PHOSALACINE, A NEW HERBICIDAL ANTIBIOTIC CONTAINING PHOSPHINOTHRICIN

FERMENTATION, ISOLATION, BIOLOGICAL ACTIVITY AND MECHANISM OF ACTION

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Phosalacine, a new herbicidal antibiotic containing phosphinothricin was isolated from the culture filtrate of a soil isolate *Kitasatosporia phosalacinea* KA-338. It was a water soluble, amphoteric compound obtained as an amorphous powder ($C_{14}H_{28}N_3O_6P$, MW 365). The antibiotic exhibited antimicrobial activity against Gram-positive and Gram-negative bacteria and some fungi on a minimal medium and the activity was reversed by L-glutamine. It also showed herbicidal activity against alfalfa. It is suggested that phosalacine was decomposed to provide phosphinothricin after its incorporation into microbial or plant cells, and exhibited the antimicrobial and herbicidal activities by inhibiting glutamine synthetase with phosphinothricin although phosalacine itself hardly inhibited the enzyme.

Screening of actinomycetes for antimetabolites competitive with L-glutamine was conducted to obtain antitumor and herbicidal antibiotics. Among about 2,500 soil isolates, strain KA-338 was found to produce a new antibiotic possessing herbicidal activity. The antibiotic was isolated from the culture filtrate of the strain, which was named *Kitasatosporia phosalacinea* sp. nov. KA-338, and then the structure was elucidated to be phosphinothricylalanylleucine, after which the antibiotic was named phosalacine.

The present paper deals with the fermentation, isolation, biological activities and mechanism of action of phosalacine. The taxonomy of the producing strain[1] and the structure elucidation[2] will be reported elsewhere.

Materials and Methods

Phosalacine Production

A loopful of the aerial mycelia of a slant culture of *K. phosalacinea* KA-338[1] was transferred into a seed medium (pH 7.0, 100 ml) containing glucose 0.1%, starch 2.4%, yeast extract 0.5% and $CaCO_3$ 0.4% in a 500-ml Sakaguchi flask, and incubated with reciprocal shaking for 2 days at 27°C to give a seed culture for production of phosalacine. The seed culture (2 ml) was transferred into a production medium (pH 6.0, 100 ml) containing tomato paste 4.0%, oatmeal 1.5% and glucose 0.2% in a 500-ml Sakaguchi flask, and then incubated with reciprocal shaking at 27°C.

Assay of Phosalacine and Phosphinothricin

Phosalacine was assayed by a paper disc method using *Bacillus subtilis* KB-211 (PCI 219) and DAVIS' minimal medium. Since phosphinothricin possesses little antimicrobial activity, it was assayed by TLC method. After a sample solution containing phosphinothricin was passed through a column of Amberlite IR-120 (H^+), the adsorbed material was eluted with 2 N NH_4OH and submitted to TLC using BuOH - AcOH - H_2O (3: 1: 2) as developing solvent. The spots visualized with ninhydrin cor-

responding to phosphinothricin were scanned at 480 nm with a dual wavelength chromatogram scanner.

Herbicidal Activity

Several seeds of alfalfa (*Medicago sativa* L.) were put on some wet cotton in a test tube (1.6×10 cm) and germinated in a dark room at 27°C. When the seedlings grew to a height of 4~5 cm, 0.2 ml of an aqueous solution of a test compound was applied for foliage treatment, and the treated plants were exposed to continuous illumination. Four and seven days after treatment, damage to plants was visually assessed and expressed in terms of symbols ranging from (−) to (+++); (−) indicates no effect and (+++) complete kill.

Growth Inhibition and Its Reversion

B. subtilis KB-211 was grown in a liquid medium (10 ml) of Yeast Nitrogen Base (Difco) in an L-tube with shaking at 37°C. The logarithmic phase culture (2.0%) was transferred into SPIZIZEN's medium (10 ml) in an L-tube and then incubated at 37°C. The growth was monitored at A_{660} measured with a Coleman photometer.

Preparation of Glutamine Synthetase

Preparation of the enzymes from *B. subtilis* and spinach leaves was carried out with some modifications according to the methods of DEUEL *et al.*[3] and LEASON *et al.*[4], respectively.

The wet cells (54 g) of *B. subtilis* KB-211 obtained by the incubation in 20 liters of nutrient broth using a 30-liter jar fermentor were disrupted with quartz sand (108 g), and 50 mM imidazole - HCl buffer (pH 7.0, 378 ml) containing 0.2 mM EDTA and 1.0 mM 2-mercaptoethanol was added to the disrupted cells. The supernatant obtained by centrifugation at 27,000×*g* for 30 minutes was applied to $(NH_4)_2SO_4$ fractionation after the treatment with streptomycin sulfate (final concentration, 1.0%). The 55~75% saturated $(NH_4)_2SO_4$ precipitated fraction was dialyzed against the above buffer and then used as an enzyme preparation for glutamine synthetase from the organism.

