Phosphonothrixin, a Novel Herbicidal Antibiotic Produced by *Saccharothrix* sp. ST-888

I. Taxonomy, Fermentation, Isolation and Biological Properties

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A novel herbicidal compound, phosphonothrixin, was found in the fermentation broth of *Saccharothrix* sp. ST-888 cultured on a vegetable juice medium. The compound exhibiting acidic and hydrophilic properties was obtained when the fermentation broth of ST-888 was subjected to ion exchange chromatography, gel filtration chromatography and ion-pair chromatography. Phosphonothrixin significantly inhibited germination of gramineous and broadleaf weeds. Foliar application of this antibiotic gave rise to chlorosis in all of the plants tested.

Microbial metabolites are known to provide new herbicidal substances. Bialaphos[1], phosphinothricin[2], and phosalacine[3] are herbicidal substances from the fermentation broths of actinomycetes and have a C-P bond in their structures.

In the course of our screening for new herbicidal antibiotics, we found that a soil isolate, collected at Iwaki-city, Japan, produced an active compound, phosphonothrixin. The strain was identified as *Saccharothrix* sp. ST-888.

The present paper deals with the taxonomy and fermentation of *Saccharothrix* sp. ST-888 as well as the purification and the biological properties of phosphonothrixin. The chemical characteristics, the molecular structure and the chemical synthesis of phosphonothrixin are described in separate papers[4,5].

Materials and Methods

Microorganism

The strain ST-888, was isolated from a soil sample collected at Nakoso, Iwaki, Japan. This organism has been deposited at the National Institute of Bioscience and Human Technology, Agency of Industrial Science &Technology, Japan, as *Saccharothrix* sp. ST-888 with the accession No. FERM BP-3053.

Screening Procedures

Microorganisms isolated from soil samples were cultured on V-8 juice agar slants at 26°C for 2 weeks and then maintained at 5°C until used. Selected strains were inoculated into Erlenmeyer flasks (300 ml) each containing 100 ml of the screening medium consisting of V-8 vegetable juice (Suntory, Japan) 100 ml, soluble starch 5 g, glycerol 5 g, yeast extract 1 g, Pharmamedia 10 g, $MgSO_4 \cdot 7H_2O$ 1 g, KH_2PO_4 1 g, K_2HPO_4 2 g, $CoCl_2 \cdot 6H_2O$ 10 mg, $(NH_4)_2SO_4$ 1 g and DL-alanine 0.5 g in 900 ml of deionized water. The pH was adjusted to 6.8~7.0 with 5N NaOH before sterilization. After cultivation at 28°C for 5 days on a rotary shaker (150 rpm), the broth filtrates were examined by a germination test as described later.

Taxonomy

Morphological and physiological characteristics were performed by the methods of SHIRLING and GOTTLIEB[6]. For experiments on cultural characteristics, all the cultures were incubated at 27°C and were observed for 14~21 days. The color names and numbers indicated Table 1 are those of the Color Harmony Manual (4th Ed.)[7]. The whole-cell preparation was analyzed by the method of KAWAMOTO[8]. We also referred to the Bergey's Manual of Systematic Bacteriology[9].

Fermentation

The fermentation medium: V-8 vegetable juice (Suntory, Japan) 100 ml, soluble starch 5 g, glycerol 5 g, yeast extract (Difco) 1 g, Pharmamedia 10 g, $MgSO_4 \cdot 7H_2O$ 1 g, KH_2PO_4 2 g, K_2HPO_4 4 g, $(NH_4)_2SO_4$ 5 g and DL-alanine 0.5 g in 900 ml of deionized water. The pH was adjusted to 6.8 with 5N NaOH before steriliza-

tion. An antifoam (TSA737F, Toshiba Silicon, Japan) was added when necessary.

