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SYNTHESIS AND BIOLOGICAL CHARACTERIZATION OF (*Z*)-9-HEPTADECENOIC AND (*Z*)-6-METHYL-9-HEPTADECENOIC ACIDS: FATTY ACIDS WITH ANTIBIOTIC ACTIVITY PRODUCED BY *Pseudozyma flocculosa*

T. J. AVIS, R. R. BOULANGER, and R. R. BÉLANGER*

Département de phytologie
Faculté des sciences de l'agriculture et de l'alimentation
Centre de recherche en horticulture
Université Laval
Ste-Foy, Québec, Canada G1K 7P4

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Abstract—Difficulties in isolating and purifying antibiotic fatty acids from culture filtrates of *Pseudozyma flocculosa*, a biocontrol agent against powdery mildew, have been limiting factors in studying the properties and understanding the mode of action of the biocontrol agent. We report a new protocol for synthesizing (*Z*)-9-heptadecenoic and for the first time synthesis of (*Z*)-6-methyl-9-heptadecenoic acids, two antibiotic fatty acids produced by *P. flocculosa.* This allowed reproducible and quantifiable means of assaying biological activity of the molecules. In these bioassays, both molecules exhibited antifungal activity corresponding to their expected potency. These new developments should facilitate further studies aimed at deciphering the ecological properties of *P. flocculosa.*

Key Words—Fatty acids, *Pseudozyma flocculosa*, powdery mildew, antibiosis, biocontrol.

INTRODUCTION

Pseudozyma flocculosa (Traquair, L. A. Shaw & Jarvis) Boekhout & Traquair (syn.: *Sporothrix flocculosa* Traquair, Shaw & Jarvis) (Boekhout, 1995) is a yeastlike fungus with antagonistic activity against powdery mildew fungi (Jarvis

*To whom correspondence should be addressed.

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et al., 1989; Hajlaoui and Bélanger, 1991, 1993; Bélanger et al., 1994). Microscopic and cytochemical observations revealed that the antagonist induces rapid collapse of powdery mildew conidial chains and cytoplasmic disintegration of fungal cells without hyphal penetration (Hajlaoui et al., 1992). These findings suggested that antibiosis was the main mode of action by which *P. flocculosa* exerted its biocontrol activity.

Recently, our laboratory isolated extracellular fatty acids with antimicrobial properties produced by *P. flocculosa*, two of which were reported for the first time (Benyagoub et al., 1996a). These molecules, identified as 9-heptadecenoic acid and 6-methyl-9-heptadecenoic acid, exhibited toxic activity against several fungi, but had little effect on *P. flocculosa* itself (Benyagoub et al., 1996b). Bélanger and Deacon (1996) found a correlation between fungal sensitivity to the fatty acids and fungal susceptibility to *P. flocculosa*, suggesting that these fatty acids are the active principles behind the biocontrol potential of the antagonist.

Isolation and manipulation of these fatty acids from culture filtrates has proven difficult as extraction and purification procedures resulted in low and unreliable yields. This has hampered our efforts to learn more about the ecological and molecular properties of these compounds. As a result, we sought a way to obtain large and stable amounts of the antibiotics. Therefore the objective of this work was to develop a synthesis method for both (*Z*)-9-heptadecenoic and (*Z*)-6-methyl-9-heptadecenoic acids in order to facilitate biological studies of the antibiotics.

METHODS AND MATERIALS

General Chemical Procedures and Instruments

All oxygen- or moisture-sensitive reactions were performed in flame- or oven-dried glassware under pressure of nitrogen or argon. Air-sensitive liquids and solutions were transferred by syringe or cannula. Ether, tetrahydrofuran, and 1,4-dioxane were dried before use. 1,7-Dibromoheptane, 3-methylglutaric anhydride, and 1-nonyne were obtained from commercial sources (Sigma-Aldrich, Mississauga, Ontario, Canada). Flash column chromatography was performed with silica gel EM science 200-400 mesh (40–63 μm). All nuclear magnetic resonance spectroscopy (^{1}H, ^{13}C) was recorded in deuterium chloroform with a Bruker 300 MHz. Peaks positions are expressed as downfield shifts in parts per million (δ) from tetramethylsilane internal standards. Split patterns are designed as singlet (s); doublet (d); doublet–triplet (dt); triplet (t); quartet (q); multiplet (m); broad signal (br); *J* is coupling in Hertz. GC-MS data were recorded with a Hewlett Packard 5890 series II gas chromatography coupled to a Hewlett Packard 5972 mass selective detector. Mass spectra were obtained with electron impact (EI) (70 eV, energy beam).

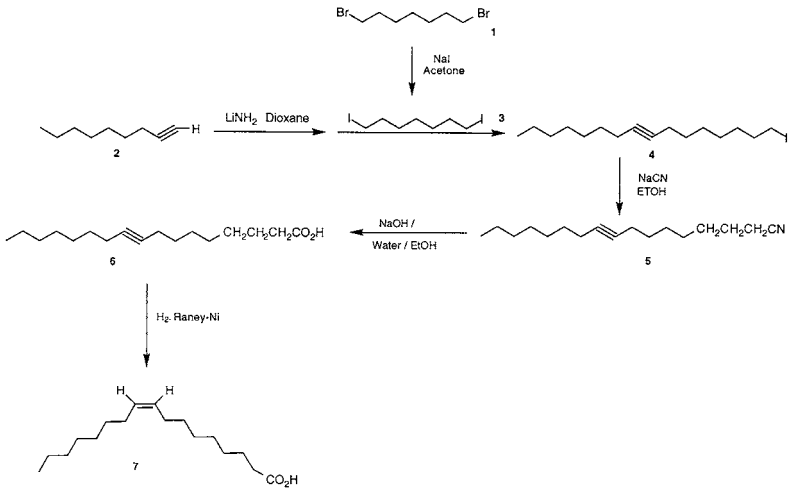

FIG. 1. Synthetic scheme for the synthesis of (*Z*)-9-heptadecenoic acid.

