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PURIFICATION AND CHARACTERIZATION OF NEW FATTY ACIDS WITH ANTIBIOTIC ACTIVITY PRODUCED BY *Sporothrix flocculosa*[1]

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Abstract—Chloroform extracts from a culture broth of the biocontrol fungus *Sporothrix flocculosa* were separated by thin-layer chromatography (TLC). Compounds with antifungal activity were revealed with the *Cladosporium cucumerinum* bioassay on TLC plates and were isolated by using silica gel column, preparative TLC, and reverse-phase high-performance liquid chromatography (RP-HPLC). They were identified by standard methods of nuclear magnetic resonance (NMR), gas chromatography (GC), mass spectrometry (MS), and infrared spectrum (IR). In addition to an active molecule previously described, two new fatty acids with antibiotic activity were characterized, 9-heptadecenoic acid and 6-methyl-9-heptadecenoic acid, the latter displaying the strongest activity among all three active molecules. This high number of active metabolites produced by *S. flocculosa* would explain its rapid and strong activity as a biocontrol agent of powdery mildews. In this context, screening of *S. flocculosa* isolates for increased production of these molecules should help in selection of candidates with superior biocontrol properties.

Key Words—*Sporothrix flocculosa*, antibiotics, fatty acids, biocontrol.

INTRODUCTION

Sporothrix flocculosa (Traquair, Shaw & Jarvis) has been reported in numerous studies as a potential antagonist against powdery mildew fungi (Jarvis et al., 1989; Hajlaoui and Bélanger, 1991, 1993; Bélanger et al., 1994). Practical exploitation of *S. flocculosa* as a biocontrol agent presupposes a thorough knowl-

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edge of its mode of action. Only through an understanding of the mechanisms of activity of *S. flocculosa*, will it be possible to achieve consistency of efficacy and selection of superior strains. To this end, cytochemical studies of the interaction between *S. flocculosa* and *Sphaerotheca pannosa* var. *rosae* have revealed that disintegration of host cytoplasm occurred in the absence of hyphal penetration, suggesting the involvement of extracellular diffusible molecules (Hajlaoui et al., 1992). In addition, it was shown that *S. flocculosa* produced antimicrobial compounds active against several microorganisms such as *Fusarium oxysporum*, *Botrytis cinerea*, *Trichoderma viride*, and *Bacillus subtilis* (Hajlaoui et al., 1994; Choudhury et al., 1994). Recently, two aliphatic molecules (4-methyl-7,11-heptadecadienoic acid and 4-methyl-7,11-heptadecadienal), were isolated from liquid cultures of *S. flocculosa* and *S. rugulosa*, with the former compound displaying antimicrobial activity (Choudhury et al., 1994). It thus appears evident that antibiosis is the main mode of action by which *S. flocculosa* exerts its biocontrol activity. However, it is still unclear whether this activity is mediated by one or several molecules, and if the latter, whether those molecules can be isolated and characterized. In this context the objectives of the present study were twofold: (1) to screen extracellular metabolites produced by *S. flocculosa* for potential antifungal activity, and (2) to purify and characterize active molecules.

METHODS AND MATERIALS

Fungal Culture. Single-spore isolates of *Sporothrix flocculosa* were obtained by reisolating the fungus from rose leaves that were submitted to weekly applications of a fungal suspension initiated from an isolate of *S. flocculosa* graciously provided by Dr. W. R. Jarvis, Agriculture and Agri-food Canada (Harrow, Ontario, Canada). Stock cultures of the isolates were maintained at 4°C on yeast–malt–peptone–dextrose agar (YMPDA) containing yeast extract (3 g/liter), malt extract (3 g/liter), peptone (5 g/liter), dextrose (10 g/liter), and bacto-agar (15 g/liter). Erlenmeyer flasks (500 ml) each containing 100 ml YMPD broth were inoculated with 10^6 conidia/ml of *S. flocculosa*. The fungus was grown in still culture at 26°C in the dark for 28 days.

Cladosporium cucumerinum Ellis and Arth was obtained from Biosystematics Research Centre (Ottawa, Ontario, Canada) and was maintained on potato dextrose agar (PDA) at 25°C.