A loopful of a slant culture (V-8 juice agar) was inoculated into an Erlenmeyer flask (300 ml) containing 100 ml of a seed medium consisting of glucose 10 g, meat extract (Kyokuto, Japan) 1 g, yeast extract (Daigo, Japan) 1 g and polypeptone (Daigo, Japan) 2 g in 1000 ml of deionized water. The pH was adjusted to 7.2 with 1 N NaOH before sterilization. This seed culture was shaken on a rotary shaker at 150 rpm at 28°C for 48 hours. Ten milliliters of this seed culture were added to 100 ml of the fermentation medium contained in a 300 ml Erlenmeyer flask, and incubated for 5~10 days under the same conditions as described above and the broth filtrates were examined by the germination test.

For jar fermentation, the resultant seed culture (150 ml) prepared as described above was inoculated into a 3-liter jar fermenter containing 1.5 liters of the fermentation medium and fermented at 28°C with an agitation rate of 500 rpm, air flow rate of 1.5 liters/minute, and internal pressure of 0.5 kgG/cm^2. Herbicidal activity was examined by germination testing. Cell growth was expressed as g dry weight/liter.

Isolation

During the isolation procedures the herbicidal activity was monitored by germination testing.

HPLC-analysis of fractions was performed with a C-18 Nova-pack (6 mm × 150 mm, Nippon Millipore, Japan) using 10% methanol in PIC-A (Nippon Millipore, Japan) solution as the mobile phase. The flow rate was 1 ml/minute and the wave-length for detection was 210 nm.

Herbicidal Test

The germination test was performed as follows. Green foxtail seeds (*Setaria viridis*) were placed on two sheets of filter paper (No. 2) in a petri dish (i.d. 90 mm) containing 1 ml of a test sample solution and 5 ml of deionized water. The treated seeds were kept at 28°C for 2 weeks under fluorescent lamps. Herbicidal activity was examined through a visual observation of the degrees of germination and growth inhibition of the coleoptile.

Herbicidal testing by foliar application was performed as follows. Four gramineous weeds *i.e. S. viridis* L. (Green foxtail), *Echinochloa frumentaceum* (Barnyard grass), *Avena fatua* L. (Wild oat), and *Digitaria adscendes* (H.B.K) HENR. (Henry crabgrass) and 8 broadleaf weeds *i.e. Amaranthus retroflexus* L. (Redroot pigweed), *Bidens pilosa* L. (Common blackjack), *Sinapis arvensis* L. (Wild mustard), *Stellaria media* (L.) VILL./CYR. (Common chickweed), *Cassia obtusifolia* L. (Sicklepod), *Solanum nigrum* L. (Black nightshade), *Abutilon theophrasti* MEDIK. (Velvetleaf), and *Convolvulus arvensis* L. (Field bindweed) were allowed to grow to the 2- to 4- leaf stage in pots, followed by foliar application of the solution of phosphonothrixin. Fourteen days later, the herbicidal activity was evaluated through visual observation.

Antimicrobial Activity

Antimicrobial activity was tested by the conventional paper disk method. Bacteria were grown on Nutrient agar medium (Difco), and the filamentous fungus and yeast were grown on Sabouraud dextrose agar medium. The activity was examined after incubation of the test organisms for 24 hours at 30°C for bacteria and for 3 days at 30°C for the filamentous fungus and yeast.

Results

Taxonomy

The cultural characteristics of strain ST-888 on various media are shown in Table 1.

The substrate mycelia did not show fragmentation into bacillary or coccoid elements on most agar media and in broth media. Sometimes, the substrate hyphae fragment into coccoid to bacillary elements and formed loops to closed spirals or irregular shapes on the surface or in the surface layer of agar media such as yeast extract-malt extract agar and inorganic salts-starch agar. Strain ST-888 produced a pale yellowish brown soluble pigment on most agar media, but no melanoid pigment was produced on ISP medium No. 7.

The aerial mass color was gray or white and the aerial mycelia bore long spore chains which had 10 to 50 or more spores per chain. The morphology of spore chains was open loops, hooks, terminal closed spirals or very irregular spirals. The spores were nonmotile, and ellipsoidal to oblong of 0.6 × 1.4~0.8 × 2.0 μm (Photo 1) in most cases and lenticular, reniform or irregular forms in the rest. Scanning electron micrographs of the spores exhibited smooth but slightly irregular surfaces. Sclerotic granules were not observed.

