Gopalamicin, an Antifungal Macrodiolide Produced by Soil Actinomycetes

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Gopalamicin, an antifungal antibiotic, was isolated from the cells of two actinomycetes, MSU-625 and MSU-616. Gopalamicin is structurally similar to salbomycin and elaiophylin. Gopalamicin completely inhibited the growth of all pathogenic fungi tested in vitro at 12–16 ppm. Similarly, it was somewhat effective in controlling wheat powdery mildew, grape downy mildew, and rice blast pathogens in greenhouse experiments. Gopalamicin was ineffective against all Gram-positive and -negative bacteria tested.

Keywords: *Antifungal; protective; curative; wheat; grape; rice; cucumber; green pepper; rice; barley; gopalamicin; salbomicin; elaiophylin; Botrytis cinerea; Erysiphe cichoracearum; Erysiphe graminis tritici; Fusarium culmorum; Plasmopara viticola; Puccinia recondita; Pyricularia oryzae; Pyrenophora teres.*

INTRODUCTION

Our continued search for antifungal compounds from soil-borne microorganisms for plant protection has resulted in discovery of a macrodiolide, gopalamicin, with broad spectrum antifungal activities. Gopalamicin was found to be identical to elaiophylin or salbomycin (Arai, 1960a,b; Arcamone et al., 1959; Kaiser and Keller-Schierlein, 1981; Fielder et al., 1981; Paulus et al., 1984) from its ^{1}H- and ^{13}C-NMR and X-ray crystal structure and showed novel antifungal activity.

MATERIALS AND METHODS

Fermentation, Isolation, and Purification. Michigan State University varieties of *Streptomyces hygroscopicus*, MSU-625 and MSU-616, were grown on YMG plates (yeast extract, 4 g; malt extract, 10 g; glucose, 4 g; agar, 18 g; in 1 L of distilled H_2O) for 7 days at 26 °C. Agar plugs from these plates were inoculated into 500 mL baffle-bottom Erlenmeyer flasks containing 100 mL of liquid YMG medium (yeast extract, 4 g; malt extract, 10 g; glucose, 4 g; in 1 L of distilled H_2O). All media were sterilized for 20 min at 120 °C and 17 atm of pressure prior to inoculation. The inoculated flasks (eight) were placed on a rotary shaker (150 rpm) at 26 °C. After 7 days, the cultures (800 mL) were inoculated into 2 L baffle-bottom flasks (15), each containing 400 mL of A-9 medium (peptone, 4 g; glucose, 10 g; Brer Rabbit (green label) molasses, 15 g; in 1 L of distilled H_2O). The inoculated flasks were placed on a rotary shaker at 150 rpm at 26 °C for 7 days. Larger batches were grown in a 130 L fermenter containing 100 L of A-9 medium, aerated at 100 L/min, and stirred at 100 rpm at 26 °C. The 6 L of A-9 fermentation broth was used as the inoculum. After 5 days of fermentation, the culture broth was centrifuged and the mycelial cake homogenized with MeOH and filtered. The MeOH extract was evaporated to dryness under reduced pressure, and the resulting crude was purified by vacuum liquid chromatography (VLC) and thin layer chromatography (TLC; Si gel, $CHCl_3$/MeOH 6:1 v/v, R_f 0.35). The active fraction was recrystallized from hot MeOH to afford colorless crystals of gopalamicin. The yields of gopalamicin from MSU-616 and MSU-625 were 83 and 110 mg/L of culture broth, respectively.

Gopalamicin. $C_{54}H_{88}O_{18}$·2MeOH; mp 156–157 °C; UV λ_{max} (MeOH) 253 (ϵ 67 680), 209 (ϵ 14 342) nm; MS (FAB) *m/z* (% int, MF) 1048 (67, $C_{54}H_{88}O_{18}$ + Na), 649 (2, $C_{35}H_{53}O_{11}$, $M^+ - C_{19}H_{35}O_7$), 635 (0.5, $C_{34}H_{51}O_{11}$), 555 (10, $C_{29}H_{47}O_{10}$), 541 (27, $C_{28}H_{45}O_{10}$), 523 (20, $C_{28}H_{43}O_9$), 417 (4, $C_{22}H_{41}O_7$), 403 (25,$C_{21}H_{39}O_7$), 389 (100, $C_{20}H_{37}O_7$), 273 (5), 209 (23), 195 (40), 177 (100), 137 (50), 131 (58), 121 (35), 113 (100); ^{1}H- and ^{13}C-NMR data (Table 1).

