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# PHENOLIC AND OTHER METABOLITES OF *PHELLINUS PINI*, A FUNGUS PATHOGENIC TO PINE

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**Key Word Index**—*Pinus* spp.; Pinaceae; white pocket rot; *Phellinus pini*; natural product isolation; antifungal metabolite; 2D NMR.

**Abstract**—Metabolites from *Phellinus pini*, a fungus pathogenic to conifer trees, were isolated and tested for antifungal activity against several tree pathogens. These compounds are 2,4,6-triphenylhex-1-ene, 2-farnesyl-5-methylbenzoquinone, 8-methyl-13-phenyltri-deca-4,6,8,10,12-pentaene-3-one, 4-vinylphenol and 4-vinylresorcinol. 4-Vinylphenol showed activity against most fungi examined.

## INTRODUCTION

For several years now, we have been involved in a study of the fungi associated with decay and stain of aspen [1]. Many of these pathogens have been reported to be mutually antagonistic. The two most prevalent aspen decay fungi, *Phellinus tremullae* and *Peniophora polygonia*, show this mutual inhibition [2], suggesting the possibility that fungal metabolites are responsible for the antagonism. In this case, it was shown that *P. polygonia* produces the inhibitory metabolites [3]. We are now involved in a similar study of fungi pathogenic to pine. The most economically important conifer decay fungus in western Canada is *Phellinus pini*, which causes white pocket rot in many species of pine [4]. Antagonism between this fungus and several other conifer pathogens has been observed. By analogy with the aspen project, we decided to begin this project with a chemical investigation of the metabolites from *P. pini*. In this report, we wish to describe the structures and antifungal properties of the compounds isolated from this disease causing fungus.

## RESULTS AND DISCUSSION

The fungus *P. pini* (Tho. ex Fr.) Pil. was isolated from a fruiting body on lodgepole pine (*P. contorta* var. *latifolia*) and grown on malt extract medium in the presence of Diaion HP-20 exchange resin as a metabolite absorbent [5]. After several weeks growth at room temperature, the resin and mycelium were separated by filtration and extracted with methylene chloride. The crude yellow extract was then separated into six fractions by chromatography on silica gel. The first fraction provided a liquid hydrocarbon, which was shown to possess the formula $C_{24}H_{24}$ by HREI mass spectrometry. The NMR data indicated three monosubstituted aromatic rings and a *gem*-disubstituted double bond; three methylenes and one methine accounted for the remaining $^{13}C$ and $^{1}H$ NMR signals. These data indicated that this compound was 2,4,6-triphenylhex-1-ene (**1**). This assignment was verified by a $^{1}H$–$^{1}H$ COSY experiment. The olefinic and certain of the aromatic protons correlated with each other and with the two proton methylene signals centred at $\delta$ 2.8 (H-3). The latter protons also correlated with the H-4 methine ($\delta$ 2.67), which in turn correlated with the methylene protons at $\delta$ 1.86 and 2.02 (H-5). The second-order multiplet at $\delta$ 2.34 (H-6) showed correlation with only the H-5 protons. This compound has been reported previously as one of the products from the thermolysis of styrene [6, 7]. In the present case, **1** also may arise from the trimerization of styrene.

The second fraction contained a single compound with the molecular formula $C_{22}H_{30}O_2$. The IR and NMR spectra suggested a substituted benzoquinone ($\delta_C$ 187.2 and 187.6; $\nu_{max}$ 1657 $cm^{-1}$). In addition to the two carbonyls, the $^{13}C$ NMR spectrum contained signals for five methyls, five methylenes and five trisubstituted double bonds. Two benzoquinone protons were evident in the $^{1}H$ NMR spectrum, giving rise to signals at 6.09 and 6.35 ppm; due to homoallylic coupling, these signals appeared as a quartet and triplet, respectively. In the $^{1}H$ NMR spectrum, the three remaining olefinic protons absorbed at higher field, and each was observed as a triplet of quartets, indicative of three isoprene units. These data suggested a farnesyl chain attached to C-2 of 5-methylbenzoquinone; structure **2** was confirmed on the basis of HMBC experiments (see Experimental). After unambiguous assignments of the $^{1}H$ NMR signals and correlation with the $^{13}C$ NMR signals in an HMQC experiment, the $^{13}C$

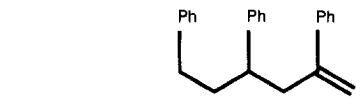
NMR data were found to be identical to those in the literature [18].

