Screening Method for Cellulose Biosynthesis Inhibitors with Herbicidal Activity

YOSHITAKE TANAKA, MASAKO SUGOH, WEI JI,† JUN IWABUCHI,††
HIROSHI YOSHIDA and SATOSHI ŌMURA*

Research Center for Biological Function,
The Kitasato Institute,
Minato-ku, Tokyo 108, Japan

(Received for publication February 6, 1995)

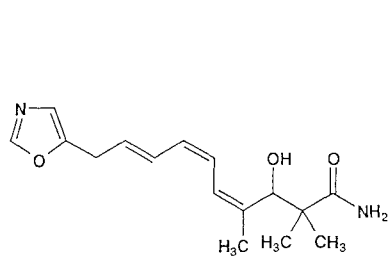
A new screening method for inhibitors of cellulose biosynthesis is described. This method utilized three microbial strains; a cellulose-containing fungus *Phytophthora*, and a cellulose non-containing fungus *Candida*, and a bacterial strain of *Acetobacter*, a cellulose-producing acetic acid bacterium. The primary screen examined microbial cultures for selective growth inhibition against *Phytophthora* with no inhibition against *Candida*. The secondary screen tested for herbicidal activity. Thirdly, the active cultures were examined for their inhibition of cellulose biosynthesis by an *Acetobacter* strain. A screening trial with this new method led to the discovery of two microbial metabolites named phthoxazolin A and phthoramycin as new inhibitors of cellulose biosynthesis with herbicidal activity.

Herbicides are useful in agriculture for crop production. Today they comprise the largest portion of agricultural pesticides. They are required to possess potent activity and safety to animals and humans as well as to the ecosystems.[1] Microbial metabolites are biodegraded in nature, giving less stress to the ecosystems, and are more likely to meet the above requirements than synthetic pesticides. Although many microbial metabolites show herbicidal activity, few of them, *e.g.* bialaphos, have found practical usefulness as herbicides, largely because of insufficient cost-effectiveness and of instability under agricultural weathering conditions.

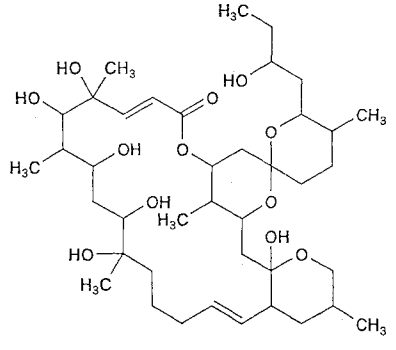
Cellulose is contained in the cell envelopes of all the plant and alga systems, but is not at all in any animal systems. Therefore cellulose biosynthesis provides a promissing target site for safe and non-selective herbicides. No reports have described trials of finding cellulose biosynthesis inhibitors from microbial metabolites, although coumarin[2] and synthetic chemicals 2,6-dichlorobenzonitrile[2] and isoxaben[3] have been suggested to have this type of activity *in vivo*.

The present paper describes an attempt at the construction of a screening method for inhibitors of cellulose biosynthesis of microbial origin. The discovery of two new compounds phthoxazolin A[4~6] and phthoramycin[7] (Fig. 1) by the use of this screening method will also be presented.

Fig. 1. Structure of phthoxazolin A and phthoramycin.



Phthoxazolin



Phthoramycin

Present address:
† Antibiotic Research Institute, North China Pharmaceuticals Co., Shijiazhuang, Hebei Prov., China.
†† Ageo Research Laboratory, Nippon Kayaku Co., Ageo-city, Saitama, Japan.

Materials and Methods

Microorganisms

Microorganisms used in the present work were: *Phytophthora parasitica* subsp. *nicotianae* IFO-4873, *P. palmivora* IFO-30285, *P. capsici* KF-278, *P. cactorum* KF-279, *Candida albicans* KF-1, and *Acetobacter aceti* subsp. *xylinum* IFO-3288. Other fungi described in Table 1 are laboratory strains of The Kitasato Institute.

Growth of Microorganisms

P. parasitica subsp. *nicotianae* IFO-4873 and other *Phytophthora* strains were grown at 27°C for two days with shaking (110 rpm) in a liquid V8 medium[8] (10 ml) which contained V8 vegetable juice (Suntory Co., Japan) 14% (v/v), glucose 1%, and agar 0.1% (pH 6.0). The vegetative cultures were poured into a teflon homogenizer. Mycelial pellets were homogenized softly, and a portion of the mycelial suspension was used to seed at 1% (v/v) to prepare V8 agar plates. Leucomycin (50 μml) was supplemented to V8 agar. The plates were incubated at 27°C for two days under lightening with a fluorescent lamp.