Table 1. ^{1}H- and ^{13}C-NMR Chemical Shifts for Gopalamicin in $CDCl_3$

atom	H	$^3J_{HH}$ (ppm)	C
1,1′			169.91
2,2′	5.62		120.93
3,3′	6.99	11.5	145.00
4,4′	6.14	12	131.95
5,5′	5.68		144.31
6,6′	2.18	7.5, 12	41.58
7,7′	5.06		77.83
8,8′	1.8		35.88
9,9′	4.10		70.58
10,10′	1.62		41.63
11,11′			99.03
12,12′	2.50		40.80
13,13′	3.90		66.02
14,14′	1.60		48.38
15,15′	3.85		65.93
16,16′	1.50	6.1	19.34
17,17′	1.45	6.1	14.88
18,18′	0.81	6.5	8.70
19,19′	1.0	6.5	7.02
20,20′	1.43		19.11
21,21′	0.85	5, 2.5	9.05
1″,1‴	5.24		93.21
2″,2‴	1.78	3.5	33.49
3″,3‴	3.85		65.87
4″,4‴	3.60		71.44
5″,5‴	3.85		66.53
6″,6‴	1.60		16.78

Circular Dichroism (CD) of Gopalamicin. The experimental conditions were as follows: scan mode, wavelength; bandwidth, 2 nm; sensitivity, 5 mdeg; response, 4 s; wavelength range, 200–300 nm; step resolution, 1 nm/data; scan speed, 20 nm/min; and accumulation, 1 (Figure 1).

X-ray crystal structure was determined on a Siemens P4 instrument: crystal size, 0.30, 0.32, 0.15 mm; crystallization solvent, MeOH; lattice constants, a = 9.8366 (010), b = 10.0620 (012), c = 31.1387 (034) Å; α = 90 (00)°, β = 93.2831 (07)°, γ

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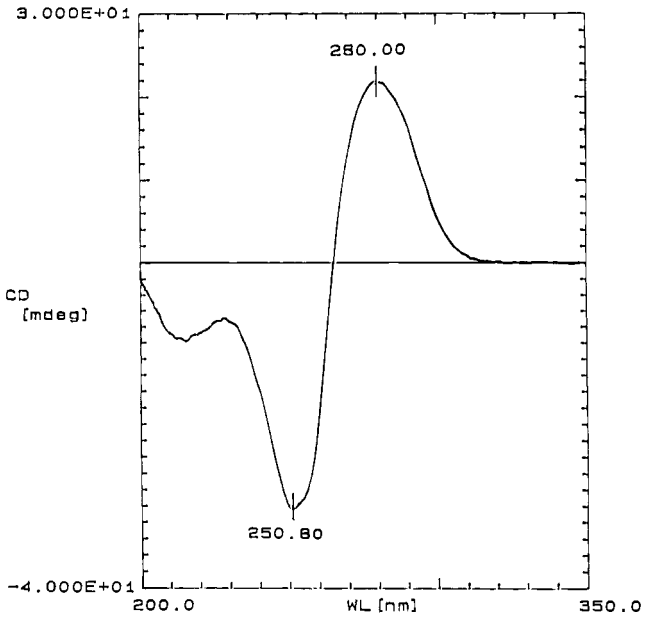

Figure 1. CD curve of gopalamicin.