The compound obtained from fraction three accounted for the yellow colour observed in the extract. This compound readily crystallized from methanol and was shown to have the molecular formula $C_{20}H_{22}O$. The NMR data for this ketone ($\delta_C$ 200.8; $\nu_{max}$ 1685 $cm^{-1}$) confirmed the presence of a monosubstituted aromatic ring, five double bonds, one of which bore a methyl group, and an ethyl group adjacent to the carbonyl. The yellow colour ($\lambda_{max}$ 404 nm) suggested that the phenyl group, the double bonds and the ketone were in conjugation. Thus, the pigment was a methyl substituted 13-phenyltrideca-4,6,8,10,12-pentaen-3-one. A literature search revealed that the identical compound had been isolated from *Aspergillus niger* independently by two groups [9, 10]. Primarily on the basis of the mass spectral fragmentation pattern, it was suggested that the methyl group could be at the 6-, 7- or 8-position. The problem was later solved by the synthesis of the 8-methyl compound [11], which was identical in all respects with the natural product, and asperenone was assigned structure **3**. In the present work, the position of the methyl group was determined on the basis of 2D NMR experiments. The highest field olefinic proton, a doublet at $\delta$ 6.22, was shown through HMBC experiments to be $\alpha$- to the carbonyl. This proton showed three correlations in a $^1H$–$^1H$ TOCSY experiment, which indicated a four-proton spin system adjacent to the carbonyl. Therefore, the methyl group must be five carbons removed from the carbonyl. This result was verified by complete assignment of the $^1H$ NMR signals (see Experimental). The $^{13}C$ NMR signals were assigned after correlation with the $^1H$ NMR signals in a $^1H$–$^{13}C$ COSY experiment, using the HETCOR sequence of the Varian software.

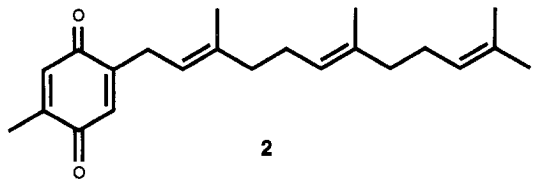
Fraction four was the largest fraction by mass and contained one pure compound. This major component was characterized as 4-vinylphenol (**4**) on the basis of the spectral data. Compound **4** is a very common metabolite of fruits, flowers and bacteria, especially those that contaminate beer and wine. Since the presence of styrene may be inferred from the isolation of trimer **1**, it is assumed that **4** arises by oxidation of styrene, likely through its 3,4-epoxide [12].

The fifth fraction contained a very closely related styrene, identified by $^1H$ and $^{13}C$ NMR and HREI mass spectrometry as 4-vinylresorcinol (**5**). In the literature, the only report of a 'natural' source of 4-vinylresorcinol is from cigarette smoke, and in this case **5** was identified only by GC–mass spectrometry [13]. In the present work, the phenolic $^{13}C$ signals were not revealed in the $^{13}C$ NMR spectrum, and the sample polymerized before a more concentrated solution could be examined.

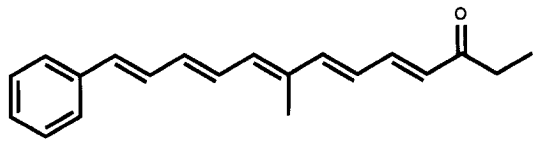
Since one of the objectives of this work was to discover potential antifungal agents, the above compounds were tested for biological activity against a number of pathogens of both pine and aspen (Table 1). Both **3** and **4** were active against two blue-stain fungi



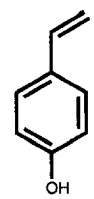

**1**



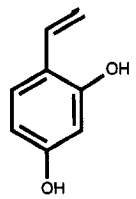

**2**




**3**




**4**




**5**

on aspen (*Ophiostoma crassivaginatum* and *O. piliferum*), although they showed no activity against *O. ulmi* (the cause of Dutch Elm disease). 4-Vinylphenol inhibited growth of *P. tremullae*, a fungus causing serious decay to aspen. Regrettably, the other phenolic styrene, **5**, underwent polymerization before it could be subjected to bioassay.

## EXPERIMENTAL

*General.* Mps are uncorr. FTIR and UV spectra were recorded in $CHCl_3$. Routine NMR spectra were recorded on Bruker AM-300 or AM-360 spectrometers, chemical shifts are reported in ppm downfield of TMS, and carbon multiplicities were measured with the APT sequence. 2D NMR spectra were run on a Varian Unity 500, using the standard pulse sequences of the Varian software. HREIMS were recorded on an AEI MS-50 mass spectrometer using an ionizing voltage of 70 eV. Prep. TLC was run on 0.25 mm thick silica gel $UV_{254}$ plates. All solvents were redistilled before use.