Synthesis of (Z)-9-heptadecenoic Acid

Preparation of various homologs and positional isomers of the C-13–C-19 odd-carbon ethenoic acids were synthesized according to Grimmer and Kracht (1963) and Jacob and Grimmer (1966) (Figure 1). These acids were prepared by reaction of the appropriate lithium acetylide and dihaloalkane in 1,4-dioxane and followed by reaction of the resulting acetylenic halide with sodium cynanide to obtain the corresponding acid **7**, which was half-hydrogenated to the ethenoic according to Jacob and Grimmer (1966).

*1,7-Diiodoheptane (**3**).* A solution of 1,7-dibromoheptane **1** (5.17 g, 0.022 mol) in 20 ml of dry acetone was added dropwise to a 250-ml flask equipped with a condenser, containing a suspension of sodium iodide (13.3 g, 0.09 mol) with 100 ml of dry acetone at room temperature. The mixture was heated to reflux for 3 hr. After cooling, the reaction mixture was filtered and the solvent was evaporated in vacuo. The crude residue was purified by flash chromatography with hexane as eluent. The yield was 96%, 7.6 g. $C_7H_{14}I_2$: ^{1}H NMR ($CDCl_3$: δ 1.30 (m, 6H), 1.39 (m, 4H), 3.17 (t, 4H) ppm: ^{13}C NMR ($CDCl_3$): δ 6.91 (C-4), 27.32 (C-3), 27.81 (C-3′), 30.14 (2 carbons) (C-2), 33.21 (2 carbons) (C-1); EI-MS (*m/z*, loss fragment): 352 (M^+), 225 (M^+-HI), 183 (M^+-HI-C_3H_6), 155 (M^+-$C_5H_{11}I$), 97 (C_7H_{13}).

*9-Hexadecyne Iodide (**4**).* Under an atmosphere of argon, 1-nonyne **2** (2.5 g, 0.02 mole) in 500 ml of dry 1,4-dioxane was refluxed with lithium amide (0.414 g, 0.018 mole) for 3.5 hr in a tricol equipped with a condenser and a dropping funnel with pressure equalization arm. To the organolithium compound

thus formed, 1,7-diiodoheptane **3** (8 g, 0.022 mol) in 50 ml of dry 1,4-dioxane was added dropwise. The mixture was refluxed an additional 2 hr, allowed to cool to room temperature, and then poured into a mixture of ice–water and stirred 15 min. The mixture was extracted with diethylether (3 × 50 ml). The combined organic fractions were dried over magnesium sulfate, filtered, and the solvent was evaporated in vacuo. The residue was purified by flash chromatography with hexane as eluent to give the corresponding iodoalkyne **4** with a 95% yield (6.6 g). $C_{16}H_{29}I$: ^1H NMR ($CDCl_3$): δ 0.87(t, 3H), 1.27–1.54 (m, 18H), 1.79–1.92 (m, 2H), 2.13 (t, 4H), 3.18 (t, 2H) ppm; ^{13}C NMR ($CDCl_3$): δ 7.11 ($\underline{C}H_3CH_2$), 14.11, 18.72, 18.78, 22.65, 28.07, 28.58, 28.85 (2 carbons), 29.00, 29.19, 30.42, 31.79, 33.52, 80.00 ($\underline{C}$, alkyne (80.42 ($\underline{C}$, alkyne) ppm; EI-MS (*m/z*, loss fragment): 348 (M^+), 305 (M^+-C_3H_9), 291 (M^+-C_4H_9), 263 (M^+-C_6H_{13}), 249 (M^3-C_7H_{15}).

*9-Heptadecyne Cyanide (**5**).* A solution of iodide compound **4** (4 g, 0.0115 mol) with 200 ml of 92% ethanol was added to a 250-ml flask equipped with a condenser and was refluxed 5 hr with sodium cyanide (2.8 g, 0.06 mol). After cooling to room temperature the mixture was poured into 50 ml water and extracted with hexane (3 × 50 ml). The combined organic fraction was dried over magnesium sulfate, filtered, and the solvent was evaporated in vacuo. The residue was purified by flash chromatography with hexane as eluent. The yield was 2.5 g (88%). $C_{17}H_{29}N$: ^1H NMR ($CDCl_3$): δ 0.86 (t, 3H), 1.26–1.49 (m, 18H), 1.59–1.69 (m, 2H), 2.11 (t, 4H), 2.31 (t, 2H) ppm; ^{13}C NMR ($CDCl_3$): δ 13.92 ($\underline{C}H_3CH_2$), 16.95, 18.52, 18.59, 22.97, 25.19, 28.14, 28.27 (two carbons), 28.42, 28.68, 28.77, 29.01, 31.63, 79.71 ($\underline{C}$, alkyne), 80.31 ($\underline{C}$, alkyne) 119.59 ($\underline{C}$, nitrile) ppm; EI-MS (*m/z*, loss fragment): 247 (M^+), 176 (M^+-C_5H_{11}), 162 M^+-C_6H_{13}), 148 (M^+-C_7H_{15}), 134 (M^+-C_8H_{17}), 81 (C_5H_7N), 67 (C_4H_5N).

