5-Hydroxy-3-vinyl-2(5H)-furanone – a New Inhibitor of Human Synovial Phospholipase A_2 and Platelet Aggregation from Fermentations of a *Calyptella* Species (Basidiomycetes)

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5-Hydroxy-3-vinyl-2(5H)-furanone, a potent and selective inhibitor of human synovial phospholipase A_2 was isolated from fermentations of a *Calyptella* species. Its structure as identified by spectroscopic methods is identical to PA 147, an antibiotic previously isolated from a streptomycete. 5-hydroxy-3-vinyl-2(5H)-furanone inhibits the aggregation of human and bovine platelets stimulated by different inducers and exhibits weak antimicrobial activities.

Introduction

It is generally accepted that platelets play a major role in the pathogenesis of several vascular disorders as myocardial infarction and arteriosclerosis (Holmsen, 1982). Their receptors for different agonists as well as the following pathways of signal transduction offer a number of interesting pharmacological targets e.g. phospholipases A_2 (PLA_2) and C (PLC), proteinkinase C (PKC) and adenylate cyclase (ADC). PLA_2 of platelets is one central enzyme in the cascade of signals leading to complete aggregation (Kramer *et al.*, 1989).

Arachidonic acid liberated by PLA_2's of different tissues serves as a precursor of prostaglandines, leukotrienes and thromboxanes, mediators that play a central role in diseases like arthritis or inflammation (Decker, 1991).

In the course of a screening several hundred extracts derived from submerged cultures of basidiomycetes were tested for the presence of inhibitors of collagen-induced aggregation of bovine platelets.

In the following we describe the fermentation, isolation, structure elucidation and new biological properties of 5-hydroxy-3-vinyl-2(5H)-furanone **1** from fermentations of *Calyptella* sp. 9039.

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Material and Methods

General

IR and UV spectra were measured with a Bruker ISF 48 and a Perkin-Elmer Lambda 16 UV/VIS spectrometer, respectively. For analytical HPLC a Hewlett-Packard 1090 series II instrument was used.

All NMR spectra were recorded on a Bruker AMX500 spectrometer working at 500.14 MHz for ^1H and at 125.77 MHz for ^{13}C. The solution of 5-hydroxy-3-vinyl-2(5H)-furanone (**1**) was 24 mM in D_2O.

High resolution mass spectra were measured with a Finnigan MAT 312 spectrometer (ion energy 70 eV, source temperature 250 °C, acc. volt 3 kV).

For LC coupled thermospray mass spectroscopy (LC-TSPMS) a Finnigan MAT TSQ45 instrument was used (source temperature 140 °C, nebulizer temperature 140 °C, repeller 130 V; MS/MS: collision gas N_2, collision energy 5.1 V; chromatographic conditions: HPLC: LiChropher RP 18, 10 µm, Merck, column 250×4 mm, water–methanol–trifluoroacetic acid (85:15:0.05), flow rate 1.2 ml/min).

Calyptella sp. strain 9039

Calyptella sp. strain 9039 was isolated from the spore print of a fruiting body collected on wood in Mossman/Cairns, Australia. The specimen

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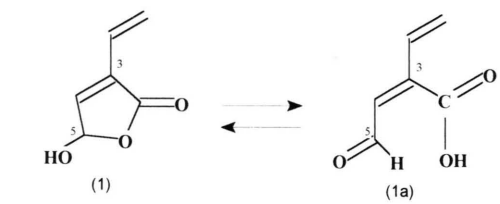
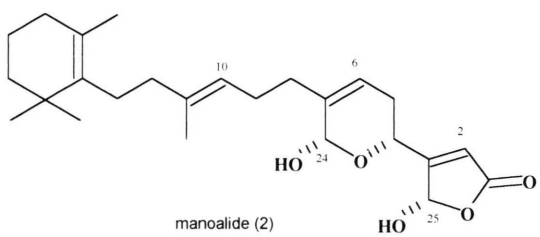
showed all characteristics of the genus *Calyptella* (Singer, 1986) (Agaricales, Tricholomataceae, Collybieae), the species, however, could not be identified. Herbarium specimen and mycelial cultures are deposited in the culture collection of the LB Biotechnologie, University of Kaiserslautern. For maintenance on agar slants the fungus was grown on YMG medium (yeast extract 0.4 %, malt extract 1.0 %, glucose 0.4 %, pH 5.5).