*Fungal strains and isolation of metabolites. Phellinus pini* (Tho. ex Fr.) Pil. (strain NOF 2232) was isolated from lodgepole pine (*Pinus contorta* var. *latifolia*) at the Northern Forestry Centre, Canadian

Table 1. Effect of compounds **1–4** on the *in vitro* growth of four tree pathogens

| | *Ophiostoma ulmi* | *O. crassivaginatum* | *O. piliferum* | *P. tremulla* |
|---|---|---|---|---|
| **1** | – | – | – | – |
| **2** | – | – | – | – |
| **3** | – | ++ | + | – |
| **4** | – | ++ | ++ | ++ |

–, No activity observed after impregnated disc applied to carrot agar culture.
+, Complete inhibition of fungal growth at 1000 ppm.
++, Complete inhibition at 100 ppm.

Forestry Service, Edmonton. A voucher specimen of the fungus was deposited at the University of Alberta Microfungus Herbarium as UAMH 8229. The fungus was grown in liquid shake culture using 2% malt extract (Difco), 2% (w/w) Diaion HP-20 ion-exchange resin and 0.1% bacto-peptone (Difco). After 24–26 days of shaking at room temp., the cultures were filtered and combined resin and mycelia were washed exhaustively with $CH_2Cl_2$ to yield a yellow oil. One 20-l batch yielded 860 mg crude extract, from which ergosterol was removed by crystallization from EtOH, and the remaining oil (725 mg) was chromatographed on silica gel using hexane–EtOAc (20:1) to provide 6 frs: A (65 mg), B (24 mg), C (142 mg), D (200 mg), E (18 mg), F (170 mg). All compounds reported by number gave single spots in at least two solvent systems and showed no substantial extraneous peaks in the $^1H$ and $^{13}C$ NMR spectra.

2,4,6-*Triphenylhex*-1-*ene* (**1**). Fr. A (65 mg) was purified by prep. TLC to yield **1** as an oil (18 mg). $\nu_{max}$ $cm^{-1}$: 3082, 3060, 3025, 2928, 2856, 1494, 1452; HREIMS *m/z* (rel. int.): 312.18818 (37), $C_{24}H_{24}$; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$: 1.86 (*dtd*, $J$ = 13.5, 10.0, 5.2 Hz, H-5), 2.02 (*dddd*, $J$ = 13.5, 10.1, 6.2, 4.6 Hz, H-5), 2.34–2.38 (2H, *m*, H-6), 2.67 (*m*, H-4), 2.78 (*ddd*, $J$ = 14.0, 7.0, 1.0 Hz, H-3), 2.83 (*ddd*, $J$ = 14.0, 7.6, 1.0 Hz, H-3), 4.88 (*dd*, $J$ = 2.6, 1.0 Hz, H-1), 5.14 (*d*, $J$ = 2.6 Hz, H-1), 7.00–7.32 (15H); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$: 33.7 (C-6), 37.4 (C-5), 43.4 (C-3), 43.7 (C-4), 114.4 (C-1), 125.6, 126.1, 126.4 (2), 127.3, 127.8 (2), 128.2 (2), 128.3 (2), 128.4 (4) (phenyl CH), 141.2 (C-2), 142.5, 145.0, 146.8 (phenyl C).

5-*Methyl*-2-(3,7,11-*trimethyldodeca*-2,6,10-*trienyl*)*benzoquinone* (**2**). Fr. B contained mainly one compound, which was purified by prep. TLC to yield **2** (12 mg) as an oil. HREIMS: *m/z* (rel. int.) 326.22468 (6.9), $C_{22}H_{30}O_2$; $^1H$ NMR (500 MHz, $C_6D_6$) $\delta$: 1.44 (3H, *d*, $J$ = 1.3 Hz, 3-Me), 1.56 (9H, 5-Me, 7′-Me, 11′-Me), 1.61 (3H, *d*, $J$ = 1.3 Hz, H-12′), 1.99 (2H, *t*, $J$ = 7.3 Hz, H-4), 2.10 (4H, *m*, H-5′, H-8′), 2.17 (2H, *t*, $J$ = 7 Hz, H-9′), 3.00 (2H, *d*, $J$ = 7.3 Hz, H-1′), 5.08 (1H, *tq*, $J$ = 7.3, 1.3 Hz, H-2′), 5.17 (1H, *br t*, $J$ = 6.8 Hz, H-6′), 5.21 (1H, *br t*, $J$ = 6.9 Hz, H-10′), 6.09 (1H, *q*, $J$ = 1.3 Hz, H-6), 6.35 (1H, *t*, $J$ = 1.3 Hz, H-2); $^{13}C$ NMR (125 MHz, $C_6D_6$) $\delta$: 15.1 (5-Me), 16.0 (3′-Me), 16.1 (7′-Me), 17.7 (11′-Me), 25.8 (C-12′), 26.7 (C-5′), 27.2 (C-9′), 27.5 (C-1′), 39.9 (C-4′), 40.1 (C-8′), 119.0 (C-2′), 124.3 (C-6′), 124.9 (C-10′), 132.3 (C-3), 133.3 (C-6), 131.1 (C-11′), 135.4 (C-7′), 139.3 (C-3′), 145.1 (C-5), 148.0 (C-2), 187.2 (C-1), 187.6 (C-4).