*9-Heptadecynoic Acid (**6**).* In a 250-ml flask equipped with a condenser, the cyanide **5** (2.5 g, 0.0094 mol) was diluted with 50 ml of 92% ethanol. Sodium hydroxide (2.28 g, 0.06 mol) in 25 ml of water was added in one portion, and the mixture was heated to reflux for 10 hr. The reaction was cooled to room temperature, diluted with 200 ml of water, acidified with hydrochloric acid 2 N, and extracted with diethylether (3 × 50 ml). The crude residue 3.8 g was purified by flash chromatography with a gradient of 10–30% ethylacetate–hexane as eluent to yield 2 g (66%) of 9-heptadecynoic acid **6**. $C_{17}H_{30}O_2$: ^1H NMR ($CDCl_3$): δ 0.81 (t, 3H), 1.21–1.40 (m, 18H), 1.52–1.60 (m, 2H), 1.98–2.09 (t, 4H), 2.26–2.31 (t, 2H) 8.75 (br, COO$\underline{H}$, 1H) ppm; ^{13}C NMR ($CDCl_3$): δ 13.93 ($\underline{C}H_3CH_2$), 18.58, 22.48, 24.48, 28.46, 28.62 (two carbons), 28.68 (two carbons), 28.81, 28.92, 29.03, 31.63, 33.81, 79.92 ($\underline{C}$, alkyne), 80.22 ($\underline{C}$, alkyne) 179.46 ($\underline{C}$OOH) ppm; EI-MS (*m/z*, loss fragment): 248 (M^+), 195 (M^+-C_5H_{11}), 178 (M^+-$C_4H_8O_2$), 164 (M^+-$C_5H_{10}O_2$), 128 (M^+-$C_7H_{12}O_2$), 95 (C_7H_{11}), 81 (C_6H_8), 67 (C_5H_7).

*(Z)-9-heptadecenoic Acid (**7**).* 9-Heptadecynoic acid **6** (1.35 g, 0.0051 mol) was dissolved with 50 ml of methanol/pyridine (5 : 1), poured into a hydrogena-

tion tube, and 0.03 g of Raney nickel powder was added to the mixture. The tube was closed and shaken under hydrogen atmosphere until hydrogen adsorption ceased (30 min). The reaction mixture was filtered through celite, the solvent was evaporated in vacuo, and the residue was purified by flash chromatography with a gradient of 10–30% ethylacetate–hexane as eluent. Pure (*Z*)-9-heptadecenoic acid **7** was obtained. The yield was 1.1 g (81%). $C_{17}H_{32}O_2$: ^{1}H NMR ($CDCl_3$: δ 0.81 (t, 3H), 1.18–1.24 (m, 19H), 1.51–1.59 (m, 2H), 1.91–2.07 (t, 4H), 2.25–2.30 (t, 2H) 5.22–5.33 (m, 2H, J = 5.80 Hz) ppm; ^{13}C NMR ($CDCl_3$): δ 13.99 ($\underline{C}H_3CH_2$), 22.56, 24.54, 27.09, 28.94 (two carbons), 29.02, 29.15 (two carbons), 29.56 (two carbons), 29.65, 31.76, 33.96, 129.61 ($\underline{C}$, alkene), 129.90 ($\underline{C}$, alkene) 180.20 ($\underline{C}$OOH) ppm; EI-MS (m/z, loss fragment): 268 (M^+), 250 (M^+-H_2O), 196 (M^+-$C_3H_4O_2$), 182 (M^+-$C_4H_6O_2$), 138 ($C_7H_{14}O_2$), 95 (C_7H_{11}), 81 (C_6H_8), 67 (C_5H_7).

Synthesis of (Z)-6-Methyl-9-Heptadecenoic Acid

Heptadecenoic acid methyl substituted at the 6 position was synthesized by a modification of Figure 1. The corresponding α,ω-dibromopentane was prepared by reduction of methyl-3-glutaric anhydride followed by bromation with phosphorus tribromide or a Vonbraun degradation of substituted piperidine (Nguyen and Cartledge, 1986) (Figure 2).

*3-Methyl-1,5-Pentanediol (**10**).* A solution of 3-methylglutaric anhydride **9** (1.48 g, 0.008 mol) in 50 ml of anhydrous tetrahydrofuran was added dropwise to a suspension of lithium aluminum hydride (0.59 g, 0.96 mol) in 200 ml of anhydrous tetrahydrofuran. After 2 hr of stirring at room temperature, hydrolysis was accomplished by pouring the reaction mixture in an ice-cold saturated ammonium chloride solution. The mixture was extracted with diethylether (3 × 50 ml). Drying of the combined organic phase was performed over magnesium sulfate, and evaporation of solvent gave pure 3-methyl-1,5-pentanediol **10**, 0.602 g (44% yield). $C_6H_{14}O_2$: ^{1}H NMR ($CDCl_3$): δ 0.92 (d, 3H, J = 6.63 Hz), 1.36–1.45 (dt, 2H), 1.53–1.64 (dt, 2H), 1.72–1.83 (m, 1H), 3.39 (br, 2H), 3.59–3.74 (m, 4H) ppm; ^{13}C NMR ($CDCl_3$): δ 19.71, 25.69, 39.24 (two carbons), 60.18 (two carbons) ppm; EI-MS (m/z, loss (fragment): 119 (M^++1), 99 (M^+-H_3O), 82 (C_6H_{10}), 67 (C_5H_7).

*3-Methyl-1,5-dibromopentane (**11**).* Phosphorus tribromide, 1.41 g (0.0052 mol) was slowly added to a 250-ml flask equipped with a condenser, containing 0.6 g (0.0051 mol) of 3-methyl-1,5-pentanediol **9**, at −10°C. The mixture was brought to room temperature and stirred 20 hr. Ethylacetate (20 ml) was added to precipitate the oxides, followed by filtration and evaporation of solvent in vacuo. The crude residue was purified by flash chromatography with hexane as eluent or by distillation (bp 8°C/5 mm Hg), to give pure dibromide **11**. The yield was 0.9 g (73%) $C_6H_{12}Br_2$: ^{1}H NMR ($CDCl_3$): δ 0.93 (d, 3H, J = 6.26