The physiological properties are shown in Table 2. The pattern of utilization of carbon and nitrogen sources are summarized in Table 3. The whole-cell hydrolysates contained *meso*-diaminopimelic acid and cell wall contained glycine, therefore cell wall type of strain ST-888 was classified as Type III. The whole-cell sugar pattern consisted of galactose, glucose, mannose, ribose and a trace of rhamnose. We, therefore, classified the whole-cell sugar pattern as pattern C. Based on the taxonomic characteristics described above, we assigned the strain ST-888 to the genus *Saccharothrix*.

Fermentation

Utilizing the screening medium, we examined the effects of medium components and their relative concentrations on the production of phosphonothrixin in

Table 1. Cultural characteristics of strain ST-888.

Medium	Cultural characteristics
Sucrose-nitrate agar (Waksman medium No. 1)	G: Poor R: Colorless AM: Poor, grayish white SP: None
Glucose-asparagine agar (Waksman medium No. 2)	G: Moderate R: Pale yellowish brown (2gc~3gc)~light brownish gray (3ec) AM: Abundant, gray (5fe~7fe), sprinkled with whitish spots SP: Trace of pale yellowish brown
Yeast extract-malt extract agar (ISP medium No. 2)	G: Moderate R: Pale yellowish brown (2ie~3ie)~brownish gray (3lg), sprinkled with dark yellowish brown (3pl) spots AM: Moderate, gray (5fe~7fe) SP: Pale yellowish brown
Oatmeal agar (ISP medium No. 3)	G: Moderate R: Pale yellowish brown (2ie~3ie)~brownish gray (3lg) AM: Abundant, gray (3fe~5fe) SP: Pale yellowish brown
Inorganic salts-starch agar (ISP medium No. 4)	G: Moderate R: Pale yellowish brown (2gc)~light brownish gray (3ge) or brownish gray (4ig) AM: Abundant, gray (5fe~7fe), sprinkled with whitish spots SP: Pale yellowish brown
Glycerol-asparagine agar (ISP medium No. 5)	G: Moderate R: Light yellow (2ea)~light yellowish gray (2ec) AM: Trace, whitish SP: Trace of yellowish
Tyrosine agar (ISP medium No. 7)	G: Moderate R: Light yellowish gray (2ec) AM: None SP: Trace of yellowish
Nutrient agar	G: Moderate R: Pale yellowish brown (2gc) AM: None SP: None

Abbreviation: G, growth; R, reverse color; AM, aerial mycelium; SP, soluble pigment.

Photo 1. Spores of strain ST-888 grown on glucose-asparagine agar 15 days.

Scanning electron micrograph (20,000×).

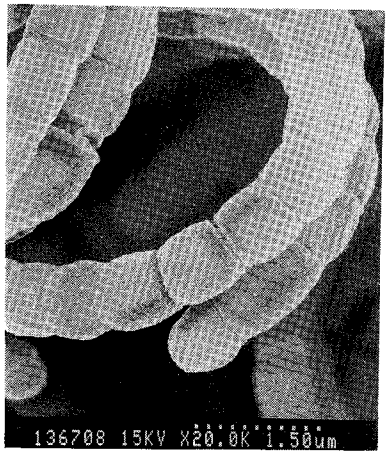


Table 2. Physiological properties of strain ST-888.

