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TRIPHENYLTIN SALICYLATE-ANTIMICROBIAL EFFECT AND RESISTANCE – THE PYROPHOSPHATASE CONNECTION

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The effect of Triphenyltin salicylate (TPS) was tested against six bacteria, *Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae and Salmonella typhi* and five fungi, *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Rhodotorula* spp. and *Saccharomyces* spp. Sensitivity tests were determined with 5–500 μg/ml of TPS. All organisms were sensitive to the compound except *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Rhodotorula* spp. and *Saccharomyces* spp. The minimum dose of TPS that can kill 50% of the susceptible microorganisms is in the range 5–50 μg/ml.

Membrane bound pyrophosphatase(s) from the organisms was non-competitively inhibited by 5 μM TPS with K_i values of 7.6, 18, 8.8 and 6.9 μM for *Escherichia coli*, *Shigella flexneri*, *Aspergillus niger*, and *Aspergillus fumigatus*, respectively.

The physiological index of efficiency of the enzyme (V_{max}/K_M) for TPS susceptible organisms was reduced by 17–68% in the presence of 5–10 μM of the compound. In contrast the index for the non-susceptible organisms was unaffected. The mode of action of TPS is discussed.

Keywords: Triphenlytin salicylate; Pyrophosphatase inhibition

INTRODUCTION

Organotin compounds are widely used in agriculture and industry. They possess one or more direct tin–carbon covalent bond(s) responsible for the specific properties of such molecules.[1] The biological properties of

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organotin compounds are affected by both the number of carbon–tin bonds in the molecule and the nature of the group(s) covalently linked to the tin atom.[2]

The antitumor, bactericidal, fungicidal and trypanocidal properties of organotin compounds have been well established.[3–5] The focus in the synthesis of organotins for these purposes is towards less toxic and more easily degradable compounds. The core structure of organotin compounds provides an excellent template for the synthesis of different derivatives with broad features and functions.

In this paper, we show the efficacy of triphenyltin salicylate (TPS) on some microorganisms and the connection between their sensitivity and pyrophosphatase inhibition.

MATERIALS AND METHODS

Materials

All growth media Potato Dextrose Agar (PDA), Mueller Hinston Agar (MHA), Nutrient Agar (NA) and Nutrient broth were products of Sigma Chemical Co. All other reagents are of analytical grade.

Methods

TPS was synthesized as described previously.[6] *Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus flavus*, *Saccharomyces* spp. and *Rhodotorula* were obtained from the Department of Microbiology, ABU Zaria, Nigeria. The bacteria were maintained in NA and the fungus on PDA slants under refrigerated condition.

Inhibitory Activity of TPS

Assay of the antimicrobial activity of TPS was done in 100×15 mm petridishes containing MHA and PDA for bacteria and fungi respectively.

TPS was dispensed into four bored wells on the plates at the following concentrations; 5, 50, 200, and 500 μg/ml. The plates were incubated for 2 h to allow for diffusion. The MHA plates were incubated for 24 h at 37°C and the PDA at room temperature (28°C) for 48–72 h. At the end of incubation, the crescent of inhibition around the disc of each well containing TPS was measured. Microbial susceptibility was done using RPMI 1640 medium for

the TPS-sensitive organisms in the presence of 25 μg/ml TPS. The effect of TPS on growth was determined by turbidity.

Solubilization of Pyrophosphatase (PPase)

About 10 ml of the microorganism culture was incubated with 20 ml of 60 mM Tris–HCl pH 8.8 (containing 0.5% w/v cholate, 20% v/v ethylene glycol, 0.5 M $MgCl_2$, and 0.5 M EDTA) at 4°C for 30 min and then centrifuged at 27,000× *g* for 2 h. The supernatant was brought to 12% w/v with 50% polyethylene glycol (PEG) 4000 in 0.1 M Tris–HCl pH 8.8, centrifuged at 27,000× *g* for 1 h, and then again raised to 14% saturation with PEG. The recovered precipitate was dissolved in 0.1 M Tris–HCl pH 8.8 (containing 0.1 M $MgCl_2$, 0.2 mM EDTA, and 2% Triton X-100). The enzyme solution was then loaded onto a DEAE-cellulose column (2 × 15 cm) pre-equilibrated with 60 mM phosphate buffer pH 6.9. The enzyme emerged in the flow through fraction and was chromatographed on Sephadex G-75. The active fraction that emerged from the void volume was utilized as the pyrophosphatase source. The protein content was determined as described by Lowry *et al.*[7]

Enzyme Assay

The enzyme activity was measured as described by Celis *et al.*[8] For inhibition studies, the enzyme was preincubated with TPS or an ethanol control for 15 min prior to addition to the reaction system. Dialysis of the Enzyme–Inhibitor complex (E–TPS) was done overnight against 0.1 M Tris–Maleate buffer pH 6.5 and the dialysate was assayed for PPase. Kinetic data were analyzed by the Cricket Graph program Version 1.3.2.