Table 2. In Vitro Antifungal Activity of Gopalamicin

organism	MIC (μg/mL)	organism	MIC (μg/mL)
Aspergillus fumigatus	16	*Fusarium oxysporum*	16
Aspergillus flavus	16	*Fusarium moniliforme*	16
Aspergillus niger	16	*Pythium ultimum*	12
Candida albicans	16	*Phialophora graminicola*	12
Alternaria solani	16	*Leptosphaeria korrae*	12

= 90 (00)°; volume, 3077.09 Å^3; space group, P_21 (International Table No. 4); molecules per unit cell, 2; per asymmetric unit, 1; density, 1.176 g/cm^3 (calcd); molecular weight, 1089.31; radiation for measurement, Cu Kα, monochromatic; scan type, theta/2theta; scan range, 2–115°; step-scan data, profile analysis applied; temperature during measurement, 203 K; measured deflections, 5884; unique reflections, 4886; sif(F) cutoff, 4*sig(F).

Antimicrobial Bioassay. In vitro MIC determination of gopalamicin for various fungi was carried out as published earlier (Nair et al., 1992). The cell suspension cultures of the test organisms were prepared in Emmons liquid medium (neopeptone 10 g, glucose 20 g in 1 L of distilled H_2O, pH 6.8) to obtain a final concentration of 10^3 colony forming units (cfu)/mL. An equal volume of uninoculated culture medium was used as a control. A known amount of gopalamicin was dissolved in DMSO, and serial dilutions were prepared in the same solvent. A 20 μL aliquot of each solution was mixed with 2 mL of Emmons liquid medium seeded with 10^3 cfu/mL of the test organisms. Also, the culture medium was mixed with 20 μL of DMSO to serve as another control. The inoculated tubes were vortexed and incubated at 26 °C. Depending on the growth characteristics of the test organisms, results were recorded after 2–4 days. The lowest concentration of the gopalamicin that totally inhibited the growth of the organisms was recorded as minimum inhibitory concentration (MIC) for that species (Table 2).

Screening of Antifungal Activity in the Greenhouse. Suitable plants, prone to fungus infection, were reared in the greenhouse and treated with the compounds prior to (protective) or after (curative) infection with test fungi. After 8 days of growth of the plants, aqueous zoospore suspensions of the test fungi were sprayed on the plants which were then kept for 2 days at 20–23 °C and high relative humidity. After the given time, the plants were rated for the degree of infection from 0 (total infection) to 8 (no infection) (Table 3). The plants were subsequently kept for 5 days in the greenhouse and again at high relative humidity and 20–23 °C for 16 h before being visually rated for disease control (Table 3).

RESULTS AND DISCUSSION

^{13}C-NMR shift values for gopalamicin in $CDCl_3$ are very similar to the chemical shift values reported for elaiophylin in pyridine (Hammann and Kretzschmar, 1991); the observed differences are due only to the solvent effect. Gopalamicin, like elaiophylin, gave only 27 signals in its ^{13}C NMR and half the proton signals are due to C_2 symmetry in the molecule (Table 1). Gopalamicin is chiral and crystallized in a chiral crystal space group where only one of the two possible configurations is possible. An attempt was made to determine the absolute configuration of gopalamicin by comparison with the published X-ray results of salbomicin (Paulus et al., 1984), and its configuration was confirmed as 6,6′*S*;7,7′*S*;8,8′*S*;9,9′*R*;10,10′*S*;11,11′*R*;13,13′*R*;14,14′*S*;15,15′*R*;1″,1‴*R*;3″,3‴*S*;4″,4‴*S*;5″,5‴*S* (Figure 2). It is similar to the known salbomicin or elaiophylin.

Biogenesis of elaiophylin was determined by Gerlitz et al. (1992) using ^{13}C-labeled precursors. They found that acetate, propionate, butyrate, and glucose are required for the biosynthesis of elaiophylin. Our preliminary feeding study with [^{13}C]-1-acetate was achieved by the addition of 200 mg/400 mL of [^{13}C]-1-acetate (Aldrich Chemical Co., Milwaukee, WI) successively during the fermentation of MSU-616/MSU-625 at 24, 48, and 72 h. The antibiotic was isolated and purified as before. Comparison of ^{13}C-NMR spectra of natural and enriched gopalamicins from MSU-616/MSU-625 indicated that the macrodiolide ring and the side-chain moieties in gopalamicin had incorporation from the acetate precursor (data not shown). Additional feeding experiments with propionate, butyrate, and glucose are required to confirm this observation.