(a) Enzyme activity	Melanin formation	−
	Lecithinase (egg yolk medium)	+
	Proteolysis (egg yolk medium)	+
	Lipolysis (egg yolk medium)	+
	H_2S production	+
	Pectin hydrolysis	−
	Chitin hydrolysis	−
	Nitrate reduction	−
(b) Decomposition of	Hypoxantine, elastin, adenine, xanthine, allantoin	−
	L-Tyrosine, starch, casein, arbutin	+
(c) Growth	Temperature range (°C)	15~36
	Optimum temperature (°C)	29~32
	pH 4.3	−
(d) Growth in the presence of	7% Sodium chloride	−
	0.01% Sodium azide	−
	0.1% Phenol	−
	0.001% Potassium tellurite	+

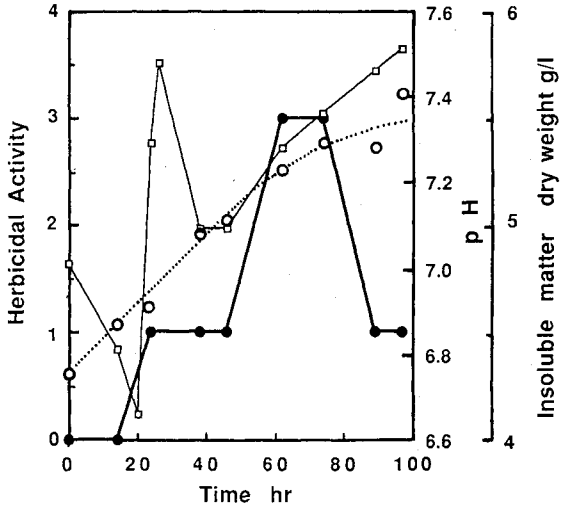
Symbols: + active; −, inactive.

Table 3. Utilization of carbon and nitrogen sources of strain ST-888.

	Carbon	Nitrogen
Utilized	Sucrose	L-Phenylalanine, L-histidine L-Hydroxyproline
Not utilized	L-Arabinose, D-galactose D-Xylose, meso-inoshitol Mannitol, raffinose, salcin Inulin, adonitol, cellobiose D-Melibiose, L-rhamnose	DL-Amino-n-butyric acid L-Valine
Not identified	D-Fructose	

Fig. 1. Time course of phosphonothrixin production in a 3-liter fermenter.

□, pH; ○, dry weight: g/liter; ●, herbicidal activity, 0: no effect, 1: weak effects, 2: moderate effects, 3: strong effects (no germination).



shake flask experiments. V-8 juice, inorganic phosphate, DL-alanine and ammonium sulfate were important components for phosphonothrixin production in the screening medium.

A typical time course of phosphonothrixin production in a 3-liter jar fermenter is illustrated in Fig. 1. The pH gradually decreased from the beginning of fermentation, reached minimum at 20 hours, then went up and down during the 20~45 hour period, and showed a gradual increase after the 50 hour period, at which time the pH was 7.1. Herbicidal activity reached maximum at about 70 hours inoculation. Insoluble matter gradually accumulated from the beginning of the fermentation to 100 hours.

Isolation

The isolation methods used for phosphonothrixin are outlined in Fig. 2. After fermentation for 70 hours in a 3-liter jar fermenter, one liter of the fermentation broth was mixed with 50 g of active carbon powder, the mixture was stirred and centrifuged to obtain supernatant. The supernatant was applied on a Dowex 1×8 (Cl^- form, 100~200 mesh, 200 ml) column, The column was washed

Fig. 2. Isolation procedures for phosphonothrixin.

Fermentation broth (1000 ml)
- added active carbon (50 g)
- centrifuge

Supernatant

Dowex 1×8 (Cl^-) column
- eluted with 0.5 N NaOH
- adjusted to 7.0

Lyophilization

MeOH extract 5 times
- cond.

Lyophilization
- dissolved in deionized water

Dowex 50W×8 (H^+) column
- passed through

DEAE-Toyopearl 650C column
- eluted with linear gradient deionized water to 0.2 N NaCl
- cond.

Sephadex G-15 column
- cond.

Preparative ion-pair HPLC

DEAE-Toyopearl 650C column
- eluted with 0.2 N NaCl
- concd.

Sephadex G-15 column
- cond.