Fermentation and isolation of 5-hydroxy-3-vinyl-2(5H)-furanone (**1**)

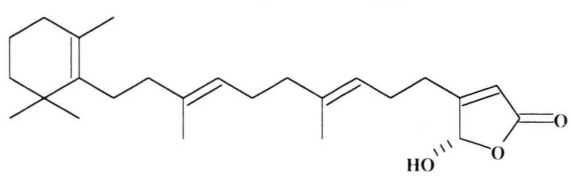
Fermentations were carried out in a Biostat U fermentor equipped with a MFCS system (B. Braun Biotech) containing 100 l of YMG medium at 135 rpm, 20 liters air/min and 22 °C. The content of oxygen in the medium and CO_2 and oxygen in the exhaust were determined on-line. During fermentation and in fractions during chromatography the content of **1** was measured by analytical HPLC (LiChrosorb RP-18, 5 μm, Merck, column 4×125 mm; 1.5 ml/min, 40 °C; H_2O : MeOH 0 → 100% in 20 min; retention time 4 min) and by platelet aggregation assay.



5-hydroxy-3-vinyl-2(5H)-furanone



manoalide (2)



luffariellolide (3)

After 70 hours the oxygen content of the medium was limited by reducing the stirrer speed to 90 rpm and the aeration to 4 litres air/min. After 116 hours of fermentation the mycelia were separated from the culture fluid by filtration. **1** was removed from the culture fluid (95 l) by adsorption to HP21 resin (Mitsubishi) and eluted with acetone. The crude extract (12.86 g) was subjected to gel permeation chromatography (LiChrogel PS1, 7 μm, Merck, column 250×25 mm, elution with 2-propanol) resulting in 2.14 g of enriched product containing approximately 25 % **1**. Pure **1** was obtained by isocratic HPLC (LiChropher RP 18, 10 μm, Bischoff, column 250×20 mm) using water–methanol–trifluoroacetic acid (85:15:0.05); retention time 26 min.

Biological assays

The platelet aggregation assay and the test for inhibitory effects on the synthesis of macromolecules was carried out as reported previously (Lorenzen *et al.*, 1994). Further tests as for cytotoxic effects, hemolytic activity and the antimicrobial activity in the serial dilution assays are described elsewhere (Zapf *et al.*, 1995). The inhibition of phospholipases A_2 from snake venom was tested as described by Nieuwenhuizen *et al.* (1974). The tests for inhibitory effects of **1** on human synovial phospholipase A_2, human pancreatic phospholipase A_2 and human cytosolic phospholipase A_2 were performed according to Scheuer *et al.* (1989) and to Rodewald *et al.* (1994).

Results and Discussion

Fermentation and isolation

Fig. 1 shows a typical fermentation of *Calyptella* sp. 9039 in 100 liters of YMG-medium. After 186 hours the content of 5-hydroxy-3-vinyl-2(5H)-furanone **1**, as detected by analytical HPLC, reached a maximum of 6.5 mg/liter and **1** was isolated as described in the experimental section.

5-Hydroxy-3-vinyl-2(5H)-furanone (**1**)

The highly unstable 5-hydroxy-3-vinyl-2(5H)-furanone (**1**) was obtained as slightly yellowish oil soluble in methanol, acetone or ethyl acetate, and moderately soluble in water. At room temperature **1** undergoes rapid polymerisation forming a com-