C. albicans KF-1 was grown in GY medium (glucose 1%, yeast extract 0.5%, pH 6.0) at 27°C for two days. This culture was used to seed into GY agar medium. Other fungi were grown in conventional manners.

Acetobacter aceti subsp. *xylinum* IFO-3288 was grown at 27°C on a Bouillon agar slant, and stored in the cold. This culture was used to inoculate into a 500-ml vol. conical flask containing 100 ml of a growth medium (Bouillon supplemented with sterile ethanol 2% and KH_2PO_4 0.3%), incubated statically at 27°C for 4 days. A portion (10 ml) of this vegetative seed culture was transferred to another conical flasks containing 100 ml of the same growth medium, and incubated statically at 27°C for 3 to 4 days. This culture was used for resting cell experiments to measure cellulose biosynthesis.

Determination of Cellulose Biosynthesis in Resting Cells of *A. aceti* subsp. *xylinum* IFO-3288

Cellulose biosynthesis was determined by measuring the incorporation of [^{14}C]glucose into the cellulose fraction of resting cells of strain IF0-3288. The method described by SWISSA *et al.*[9] was used with modifications as follows.

A. aceti subsp. *xylinum* IFO-3288 was grown at 27°C statically in a phosphate-buffered ethanol-Bouillon medium. Under these conditions, IFO-3288 grew to produce small pieces of cell pellets or pellicles like cotton wastes in 3~4 days of incubation. The cell pellets in 100 ml culture, obtained by centrifugation (4,000 rpm, 20 minutes), were washed twice with 0.05 M sodium phosphate buffer (pH 6.0) by centrifugation. The washed cell pellets were suspended in 20 ml of the same phosphate buffer, vortexed for 1 minute to crushed into fine pieces in a glass homogenizer. The cell suspension was filtered through a filter paper. The filtrate was adjusted to OD at 660 nm of 0.1~0.2, and used as the source of resting cells.

The reaction mixture contained in a total volume of 1.02 ml: *A. aceti* subsp. *xylinum* IFO-3288 cell suspension 0.9 ml, an inhibitor or an active culture at the concentrations of 10-fold strength (or water in a control run) 0.1 ml, and [^{14}C] glucose (10 μCi/ml) 0.02 ml.

The reaction was carried out at 27°C for an hour with shaking, stopped by adding 1 ml of 1 N NaOH solution, then heated for 20 minutes in a boiling water bath. Labeled cellulose that was synthesized was collected, together with disrupted cellular particles, on a glass filter (GF/C, pore size of 1.2 μm, Whatman). After washing with water, the filter was used to count for radioactivity.

Herbicidal Activity

Herbicidal effect was estimated by two methods. In one method the inhibitory effect on germination and proliferation was measured as follows. Cultured broths or solutions of known conpounds (each at 0.5 ml) were put dropwise onto pieces (1.0 cm × 1.0 cm) of defatted cotton. The cotton pieces were allowed to stand overnight at room temperature. The dried cotton pieces were then put into the bottom of test tubes (2 cm × 10 cm). Tap water (0.5 ml) was dripped onto the cotton pieces. Each five seeds of radish (*Raphanus sativus* L.) and sorghum (*Sorghum bicolor*) were layed on the wet cotton. The test tubes, the top of which was covered with a steel cap, were incubated for 4~5 days at 27°C under fluorescent lamps. Under these conditions both radish and sorghum in control tubes grew to 3~5 cm in height. Herbicidal activity was determined by comparison of plant heights with those of no drug control. Cycloheximide (10 μg/ml) was used as a positive control drug.

The second method estimated post-emergence effect. Active cultures were applied onto leaves of 4 day-grown plants by using a small brush. After a further several days of incubation, plant heights or damages on leaves were scored against no treatment control.

Results and Discussion

Growth of *Phytophthora* spp. and Anti-*Phytophthora* Activity of Known Antibiotics

Phytophthora spp. are plant-pathogenic fungi whose control is one of the most challenging problems in today's agriculture. This group of fungi is unique in that the cell walls contain cellulose as one of the essential polysaccharide components.[10] We reasoned that, by taking advantage of this physiology, it would be possible to detect inhibitors of cellulose biosynthesis by conventional growth inhibition assays with a *Phytophthora* sp. as an indicator strain.