The well-grown leaves of spinach (*Spinacia oleracea* L.) were homogenized in 50 mM imidazole - acetate buffer (pH 7.8) containing 0.5 mM EDTA, 10 mM 2-mercaptoethanol, 2 mM $MnCl_2$ and 20% glycerol at 4°C with an Ultra Turrux homogenizer and centrifuged at 27,000×*g* for 15 minutes. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation. The 30~60% saturated precipitate was dialyzed against the above buffer to give an enzyme preparation of glutamine synthetase from spinach.

Assay of Glutamine Synthetases

The assay of glutamine synthetases from *B. subtilis* and spinach were performed as described by DEUEL *et al.*[3] and LEASON *et al.*[4], respectively.

Formation of Phosphinothricin from Phosalacine by Cell-free Extracts from *B. subtilis* and Spinach Leaves

Phosalacine (0.9 mg/ml, 2.5 mM) was added to the cell-free extracts obtained as described above, and incubated at 37°C or 27°C. The formed phosphinothricin and remaining phosalacine were assayed as described above.

Results

Phosalacine Production

A typical time course of phosalacine production in a 500-ml Sakaguchi flask by *K. phosalacinea* KA-338 is shown in Fig. 1. When the production medium described in Materials and Methods was used, phosalacine production reached 95 μg/ml at 60 hours. Since the antibiotic contained alanine, leucine and phosphinothricin, the effects of these amino acids and related compounds on phosalacine production were examined. As shown in Table 1, phosphinothricin enhanced the phosalacine production when it was added to the culture medium both before and after incubation for 2 days. L-Methionine exhibited some effect only when it was added before incubation. Since it has been reported that Co^{++} ions are present in a bialaphos (phosphinothricylalanylalaine) production medium[5], the effect of the ions on phosalacine production was examined. The addition of 100 μg/liter of $CoCl_2 \cdot 2H_2O$ exhibited the

Fig. 1 Time course of phosalacine production by *K. phosalacinea* KA-388.

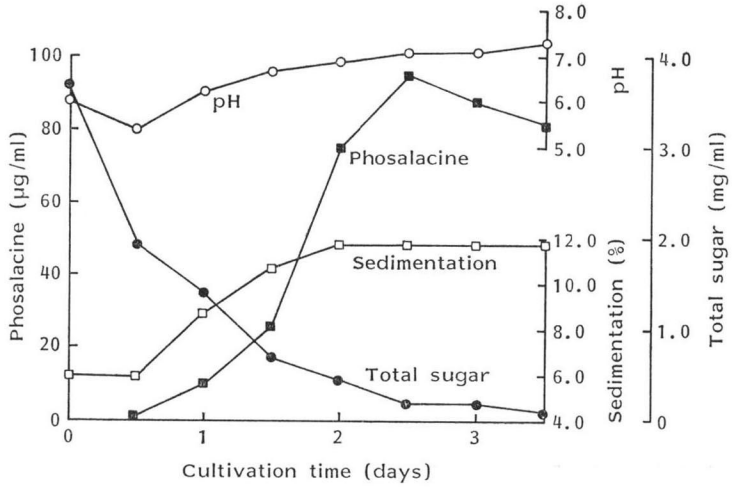


Table 1. Effect of various amino acids on phosalacine production by *K. phosalacinea* KA-338.

Addition	Phosalacine produced (μg/ml)	
	(A)	(B)
None	190	165
L-Leucine	68	110
L-Alanine	44	79
L-Asparagine	85	74
L-Valine	37	115
L-Isoleucine	105	190
L-Threonine	59	110
L-Methionine	230	91
L-Phosphinothricin	320	275
Pyruvate	145	135

Each amino acid (0.1%) was added to the culture before (A) or after (B) incubation for 2 days, and the incubation was stopped at day 3.
Basal medium (pH 6.0) contained tomato paste 4.0%, oatmeal 1.5%, glucose 0.2% and $CoCl_2 \cdot 2H_2O$ 100 μg/liter.

Table 2. Effect of Co^{++} ions on phosalacine production by *K. phosalacinea* KA-338.