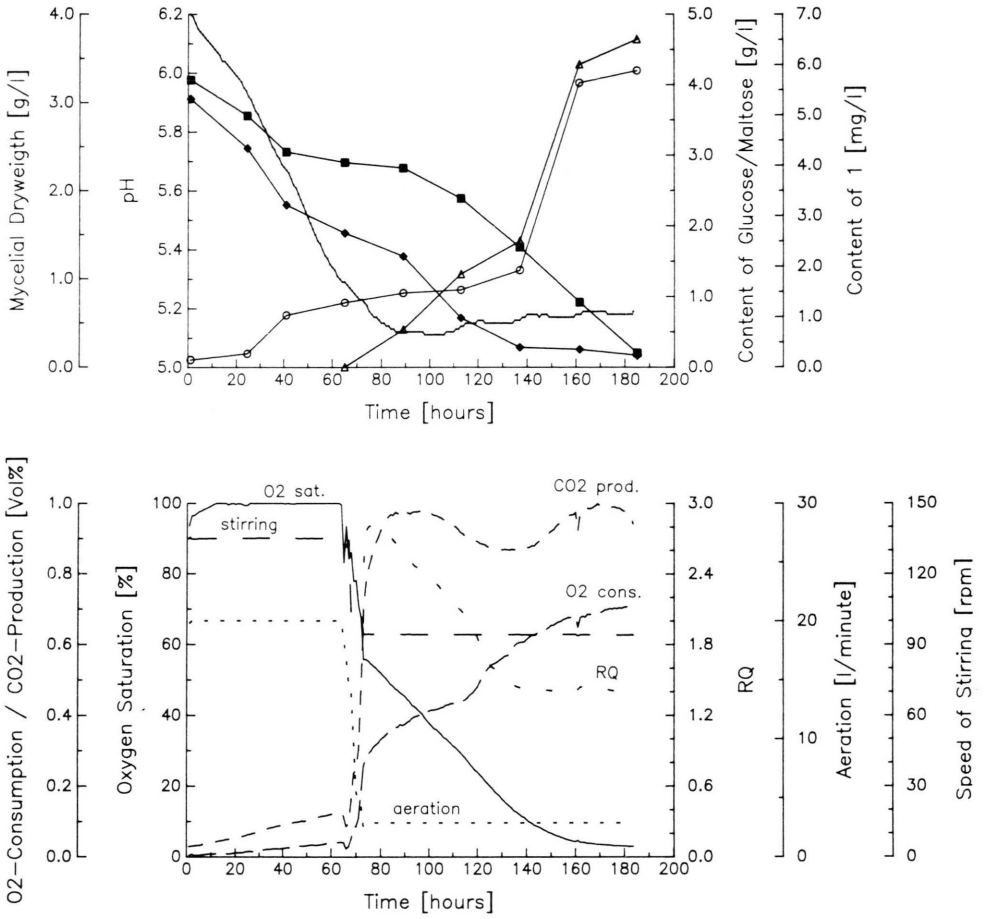


Fig. 1. Fermentation of *Calyptella* sp. TA 9039 in 100 l of YMG-medium. ◆, Content of glucose; ■, content of maltose; —, pH; ○, mycelial dry weight; △, content of **1.**

pletely insoluble polymer. Depending on the pH the 5-hydroxy-3-vinyl-2(5H)-furanone ring of **1** opens to *1a*, the predominant species in neutral methanol. The physico-chemical data of **1** (Table I) prove that the compound isolated from *Calyptella* sp. 9039 is identical to 5-hydroxy-3-vinyl-2(5H)-furanone, first isolated by Els *et al.* (1958) from a *Streptomyces* strain and published as antibiotic PA 147 (Els *et al.*, 1958). **1** is the first metabolite isolated from a *Calyptella* species.

From other authors a synthesis of **1** (Black *et al.*, 1973) starting from prop-2-ynyl vinyl ether has been described. It is intersting that the hydroxy furanone moiety of *1* also occurs in the structure of the well known PLA_2-inhibitors manoalide (**2**) and luffariellolide (**3**) which have been isolated from several marine sponges (Silva *et al.*, 1980; Potts and Faulkner, 1992).

Biological activities

The inhibitory effect of **1** on the thrombin-induced aggregation of bovine platelets is shown in Fig. 2. A complete inhibition of aggregation was obtained by 50 μg/ml (396 μM) of 5-hydroxy-3-vinyl-2(5H)-furanone, the IC_{30} value (30 % inhibition) of **1** was determined to 9 μg/ml (71 μM). The first, reversible phase, was hardly affected, the second irreversible phase of aggregation was inhibited by concentrations above 10 μg/ml (79 μM).

Figure 3 shows the influence of **1** on the thrombin-induced aggregation of human platelets. The

Table I. Physicochemical properties of 5-hydroxy-3-vinyl-2(5H)-furanone (**1**).