Because no report has described the use of *Phytophthora* spp. in antibiotic screening, we started with

testing antibiotic sensitivity and the growth of *Phytophthora* spp. under laboratory conditions. *P. parasitica* subsp. *nicotianae* IFO-4873 and three other *Phytophthora* strains, *P. palmivora* IFO-30285 and *P. capscici* KF-278, and *P. cactorum* KF-279 were compared for their growth on V8 agar at 27°C. *P. parasitica* subsp. *nicotianae* IFO-4873 grew best and most steadily under the conditions employed. It grew better under lightening with fluorescent lamps than in the dark. V8 agar was a good growth medium for *P. parasitica* IFO-4873, although other complex media containing other types of vegetable juice than V8 juice, or a chemically defined medium composed of glucose, ammonium sulfate and mineral salts also supported growth of this fungus to lesser extents. Its growth increased gradually on successive transfers on the latter media.

A supplement of leucomycin (50 ~ 100 μg/ml) or other antibacterial agents to the V8 agar was effective to suppress occasional bacterial contamination, particularly in a screening run.

Anti-*Phytophthora* activity of known antibiotics shown in Table 1 was determined with *P. parasitica* subsp. *nicotianae* IFO-4873 grown on V8 agar. Table 1 also reports antifungal activities of the same antibiotics against *Candida*, *Saccharomyces*, *Mucor*, and *Pyricularia*. *P. parasitica* subsp. *nicotianae* IFO-4873 was sensitive to many antifungal agents. These include cycloheximide, antimycin, nanaomycin, rapamycin, but was insensitive to polyenes. This insensitiveness is because *Phytophthora* and other oomycetes group of fungi lack squalene epoxidase, and do not synthesize *de novo* cholesterol and ergosterol,[10] which are the target molecules of polyene antifungals.

Herbicidal Activity of Known Antibiotics against Radish

Table 1 shows the herbicidal activity of known antibiotics, mostly antifungals, as estimated by germination and proliferation method with radish (*Raphanus sativus*) as test plant. Among 30 antibiotics tested, several compounds showed herbicidal activity. The herbicidal activity did not correlate with antifungal potency.

Table 1. Antimicrobial, herbicidal, and cellulose synthesis-inhibitory activities of known antibiotics[a].

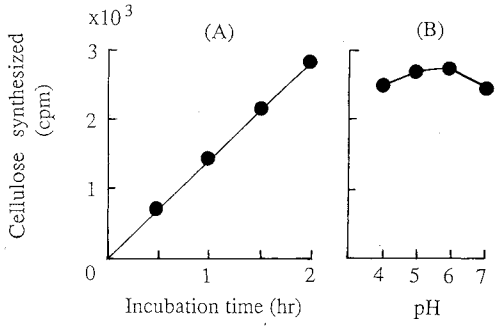
Structural group	Antibiotic	Conc. (μg/ml)	Antimicrobial activity (mm)				Herbicidal activity against radish[c]	Inhibition (%) of cellulose biosynthesis
			Phy[b]	Ca[b]	MU[b]	Py[b]		
Quinone	Antimycin	100	49	25	13	42	−	56
		10	41	22	10	36	−	48
	Nanaomycin A	100	40	17	25	42	+	42
		10	20	10	17	36	−	6
	Oligomycin	100	30	−	14	43		0
		10	18	−	10	34	−	0
	Ikutamycin	100	29	12	27	44	+	27
		10	20	10	18	33	−	0
	Kinamycin D	100	26	13	14	32	+	25
		10	17	−	−	19	−	6
Amino glycoside	Destomycin	100	−	−	10	−	++	0
		10	−	−	−	−	++	0
Polyether	Lysocellin	1000	20				ND	ND
Disulphide	Aspirochlorin	100	43	31	10	14	−	0
		10	32	22	−	−	−	0
	Gliotoxin	100	30	−	−	−	−	0
		10	21	−	−	−	−	0
Thricin	Racemomycin D	100	30	ND	ND	ND	++	0
		10	17	ND	ND	ND	+	0
Other	Rapamycin	100	45	28	28	55	+	6
		10	42	21	25	50	−	0
	Cycloheximide	100	32	−	17	20	++	3
		10	20	−	−	12	+	0
	Coumarin	100	19	−	−	−	++	23

[a] The following compounds (each at 1 mg/ml) were inactive against *Phytophthora parasitica* IFO-4873 on a paper dise assay: (Cell wall-actives) polyoxins, ezomycin, globopeptin, aculeacin A; (polyenes and polyols) amphotericin B, nystatin, eurocidin, fungichrome, copiamycin, azalomycin F; (polyethers) valinomycin, monensin A, dianemycin, (miscellaneous) cerulenin, pyrrolnitrin, siccanin, griseofulvin, irumamycin, and chatreusin.