Phosphonothrixin 20 mg

with deionized water and then eluted with 0.5 N NaOH. The active fractions were pooled and neutralized with 5 N HCl and then lyophilized to give a white powder. The powder was extracted with MeOH (50 ml) five times. The extracts were combined and concentrated under reduced pressure to give a small amount of solution. The solution was lyophilized to give 1.3 g of a white powder. The white powder was dissolved in deionized water and was passed through a Dowex 50W × 8 (H^+ form, 100~200 mesh, 100 ml) column. The non-adsorbed fractions were combined and diluted to 1.2 liters with deionized water and then applied on a DEAE-Toyopearl 650C (500 ml) column. After washing with deionized water, the metabolite was eluted with a linear gradient of deionized water (600 ml) to 0.2 M NaCl solution (600 ml). The active fractions were combined and concentrated under reduced pressure. The concentrate was chromatographed over a Sephadex G-15 column (40 i.d. × 830 mm) and developed with deionized water to remove undesired salts. The active fractions were combined and concentrated under reduced pressure and then purified by preparative ion-pair HPLC on a reverse-phased silica gel column (μBondasphere C-18, 20 mm × 300 mm, Nippon Millopore, Japan) with PIC-A (Nippon Millipore, Japan) solution containing 10% MeOH. The flow rate of the mobile phase was 5 ml/minute, the detection wavelength was 220 nm. The active fractions were combined and applied on a DEAE-Toyopearl 650C column to remove the PIC-A components. The active metabolite was eluted with 0.2 M NaCl solution and the elute was concentrated and chromatographed over a Sephadex G-15 column and developed with deionized water. Purified phosphonothrixin fractions were combined and concentrated to give 20 mg of a white powder, pure phosphonothrixin.

Biological Properties

The coleoptile of green foxtail seeds treated with phosphonothrixin at a concentration of 0.3 μg/ml turned white, while the growth of the coleoptile was not affected. Herbicidal activity by foliar application on the 2- to 4-leaf stage of plants is shown in Table 4. Phosphonothrixin showed herbicidal activity against various gramineous as well as broadleaf weeds. In addition, phosphonothrixin induced chlorosis at the rate of 50 g/10a against all of the tested plants.

The features of the herbicidal activity of phosphonothrixin are summarized as follows. First, phosphonothrixin induces chlorosis. Second, phosphonothrixin is a non-selective herbicide. Third, herbicidal effects of phosphonothrixin are observed by foliar application, but not by soil application. Last, phosphonothrixin is more effective with early-post than late-post emergency.

Table 4. Herbicidal activity of phosphonothrixin by foliar application.

Plants	Application dosage (g/10a)	
	12.5	50
S. viridis L.	4.5	4.9
E. frumentaceum	2.5	3.5
A. fatua L.	3.0	3.5
D. adecendes (H.B.K) HENR.	3.5	4.0
A. retroflexus L.	4.5	4.5
B. pilosa L.	3.75	4.0
S. arvensis L.	4.0	4.5
S. media (L.) VILL./CYR.	4.0	4.0
C. obtusifolia L.	3.5	4.0
S. nigrum L.	4.0	4.5
A. theophrasti MEDIK.	4.0	4.5
C. arvensis L.	3.0	4.0

Herbicidal activity was evaluated through visual obserbation of the degrees of foliar killing as follows: 0, no kills; 1, less than 30% killed; 2, 31~50% killed; 3, 51~70% killed; 4, 71~90% killed; 5, 91~100% killed.

No effects were noted in paper-disk assay against *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* FDA 209P, *Escherichia coli* K12, *Pseudomonas aeruginosa* IFO 3445, *Aspergillus niger* IFO 6342 or *Candida albicans* TIMM 0134 with phosphonothrixin at a concentration of 30 μg per disk.

Discussion

Much interest has centered on possible exploitation of novel natural products as safe and selective herbicides. Dozens of secondary natural products are known to have herbicidal activity. However, only two compounds, bialaphos and glyphosate, which have a C-P bond, are employed as herbicides.