Screening of Molecules with Antifungal Activity. The liquid medium was separated from the biomass through a filter paper (Whatman No. 1) and the mycelium discarded. The filtrates were further extracted with chloroform ($CHCl_3$). The organic fraction was dehydrated with Na_2SO_4 and dried under vacuum. The residue was redissolved in chloroform.

Thin-layer chromatography (TLC) of the culture filtrate extracts was carried out on silica gel plates (Merck 60 F 254, 0.1 mm thick, 10 × 5 cm). The extracts were deposited as spots of 80 μl, and after drying, the chromatograms were developed in ethyl acetate–hexane (1 : 4, v/v). The spots were viewed under ultraviolet radiation at 254 nm and 366 nm. To localize zones of antifungal activity, silica gel plates were seeded with a sporal suspension of *Cladosporium cucumerinum* as described by Homans and Fuchs (1970). Because of the dark pigmentation of spores and mycelium of this fungus, zones of antifungal activity can be readily identified as white spots, each one representing zones where spore germination is inhibited.

Purification Procedures. The chloroform extracts from the filtrates were applied to a silica gel column (Silica Gel; 230–400 mesh, Baker Analyzed reagent) and flash-chromatographed by successive elution with 100% hexane (200 ml); 150 ml each of 1%, 5%, 8%, and 15% of diethyl ether in hexane; 250 ml of 100% diethyl ether; and 100% of methanol. For each eluted fraction, assessment of antifungal activity was carried out with the TLC bioassay as described above. The active fractions were purified with a combination of preparative TLC and high-performance liquid chromatography (HPLC). Preparative TLC was performed with Silica Gel 60 G (0.30 mm thick). Chloroform concentrates from the active fractions were spotted in a line along the bottom of the plate and developed in ethyl acetate–hexane (1 : 4, v/v). The bands corresponding to active spots were scraped off the plates, and the active molecules were extracted from silica gel with chloroform. These chloroform extracts were separated by HPLC with C_{18} reverse-phase columns (Millipore-Waters, 8 × 10 cm). Elution was performed with methanol–water–acetonitrile in gradient mode at a flow rate of 1.0 ml/min. Absorbance of the effluent was monitored by a photodiode array scanning between 220 and 400 nm.

Identification of Active Fractions. FT-IR spectra of the fractions were obtained on a Bruker FT-IR instrument. All NMR spectroscopy (^1H, ^{13}C, 2D, Hector) were recorded in deuterated chloroform ($CDCl_3$) with tetramethylsilane (TMS) as an internal standard using a Bruker 300 MHz. GC-MS data were recorded with a Hewlett Packard (HP) 5890 series II gas chromatograph coupled with MS HP mass selective detector 5972. Precise location of double bonds was obtained by ozonolysis using O_3/HCO_2H according to the method described by Frans et al. (1965). The position of methyl branching was confirmed by using acylpyrrolidines according to Andersson and Holtman (1975).

RESULTS AND DISCUSSION

On silica gel thin-layer chromatograms, chloroform extracts were separated into several spots as visualized under ultraviolet radiation at 254 and 366 nm.

Spraying of a sporal suspension of *C. cucumerinum* revealed only one major active spot in the chloroform extract with an approximate R_f value of 0.8 (Figure 1). However, when the active spot was viewed under low (254 nm) and high ultraviolet (366 nm) radiation, further bands were observed, indicating the presence of several molecules at this spot.

Fractionation of the chloroform extract by column chromatography and preparative TLC followed by reverse-phase HPLC revealed three active compounds on TLC based on the bioassay with *C. cucumerinum* (Figure 2). The physicochemical analysis (^{1}H NMR, ^{13}C NMR, IR, GC-MS) of these compounds revealed two new active fatty acids: 6-methyl-9-heptadecenoic acid ($C_{18}H_{34}O_2$) (Figure 3a) and 9-heptadecenoic acid ($C_{17}H_{32}O_2$) (Figure 3b) and another active compound corresponding to 4-methyl-7,11-heptadecadienoic acid previously isolated and characterized by Choudhury et al. (1994). Based on our bioassays, the 6-methyl-9-heptadecenoic acid appeared to display the highest antifungal activity among the three isolated compounds (Figure 2).

