Journal of Chemical Ecology, Vol. 18, No. 10, 1992

ANTIMICROBIAL TERPENES FROM OLEORESIN OF PONDEROSA PINE TREE *Pinus ponderosa:* A DEFENSE MECHANISM AGAINST MICROBIAL INVASION

MASAKI HIMEJIMA, KENNETH R. HOBSON, TOSHIKAZU OTSUKA, DAVID L. WOOD, and ISAO KUBO*

Division of Entomology and Parasitology
College of Natural Resources, University of California
Berkeley, California 94720

(Received March 2, 1992; accepted June 8, 1992)

Abstract—The oleoresin of the ponderosa pine, *Pinus ponderosa* (Pinaceae) exhibited broad antimicrobial activity. In order to identify the active compounds, the oleoresin was steam distilled to give a distillate and residue. The distillate contained mainly monoterpenes and some sesquiterpenes, while the residue consisted chiefly of four structurally related diterpene acids. An antimicrobial assay with the pure compounds indicated that the monoterpenes were active primarily against fungi, but there was also some activity against gram-positive bacteria. The diterpene acids, in contrast, only exhibited activity against gram-positive bacteria. Although not all of the identified sesquiterpenes could be tested, longifolene showed activity only against gram-positive bacteria. Therefore, it appears that the oleoresin of *P. ponderosa* functions as a biochemical defense against microbial invasion.

Key Words—Antimicrobial activity, gram-positive bacteria, fungi, Pinus ponderosa, oleoresin, monoterpene, sesquiterpene, diterpene acid, defense mechanism.

INTRODUCTION

When pine trees are physically damaged or attacked by insects, they exude resins that apparently protect them from further damage. This damage is usually caused by microorganisms that may be transmitted by insects as well as by other

*To whom correspondence should be addressed.

0098-0331/92/1000-1809$06.50/0

means (Harris, 1948). Paradoxically, bark beetles are known to be attracted by pine oleoresins (Wood, 1982; Miller et al., 1986; Phillips et al., 1988). Thus, the response of bark beetles to attractants in oleoresins is a critical, initial step in the colonization of host tissues. However, as long as trees are healthy, the insects are probably expelled by resin flow before they can colonize the phloem tissue (Vité, 1961; Berryman, 1972; Wood, 1972). Often, the trees are damaged more by insect-transmitted microorganisms than by insect action itself (Raffa and Berryman, 1982; Cook and Hain, 1985; Pain and Stephen, 1987; Owen et al., 1987; Raffa and Smalley, 1988; Parmeter et al., 1989). Moreover, microorganisms may be transmitted by other means as well, since trees are continually exposed to microorganisms in the environment (Andrews et al., 1980). Therefore, we might expect that oleoresins have broad antimicrobial activity that protects the trees from these nonspecific microorganisms (Smirnoff, 1972; Morris, 1972).

This paper describes identification of the principal components in the distillate and residue of the ponderosa pine oleoresin, and their antimicrobial activities.

METHODS AND MATERIALS

General. All NMR spectra were run in $CDCl_3$ on a Nicolet spectrometer equipped with an Oxford superconducting magnet at 300 MHz for ^1H and 75 MHz for ^{13}C. Mass spectra were obtained with a Hitachi RMU 6-MG spectrometer in electron impact mode (EI-MS). UV spectra were recorded in ethanol on a Hitachi 100-80 spectrometer. IR spectra were recorded on a Perkin-Elmer 1310 spectrometer. Merck 35–70 mesh silica gel was used for column chromatography. The solvents used were of reagent or HPLC grade.