Appearance	yellowish oil
Molecular formula	$C_6H_6O_3$
IR ν_{max} (KBr) cm^{-1}	3400 (OH), 1750 (C=O), 1640 (C=C)
UV $\lambda_{max}^{0.1\ N\ HCl}$ nm (ε)	240 (10,900)
UV λ_{max}^{MeOH} nm (ε)	272 (17,800) ⇒ **1a**
LC-TSPMS *(m/e)*	127 (17%) MH^+, 159 (100%) $[MH+MeOH]^+$, 173 (15%) $[MH+2MeOH-H_2O]^+$, 191 (15%) $[MH+2MeOH]^+$
MS/MS *(m/e)*[a]	191 → 159, 127; 159 → 127, 109 $[MH-H_2O]^+$; 127 → 109 ⇒ 126 Da
HR-EIMS *(m/e)*[b]	468 (rel. int. 1%) M^+ (tri-TMS derivative of the dimer); 378.1305 (rel. int. 8%) → $C_{18}H_{26}O_5Si_2$ (D 3.7 ppm) $[M-HOTMS]^+$ molecular formula of dimer: $C_{12}H_{12}O_6$ (252 Da)
^1H NMR (500 MHz, D_2O)	δ 7.28 (s, H-4), 6.48 (dd, H-6, J = 11.4 Hz, 17.8 Hz), 6.24 (s, H-5), 6.16 (d, H-7 (Z), J = 17.8 Hz), 5.59 (d, H-7 (E), J = 11.4 Hz)
^{13}C NMR (125 MHz, D_2O)	δ 174.3 (C-2), 147.1, 133.79, 126.61, 124.63, 99.11 (C-5)
TLC R_f value (silica gel, Merck), toluene–acetone (70:30)	0.43
Cyclohexane–ethyl acetate (50:50)	0.30

[a] Parent ion → daughter ions.
[b] After TMS derivatization.

Table II. IC_{30} values of **1** for the inhibition of aggregation of human and bovine platelets stimulated by different inducers.

Inducer	IC_{30} value[a] [μM] Bovine platelets	Human platelets
Collagen (0.3 mg/ml)	142	198
ADP (2.5 μM)	396	198
Thrombin (0.1 U/ml)	71	79
Ristocetin (0.4 mg/ml)	–	198
Arachidonic acid (0.6 μg/ml)	–	59.5
U 46619 (0.45 μM)[b]	–	158

[a] 30% inbibition of aggregation.
[b] Thromboxane A_2 analogue (UpJohn).
– Inducer not suitable.

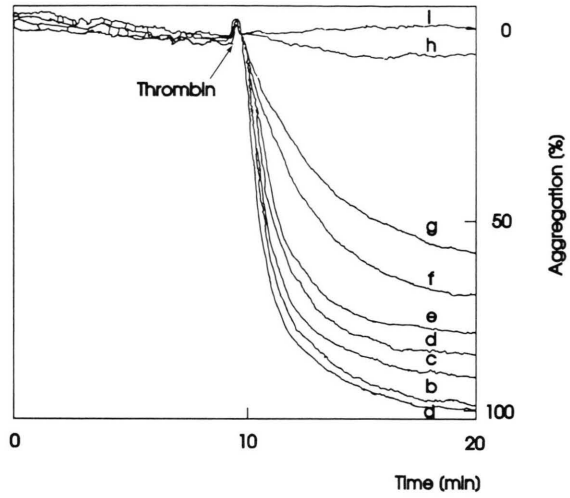


Fig. 2. Effect of **1** to the thrombin-induced aggregation of bovine platelets (thrombin 0.1 U/ml). (a) Control, (b) 7.9 μM, (c) 39 μM, (d) 48.5 μM, (e) 71 μM, (f) 79 μM, (g) 158 μM, (h) 276 μM, (i) 396 μM.