[b] Phy, *Phytophthera parasitica*; Ca, *Candida albicans*; Mu, *Mucor racemosus*; Py, *Pyricularia oryzae*.

[c] −, 0 ~ 30% inhibition of plant growth ; +, 30 ~ 80% inhibition; ++, 80 ~ 100% inhibition.

Fig. 2. Effects of reaction period (A) and pH (B) on cellulose biosynthesis by resting cells of *Acetbacter aceti* subsp. *xylinum* IFO-3288.



Inhibition of Cellulose Biosynthesis by Known Antibiotics

Inhibition of cellulose biosynthesis by known antibiotics was determined by the method described in Materials and Methods, using the resting cells of *A. aceti* subsp. *xylinum* IFO-3288.

The incorporation of [^{14}C] glucose into the cellulose fraction of IFO-3288 proceeded linearly to the reaction period of time (Fig. 2-A) at its optimal pH of 6 (Fig. 2-B). The effect of known antibiotics on cellulose biosynthesis is shown in Table 1. None of the antibiotics tested, except for antimycin A, inhibited cellulose biosynthesis by more than 50% at 100 μg/ml.

Whereas respiratory inhibitors such as antimycin A inhibited it, possibly because ATP generation was inhibited by these antibiotics in *Acetobacter* cells. Noteworthy is that coumarin, which is postulated as a cellulose biosynthesis inhibitor in plant systems,[2] showed moderate inhibition in all the three assays; growth inhibition against *Phytophthora* and *Candida*, herbicidal activity, and cellulose biosynthesis inhibition. Unfortunately, 2,6-dichlorobenzonitrile and isoxaben, proposed cellulose biosynthesis inhibitors,[2,3] were not active in these assays. All the other compounds tested that are not proved to be cellulose biosynthesis inhibitors were inactive, or active either in one or two of the three assays. It is suggested that microbial cultures showing positive responses in all the three assays can be assumed to contain cellulose biosynthesis inhibitors.

Screening of Microbial Cultures in Search for Inhibitors of Cellulose Biosynthesis

A screening trial was carried out with the three screens established as above in which active microbial cultures were selected in the order and with criteria as follows:

1. Positive anti-*Phytophthora* activity but negative (or weak) anti-*Candida* activity with a difference of 10 mm or more in diameter of inhibition zones.

2. Herbicidal activity of more than 80% as determined by the reduction of plant heights in either one or both of radish and sorghum.

3. More than 10% of inhibition of cellulose biosynthesis by 10-fold concentrated culture extracts (final concentration in resting cell reaction mixture was the same as the cultured broth).

Table 2. Herbicidal activity and bacterial cellulose biosynthesis inhibitory activity of phthoxazolin A and phthoramycin.

Compound	Conc.[a] (μg/ml)	Inhibition (%) of cellulose biosynthesis	Growth inhibition (%) against	
			Radish	Rice plant
Phthoxazolin A	1000	83	100	ND[b]
	100	42	90	ND
Phthoramycin	1000	63	100	95
	100	23	90	85
Coumarin	100	19	30	ND

[a] Concentrations of the solutions used for assay are shown. A half ml of each solution was taken into test tubes for herbicidal assay (see Materials and Methods).
[b] ND; Not determined.

About 30,000 cultures of actinomycetes and fungi were subjected to this screening system. This trial led to the discovery of two new compounds, phthoxazolin A and phthoramycin (Fig. 1). Phthoxazolin A was produced by *Streptomyces* sp. OM-5714[4~6] and three other unidentified actinomycete strains, OM-5739, K93-615 and K93-5455. Phthoxazolin A was active *in vitro* against *Phytophthora* spp., but was inactive against other fungi and bacterea tested, none of which contain cellulose in the cells. Phthoramycin was produced by *Streptomyces* sp. WK-1875.[7] It showed *in vitro* antifungal activity against *Phytophthora*, as well as *Mucor* and *Pyricularia*.[7]

The two compounds inhibited cellulose biosynthesis in a resting cell system of *A. aceti* subsp. *xylinum* IFO-3288 (Table 2). They were potently herbicidal. These results demonstrate that the screening system described in this paper is useful in search for new cellulose biosynthesis inhibiters of both microbial and synthetic origins. This screening method is characterized by the use of microorganisms for herbicidal screening. This is the first example of the use of *Phytophthora* in antibiotic screening on a mass scale. Other fungi and bacteria containing or producing cellulose would also be utilized.