8-*Methyl*-13-*phenyltrideca*-4,6,8,10,12-*pentaen*-3-*one* (**3**). After standing in MeOH in the cold overnight, yellow crystals appeared in fr. C. These were collected to yield 15 mg of crystals, mp 114–117° (lit. [11] 128–130°), which were characterized as **3** based on physical data. $\nu_{max}$ $cm^{-1}$: 1659, 1590, 1560, 1188, 1113, 1032, 991; $\lambda_{max}$ nm (log $\varepsilon$): 424 (sh), 404 (4.83), 381 (sh); HREIMS *m/z* (rel. int.): 278.16607 (62), $C_{20}H_{22}O$; $^1H$ NMR (360 MHz, $CD_2Cl_2$) $\delta$: 1.14 (3H, *t*, $J$ = 7.3 Hz, H-1), 1.97 (3H, *s*, 8-Me), 2.60 (2H, *t*, $J$ = 7.3 Hz, H-2), 6.22 (1H, *d*, $J$ = 15.4 Hz, H-4), 6.39 (1H, *d*, $J$ = 11.7 Hz, H-9), 6.44 (1H, *dd*, $J$ = 14.8, 11 Hz, H-6), 6.55 (1H, *dd*, $J$ = 14.5, 10.8 Hz, H-11), 6.65 (1H, *d*, $J$ = 15.5 Hz, H-13), 6.72 (1H, *d*, $J$ = 14.8 Hz, H-7), 6.75 (1H, *dd*, $J$ = 14.5, 11.7 Hz, H-10), 6.97 (1H, *dd*, $J$ = 15.5, 10.8 Hz, H-12), 7.23 (1H, *tt*, $J$ = 7.5, 2.0 Hz, H-4′), 7.27 (1H, *dd*, $J$ = 15.4, 11 Hz, H-5), 7.33 (2H, *t*, $J$ = 7.5 Hz, H-3′), 7.44 (2H, *dd*, $J$ = 7.5, 2.0 Hz, H-2′); $^{13}C$ NMR (125 MHz, $CDCl_3$) $\delta$: 8.4 (C-1), 12.6 (8-Me), 33.9 (C-2), 126.2 (C-6), 126.5 (2, C-2′), 127.8 (C-4′), 128.5 (C-4), 128.7 (2, C-3′), 129.2 (C-12), 129.4 (C-10), 134.0 (C-13), 135.4 (C-8), 136.0 (C-9), 136.1 (C-11), 137.3 (C-1′), 142.7 (C-5), 145.8 (C-7), 200.8 (C-3).

4-*Vinylphenol* (**4**). Fr. D crystallized on evapn to yield a pure sample of **4**. HREIMS *m/z* (rel. int.): 120.05728 (100), $C_8H_8O$; $^1H$ NMR (500 MHz, $CD_3OD$) $\delta$: 5.16 (1H, *d*, $J$ = 10.9 Hz, *trans*-H-8), 5.60 (1H, *d*, $J$ = 17.6 Hz, *cis*-H-8), 6.63 (1H, *dd*, $J$ = 17.6, 10.9 Hz, H-7), 6.80 (2H, *d*, $J$ = 7.8 Hz, H-2), 7.31 (2H, *d*, $J$ = 7.8 Hz, H-3); $^{13}C$ NMR (125 MHz, $CD_3OD$) $\delta$: 110.8 (C-8), 116.2 (C-3), 128.4 (C-2), 130.8 (C-1), 137.8 (C-7), 158.3 (C-4).

4-*Vinylresorcinol* (**5**). Fr. E was purified by prep. TLC to yield **5** (12 mg). HREIMS *m/z* (rel. int.): 136.05274 (89), $C_8H_8O$; $^1H$ NMR (500 MHz, $CD_3OD$) $\delta$: 4.99 (1H, *dd*, $J$ = 10.9, 1.2 Hz, *trans*-H-8), 5.50 (1H, *dd*, $J$ = 17.6, 1.2 Hz, *cis*-H-8), 6.54 (1H, *dd*, $J$ = 17.6, 10.9 Hz, H-7), 6.69 (1H, *d*, $J$ = 8.0 Hz, H-6), 6.72 (1H, *dd*, $J$ = 8.0, 2.0 Hz, H-5), 6.87 (1H, *d*, $J$ = 2.0 Hz, H-3); $^{13}C$ NMR (75 MHz, $CD_3OD$) $\delta$: 110.7 (C-8), 113.6 (C-3), 116.2 (C-5), 119.7 (C-6), 131.4 (C-1), 138.1 (C-7).

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