FIG. 1. Fungitoxicity of chloroform extracts from *Sporothrix flocculosa* culture filtrates on thin layer chromatograms (TLC). Eighty microliters were spotted on a silica gel TLC plate and developed with ethyl acetate–hexane (1:4, v/v). After drying, the plate was sprayed with a conidial suspension of *Cladosporium cucumerinum* and incubated at room temperature. After 48 hr of incubation, one major zone of inhibition was revealed with R_f value of approximately 0.8.

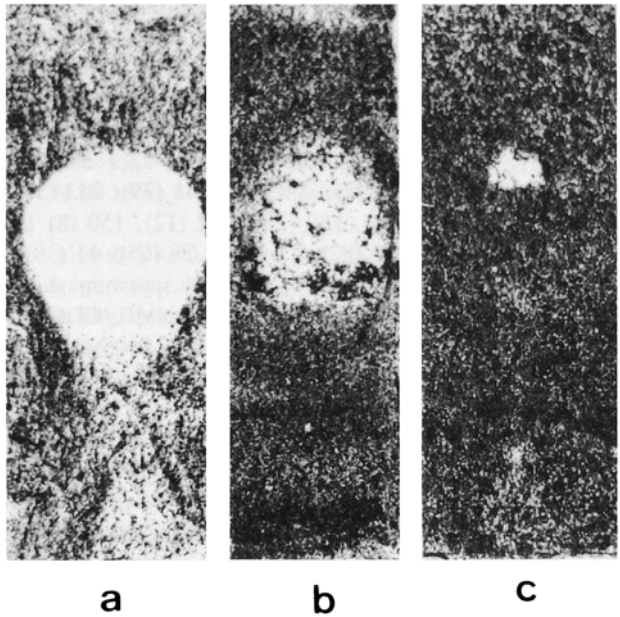

FIG. 2. Fungitoxicity of fatty acids from *Sporothrix flocculosa* culture filtrates on thin layer chromatograms (TLC). After purification with silica gel column, preparative TLC, and HPLC, aliquots (120 μl) from (a) 6-methyl-9-heptadecenoic acid, (b) 4-methyl-7,11-heptadecadienoic acid, and (c) 9-heptadecenoic acid were spotted on silica gel TLC and developed with ethyl acetate–hexane (1:4; v/v). After drying, the plates were sprayed with a conidial suspension of *Cladosporium cucumerinum* and incubated for 48 hr at room temperature.

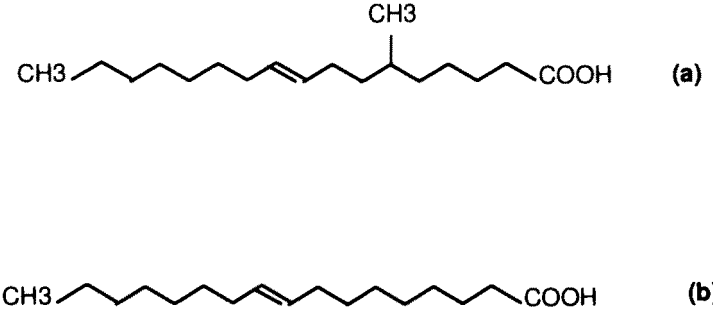

FIG. 3. Structure of the new fatty acid antibiotics: (a) 6-methyl-9-heptadecenoic acid and (b) 9-heptadecenoic acid.

The 6-methyl-9-heptadecenoic acid (Figure 3b) infrared spectrum exhibited strong absorption at 3350, 2680, 1720, 1660, 1420 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) was: $\underline{\delta}$ppm 10.80 (s, 1H), 5.35 (m, 2H), 2.30 (t, 2H), 2.18 (m, 4H), 1.95–2.0 (m, 18H), 0.89 (d, 3H), 0.83 (d, 3H). Cosy NMR (C-H coupling) ($CDCl_3$, 300 MHz) was: δppm 180.2 (s, COOH), 130 (d, CH=CH), 129.56 (d, CH=CH), 45.3 (q, CH_3), 38.3 (s, CH), 22.4–34.0 (t, $12CH_2$), 13.90 (q, CH_3). El-MS: *m/z*: 282 (92), 260 (58), 245 (39), 243 (35), 232 (2), 220 (3), 201 (8), 199 (10), 192 (4), 180 (28), 162 (12), 150 (8) 136 (4) 122 (47) 111 (48), 93 (50), 83 (25), 73 (82), 71 (100), 58 (65), 41 (30).