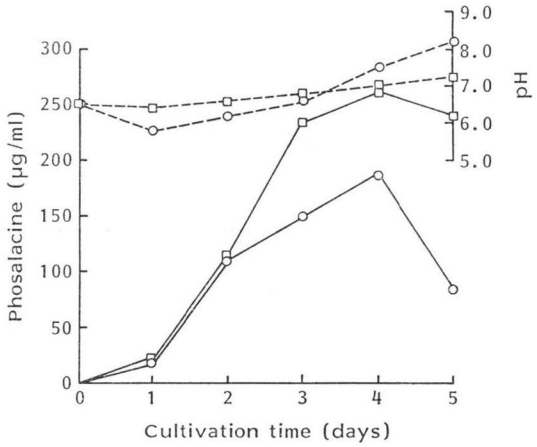
$CoCl_2 \cdot 2H_2O$ (μg/liter)	Phosalacine produced (μg/ml)
0	96
10	108
20	150
50	150
100	160
500	92

The organism was incubated at 27°C for 4 days. Basal medium (pH 6.0) contained tomato paste 4.0%, oatmeal 1.5% and glucose 0.2%.

Fig. 2. Effect of KH_2PO_4 and K_2HPO_4 on phosalacine production by *K. phosalacinea* KA-338.

Basal medium: Tomato paste 4.0%, oatmeal 1.5%, glucose 0.2% and $CoCl_2 \cdot 2H_2O$ 100 μg/liter, pH 6.0.

---- pH, —— phosalacine production, ○ control (no addition), □ KH_2PO_4 and K_2HPO_4, 0.2%, respectively.



best effect, and under these conditions the phosalacine concentration reached 160 μg/ml (Table 2).

The decrease of phosalacine concentration in the later stage of the cultivation was often observed together with the increase of pH. The observation seemed to reflect the decomposition of phosalacine under alkaline conditions. The addition of relatively high concentrations of

Table 3. Effect of KH_2PO_4 and K_2HPO_4 on phosalacine production by *K. phosalacinea* KA-338.

Both KH_2PO_4 and K_2HPO_4 (%)	Phosalacine produced (μg/ml)
0	185
0.01	200
0.05	210
0.10	245
0.20	260
0.50	230

The incubation was stopped after 4 days. Basal medium (pH 6.0) contained tomato paste 4.0%, oatmeal 1 5%, glucose 0.2% and $CoCl_2 \cdot 2H_2O$ 100 μg/liter.

KH_2PO_4 and K_2HPO_4 was tried to increase the buffer action of the medium. As shown in Table 3 and Fig. 2, when 0.2% KH_2PO_4 and 0.2% K_2HPO_4 were added, the increase of pH of the medium in the later stage was suppressed and the phosalacine production reached 260 μg/ml.

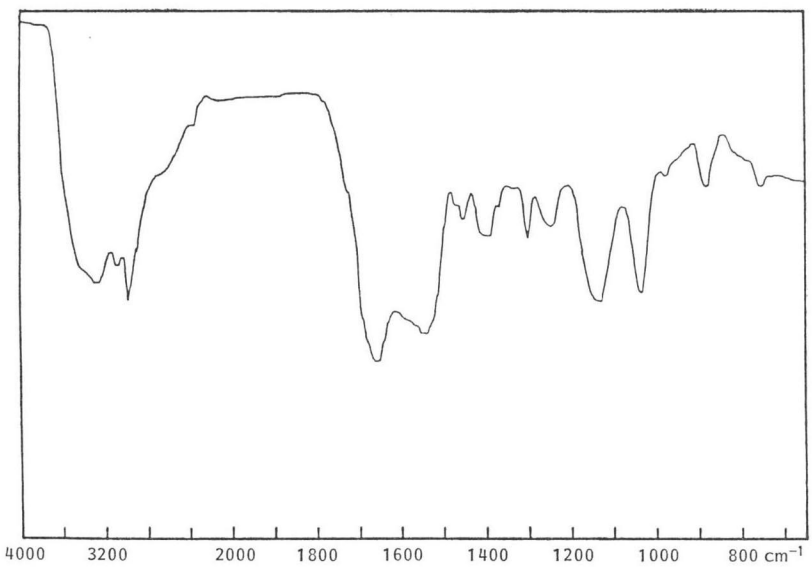
Isolation and Characterization

Cultured broth (70 liters, phosalacine, 125 μg/ml) of *K. phosalacinea* KA-338, obtained by incubation in a 100-liter tank for 2.5 days at 27°C, was used as a starting material for the

Table 4. Physico-chemical properties of phosalacine.

