorporation of [^{14}C]glucose into the alkali-insoluble fraction of resting cells of *Acetobacter xylinum*, an acetic acid bacterium known to produce extracellular cellulose from glucose.[5]

Fermentation was carried out at 27°C for 4 days with agitation and aeration in a 50-liter fermenter containing 30 liters of production medium (soluble starch 2%, glycerol 0.5%, wheat germ 1%, meat extract 0.3%, dried yeast cells 0.3%, $CaCO_3$ 0.4%, and Allophane 0.5%, presterile pH of 7.2). The addition of Allophane, a non-crystalline aluminosilicic clay with phosphate-trapping activity,[6] to the medium was absolutely required for efficient production of the herbicide. Phthoxazolin production was monitored by its antifungal activity vs. *P. parasitica* KF-265 using a conventional paper-disc method.

The active principle accumulated both extra- and intracellularly was extracted with ethyl acetate (18 liters) from the whole broth (28 liters) of 4-day culture of strain OM-5714. After evaporation of the organic layer, the residual oily material (20 g) was applied on a SiO_2 column (330 ml). The active substance was eluted with mixtures of chloroform-methanol (100 : 1 ~ 10 : 1). Active fractions, pooled and concentrated, was chromatographed again on a SiO_2 column (140 ml) with mixtures of benzene-acetone (100 : 1 ~ 1 : 1) as elution solvents. The active fractions gave a brown oily powder (310 mg) after evaporation. A 150 mg portion of this powder was purified by HPLC (column: YMC D-ODS-5, 20 × 250 mm, Yamamura Chemicals, Kyoto; elution solvent: 30% aq acetonitrile) to give a pale yellow powder (17 mg) of pure phthoxazolin. The purified material gave a single peak on HPLC eluted with two solvent systems as detected by UV both at 210 and 275 nm, and a single spot on SiO_2 TLC developed with four solvent systems and visualized by iodine, H_2SO_4 and phosphomolybdate reagent.

The physico-chemical properties of phthoxazolin are as follows: $C_{16}H_{22}N_2O_3$, MW *m/z* 290.163 (Calcd: 290.163), mp 58 ~ 62°C, $[\alpha]_D^{18}$ +37.4° (*c* 1, CH_2Cl_2), UV absorption maximum (MeOH) at 275 nm (ε 7.0 × 10^3), the profile of which suggested a triene moiety in the structure. It was readily soluble in methanol, acetone, ethyl acetate, chloroform, but was sparingly soluble in water and *n*-hexane. The ^{13}C and ^{1}H NMR, and ^{13}C-^{1}H and ^{1}H-^{1}H COSY as well as long range ^{1}H-^{1}H COSY spectroscopies revealed the structure for phthoxazolin as depicted in Fig. 1. The name "phthoxazolin" was given after its oxazole moiety and the selective activity against *Phytophthora* spp. The

Fig. 1. Structure of phthoxazolin.

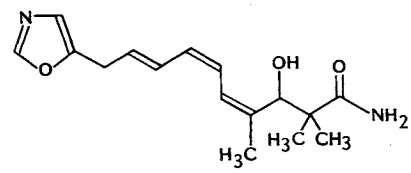

Table 1. Inhibitory activities of phthoxazolin and coumarin.

Compound	Amount	Anti-*Phytophthora* activity (inhibition zone, mm)[b]	Growth inhibition of radish seedlings (%)[c]	Inhibition (%) of cellulose biosynthesis in *Acetobacter xylinum*	
	μg/ml (mM)[a]			Resting cell system[d]	Cell-free system[e]
Phthoxazolin	100 (0.34)	31	100	47	69
	10 (0.03)	11	30	30	27
Coumarin	100 (0.68)	No activity	20	14	57
	10 (0.07)	No activity	No inhibition	<5	43

[a] To be read as μg per tube for herbicidal activity.

[b] *Phytophthora parasitica* was grown on glucose (1%)-supplemented V8 agar[12] at 27°C for 2 days. Paper discs of 8 mm i.d. were used.