The 9-heptadecenoic acid (Figure 3b) infrared spectrum showed strong absorption at 3400, 2700, 1710, 1650, 1420 cm^{-1}. ^1H NMR ($CDCl_3$, 300 MHz) was: δppm; 10.85 (s, 1H), 5.38 (m, 2H), 2.30 (t, 2H), 2.05 (m, 4H), 1.6 (m, 2H), 1.2 (m, 18H), 0.85 (t, 3H). Cosy NMR (couplage C—H) ($CDCl_3$, 300 MHz) was: δppm 180.26 (s, COOH), 129.85 (d, CH=CH), 129.56 (d, CH=CH), 22.5–33.9 (t, $13CH_2$), 13.91 (q, CH_3). El-MS: *m/z* 268 (85), 246 (70), 219 (35), 219 (3), 194 (5), 152 (4), 138 (5), 111 (25), 83 (32), 55 (92), 41 (82), 28 (25).

The number of double bonds was confirmed by catalytic hydrogenation using Pt/H_2 and by ^1H and ^{13}C NMR based on integration of ethynilic protons, which indicated the presence of one double bond in both compounds. Ozonolysis of 6-methyl-9-heptadecenoic acid gave a mixture of a monoacid that was identified as octanoic acid by comparison of its analytical data (^1H and ^{13}C NMR) with a commercial product (Aldrich, Milwaukee, Wisconsin), and a decadioic acid branch for which the number of carbons was determined by ^{13}C NMR. Ozonolysis of 9-heptadecenoic acid gave a mixture of two compounds that were identified as octanoic and azelaic acids by comparison of their analytical data with commercial products (Aldrich). These analytical and transformation techniques indicated the presence of a unique double bond between carbons 9 and 10 in the two compounds described. The saturated pyrrolidide derivative of 6-methyl-9-heptadecenoic acid showed a minimum between the fragment $[M\text{-}C_{11}H_{23}]^+$ (molecular ion 182) and the fragment $[M\text{-}C_{13}H_{27}]^+$ (molecular ion 154). This minimum had a molecular ion of 168 corresponding to the fragment $[M\text{-}C_{10}H_{21}]^+$ minus $[CH\text{—}CH_3]$, which confirmed the position of the methyl branch on carbon 6.

It was thus possible to isolate and purify three active compounds from the culture filtrates of *S. flocculosa*, two of which are reported for the first time. In a previous study, Choudhury et al. (1994) had isolated and identified two molecules with antimicrobial activity from *S. flocculosa* and *S. rugulosa*. They were both observed here, but the 4-methyl-7,11-heptadecadienal, which rapidly oxidizes into 4-methyl-7,11-heptadecadienoic acid could only be detected in trace amounts because of its instability at room conditions. The latter compound (4-methyl-7,11-heptadecadienoic acid) was determined to be active to varying

degrees against a number of fungi based on an agar diffusion assay (Choudhury et al., 1994). However, reliability of this bioassay may be variable with apolar compounds such as fatty acids, which do not diffuse readily in agar. In addition, such an assay is not amenable to rapid screening. By a more discriminating bioassay directly on TLC, it was possible to identify two additional fatty acids with antibiotic activity, 9-heptadecenoic acid and 6-methyl-9-heptadecenoic acid, which appear to be more active than 4-methyl-7,11-heptadecenoic acid (Figure 3).