RESULTS

The effects of TPS on bacteria and fungi are summarized in Table I. When the bacteria and fungi were exposed to TPS, inhibition was observed in a dose-dependent fashion. *Shigella flexneri*, a gram negative bacillus, was sensitive even at 5 μg/ml. *Staphylococcus aureus*, a gram positive cocci, was apparently resistant at 5 μg/ml but sensitive at all other levels of TPS. However, *Salmonella typhi*, a gram negative typhoid bacillus, was sensitive only at 500 μg/ml. *Escherichia coli* was sensitive at all concentrations of TPS used whilst *Klebsiella pneumoniae*, a gram negative diplobaccili, was resistant at all tested levels of TPS. The compound also exhibited fungicidal effects

TABLE I Effect of TPS on bacteria and fungi in the presence of 5–500 μg/ml of TPS

Organism	*Zone of Inhibition* (mm)			
	5 (μg/ml)	50 (μg/ml)	200 (μg/ml)	500 (μg/ml)
Shigella flexneri	20	25	27	30
Staphylococcus aureus	0	18	22	23
Salmonella typhi	0	0	0	26
Escherichia coli	9	18	20	22
Pseudomonas aeruginosa	0	0	0	0
Klebsiella pneumoniae	0	0	0	0
Aspergillus niger	9	16	18	20
Aspergillus fumigatus	7	15	18	20
Aspergillus flavus	0	14	16	20
Saccharomyces spp.	0	0	0	0
Rhodotorula spp.	0	0	0	0

The MHA and PDA plates were inoculated with bacteria and fungi respectively. Four wells on each plate were bored with punch No. 3. To each well 5–500 μg/ml of TPS were dispensed. The plates were kept for 2 h to allow for diffusion. Incubation of MHA was done at 37°C and the PDA plates at room temperature (28°C). The zone of inhibition was measured in millimeters. Results are the average of three experiments.

TABLE II Inhibitory effect of 5 μg/ml TPS on bacteria and fungi over a 5–25 h period

Organism	*Bactericidal/fungicidal activity*				
	5 h	10 h	15 h	20 h	25 h
Bacteria					
Escherichia coli	++	++	–	–	–
Staphylococcus aureus	+	+	–	–	–
Shigella flexneri	–	–	–	–	–
Salmonella typhi	+	+	–	–	–
Control (negative)	–	–	–	–	–
Control (positive)					
Shigella flexneri	+	+	++	+++	+++
Fungi					
Aspergillus niger	–	–	–	–	–
Aspergillus fumigatus	–	–	–	–	–
Aspergillus flavus	–	–	–	–	–
Control (negative)	–	–	–	–	–
Control (positive)					
Aspergillus niger	++	++	+++	+++	+++

Organisms were inoculated into RPMI media and the growth noted at intervals of 5 h using turbidity measurements. (+) Little growth (no inhibition). (++) High growth (no inhibition). (+++) Very high growth (no inhibition). (–) No growth observed implying TPS inhibition. Control (negative) Contains the culture only. Control (positive) Culture with microorganism but without TPS. Results are the average of three experiments.

except for *Saccharomyces* spp. and *Rhodotorula* that were resistant even at 500 μg/ml of TPS (Table I).

Results of bactericidal and fungicidal activity of TPS on the selected organisms that were TPS sensitive are shown in Table II. There was no

observable growth by all organisms after 10 h of cultivation. The partially purified membrane bound pyrophosphatase(s) from the organisms was assayed in the absence and presence of 5, 10 and 25 μM TPS. Figure 1 is a representative plot of initial velocity plot for *Escherichia coli* pyrophosphatase with increasing substrate concentration under the given conditions. The plot indicates a dose-dependent pattern for inhibition by TPS.

Dialysis of the Enzyme–Inhibitor complex (E–TPS) did not restore the activity of the enzyme (Figure 1).

Kinetic analysis of the inhibition revealed a non-competitive patterns. (Figures 2–5). Computed inhibition binding constants, K_i, at 5 μM TPS (10 μM) against pyrophosphatase from *Escherichia coli*, *Shigella flexneri*, *Aspergillus niger* and *Aspergillus fumigatus* were 7.6 (4.6), 18 (17), 8.8 (6.6) and 6.9 (5.0) μM respectively.