[c] Five radish seeds were incubated at 27°C for 3 days under lightening on inhibitor-supplemented wet cotton in test tubes. Percent decrease in plant height against no drug control (3~5 cm, average 3.8 cm) is shown.

[d] *A. xylinum* cells (1 mg/ml in dry weight) were incubated with [^{14}C]glucose (0.2 μCi/ml) and indicated inhibitors in 1 ml in total of 50 mM phosphate buffer (pH 6.0) at 27°C for 1 hour. The cells were boiled in alkaline conditions as described.[5] Percent inhibition of formation of radioactive alkali-insoluble materials is shown.

[e] A cell-free extract from *A. xylinum* cells was prepared in 50 mM Tris-HCl (pH 7.5) containing 20% polyethylene glycol No. 6000. The cell free preparation (0.01 mg protein/ml) was incubated at 27°C with UDP-[^{14}C]glucose (1 μCi/ml), indicated inhibitors and GTP (0.25 μmol/ml) in 0.2 ml in total of 50 mM Tris-HCl (pH 8.5), as described.[8] Percent inhibition of formation of radioactive alkali-insoluble materials is shown.

structure determination will be reported elsewhere. None of known antibiotics share the physicochemical characteristics of phthoxazolin, and therefore this is a new antibiotic. Oxazolomycin, an antibacterial compound reported by MORI *et al.*[7] possesses the phthoxazolin moiety in the molecule.

Phthoxazolin showed selective antimicrobial activity against cellulose-containing *P. parasitica* and *P. cactorum* only moderately with MICs of 125 and 31.3 μg/ml, respectively. No growth inhibition was observed at 200 μg/ml of phthoxazolin against cellulose-noncontaining 22 strains of Gram-positive and Gram-negative bacteria such as *Bacillus*, *Staphylococcus*, *Escherichia*, *Pseudomonas*, *Acetobacter* spp. and yeasts and filamentous fungi including *Piricularia*, *Botrytis*, *Microsporum* and *Trichophyton* spp. Phthoxazolin showed herbicidal activity. It inhibited the growth of radish seedlings (*Rhaphanus sativus* L.) grown in laboratory test tubes under lightening with MIC of 25 μg/tube. The toxicity of phthoxazolin seems to be low. It showed no or marginal effect at 100 μg/ml on the growth of melanoma B-16 cells and Vero cells incubated at 37°C in EAGLE's MEM medium supplemented with 10% fetal bovine serum. No mice died after oral administration of phthoxazolin at 100 mg/kg.

The mode of action of phthoxazolin was studied. A microscopic observation revealed that phthoxazolin induced round-shaped morphology of *P. parasitica* at the mycelial tips, which resembled the spheroplast-like morphology of *P. oryzae* induced by the chitin synthesis inhibitors polyoxin and nikkomycin. With resting cells of *A. xylinum* phthoxazolin at 100 μg/ml inhibited the incorporation of [^{14}C]glucose into the cellulose fraction (Table 1). It also inhibited the GTP- and polyethylene glycol-stimulated cellulose synthesis from UDP-[^{14}C]glucose by a cell-free extract from *A. xylinum*, which was prepared according to the method of BENZIMAN *et al.*[8,9] (Table 1). A known cellulose synthesis inhibitor, coumarin, inhibited cell-free synthesis of cellulose at the same degree, but did to a lesser extent in resting cell system. Because the cellulose-synthesizing machinery of *A. xylinum* resembles that of plants,[10,11] the herbicidal effect of phthoxazolin is assumed to be due to inhibition of cellulose synthesis. All of these findings, together with the highly selective antimicrobial activity, suggest that phthoxazolin is a specific inhibitor of cellulose synthesis in bacterial, fungal and plant systems.

Phthoxazolin is expected to be useful as a new type of herbicide, plant growth regulator, and as a biochemical reagent for the studies on cellulose biosynthesis. The importance of methodological devices in assay and fermentation is also emphasized for screening of new bioactive microbial metabolites.

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