Chemicals. The xylem oleoresin was collected in vials screwed into holes bored into the stems of the ponderosa pine *P. ponderosa*, near Blodgett Research Forest (El Dorado County, California) in 1987. The oleoresin (850 g) was steam distilled for 2 hr to yield a distillate (126 g) and residue (690 g). The fractions were submitted to GC-MS analysis and R-HPLC separation, respectively. α-Pinene, β-pinene, 3-carene, and limonene were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin). Terpinolene was purchased from Glidco Organics (Jacksonville, Florida), and abietic acid was purchased from Sigma Chemical Co. (St. Louis, Missouri). Longifolene was a gift from Takasago International Corporation (Tokyo, Japan). These compounds were all used for the assay without purification. *N,N*-Dimethylformamide (DMF) was purchased from EM Science (Gibbstown, New Jersey).

Microorganisms and Media. All microorganisms used for the antimicrobial assay were purchased from American Type Culture Collection (Rockville,

Maryland). They are *Bacillus subtilis* ATCC 9372, *Brevibacterium ammoniagenes* ATCC 6872, *Staphylococcus aureus* ATCC 12598 (a methicillin-susceptible strain), *Escherichia coli* ATCC 9637, *Pseudomonas aeruginosa* ATCC 10145, *Saccharomyces cerevisiae* ATCC 7754, *Candida utilis* ATCC 9226, *Penicillium chrysogenum* ATCC 10106, *Rhizopus stolonifer* ATCC 6227b, *Aspergillus niger* ATCC 16404, and *Mucor mucedo* ATCC 20094.

The culture media for bacteria consisted of 0.8% nutrient broth (BBL), 0.5% yeast extract (Difco) and 0.1% glucose. The culture media for fungi consisted of 2.5% malt extract broth (BBL).

Cultivation of Microorganisms. The freeze-dried microorganisms were reactivated as follows. *B. subtilis*, *S. cerevisiae*, *C. utilis*, *P. chrysogenum*, *R. stolonifer*, *A. niger*, and *M. mucedo* were cultured with shaking at 30°C. *B. ammoniagenes* was cultured stationarily at 30°C, and *S. aureus*, *E. coli*, and *P. aeruginosa* were cultured stationarily at 37°C.

Antimicrobial Assay. Preliminary routine antimicrobial assay was performed by a paper disk method. The test compound was dissolved in chloroform at a concentration of 8 mg/ml, and an aliquot of 0.1 ml of the sample solution was applied to a disk with a diameter of 8 mm (Toyo disk). This disk was placed onto the agar medium, which had been previously inoculated with a test microorganism. After incubation for two days at 37°C for bacteria and 30°C for fungi, the diameter of the inhibitory zone was measured with a caliper.

Since the *P. ponderosa* oleoresin was only slightly soluble in water, the paper disk method was an inadequate method because the water-insoluble oleoresin cannot disperse into the media. Therefore, throughout this report, the assay was performed by a broth dilution method (Taniguchi and Satomura, 1972) unless otherwise specified. For the broth dilution method, the test compound was dissolved in DMF and diluted in a twofold series with DMF, and 1% of sample solution was added to the broth medium. Microorganisms were cultured in a series of tubes with the different concentrations of the test compound in the broth medium. For the assay, the four common molds, *P. chrysogenum*, *R. stolonifer*, *A. niger*, and *M. mucedo*, were cultured with shaking, and other microorganisms were cultured stationarily. After two days (five days for molds), the growth was examined as a function of turbidity (OD at 660 nm) or visually in the case of molds. The lowest concentration of the test compound in which no growth occurred was defined as the minimal inhibitory concentration (MIC). It should be noted that the concentration of DMF in each test medium was always 1%. This did not affect the growth of any of the microorganisms tested.

GC and GC-MS Analysis. Analytical gas chromatography (GC) of the distillate was performed on a Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector (FID) and fitted with a glass capillary column (0.25 mm ID × 30 m) coated with Carbowax bonded (20 m). The carrier gas was helium at 1.5 ml/min, and the oven temperature was programmed from 50 to 230°C at 4°C/min.