By raising the inhibitor level from 5–10 μM, the percent inhibition increased about two fold (34–65%), (17–35%) and (35–60%) for *Escherichia coli*, *Shigella flexneri* and *Aspergillus niger* respectively. In the case of *Aspergillus fumigatus* the inhibition quadrupled (17–68%) from the two fold increase in the concentration of TPS.

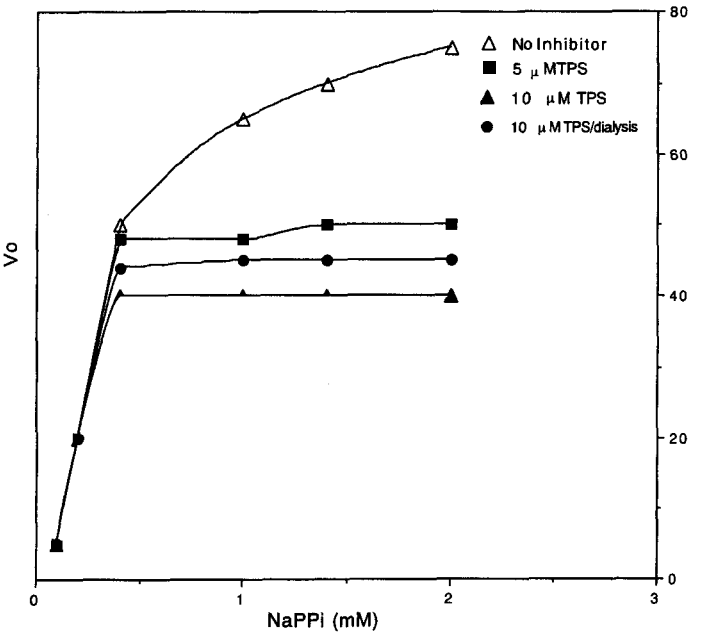

FIGURE 1 Initial velocity plot of V_0 versus NaPPi (mM) for *Escherichia coli* pyrophosphatase in the presence of TPS. The activity of the enzyme is expressed in terms of the production of inorganic phosphate Pi μmol/h. Results are the average of three experiments.

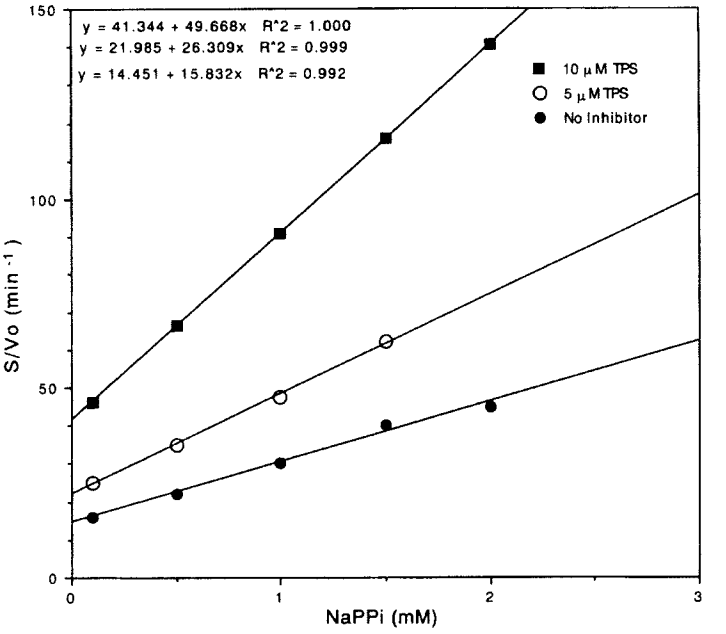

FIGURE 2 Hanes Wolf Plot of S/V_0 versus S for *Escherichia coli* in the presence and absence of TPS. Results are the average of three experiments.