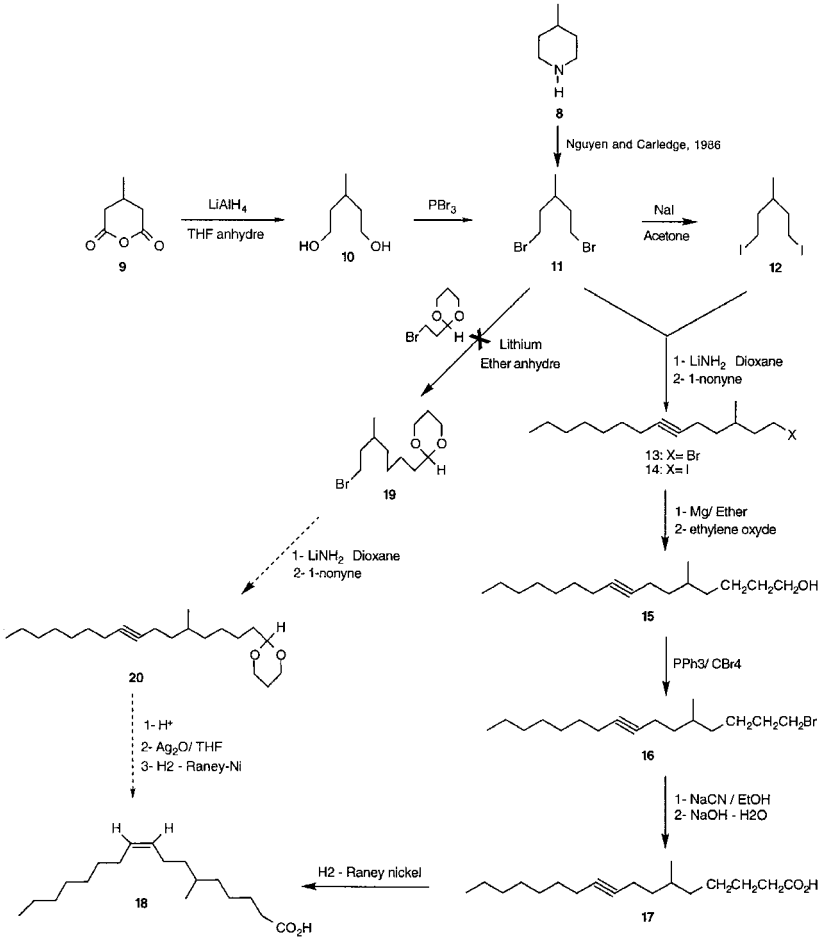

FIG. 2. Synthetic scheme for the synthesis of (*Z*)-6-methyl-9-heptadecenoic acid.

Hz), 1.65–1.77 (m, 2H), 1.82–1.97 (m, 3H), 3.36–3.49 (m, 4H) ppm; ^{13}C NMR ($CDCl_3$): δ 18.05, 30.47, 31.12 (two carbons), 39.28 (two carbons) ppm; EI-MS (*m/z*, loss fragment): 244 (M^+, ^{79}Br), 246 (M^+, ^{81}Br), 162 (M^+-^{81}Br), 164 (M^+-^{79}Br), 135 (M^+-$C_2H_4{}^{81}$Br), 137 (M^+-$C_2H_4{}^{79}$Br), 83 (C_6H_{11}).

*3-Methyl-1,5-diiodopentane (**12**).* A solution of 3-methyl-1,5-dibromopentane **11** (1.15 g, 0.045 mol) in 20 ml of dry acetone was added dropwise to a 250-ml flask equipped with a condenser and containing a suspension of sodium iodide (1.8 g, 0.012 mol) with 25 ml of dry acetone at room temperature. The mixture was heated to reflux for 3 hr. After cooling, the reaction mixture was

filtered and the solvent was evaporated in vacuo. The crude residue was purified by flash chromatography with hexane to give pure diiodide **12**. The yield was 1.65 g (89%). $C_6H_{12}I_2$: ^{1}H NMR ($CDCl_3$): δ 0.90 (d, 3H, J = 5.32 Hz), 1.71 (m, 3H), 1.90 (m, 2H), 3.30 (m, 4H) ppm; ^{13}C NMR ($CDCl_3$): δ 3.87, 17.69, 34.91, 40.00 (two carbons) ppm; EI-MS (m/z, loss fragment): 338 (M^+), 211 (M+-I), 169 (M^+-C_3H_6I), 155 (M^+-C_4H_8I), 127 (M^+-$C_6H_{12}I$), 83 (C_6H_{11}), 55 (C_4H_7).

*3-Methyl-6-tetradecyne Bromide (**13**) or 3-Methyl-6-tetradecyne Iodide (**14**).* Under an atmosphere of argon, 1-nonyne **2** (3.72 g, 0.03 mol) in 100 ml of dry 1,4-dioxane was refluxed with lithium amide (0.762 g, 0.033 mol) for 3.5 hr in a tricol equipped with a condenser and a dropping funnel with pressure equalization arm. To the organolithium compound thus formed, 0.0047 mol of the 3-methyl-1,5-halopentane **11** (1.14 g) or **12** (1.59 g) in 50 ml of dry 1,4-dioxane was added dropwise, and the mixture was heated to reflux another 2 hr. The mixture was allowed to cool to room temperature and poured into a mixture of ice–water and stirred 15 min. The mixture was extracted with diethylether (3 × 50 ml). The combined organic fractions were dried over magnesium sulfate, filtered, and the solvent was evaporated in vacuo. The residue was purified by flash chromatography with light petroleum ether as eluent to give the corresponding haloalkyne **13** (0.897 g, 76% yield) or **14** (1.00 g, 64% yield). $C_{15}H_{27}Br$: ^{1}H NMR ($CDCl_3$): δ 0.89 (d, J = 6.47 Hz, 3H), 0.92 (t, 3H), 1.10–1.49 (m, 11H), 1.52–1.94 (m, 4H), 2.10–2.27 (m, 4H), 3.47 (m, 2H, CH_2-Br) ppm; $_{13}$C NMR ($CDCl_3$): δ 13.94 ($\underline{C}H_3CH_2$), 16.22, 18.37, 18.61, 22.49, 28.70, 28.99 (2 carbons), 30.49, 31.56, 31.64, 35.57, 39.57, 79.46 (C, alkyne), 80.45 (C, alkyne) ppm; EI-MS (m/z, loss fragment): 288 (M^+), 207 (M^+-^{81}Br), 189 ($C_8H_{12}{}^{81}$Br), 187 ($C_8H_{12}{}^{79}$Br), 125 (C_9H_{17}), 95 (C_7H_{11}), 81 (C_6H_9), 67 (C_5H_7).