GC-MS analysis was carried out on a Hitachi M-808 double-focusing instrument equipped with a Hewlett-Packard 5890 gas chromatograph. The GC conditions were identical to the above analytical GC runs. Mass spectral data were acquired and processed by a built-in computer system (M-0101) (Takasago International Corporation, Tokyo, Japan). The distillate was composed of a mixture of almost exclusively mono- and sesquiterpene hydrocarbons. Components were identified on the basis of a comparison of the GC retention time and MS fragmentation with those of the authentic samples.

R-HPLC Analysis. Recycle HPLC was performed on a JAI LC-09 (Japan Analytical Industry, Tokyo, Japan) equipped with a JAI refractive index detector and a JAI UV detector operating 235 nm. An ODS column (25 cm × 25 mm ID) was employed. The residue (19 g) was first further purified by silica-gel chromatography with *n*-hexane–ethyl acetate eluant to obtain a major fraction (14.5 g), which showed a single spot on a TLC plate. This fraction (40 mg) was injected into the R-HPLC system. The mobile phase was methanol at a flow rate of 3 ml/min. R-HPLC was repeated until four known diterpene acids were separated (Figure 1). Their UV and [^{1}H]NMR were identical with those of the authentic samples.

RESULTS AND DISCUSSION

Preliminary antimicrobial assays of *P. ponderosa* oleoresin exhibited activity against the gram-positive bacteria, *B. subtilis* and *B. ammoniagenes*, but not against the gram-negative bacteria, *E. coli* and *P. aeruginosa* up to 500 μg/ml. For the fungi, the oleoresin showed activity against *C. utilis* but not against *S.*

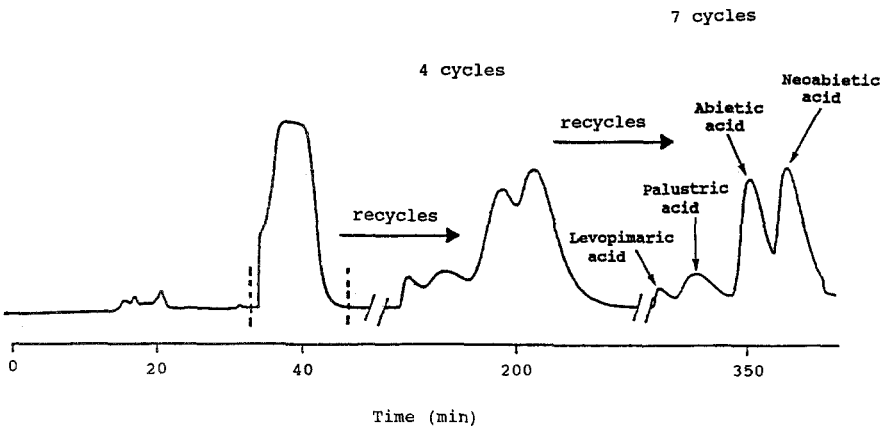

FIG. 1. Separation of diterpenoids by recycle HPLC.

cerevisiae at 10,000 ; gmg/ml (Table 1). As shown in Table 1, the distillate maintained almost the same activity as the oleoresin itself and showed additional activity against *S. cerevisiae*, but the residue lost activity against fungi. Thus, the distillate showed activity against fungi and gram-positive bacteria, while the residue exhibited activity only against gram-positive bacteria. Neither fraction showed any activity against gram-negative bacteria. It seems that the distillate and residue combine to yield the original antimicrobial activity of the oleoresin. It should be noted that none of these fractions showed any activity by the paper disk method. A possible reason for this is discussed later.

In order to identify the antimicrobial principles in both the distillate and residue, further analysis was performed. In the distillate, seven monoterpenes (α-pinene, β-pinene, 3-carene, myrcene, limonene, β-phellandrene and terpinolene), three sesquiterpenes (α-longipinene, longifolene, and germacrene D), and one phenolic compound (estragole) were identified by GC-MS analysis. This result is in general agreement with those reported earlier (Anderson et al., 1969; Smith, 1964).