Gopalamicin showed good antifungal activities against a variety of plant pathogens under laboratory conditions (Table 2). Further evaluation of this compound for fungal disease management in crop plants was carried out in the greenhouse on plants infected with the respective pathogen (Table 3). The best efficacy was

Table 3. Greenhouse Fungicide Screening Data for Gopalamicin[a]

organism	disease	crop	trial design	rating at 1000 ppm
Botrytis cinerea	gray mould	green pepper	protective	2
Erysiphe cichoracearum	powdery mildew	cucumber	curative	2
Erysiphe graminis tritici	powdery mildew	wheat	protective	5
Fusarium culmorum	culm rot	wheat	protective	2
Plasmopara viticola	downy mildew	grape	protective	7, 6[b]
Puccinia recondita	brown rust	wheat	curative	2
Pyricularia oryzae	rice blast	rice	protective	5[b]
Pyrenophora teres (*Helmenthosporium teres*)	net blotch	barley	protective	2

[a] Compounds were applied as sprays and rated for infection on a scale of 0–8: 8, very good efficacy, no infection; 7, intermittent infection; 6, good efficacy, light infection; 5, moderate efficacy, light infection; 4, slight efficacy, moderate infection; 3, moderate infection; 2, heavy infection; 0, no effect, total infection. [b] Tested at 500 ppm.