$C_{15}H_{27}I$: ^{1}H NMR ($CDCl_3$): δ 0.89 (d, J = 6.10 Hz, 3H), 0.92 (t, 3H), 1.25 (m, 14H), 1.55 (m, 2H), 1.63–1.80 (m, 1H), 1.83–2.20 (m, 2H), 3.20 (t, 2H, CH_2-I) ppm; ^{13}C NMR ($CDCl_3$): δ 4.53 ($\underline{C}H_3CH_2$), 13.97, 16.22, 18.18, 18.63, 22.51, 28.71 (two carbons), 29.00, 31.65, 32.98, 35.32, 40.50, 79.48 (C, alkyne), 80.58 (C, alkyne) ppm; EI-MS (m/z, loss fragment): 334 (M^+), 207 (M^+-I), 155 (M^+-C_4H_8I), 123 ($C_6H_{12}I$), 55 (C_4H_7), 41 (C_3H_5).

*5-Methyl-8-hexadecyn-1-ol (**5**).* Under an atmosphere of argon, 0.087 g (3.6 mmol) of magnesium turnings were introduced into a three-necked 100-ml round-bottomed flask equipped with a pressure-equalizing addition funnel, a condenser, and a magnetic stir bar. The magnesium was covered by 10 ml of anhydrous diethylether (freshly distilled on sodium). A solution of 3-methyl-6-tetradecyn iodide **14** (0.800 g, 0.0024 mol) in 20 ml of anhydrous tetrahydrofuran was added dropwise at 20°C. The formation of the Grignard reagent was accomplished after 2 hr of stirring. The solution was then cooled to 0°C in an ice water bath and a flow of ethylene oxide was bubbled into the Grignard solution for a period of 45 min. The resulting suspension was stirred for an additional 45 min at 0°C. The reaction was allowed to warm at room temperature and treated with

cold saturated ammonium chloride. The resulting mixture was extracted with diethylether, and the combined ether extract was washed with brine and dried over anhydrous magnesium sulfate. Removal of the solvent by distillation under pressure afforded of the desired product. The alcohol was purified by flash chromatography and eluted with light petroleum ether–ethylacetate (70 : 30). Pure hydroxyalcyne **14** was obtained (0.100 g, 13%). $C_{17}H_{32}O$: ^{1}H NMR ($CDCl_3$): δ 0.89 (t, 3H), 0.92 (d, J = 6.57 Hz, 3H), 1.11–1.75 (m, 20H), 2.09–2.27 (m, 4H), 3.62–3.76 (m, 2H, C$\underline{H}_2$OH) ppm; ^{13}C NMR ($CDCl_3$): δ 13.93, 16.32, 18.60, 19.00, 19.21, 22.95, 28.62 (two carbons), 28.69, 29.01, 29.55, 31.63, 36.26, 39.39, 60.91, 79.81 ($\underline{C}$, alkyne), 80.26 ($\underline{C}$, alkyne) ppm; EI-MS (m/z, loss fragment): 223 (M^+-C_2H_5), 209 (M^+-C_3H_8), 193 (M^+-C_3H_7O), 179 (C_4H_9O), 95 (C_7H_{11}), 80 (C_6H_8), 67 (C_5H_7).

*5-Methyl-8-hexadecyne Bromide (**16**).* Freshly recrystallized from hexane, triphenylphosphine (0.155 mg, 0.00059 mol) was added in one portion to a cold solution of hydroxyalcyne **15** (0.135 g, 0.00053 mol) with 5 ml of dry dichloromethane. After a 1-hr reaction period, carbon tetrabromide, 0.197 mg (0.00059 mol) was added in one portion and the mixture was stirred at 0°C for 5 hr. A mixture of ethylacetate and petroleum ether (20 : 80) was added, and the mixture was filtered on a bed of silica gel and washed with the same solvent. After evaporation of the solvent in vacuo, the bromoalkyne **16** was purified by flash chromatography with light petroleum ether as eluent to give 0.138 mg, 82% yield. $C_{17}H_{31}Br$: ^{1}H NMR ($CDCl_3$): δ 0.87 (t, 3H), 0.98 (d, J = 6.40 Hz, 3H), 1.10–1.96 (m, 19H), 2.11–2.25 (m, 4H), 3.36–3.51 (m, 2H, C$\underline{H}_2$Br) ppm; ^{13}C NMR ($CDCl_3$): δ 13.94, 16.22, 18.38 (two carbons), 18.61, 22.49, 28.71, 28.99 (three carbons), 30.79, 31.60, 31.64, 35.57, 39.57, 79.48 ($\underline{C}$, alkyne), 80.48 ($\underline{C}$, alkyne) ppm; EI-MS (m/z, loss fragment): 273 (M^+-C_3H_6), 235 (M^+-^{79}Br), 179 (M^+-C_4H_7Br), 95 (C_7H_{11}), 81 (C_6H_9).