On the other hand, the residue was found to be a mixture of primarily diterpene acids. The residue was first further fractionated by silica-gel column chromatography to remove the remaining volatile compounds resulting in a nondistillable fraction in 65% yield from the original oleoresin. Although this fraction showed a single spot on TLC plate, its NMR (^{1}H and ^{13}C) spectra indicated that it was still a mixture of abietic acid-type diterpenes. These structurally related diterpene acids were efficiently separated by R-HPLC (Kubo and Nakatsu, 1990). The mixture was injected into an R-HPLC system using an ODS column with the recycling carried out at intervals of about 50 min. By cycle 4 it was evident that this mixture contained four components as shown in

TABLE 1. ANTIMICROBIAL ACTIVITY OF OLEORESIN AND ITS DISTILLATE AND RESIDUE BY STEAM DISTILLATION

Microorganisms tested	Activity[a]		
	Oleoresin	Distillate	Residue
Bacillus subtilis ATCC 9372	+	+	+
Brevibacterium ammoniagenes ATCC 6872	+	+	+
Escherichia coli ATCC 9637	−	−	−
Pseudomonas aeruginosa ATCC 10145	−	−	−
Saccharomyces cerevisiae ATCC 7754	−[b]	+	−
Candida utilis ATCC 9226	+[b]	+	−

[a] +, Active at a concentration of 500 μg/ml; −, inactive at the concentration of 500 μg/ml.
[b] The concentration was 10,000 μg/ml.

Figure 1. Each fraction was collected, and R-HPLC was repeated under the same conditions to give four pure structurally similar diterpene acids; abietic acid, levopimaric acid, neoabietic acid, and palustric acid were isolated in natural form and identified by spectroscopic studies. These diterpene acids were previously identified in the same source by various methods (Anderson et al., 1969) including GC-MS analysis after methylation with diazomethane (Fujii and Zinkel, 1984).

Various biological activities of abietic acid-type diterpenes have been reported. For example, ingestion of the hexane extract of *P. ponderosa* needles, composed chiefly of eight diterpene acids, caused reproductive failure in mice during the early stages of gestation (Kubik and Jackson, 1981). The diterpene acids from various coniferous trees are also known as antifeedants against insects (Wagner et al., 1983; Schuh and Benjamin, 1984) and aphids (Rose et al., 1981).

Among the volatile hydrocarbons identified in the distillate, α-pinene, β-pinene, 3-carene, limonene, terpinolene, and longifolene were tested for antimicrobial activity. However, we were unable to test the antimicrobial activity of several compounds for the following reasons: (1) myrcene was barely soluble in DMF, so that we could not prepare the necessary sample solution for the assay; (2) germacrene D was unstable under the sample preparation conditions for the assay; and (3) some substances were minor and available only in small quantities. None of the volatile components tested showed activity against gram-negative bacteria up to 800 μg/ml (Table 2). Most monoterpenes showed activity against fungi, especially against *S. cerevisiae*, but almost no activity against gram-positive bacteria at concentrations up to 800 μg/ml. However, longifolene, a sesquiterpene hydrocarbon identified in the distillate showed no activity against fungi but exhibited activity against gram-positive bacteria, among

TABLE 2. ANTIMICROBIAL ACTIVITY OF PRINCIPAL VOLATILE IN DISTILLATE

	MICs against microorganisms[a] tested (μg/ml)					
Volatile	Bs	Ba	Ec	Pa	Sc	Cu
α-Pinene	>800	>800	>800	>800	50	800
β-Pinene	>800	>800	>800	>800	100	800
3-Carene	>800	>800	>800	>800	50	100
Limonene	800	>800	>800	>800	25	200
Terpinolene	800	800	>800	>800	50	100
Longifolene	3.13	200	>800	>800	>800	>800

[a]Bs, *B. subtilis*; Ba, *B. ammoniagenes*; Ec, *E. coli*; Pa, *P. aeruginosa*; Sc, *S. cerevisiae*; Cu, *C. utilis*.