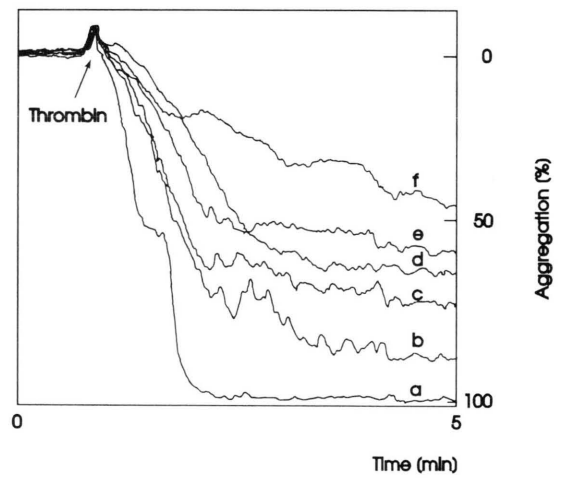


Fig. 3. Effect of **1** to the thrombin-induced aggregation of human platelets (thrombin 0.1 U/ml). (a) Control, (b) 39 μM, (c) 79 μM, (d) 158 μM, (e) 396 μM, (f) 793 μM.

IC_{30} was determined to 10 μg/ml (79 μM), a complete inhibition could not be observed at concentrations up to 100 μg/ml (793 μM).

The effects of **1** on the aggregation of human and bovine platelets stimulated with different inducers are compared in Table II. **1** inhibited preferentially the aggregation induced by thrombin and arachidonic acid (AA). In the latter case AA can act both as stimulator of PLA_2 and substrate

for cyclooxygenase. The IC_{30} values of **1** for the other inducers are significantly higher. A specific interference of **1** with one of the receptors for the agonists seems unlikely.

The receptor for thrombin couples directly to PLA_2 (Lapetina *et al.*, 1990). Therefore an inhibition of membrane bound PLA_2 of platelets seemed possible. If this were true, **1** would interfere with the stimulatory activity of AA under the the chosen conditions.

With this in view, the inhibitory activity of **1** was tested against PLA_2's from *Naja mosambique* (type I), *Crotalus atrox* (type II), U937 cells (type II), human pancreas (type I) and human synovial fluid (type II). The IC_{50} values presented in Table III show a preferential inhibition of human synovial PLA_2 at nanomolar concentrations, a value which is in good accordance with the inhibition of PLA_2's (bee venom, cobra venoms, human synovial fluid) observed with manoalide (**2**) and luffariellolide **3** (Potts and Faulkner, 1992).

1, **2**, and **3** differ from each other by the length and position of the hydroxy furanone side chain. **2** and **3** are potent inhibitors of several phospholipases A_2 by forming a Schiff base (imine) between the aldehyde group of the open hydroxybutenolide ring and lysine residues not located in the active centre of the enzymes (Potts *et al.*, 1992).

Because of the described blockage of Ca^{2+} channels by **2** and **3**, the effect of **1** on the secretion of Ca^{2+} by stimulated platelets was tested with the fluorescence indicator Quin-2-acetoxymethylester (Tsien *et al.*, 1982; Hallam *et al.*, 1984). For **1** no decrease of intracellular Ca^{2+} after stimulation of platelets was observed.

Table III. Inhibition of phospholipases A_2 from different sources by **1**.

PL A_2	IC_{50} value [μM]
Human synovial PL A_2, type II	0.3
Human pancreatic PLA_2, type I	32
Human cytosolic PL A_2 from U 937 cells, type II	<793
PL A_2 from *Naja mosambique*, type I	24
PL A_2 from *Crotalus atrox*, type II	39

Other biological activities

Els *et al.* had reported antibacterial (*Staphylococcus aureus*, *Xanthomonas oryzae*) and cytotoxic (HeLa) effects for PA 147 **1** (Els *et al.*, 1958). In mice bearing Ehrlich carcinoma a pronounced antitumor activity was observed.

The cytotoxic activities of **1** against BHK-, HeLa S3-, L 1210-, HL 60- and U 937-cell cultures were tested as described previously (Zapf *et al.*, 1995). A lytic action of **1** on BHK- and HL 60-cells could be observed at concentrations higher than 20 μg/ml (158 μM), while HeLa S3 cells were affected by concentrations starting from 50 μg/ml (396 μM) (Table IV). With L 1210- and U 937 cells a weak inhibition of cell growth at concentrations of 100 μg/ml (793 μM) was observed.

The incorporation of ^{14}C-labeled thymidine, uridine and leucine into DNA, RNA and proteins of HL 60 cells was tested as described previously (Lorenzen *et al.*, 1994). Figure 4 shows a preferen-

Table IV. Cytotoxic activities of **1** on different cells.