*6-Methyl-9-heptadecynoic Acid (**17**).* A solution of bromide compound **16** (0.120 g, 0.00038 mol) in 20 ml of 92% ethanol was added to a 50-ml flask equipped with a condenser and was heated to reflux for 5 hr with sodium cyanide (0.093 g, 0.00191 mol). The cyanide formed was not isolated and was immediately treated with sodium hydroxide (0.0076 g, 0.00199 mol) in 25 ml of water. The mixture was heated to reflux for 14 hr. The reaction was cooled to room temperature, diluted with 200 ml of water, acidified with 2 N hydrochloric acid and extracted with diethylether (3 × 50 ml). The crude residue was purified by flash chromatography with a gradient mixture of 10–30% ethylacetate–light petroleum ether as eluent to give 0.060 g, 57% yield of 6-methyl-9-heptadecynoic acid **17**. $C_{18}H_{32}O_2$: ^{1}H NMR ($CDCl_3$): δ 0.79 (t, 3H), 0.82 (d, J = 6.47 Hz), 1.19–1.68 (m, 19H), 2.03–2.17 (t, 4H), 2.26–2.39 (t, 2H) 9.75 (br, COO$\underline{H}$, 1H) ppm; ^{13}C NMR ($CDCl_3$): δ 13.95 ($\underline{C}H_3CH_2$), 18.59, 18.59, 18.63, 22.49, 28.69 (three carbons), 28.99, 29.57, 31.11 (two carbons), 31.37, 31.64, 35.70, 79.62 ($\underline{C}$, alkyne), 80.31 ($\underline{C}$, alkyne) 180.25 ($\underline{C}$OOH) ppm; EI-MS (m/z, loss

fragment): 281 (M^++1), 236 (M^+-CO_2), 169 (M^+-$C_6H_8O_2$), 108 (M^+-C_8H_{12}), 95 (C_7H_{11}), 81 (C_6H_9), 67 (C_5H_7).

*(Z)-6-Methyl-9-heptadecenoic Acid (**18**).* 6-methyl-9-heptadecynoic acid **17** (0.127 g, 0.00045 mol) was dissolved in 50 ml of methanol–pyridine (5 : 1), poured into a hydrogenation tube, and 0.03 g of Raney nickel powder was added to the mixture. The tube was closed and shaken under hydrogen atmosphere until adsorption ceased (15 min). The reaction mixture was filtered through celite, the solvent was evaporated in vacuo, and the residue was purified by flash chromatography with a gradient of 10–30% ethylacetate–light petroleum ether as eluent. Pure (*Z*)-6-methyl-9-heptadecenoic acid **18** was obtained (0.117 g, 91% yield). $C_{18}H_{34}O_2$: ^{1}H NMR ($CDCl_3$): δ 0.86 (t, 3H), 0.89 (d, J = 6.19 Hz, 3H), 1.12–1.76 (m, 17H), 1.99–2.18 (t, 4H), 2.27–2.45 (m, 2H) 5.28–5.40 (m, 2H, J = 5.80 Hz) 9.81 ($CH_2\underline{C}OOH$) ppm; ^{13}C NMR ($CDCl_3$): δ 13.94 ($\underline{C}H_3CH_2$), 18.96, 19.11, 22.53, 24.48, 27.06, 29.08, 29.13, 29.22, 29.56, 29.61, 31.45, 31.73, 31.87, 36.51, 129.39 (C, alkene), 129.97 (C, alkene), 180.08 (COOH) ppm; EI-MS (*m/z*, loss fragment): 281 (M^+-1), 267 (M^+-CH_3), 254 (M^+-C_2H_4), 238 (M^+-CO_2), 236 (M^+-H_2O, -C_2H_4), 221 (M^+-$C_2H_5O_2$), 114 ($C_6H_{10}O_2$), 97 (C_7H_{13}), 83 (C_6H_{11}).

Biological Activity of Synthesized Antibiotics on Fungal Material. Cladosporium cucumerinum Ellis and Arth. was obtained from Biosystemics Research Centre (Ottawa, Ontario, Canada) and maintained on potato dextrose agar (PDA). *Sphaerotheca fuliginea* (Schlechtend.: Fr.) Pollacci was maintained in axenic conditions on long English cucumber (*Cucumis sativus* L. cv. Corona).

Two agar disks (15 mm) of *C. cucumerinum* were suspended in 100 ml of potato dextrose broth (PDB). The antibiotics were tested at concentrations of 0.1, 0.2, 0.4, 0.6, 0.8, and 1 mg/ml. The fatty acids were solubilized in *N*,*N*-dimethylformamide (DMF) prior to being added to the medium. DMF alone (0.5%) was added to the culture media to serve as control. Growth was quantified after a three-day culture at 25°C on a rotary shaker (150 rpm) by dry weight measurement following lyophilization. Further concentrations of each antibiotic were tested to determine minimum inhibitory concentration (MIC) of *C. cucumerinum.* MIC was defined as the lowest concentration of each antibiotic at which no macroscopic evidence of growth was observed. For each concentration, the experiment was repeated three times. In order to determine the dose of each antibiotic which reduced growth of *C. cucumerinum* by 50%, probit analysis was performed with the PROC Probit procedure in SAS System (SAS Institute Inc., Cary, North Carolina).

Foliar disks (5 cm) were cut from *S. fuliginea*-infected cucumber leaves and placed on 20-20-20 agar containing 2 g/liter 20-20-20 and 8 g/liter bacto-agar. Disks were cut from the leaf portion covered by at least 95% with colonies of *S. fuliginea.* The antibiotics were sprayed on leaf disks as aqueous solutions of 0.1 and 0.2 mg/ml. The antibiotics were solubilized in DMF prior to adding to ster-

ile water. DMF alone (0.5%) was added to the sterile water to serve as control. Evaluation of antibiotic effects on *S. fuliginea* was determined following incubation periods of 0, 12, and 24 hr at 25°C. Further evaluation of the antibiotics was not possible because of their rapid degradation under these conditions. Antifungal activity was determined using an arbitrary scale of 0 to 4, where 0 = no effect on *S. fuliginea* conidial chains, 1 = 1 to 25% collapse of conidial chains, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100% collapse. For each treatment, the experiment was repeated three times with two replicates per experiment.