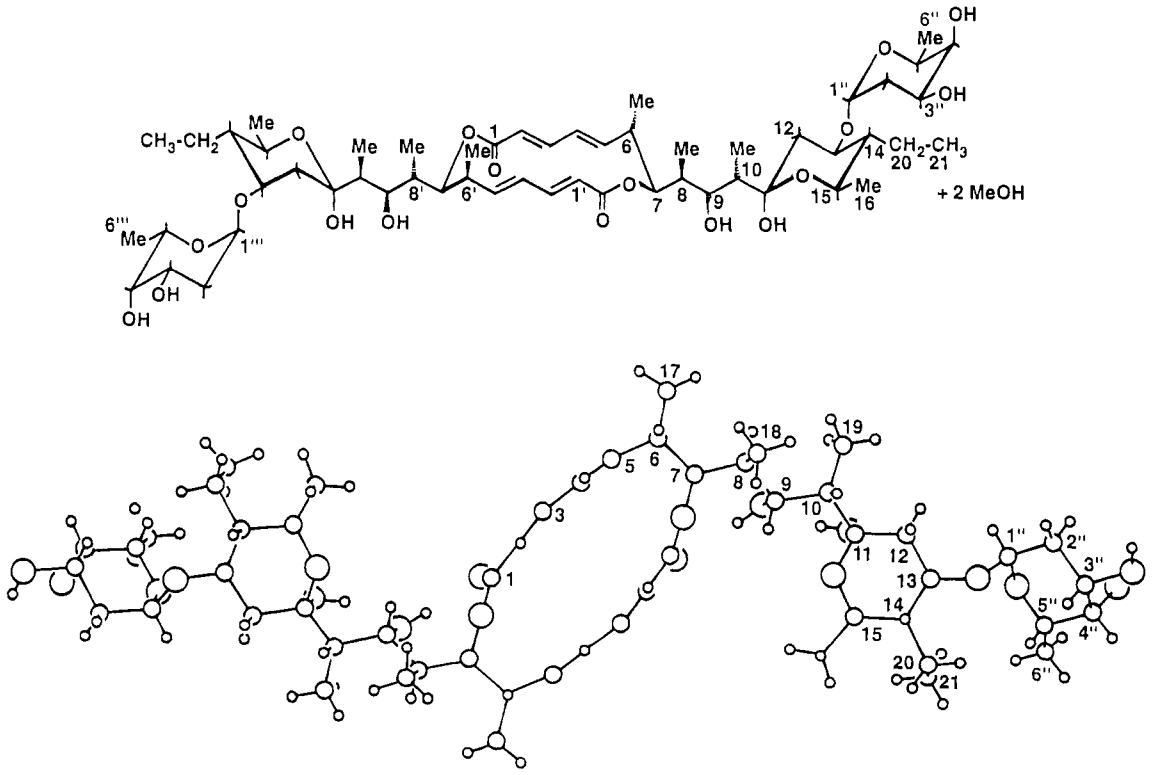

Figure 2. Gopalamicin.

observed for the control of *Plasmopara viticola*, the downy mildew pathogen on grape vine, at 500 ppm spray application (Table 3). At 250 and 16 ppm applications the ratings were 6 and 3, respectively. Similarly, the rice blast pathogen, *Pyricularia oryzae*, was inhibited by this compound and the ratings were 5 and 4, respectively, at 500 and 16 ppm spray applications. Almost all fungi tested in Table 3 gave a rating of 2 at 16 ppm.

Gopalamicin was not further investigated as a potential fungicidal compound for human or agricultural use since elaiophylin was studied for its use in human medicine by Hoechst A.G., Germany. Elaiophylin was reported to be active on *Staphylococcus aureus* and *Mycobacterium smegmatis* by Arcamone et al. (1959). However, bioassays with gopalamicin in our laboratory did not show any antibacterial activity on a variety of Gram-positive and -negative bacteria including *Staphylococcus aureus*. Therefore, we did not refer our compound to elaiophylin or salbomycin. A literature search revealed that CD and antifungal activities of macrodiolides, elaiophylin or salbomicin, are not reported. This is the first report of the CD and antifungal activity for a macrodiolide of this nature.

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LITERATURE CITED

Arai, M. Azalomycins B and F, two new antibiotics. I. Production and isolation. *J. Antibiot. A* **1960a**, *13*, 46–50.

Arai, M. Azalomycins B and F, two new antibiotics. II. Properties of azalomycins B and F. *J. Antibiot. A* **1960b**, *13*, 51–56.

Arcamone, M.; Bertazzoli, C.; Ghione, M.; Scotti, T. Melanosporin and elaiophylin, new antibiotics from *Streptomyces melanosporus*. *G. Microbiol.* **1959**, *7*, 207–216.

Fielder, H.-P.; Worner, W.; Zahner, H.; Kaiser, H. P.; Keller-Schierlein, W.; Muller, A. Metabolic products of microorganisms. 200: Isolation and characterization of niphithricins A,B, and elaiophylin, antibiotics produced by *Streptomyces violaceoniger*. *J. Antibiot.* **1981**, *34*, 1107–1118.

Gerlitz, M.; Hammann, P.; Thiericke, R.; Rohr, Jurgen. The biogenetic origin of the carbon skeleton and the oxygen atoms of elaiophylin, a symmetric macrodiolide antibiotic. *J. Org. Chem.* **1992**, *57*, 4030–4033.

Hammann, P.; Kretzschmar, G. Secondary metabolites by chemical screening. 12. ^{13}C NMR studies of Elaiophylin derivatives. *Magn. Reson. Chem.* **1991**, *29*, 667–670.

Kaiser, H.; Keller-Schierlein, W. Structure elucidation of elaiophylin: Spectroscopy and chemical degradation. *Helv. Chim. Acta* **1981**, *64*, 407–424.

Nair, M. G.; Mishra, S. K.; Putnam, A. R.; Pandey, R. A. Antifungal anthracycline antibiotics, spartanamicins A and B from *Micromonospora* spp. *J. Antibiot.* **1992**, *45*, 1738–1745.

Paulus, E. F.; Vertesy, L.; Sheldrick, G. M. Structure of Salbomycin, $C_{54}H_{88}O_{18}\cdot 2H_2O$. *Acta Crystallogr.* **1984**, *40*, 700–703.

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