which *B. subtilis* was much more sensitive than *B. ammoniagenes*. These results indicate that the original activity of the distillate against fungi was due to monoterpenes and the activity against gram-positive bacteria was due to sesquiterpenes. Therefore, even among the components of the distillate, monoterpenes and sesquiterpenes may share antimicrobial activity. Interestingly, none of these antimicrobial terpenes showed any activity by the paper disk method. There are two possible explanations for this: (1) Since these volatiles are not soluble in water they might not disperse into the media, and (2) they might have been partially if not entirely evaporated from the disk when the solvent was removed from the disk under reduced pressure. However, when these volatile monoterpenes were tested against insect-transmitted fungi in a closed system with the saturated vapor, all of them exhibited fungistatic activity (Cobb et al., 1968). Therefore, we retested the five monoterpenes against four common molds, namely *P. chrysogenum* (blue-green mold), *R. stolonifer* (black bread mold), *A. niger* (black mold), and *M. mucedo* in a broth media at 800 μg/ml. Results showed that only limonene exhibited activity against *M. mucedo* with a MIC of 400 μg/ml (Table 3).

In the case of the residue, the antimicrobial activity of abietic acid was extensively studied as a representative component, because the others were rather unstable and available only in minute quantities. Abietic acid exhibited strong activity against gram-positive bacteria (Table 4), comparable to the original activity of the residue itself. Since the three other diterpene acids are structurally similar to abietic acid, and the antimicrobial activity of pure abietic acid did not increase compared with the residue itself, they might be expected to show the same activity as abietic acid. Among gram-positive bacteria tested, *S. aureus* is an important human pathogenic bacterium. Based on this activity of abietic acid, the three other purified diterpene acids were also tested against this bacterium. All four diterpene acids exhibited the same antibacterial activity against *S. aureus* at MIC of 25 μg/ml.

TABLE 3. ANTIMICROBIAL ACTIVITY OF MONOTERPINES IN DISTILLATE AGAINST MOLDS

	MIC against molds[a] tested (μg/ml)			
Monoterpines	Pc	Rs	An	Mm
α-Pinene	>800	>800	>800	>800
β-Pinene	>800	>800	>800	>800
3-Carene	>800	>800	>800	>800
Limonene	>800	>800	>800	400
Terpinolene	>800	>800	>800	>800

[a]Pc, *P. chrysogenum*; Rs, *B. stolonifer*; An, *A. niger*; Mm, *M. mucedo*.

TABLE 4. ANTIMICROBIAL ACTIVITY OF RESIDUE AND ABIETIC ACID (COMMERCIAL)

Microorganisms tested	MIC (μg/ml)	
	Residue	Abietic acid
B. subtilis	12.5	12.5
B. ammoniagenes	25	12.5
S. aureus	25	25
E. coli	>800	800
P. aeruginosa	>800	>800
S. cerevisiae	>800	>800
C. utilis	>800	>800

The oleoresin of *P. ponderosa* may play an important role in protecting the tree from microbial invasion. Moreover, the viscous residue, comprised almost exclusively of diterpene acids, retains the volatile antifungal monoterpene hydrocarbons. Otherwise, these volatiles would rapidly escape into the environment and have only a short duration of antimicrobial activity. For our study, oleoresin was collected from the stems of *P. ponderosa* by boring through the bark into the xylem tissue. We speculate that the role of the oleoresin in defense against microbial invasion may not be only chemical but also biological. However, we were unable to determine if any of the substances identified in the oleoresin were a result of de novo synthesis. Furthermore, the relative amounts of these compounds in the outer and inner bark may effect microbial invasion of pines.

Acknowledgments—The study was supported in part by Resional Research Project W-110 and by USDA/SEA Research Grants Program (88-37253-411; 89-37250-4588-0892). We thank Dr. H. Tsuruta, Takasago International Corporation for GC-MS measurements and providing the authentic longifolene; Mr. H. Naoki, Suntory Institute for Bioorganic Research for NMR measurements; and Dr. S.J. Seybold for critical review of the manuscript.

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