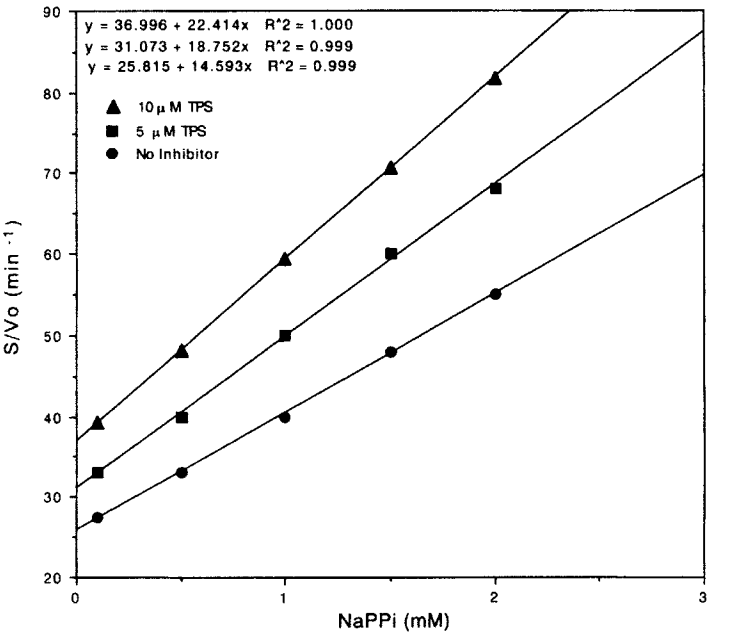

FIGURE 3 Hanes Wolf Plot of S/V_0 versus S for *Shigella flexneri* in the presence and absence of TPS. Results are the average of three experiments.

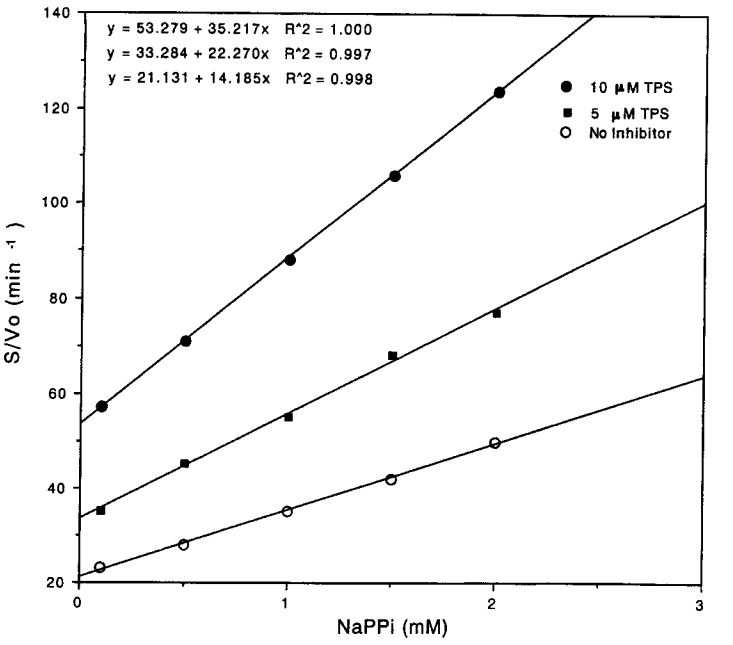

FIGURE 4 Hanes Wolf Plot of S/V_0 versus S for *Aspergillus niger* in the presence and absence of TPS. Results are the average of three experiments.

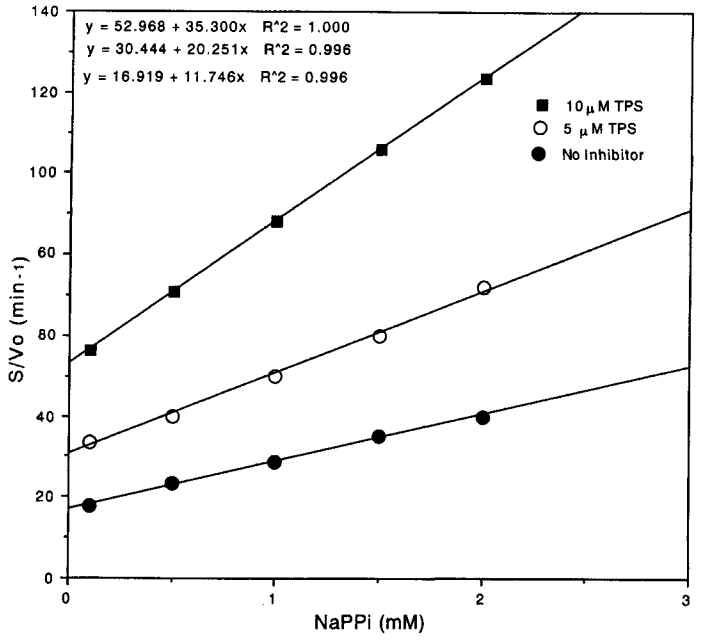

FIGURE 5 Hanes Wolf Plot of S/V_0 versus S for *Aspergillus fumigatus* in the presence and absence of TPS. Results are the average of three experiments.