During the course of the above screening run, several known compounds were found to exhibit anti-*Phytophthora* activity with or without herbicidal activity. These include toyocamycin, sangivamycin, nocardamine, saigenmycin,[11] SS-8201D[12] and BQ-180B[13] as metabo-

lites of actinomycetes, and penicillic acid, fusaric acid, dehydrofusaric acid, patulin, gliotoxin, gliovirin and *N*-methyl-gliovirin as fungal metabolites. The anti-*Phytophthora* activity of these compounds were detected in the present study for the first time. Their herbicidal activity has been described by other research groups, except for nocardamine, SS-8201D and BQ-180B, which were not herbicidal.

Among these, saigenmycin,[11] a β-lactone bonded to phthoxazolin moiety *via* an amide group, inhibited cellulose biosynthesis in the *Acetobacter* system employed here, probably due to the phthoxazolin moiety (data not shown).

Acknowledgments

Dr. IGNACIO M. LARRINUA, DowElanco Co., Indiana, USA, is gratefully acknowledged for his generous gift of isoxaben. We are indeted to Mr. AKIRA MIHARA and Mr. KEI-ICHI TAKAHASHI, Fuji plant, Kyowa Hakko Kogyo Co., Ltd. for large scale fermentations of phthoxazolin A and phthoramycin. Thanks are also due to Mr. ISAO KANAYA for excellent technical assistance.

References

1) TANAKA, Y. & S. ŌMURA: Agroactive compounds of microbial origin. Annu. Rev. Microbiol. 47: 57~87, 1993
2) DELMER, D. P.: Biosynthesis of cellulose. Adv. Carbohydr. Chem. Biochem. 41: 105~153, 1983
3) HEIM, D. R.; J. R. SKOMP, C. WALDRON & I. M. LARRINUA: Differential response to isoxaben of cellulose biosynthesis by wild-type and resistant strains of *Arabidopsis thaliana*. Pest. Biochem. 39: 93~99, 1991
4) ŌMURA, S.; Y. TANAKA, I. KANAYA, M. SHINOSE & Y. TAKAHASHI: Phthoxazolin, a specific inhibitor of cellulose biosynthesis, produced by a strain of *Streptomyces* sp. J. Antibiotics 43: 1034~1036, 1990
5) TANAKA, Y.; I. KANAYA, Y. TAKAHASHI, M. SHINOSE, H. TANAKA & S. ŌMURA: Phthoxazolin A, a specific inhibitor of cellulose biosynthesis from microbial origin. I. Discovery, taxonomy of producing microorganism, fermentation, and biological activity. J. Antibiotics 46 : 1208~1213, 1993
6) TANAKA, Y.; I. KANAYA, K. SHIOMI, H. TANAKA & S. ŌMURA: Phthoxazolin A, a specific inhibitor of cellulose biosynthesis from microbial origin. Isolation, physicochemical properties, and structure elucidation. J. Antibiotics 46 : 1214~1218, 1993
7) ŌMURA, S.; Y. TANAKA, K. HISATOME, S. MIURA, Y. TAKAHASHI, A. NAKAGAWA, H. IMAI & H. B. WOODRUFF: Phthoramycin, a new antibiotic active against a plant pathogen, *Phytophthora* sp. J. Antibiotics 41: 1910~1912, 1988
8) KO, W. H.: Heterothallic *Phytophthora*: Evidence for hormonal regulation of sexual reproduction. J. Gen. Microbiol. 107: 15~18, 1978
9) SWISSA, M.; Y. ALONI, H. WEINHOUSE & M. BENZIMAN: Intermediary step in *Acetobacter xylinum* cellulose synthesis : studies with whole cells and cell-free preparation of the wild-type and a cellulose-less mutant. J. Bacteriol. 143 : 1142~1150, 1980
10) ERWIN, D. C.; S. BARTNICKI-GARCIA & P. H. TSAO (*Eds.*): *Phytophtiora*: Its Biology, Taxonomy, Ecology, and Pathogenicity. American Phtopathology Society, Minnesota, 1983
11) DOLAK, L. A.; A. DIETZ, S. CHINTIS, T. M. CASTLE & L. SLECHTA: New antibiotics saigenmycin and deoxyenterocin produced by *Streptomyces saigensis* sp. nov. Abstracts of Papers of 26th Intersci. Conf. Antimicrob. Agents Chemother., No. 909, Atlanta, 1986
12) YOKOI, K. (Esu-esu Pharmaceuticals Co.): Manufacture of a new antibiotic SS-8201D. Jpn. Kokai S59-170092, Sept. 26, 1984
13) HAYAKAWA, Y. & Y. TODA (Kirin Brewery Co.): Manufacture of a new antibiotic BQ-180B. Jpn. Kokai H2-60596, Mar. 1, 1990