Nature		White amorphous powder, amphoteric
Mp		>225°C (dec)
$[\alpha]_D^{25}$		−38.8° (*c* 0.65, H_2O)
pKa		<3.0, 4.3, 8.3
Anal (%)	Found:	C 45.92, H 7.59, N 11.39
	Calcd:	C 46.01, H 7.73, N 11.50
FD-MS (*m/z*)		366 $(M+1)^+$
Formula		$C_{14}H_{28}N_8O_6P$
UV λ_{max}^{MeOH} (nm)		End adsorption
IR ν_{max}^{KBr} (cm^{-1})		3270, 3060, 2975, 1658, 1545, 1425, 1400
Solubility	Soluble:	H_2O
	Slightly soluble:	MeOH
	Insoluble:	EtOH, BuOH, Me_2CO, C_6H_6
Color reaction	Positive:	Ninhydrin, Rydon-Smith
	Negative:	Dragendorff, aniline phthalate

Fig. 3. IR spectrum of phosalacine (KBr method).



isolation of phosalacine. The culture supernatant was passed through a column of weakly basic anion exchange resin Diaion WA-30 (OH^-) (1.4 liters) and the adsorbed material was eluted with 0.5 N HCl. The active eluate was applied to a column of activated carbon (700 ml) and the active principle adsorbed was eluted with 30% Me_2CO. After the active eluate was passed through a column of strongly acidic cation exchange resin Amberlite IR-120 (H^+) (600 ml), the adsorbed substance was eluted with 0.5 N NH_4OH. After NH_4OH contained in the active eluate was evaporated off, 10 vol of EtOH was added to the concentrate, and centrifuged. After the supernatant was further concentrated *in vacuo*, excess Me_2CO was added to the concentrate to give a white amorphous powder. The recovery of phosalacine in isolation from the cultured broth was 25%. Further purification was performed using HPLC with LiChrosorb RP-18 column and 20% MeOH (detection: UV at 215 nm).

The physico-chemical properties of phosalacine are summarized in Table 4. IR spectrum is shown in Fig. 3. Phosalacine is presumed to be a new compound because no compounds having the above characteristics are known. Further characterization and structure of phosalacine will appear elsewhere[2].

Biological Activity

The antimicrobial spectrum of phosalacine is shown in Table 5. It inhibited Gram-positive and Gram-negative bacteria and some fungi in a chemically defined minimal medium although showing no activity in a complete medium.

Strong herbicidal activity of phosalacine against alfalfa (*Medicago sativa* L.) was observed at the concentration of 10 μg/ml (Table 6).

No toxicity of phosalacine was observed even when it was administered po to mice at a dose of 500 mg/kg.

Mode of Action

As described above, phosalacine was discovered during the screening of antimetabolites competi-

Table 5. Antimicrobial spectrum of phosalacine.

Test organism	MIC (μg/ml)*
Staphylococcus aureus KB 210 (FDA 209)	0.4
S. aureus KB 199	0.2
Bacillus subtilis KB 211 (PCI 219)	0.4
B. cereus KB 143 (IFO 3001)	0.4
Micrococcus luteus KB 212 (ATCC 9341)	6.3
Mycobacterium smegmatis KB 42 (ATCC 607)	>100
Escherichia coli KB 213 (NIHJ)	12.5
Klebsiella pneumoniae KB 214 (ATCC 10031)	25
Erwinia aroideae KB 148	<0.05
Pseudomonas aeruginosa KB 139	>100
Proteus vulgaris KB 127 (IFO 3167)	12.5
Candida albicans KF 1	>100
Saccharomyces cerevisiae KF 237 (ATCC 9763)	>100
Aspergillus niger KF 105	>100
Mucor racemosus KF 223 (IFO 4581)	6.3
Piricularia oryzae KF 180	0.1
Fusarium oxysporum KF 166	>100
Penicillium herquei KF 228 (IFO 7904)	>100

* Determined by agar dilution method using DAVIS' minimal medium for bacteria (37°C, 20 hours) and glucose CZAPEK's medium for fungi (27°C, 2 days).

Fig. 4. Restoration by L-glutamine of the growth of *B. subtilis* inhibited by phosalacine.
Basal medium: DAVIS' minimal medium.
○ Control (no addition), ● 0.1 μg/ml phosalacine, □ 0.1 μg/ml phosalacine plus 1.0 μg/ml L-glutamine, ■ 0.1 μg/ml phosalacine plus 10 μg/ml L-glutamine.