Previous ultrastructural studies had shown that cellular alterations caused in the target fungi were similar to those caused by the organism (Hajlaoui et al., 1992, 1994; Hajlaoui and Bélanger, 1993). Indeed, treatment of target fungi with the fungus or with extracts from its culture filtrates induced similar reactions, such as plasmalemma retraction and the leakage of cytoplasmic content, indicating an alteration of physiological properties of the cytoplasmic membrane (Hajlaoui et al., 1994). However, the molecular mechanism of action of fatty acids with antimicrobial activity has been little studied. It has been suggested that the fluidity of the cell membrane can be disturbed maximally by lipophilic compounds of particular chain lengths, unsaturation, or with particular hydrophilic functional groups (Kabara et al., 1977; Ko et al., 1994). In addition, alteration of the fluidity of the cell membrane by fatty acids was shown to be responsible for the inhibition of some membranous proteins such as α-mannosyl transferase (Watson and Rose, 1980), valyl-tRNA synthetase (Black, 1985) or (1,3)-β-glucan synthase (Ko et al., 1994).

The antimicrobial activity of certain fatty acids has been demonstrated in several studies (Bayliss, 1936; Nieman, 1954; Conley and Kabara, 1973; Kabara et al., 1972, 1977). For instance, some saturated and unsaturated fatty acids inhibited some bacteria such as *Pseudomonas aeruginisa*, *Streptococcus* (group A), and *Staphyloccocus aureus;* yeast fungi such as *Candida albicans* and *Saccharomyces cerevisiae* (Conley and Kabara, 1973, Kabara et al., 1972, 1977); and algae (Ikawa et al., 1984). These studies also reported on structure–function relationships and showed that the optimum antimicrobial activity for even-carbon-numbered aliphatic acids was found in C_{12} saturated fatty acids and C_{18} mono- and diunsaturated fatty acids (Bayliss, 1936; Nieman, 1964; Conley and Kabara, 1973). In addition, it was emphasized that *cis* unsaturated isomers were active while the *trans* isomers were always less active or inactive when compared to the more common unsaturation form (Kabara, 1987).

From an ecological point of view, our findings of antibiotic production by *S. flocculosa* are consequent with its description as an epiphyte. In fact, it is known that this biocontrol agent has a restricted ecological niche in the phylloplan (Traquair et al., 1988), and it would appear plausible that it is protected by antibiotics since it is neither a good competitor nor a parasite (Hajlaoui et al., 1992). In addition to the new antibiotics reported here and the one previously

described by Choudhury et al. (1994), other rare and unusual molecules have been isolated from the organism, the roles of which are still unknown (Choudhury et al., 1995).

The role of antifungal molecules in disease suppression has been shown to be of considerable importance, and the screening of mycelial culture from some biological control agents has led to the discovery of a great number of metabolites with antimicrobial activity (Fravel, 1988). It is evident from this work that *Sporothrix flocculosa*, a biocontrol agent against powdery mildew fungi, produces several antibiotics that account for its rapid and strong activity on target fungi, as demonstrated previously (Jarvis et al., 1989; Hajlaoui and Bélanger, 1991, 1993; Hajlaoui et al., 1994). In this context, screening of *S. flocculosa* isolates for increased production of these molecules should help in selection of strains with superior biocontrol properties.

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REFERENCES

ANDERSSON, B. A., and HOLMAN, R. T. 1975. Mass spectrometric localization of methyl branching in fatty acids using acylpyrrolidines. *Lipids* 10:716–718.

BAYLISS, M. 1936. Effect of the chemical constitution of soaps upon their germicidal properties. *J. Bacteriol.* 31:489–504.

BÉLANGER, R. R., LABBÉ, C., and JARVIS, W. R. 1994. Commercial-scale control of rose powdery mildew with a fungal antagonist. *Plant Dis.* 78:420–424.

BLACK, S. 1985. Inhibition of the reductive activation of valyl-tRNA synthethase from yeast by unsaturated fatty acids and associated observations on newly found lipohilic substances from yeast. *J. Biol. Chem.* 260:433–440.

CHOUDHURY, S. R., TRAQUAIR, J. A., and JARVIS, W. R. 1994. 4-Methyl-7,11-heptadecadenal and 4-methyl-7,11-heptadecadienoic acid: New antibiotics from *Sporothrix flocculosa* and *Sporothrix regulosa*. *J. Nat. Prod.* 57:700–704.