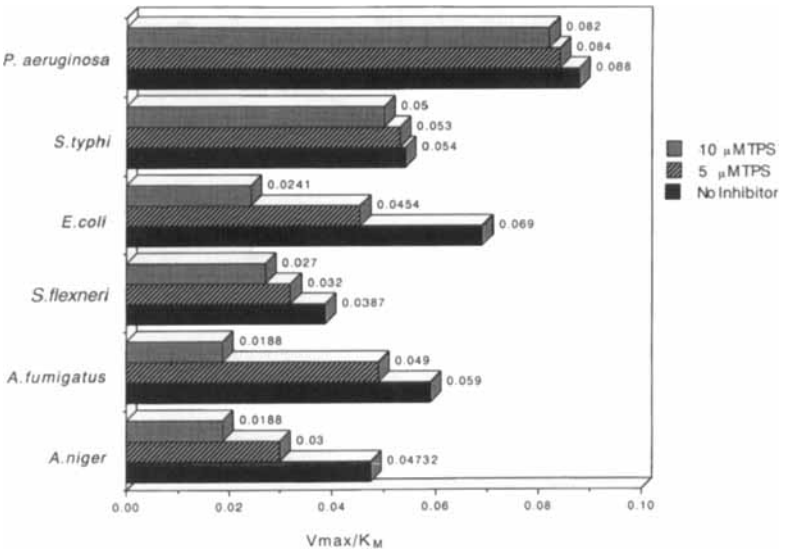

FIGURE 6 Effect of TPS on bacterial and fungal PPase index of physiological efficiency V_{max}/K_M. Results are the average of three experiments.

The index of physiological efficiency (V_{max}/K_M) for the organisms with respect to TPS is shown in Figure 6. While the *Escherichia coli* pyrophosphatase efficiency is reduced by about three fold at 10 μM of TPS, the pyrophosphatases of the TPS-insensitive *Salmonella typhi* and *Pseudomonas aeruginosa* were unaffected. Similarly, amongst the fungi group, this ratio for the pyrophosphatases from *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigatus* was reduced by about two fold, but unchanged in the TPS-insensitive fungi.

DISCUSSION

The organotin compound TPS has been shown to possess growth inhibitory properties on some bacteria and fungi. Experiments conducted at a level of up to 5 μg/ml proved very effective on most of the microorganisms that were susceptible to the compound.

The mode of action of organotin compounds is the inhibition of oxidative phosphorylation so arresting ATP synthesis as well as promoting anion–hydroxide exchange reaction across mitochondrial inner membrane.[8]

Reports have shown that the mechanism of the inhibition of ATP synthase by organotin is multisite and differs from that of oligomycin which is unisite.[9,10]

Our data suggested that the ability of TPS to inhibit the pyrophosphatases amongst the different organisms tested was related to the organism's susceptibility to TPS. The efficiency of pyrophosphatase enzyme of the sensitive *Escherichia coli*, *Aspergillus flavus*, *Aspergillus fumigatus* was reduced in the presence of 5 and 10 μM of TPS by one to three fold. Their respective activities were considerably reduced by 17–68%. However in the moderately susceptible organisms *Salmonella typhi*, and *Staphylococcus* spp. their pyrophosphatase enzymes were unaffected by the compound. The failure of dialysis of the Enzyme–Inhibitor complex (EI) in excess of Na-PP to reverse the inhibition suggests strong covalent interaction as the basis of inhibition.

TPS resistance by these organisms could imply that their pyrophosphatases are protected or modified thereby making their binding sites unavailable to TPS. Other factors could involve hydrophobic interactions of some amino acids and other cell wall components that are critical to the oligomeric stability of the enzyme.

The resistance of some microorganisms to some organotins have been linked to heavy metals toxicity resistance.[1] Whether this phenomenon applies to the TPS-resistant organisms described here remains to be elucidated.

Inorganic pyrophosphatase is an enzyme essential for the synthesis of steroids and ATP. It is a highly efficient catalyst for reversible phosphoryl transfer from the simplest phosphoric acid anhydride, pyrophosphate, to water.[11] During β-oxidation in the metabolism of fatty acid, the activation of acetyl CoA is driven by the hydrolysis of ATP to generate AMP and PP. The latter is split to form 2Pi by the enzyme making the subsequent reaction thermodynamically favourable.

Resistance to TPS implies this process is not compromised and ATP and sterol synthesis needed for cell division and growth proceeds. This is seen in the uninterrupted and spontaneous growth of *Salmonella typhi*, *Staphylococcus aureus*, *Rhodoturolla* spp. even at high levels of TPS. Furthermore, the uninhibited growth of *Shigella flexneri* and *Aspergillus niger* cultured in the absence of TPS is an attestation to the microcidal efficacy of TPS.

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