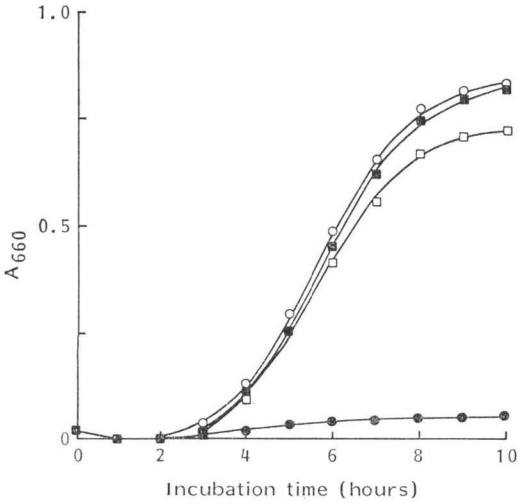
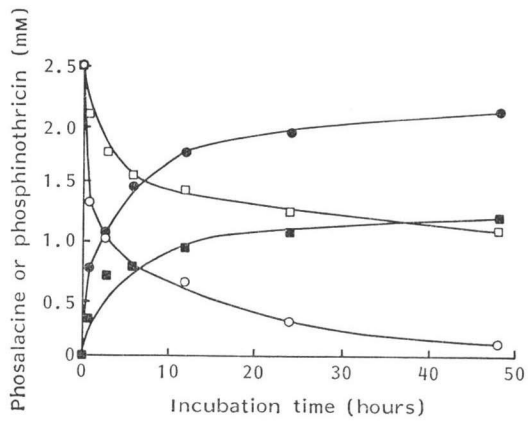


Table 6. Herbicidal effect of phosalacine on alfalfa (*Medicago sativa* L.).

Phosalacine (μg/ml)	Herbicidal effect*	
	4-day	7-day
0	−	−
10	++	+++
100	+++	+++
1,000	+++	+++

* Herbicidal effect: −, no effect; +, considerable effect; ++, medium effect; +++, complete kill.

Fig. 5. Formation of phosphinothricin (●, ■) from phosalacine (○, □) by cell-free extracts from *B. subtilis* (●, ○) and spinach leaves (■, □).



tive with L-glutamine. Phosalacine inhibited almost completely the growth of *B. subtilis* at the concentration of 0.1 μg/ml, and the inhibition was completely reversed by addition of 10 μg/ml of L-glutamine as shown in Fig. 4. The inhibition by phosphinothricin was much weaker than that by phosalacine. Phosphinothricin did not completely inhibit the organism even at the concentration of 50 μg/ml (data not shown).

In contrast, in the inhibition against glutamine synthetase, phosphinothricin was much more potent than phosalacine. Phosphinothricin inhibited competitively the glutamine synthetases from *B. subtilis* and spinach leaves: *Ki* values were 81.1 μM and 306 μM, and *Km* values were 18.2 mM and 112 mM, respectively. On the other hand, phosalacine exhibited very weak inhibition against *B. subtilis* glutamine synthetase (it exhibited 21 and 29% inhibition at 1.37 and 13.7 mM, respectively, when 25 mM glutamic acid was used as substrate), and no inhibition against the enzyme from spinach leaves even at 2.5 mM.

Since the antimicrobial and herbicidal activities of phosalacine could not be explained only from the above data, we attempted to convert phosalacine to phosphinothricin with cell-free extracts of *B. subtilis* and spinach leaves. As shown in Fig. 5, phosalacine was easily converted into phosphinothricin by the cell-free extracts.

Discussion

From the above data, it appears that after phosalacine was incorporated into bacterial or plant cells, it was converted to phosphinothricin, which inhibited the glutamine synthetases to result in antibacterial or herbicidal activities.

As described above, it was found that phosalacine isolated from the cultured broth of a soil isolate named *Kitasatosporia phosalacinea* KA-338 is a new antimetabolite competitive with L-glutamine and possesses a potent herbicidal activity. Since it was speculated that phosalacine is decomposed to phosphinothricin after the incorporation into plant cells and the phosphinothricin inhibits glutamine synthetase, phosalacine may be a nonspecific herbicide like phosphinothricylalanylalanine[6] (bialaphos[5,7~9]).

From the physico-chemical properties described above and further structure elucidation[2], the structure of phosalacine was determined to be L-phosphinothricyl-L-alanyl-L-leucine, in which L-leucine is substituted for *C*-terminal L-alanine of bialaphos. The phosalacine production was found to be stimulated by the addition of Co^{++} ions, phosphinothricin and relatively high concentrations of KH_2PO_4 and K_2HPO_4. Since the stimulation by Co^{++} ions and phosphinothricin has been also reported in bialaphos production[5], phosalacine might be biosynthesized through a similar pathway as that for bioalaphos production[10] although the producing organism differs from that of bialaphos.

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