CHOUDHURY, S. R., TRAQUAIR, J. A., and JARVIS, W. R. 1995. New extracellular fatty acids in culture filtrates of *Sporothrix flocculosa* and *S. rugulosa*. *Can. J. Chem.* 73:84–87.

CONLEY, A. J., and KABARA, J. J. 1973. Antimicrobial action of esters of polyhydric alcohols. *Antimicrob. Agents Chemother.* 4:501–506.

FRANS, J. E., KNOWLES, W. S., and OSUCH, C. 1965. Ozonolysis of cyclic olefins. *J. Org. Chem.* 30:4328–4330.

FRAVEL, D. R. 1988. Role of antibiosis in the biocontrol of plant diseases. *Annu. Rev. Phytopathol.* 26:75–91.

HAJLAOUI, M. R., and BÉLANGER, R. R. 1991. Comparative effects of temperature and humidity on the activity of three potential antagonists of rose powdery mildew. *Neth. J. Plant Pathol.* 97:203–208.

HAJLAOUI, M. R., and BÉLANGER, R. R. 1993. Antagonism of the yeast-like phylloplane fungus *Sporothrix flocculosa* against *Erysiphe graminis* var *tritici*. *Biocont. Sci. Technol.* 3:427–434.

HAJLAOUI, M. R., BENHAMOU, N., and BÉLANGER, R. R. 1992. Cytochemical study of the antag-

onistic activity of *Sporothrix flocculosa* on rose powdery mildew, *Sphaerotheca pannosa* var. *rosae*. *Phytopathology* 82:583–589.

HAJLAOUI, M. R., TRAQUAIR, J. A., JARVIS, W. R., and BÉLANGER, R. R. 1994. Antifungal activity of extracellular metabolites produced by *Sporothrix flocculosa*. *Biocont. Sci. Technol.* 4:229–237.

HOMANS, A. L., and FUCHS, A. 1970. Direct bioautography on thin layer chromatograms as a method for detecting fungitoxic substances. *J. Chromatogr.* 51:327–329.

IKAWA, M., HARTSHORNE, T., CARON, L., IANNITELLI, R. C., BARBERO, L. J., and WEGENER, K. 1984. Inhibition of growth of the green alga *Chlorella pyrenoidosa* by unsaturated fatty acids. *J. Am. Oil. Chem. Soc.* 12:1877–1878.

JARVIS, W. R., SHAW, L. A., and TRAQUAIR, J. A. 1989. Factors affecting antagonism of cucumber powdery mildew by *Stephanoascus flocculosus* and *S. rugulosus*. *Mycol. Res.* 92:162–165.

KABARA, J. J. 1987. Fatty acids and esters as antimicrobial/insecticidal agents, pp. 220–238, *in* G. F. Fuller and W. D. Nes (eds.). Ecology and Metabolism of Plant Lipids. American Chemical Society, Washington, D.C.

KABARA, J. J., SWIECZKOWSKI, D. M., CONLEY, A. J., and TRUANT, J. P. 1972. Fatty acids and derivatives as antimicrobial agents. *Antimicrob. Agents Chemother.* 2:23–28.

KABARA, J. J., VRABLE, R., and LIE KEN JIE, M. S. F. 1977. Antimicrobial lipids: natural and synthetic fatty acids and monoglycerides. *Lipids* 12:753–759.

KO, Y., FROST, D., HO, C., LUDESCHER, R., and WASSERMAN, B. 1994. Inhibition of yeast (1,3)-beta-glucan synthase by phospholipase A_2 and its reaction-products. *Biochim. Biophys. Acta* 1193:31–40.

NIEMAN, C. 1954. Influence of trace amounts of fatty acids on the growth of microorganisms. *Bacteriol. Rev.* 18:147–163.

TRAQUAIR, J. A., SHAW, L. A., and JARVIS, W. R. 1988. New species of *Stephanoascus* with *Sporothrix* anamorphs. *Can. J. Bot.* 66:926–933.

WATSON, K., and ROSE, A. 1980. Fatty-acyl composition of the lipids of *Saccharomyces cerevisiae* grown aerobically and anaerobically in media containing different fatty acids. *J. Gen. Microbiol.* 117:225–233.