Cells		IC_{100}[a] [μM]
BHK 21	ATCC CCL 10	158
HeLa S3	ATCC CCL 22	396
L 1210	ATCC CCL 219	>793[b]
HL 60	ATCC CCL 240	158
U 937	ATCC CRL 1593	>793[b]

[a] Complete lysis of cells.
[b] Inhibition of growth at this concentration detectable.

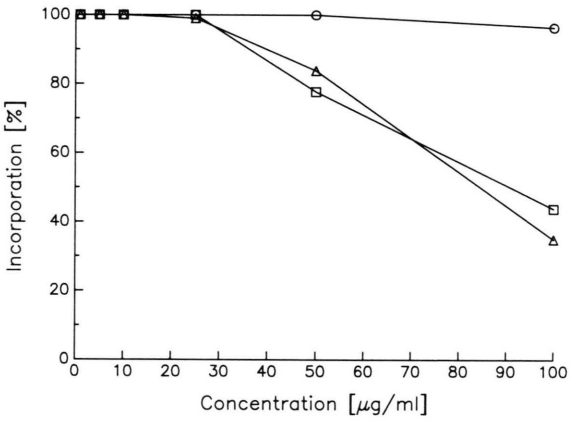


Fig. 4. Effect of **1** to the incorporation of ^{14}C-labeled precursors in macromolecules of HL 60 cells. ○, ^{14}C-labeled uridine, control 6354 cpm; □, ^{14}C-labeled thymidine, control 935 cpm; △, ^{14}C-labeled leucine, control 3596 cpm.

tial inhibition of protein and DNA syntheses by 5-hydroxy-3-vinyl-2(5H)-furanone at concentrations starting from 50 μg/ml (396 μM). The incorporation of ^{14}C-labeled uridine into RNA was not effected. The complete lack of hemolytic action of **1** on bovine erythrocytes at concentrations up to 100 μg/ml (793 μM) renders a direct action on the cytoplasmic membrane unlikely.

The antifungal and antibacterial effects of 5-hydroxy-3-vinyl-2(5H)-furanone were determined in the serial dilution assay (Table V). The organisms most sensitive to **1** were the yeast *N.* coryli and the bacteria *M. luteus* and *S. thyphimurium*, whereas other bacteria and fungi were less sensitive.

In the "pour-plate-test" for mutagenicity (without microsomes) with four strains of *S. thyphimurium* (TA 97, TA 98, TA 100 and TA 102) (Ames *et al.*, 1975) no increase of the number of revertans could be observed at concentrations from 1–20 μg/ml (8–158 μM) of 5-hydroxy-3-vinyl-2(5H)-furanone.

In conclusion **1** appears to be a highly potent and selective inhibitor of human synovial phospholipase A_2. The selectivity towards this enzyme is much higher as compared to manoalide (**2**) and luffariellolide (**3**).

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Table V. Minimal inhibitory concentration (MIC) of **1** in the serial dilution assay.

Organism		MIC [μM]
Bacteria		
Acinetobacter calcoaceticus	DSM 30005	>793
Escherichia coli K12	(BT KL)	>793
Salmonella thyphimurium TA 98	(Dr.N.B. Ames)	79 z
Athrobacter citreus	ATCC 11624	>793
Bacillus brevis	ATCC 9999	793 s
Bacillus subtilis	ATCC 6633	>793
Corynebacterium insiduosum	ATCC 10253	>793
Micrococcus luteus	ATCC 381	7.9 z
Mycobacterium phlei (Lehmann & Neumann, KL)		793 s
Streptomyces spec.	ATCC 23836	>793
Fungi		
Nadsonia fulvescens	ATCC 10645	>793
Nematospora coryli	ATCC 10647	79 z
Saccaromyces cerevisiae is 1		>793
S. cerevisiae S 288 c (Prof. Lacroute, Straßburg)		>793
Fusarium oxysporum	CBS 149.25	>793
Paecilomyces variotii	ETH 114646	>793
Penicillium notatum	(BT KL)	793 z
Mucor miehei	TÜ 284	>793
Rhodotorula glutinis	ATCC 26086	>793
Ustilago nuda	CBS 118.19	>

s, Bacterio-/fungistatic effects.
z, Bacterio-/fungizide effects.

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