RESULTS AND DISCUSSION

By following the synthetic route of Figure 1, it was possible to obtain pure (*Z*)-9-heptadecenoic acid. Ames and Bowman (1951) and Broughton et al. (1952) described the synthesis of ethylenic acids starting with an acid chloride and tribenzyltricarboxylate, which can also lead to synthesis of this fatty acid. However, that synthesis was not explored because the protocol in this study was more efficient and reduced the number of steps. Figure 2 gave pure (*Z*)-6-methyl-9-heptadecenoic acid. Global yield of α,ω-dibromopentane was low when prepared by reduction of 3-methylglutaric anhydride followed by a bromation with phosphorus tribomide. The Vonbraun degradation of substituted piperidine gave better results. In order to obtain the organolithium reagent, Brandänge et al. (1984) proposed a new technique by using lithium 4,4′-di-*tert*-butylbiphenylide (LDBB) as an intermediate reagent at −60°C in tetrahydrofuran. However, our attempts to use this synthetic route were not conclusive (Figure 2). On the other hand, the yield of 5-methyl-8-hexadecyn-1-ol (**15**), although not optimal, was obtained reproducibly by the route in Figure 2. For large-scale endeavors, this step could possibly be optimized with a modification of the halogenure structure such as hydrogenation of the haloalcyne to haloalcene prior to the Grignard reaction.

Biological testing of the synthesized fatty acids was performed to verify their toxic activity on fungi and to determine the specific characteristics of each molecule. Both compounds were assayed against *C. cucumerinum*, a cucumber pathogen that inhabits the same ecological niche as *P. flocculosa* and is sensitive to the antifungal fatty acids produced by the antagonist. Growth of *C. cucumerinum* was inhibited by both fatty acids, with 6-methyl-9-heptadecenoic acid demonstrating greater biological activity (Figure 3). Determination of MICs resulted in no growth of *C. cucumerinum* at 0.6 and 0.35 mg/ml for 9-heptadecenoic acid and 6-methyl-9-heptadecenoic acid, respectively. Probit analysis revealed that the dose of each antibiotic expected to reduce growth of *C. cucumerinum* by 50% is 0.16 and 0.10 mg/ml for 9-heptadecenoic acid and 6-methyl-9-heptadecenoic acid, respectively. These results support previous quali-

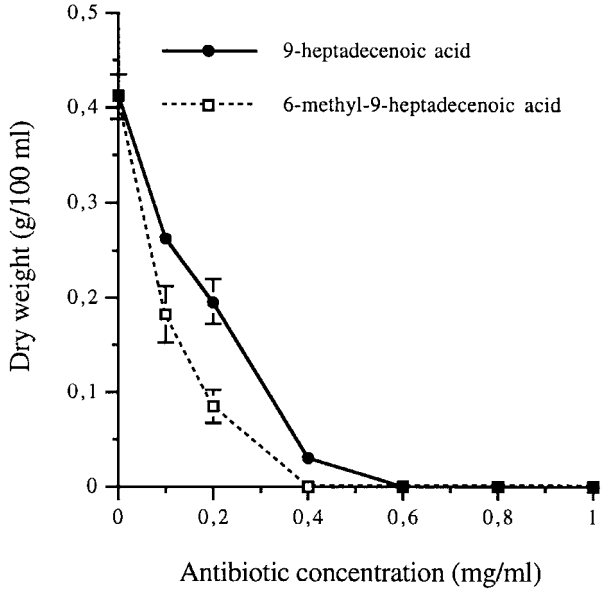

FIG. 3. Growth of three-day liquid cultures of *Cladosporium cucumerinum* following treatment with (*Z*)-9-heptadecenoic and (*Z*)-6-methyl-9-heptadecenoic acids at concentrations ranging from 0 to 1 mg/ml. Values are mean ± SD.

tative reports that 6-methyl-9-heptadecenoic acid has the higher biological activity of the two when assayed against *C. cucumerinum* (Benyagoub et al., 1996a).

The antifungal fatty acids were also tested against *S. fuliginea*, the causal agent of cucumber powdery mildew for which *P. flocculosa* is under study as a biocontrol agent. When assayed against *S. fuliginea*, both antibiotics induced a rapid collapse of the conidial chains of the pathogen, with once again a seemingly greater effect of 6-methyl-9-heptadecenoic acid (Table 1). This collapse of conidial chains was similar to the one induced by *P. flocculosa* itself. This suggests that these two fatty acids are, at least in part, responsible for the biocontrol activity. When assayed at 0.1 mg/ml, 6-methyl-9-heptadecenoic acid caused a significantly higher degree of collapse compared to 9-heptadecenoic acid 12 hr following the treatment. At 24 hr, these same treatments no longer showed differences in biological activity. This indicates that 6-methyl-9-heptadecenoic acid initially acts more rapidly on *S. fuliginea* than its nonmethylated counterpart. The fatty acids used under these conditions lost their activity when assayed at 36 hr (data not shown). This is a result of their rapid degradation, as previously demonstrated, when they are not stored under suitable conditions (appropriate temperature and protection from oxidation) (Kates, 1986). This rapid degrada-

TABLE 1. *Sphaerotheca fuliginea* CONIDIAL CHAIN COLLAPSE FOLLOWING TREATMENT WITH AQUEOUS SOLUTIONS OF ANTIBIOTIC FATTY ACIDS FROM *Pseudozyma flocculosa*[a]

Time (hr)	Control (0.0 mg/ml)	(*Z*)-9-Heptadecenoic acid		(*Z*)-6-Methyl-9-heptadecenoic acid	
		0.1 mg/ml	0.2 mg/ml	0.1 mg/ml	0.2 mg/ml
0	0.000a	0.083a	0.083a	0.167a	0.167a
12	0.000a	1.250b	1.917c	2.083c	2.167c
24	0.167a	2.917b	3.000b	3.083b	3.417b

[a] Values are means of conidial collapse where 0 = no effect on *S. fuliginea* conidial chains, 1 = 1–25% collapse of conidial chains, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100% collapse. Within a line, means followed by same letters are not significantly different by Fisher's protected LSD ($P = 0.01$).

tion indicates that *P. flocculosa* itself must be applied as the control measure of *S. fuliginea* because the fatty acids alone could not provide continuous control. Because the fatty acids degrade rapidly, there is less selection pressure on powdery mildew populations as a result of limited exposure to the compounds. This implies that the development of resistant strains of powdery mildew fungi to the antibiotics may be reduced or retarded on the leaf surface. Along these lines, it is apparent that residual activity would not be an issue, which makes *P. flocculosa* an environmentally friendly agent of powdery mildew biocontrol.