Several antibiotics are produced by *Saccharothrix* species: rebeccamycin[10], karnamicin[11] and galacardins[12], for example. However, herbicidal compound produced by *Saccharothrix* is a rarity. There is only one report on an exception, which is a carbocyclic nucleoside and does not induce chlorosis[13]. ST-888 is the first example of a *Saccharothrix* sp. being capable of producing a herbicidal compound which induces chlorosis.

Chlorosis has been attributed to several causes: absence of light, deficiency of some metals, RNA and protein synthesis inhibition, chlorophyll or chloroplast synthesis inhibition, and chlorophyll or chloroplast degradation acceleration. In a preliminary test, phosphonothrixin did not inhibit chlorophyll synthesis. The mechanism of chlorosis for phosphonothrixin is yet to be elucidated.

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References

1) KONDO, Y.; T. SHOMURA, Y. OGAWA, T. TSURUOKA, H. WATANABE, K. TOTSUKAWA, T. SUZUKI, C. MORIYAMA, J. YOSHIDA, S. INOUYE & T. NIIDA: Studies on a new antibiotic SF-1293. I. Isolation and physico-chemical and biological characterization of SF-1293 substance. Sci. Reports of Meiji Seika Kaisha 13: 34~41, 1973
2) BAYER, E.; K. H. GUGEL, K. HAGELE, H. HAGENMAIER, S. JESSIPOW, W. A. KONIG & H. ZAHNER: Stoffwechselprodukte von mikroorganismen. 98. Phosphinothricin and phosphinothricyl-alany-alanin. Helv. Chim. Acta. 55: 224~239, 1972
3) ŌMURA, S.; M. MURATA, H. HANAKI, K. HINOTOZAWA, R. ŌIWA & H. TANAKA: Phosalacine, a new herbicidal antibiotic containing phosphinothricin. Fermentation, isolation, biological activity and mechanism of action. J. Antibiotics 37: 829~835, 1984
4) KIMURA, T.; K. NAKAMURA & E. TAKAHASHI: Phosphonothrixin, a novel herbicidal antibiotic produced by *Saccharothrix* sp. ST-888. II. Structure determination. J. Antibiotics 48: 1130~1133, 1995
5) NAKAMURA, K.; T. KIMURA, H. KANNO & E. TAKAHASHI: Total synthesis of (±)-phosphonothrixin, a novel herbicidal antibiotic containing C-P bond. J. Antibiotics 48: 1134~1137, 1995
6) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1969
7) TAYLOR: Color Harmony Manual. 4th *Ed.*, TAYLOR 1948
8) KAWAMOTO, I.: *In* Identification Manual for Actinomycetes (In Japanese). *Ed.*, Japan Actinomycetes Research Association, pp. 59~79, 1988
9) LABEDA, D. P.: Section 33. Other genera. *In* Bergey's Manual Systematic Bacteriology. Volume 4. *Eds.*, S. T. WILLIAMS *et al.*, pp. 2599~2601, Williams & Wilkins Co., 1989
10) BUSH, J. A.; B. H. LONG, J. J. CATINO & W. T. BRADNER: Production and biological activity of rebeccamycin, a novel antitumor agent. J. Antibiotics 40: 668~678, 1987
11) NISHIO, M.; K. TOMATSU, M. KONISHI, K. TOMITA, T. OKI, H. KAWAGUCHI & J. CLARDY: Karnamicin, a complex of new antifungal antibiotics. I. Taxonomy, fermentation, isolation and physico-chemical and biological properties. J. Antibiotics 42: 852~868, 1989
12) TAKEUCHI, M.; S. TAKAHASHI, R. ENOKITA, Y. SAKAIDA, H. HARUYAMA, T. NAKAMURA, T. KATAYAMA & M. INUKAI: Galacardins A and B, new glycopeptide antibiotics. J. Antibiotics 45: 297~305, 1992
13) BUSH, B. D.; G. V. FITCHETT, D. A. GATES & D. LANGLEY: Carbocyclic nucleosides from a species of *Saccharothrix*. Phytochemistry 32: 737~739, 1993