From an ecological perspective, *P. flocculosa* is considered a weak competitor for nutrients (Hajlaoui et al., 1992) and would need some other mechanisms to survive on the phylloplane. Therefore, these antibiotics would provide this antagonist with a means to protect its ecological niche against powdery mildew fungi. This in turn explains its rather selective biological control efficiency against powdery mildew fungi, which compete for the same ecological niche on the leaf surface. The competition is not based on nutrients because they are biotrophs, i.e., organisms that develop only on another living organism.

We consider the synthesis of these two fatty acids produced by *P. flocculosa* as a major breakthrough in our system because it allows further experimentation of their antibiotic activity without the underlying difficulties associated with extraction and purification procedures from culture filtrates. The newly synthesized molecules may be used as standards to follow kinetics and quantify biosynthetic productions by *P. flocculosa.* This should give a better understanding of the agent's antagonistic activity on leaf surfaces and provide a tool for screening and selecting *P. flocculosa* strains with superior biocontrol potential.

It has been shown that these antibiotic fatty acids induce leakage of electrolytes and proteins (Hajlaoui et al., 1992). These and other results led to the hypothesis of a structural mode of action of *P. flocculosa* antibiotics, where fun-

gal membranes are the primary target of action. Briefly, the antibiotics would partition into fungal membranes and induce disorder due to their bulkiness caused by the *cis* double bond located near the center of these fatty acids. This in turn would cause changes in the physical properties and function of fungal membranes, which would induce electrolyte leakage and increased fluidity as shown by Benyagoub et al. (1996b). It is now apparent that the antifungal fatty acids do not directly or indirectly act upon membrane sterols, factors that influence membrane fluidity, because they are toxic to pythiaceous fungi that do not contain membrane sterols (unpublished results). Furthermore, the dose-dependent increase in both toxicity (this study) and fluidity (Benyagoub et al., 1996b) by the fatty acids would suggest that they interfere with the general membrane lipid domain rather than a localized effect on one of its components. This model is also consistent with results in this study, in which the two antibiotics show different activity in exposed fungi. An additional methyl branch would occupy a larger cross-sectional area in fungal membranes, thereby inducing greater disorder and increased fluidity. This would support the higher biological activity of 6-methyl-9-heptadecenoic acid.

The newly synthesized molecules should facilitate elucidation of the specific mode of action of this biocontrol agent. For example, immunolocalization and cytochemical observations could pinpoint sites of action of the fatty acids within fungal cells, thus providing a valuable tool to validate the model and eventually help in predicting the efficacy of the antagonist by analysis of cellular membranes of powdery mildew fungi.

Overall, the synthesis of (*Z*)-9-heptadecenoic and (*Z*)-6-methyl-9-heptadecenoic acids from *P. flocculosa* will give stable and sufficient quantities of the products for further testing with regards to specific mode of action of the antibiotics. This will be helpful in elucidating the molecular and ecological phenomena behind the antagonism by *P. flocculosa* and in helping the prediction of its biocontrol efficiency. Finally, the antibiotics can be used in repeated selection pressure experiments to determine whether target fungi can eventually develop resistance.

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REFERENCES

AMES, D. E., and BOWMAN, R. E. 1951. Synthetic long-chain aliphatic compound. Part III. A critical examination of two methods of synthesis of olefinic acids. *J. Chem. Soc.* 1951:1079–1086.

BÉLANGER, R. R., and DEACON, J. W. 1996. Interaction specificity of the biocontrol agent *Sporothrix flocculosa:* A video microscopy study. *Phytopathology* 86:1317–1323.

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.

BENYAGOUB, M., BEL RHLID, R., and BÉLANGER, R. R. 1996a. Purification and characterization of new fatty acids with antibiotic activity produced by *Sporothrix flocculosa. J. Chem. Ecol.* 22:405–413.

BENYAGOUB, M., WILLEMOT, C., and BELANGER, R. R. 1996a. Influence of a subinhibitory dose of antifungal fatty acids from *Sporothrix flocculosa* on cellular lipid composition in fungi. *Lipids* 31:1077–1082.

BOEKHOUT, T. 1995. *Pseudozyma* Bandoni emend. Boekhout, a genus for yeast-like anamorphs of Ustilaginales. *J. Gen. Appl. Microbiol.* 41:359–366.

BRANDÄNGE, S., DAHLMAN, O., LINDQUIST, B., MAHLEN, A., and MORCH, L. 1984. Absolute configuration and enantioselective synthesis of spiculisporic acid. *Acta Chem. Scand. B* 38:837–844.

BROUGHTON, B. W., BOWMAN, R. E., and AMES, D. E. 1952. Synthetic long-chain aliphatic compound. Part VII. Some mono-olefinic acids. *J. Chem. Soc.* 1952:671–677.

GRIMMER, G., and KRACHT, J. 1963. The synthesis of odd-numbered unsaturated fatty acids. *Berichte.* 96:3370–3373.

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

JACOB, V. J., and GRIMMER, G. 1966. Bildung doppelbindungsisomerer alkencarbonsäuren bei der hydrierung von 9-alkincarbonsäuren. *Tetrahedron Lett.* 24:2687–2688.

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.

KATES, M. 1986. Techniques of lipodology: isolation, analysis and identification of lipids, pp. 100–111, *in* T. S. Burton and P. H. van Knippenberg (eds.). Laboratory Techniques in Biochemistry and Molecular Biology. Elsevier, Amsterdam.

NGUYEN, B. T., and CARTLEDGE, F. K. 1986. A convenient synthetic route to methylated silacyclohexanes. *J. Org. Chem.* 51:2206–2210.