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Accumulation of Isohemigossypolone and Its Related Compounds in the Inner Bark and Heartwood of Diseased *Pachira aquatica*

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Isohemigossypolone (1) and 2-*O*-methylisohemigossypolone (2), major fungitoxins of *Pachira aquatica*, were found to accumulate locally in the outer bark of the swollen trunk, whereas the inner bark and heartwood contained only a trace amount of them. From *P. aquatica* that was infected with a phytopathogenic bacterium, we detected significant amounts of 1 and 2 from browned inner tissues of the swollen trunk. According to a quantitative analysis by a gas-chromatograph, the concentration of 1 in the diseased inner tissues was calculated to be approximately 780 μg/g f.w., which was the same level as that in the outer bark of healthy individuals. These findings suggest that the inner tissues inducibly produced and accumulated antifungals 1 and 2 during infection events, as do many plants with phytoalexins. 11-Nor-2-*O*-methylisohemigossypolone (3), showing approximately equivalent fungitoxic activity to that of 1 and 2, was also isolated from the infected inner tissues. We screened pathogenic bacteria from the infected tissue, and isolated a rod-shaped bacterium that was tentatively identified as *Pseudomonas* sp. which promoted tissue-browning on sectioned disks of *P. aquatica* trunks.

Key words: *Pachira aquatica*; isohemigossypolone; cadinane sesquiterpenes; norcadinane; phytoalexin

Family Bombacaceae, a member of order Malvales, is a relatively small family consisting of over 180 species in 20 genera and is widely distributed throughout tropical areas. Malabar (*Bombax malabaricum*), balsa (*Ochroma lagopus*), kapok tree (*Ceiba pentandra*), baobab (*Adansonia digitata*), durian (*Durio zibethinus*) and malabar chestnut (*Pachira aquatica*) belonging to family Bombacaceae are diversely used by humans.[1)] *P. aquatica* is a midium-tall tree distributed throughout Central America. Its trunk is characteristically swollen at the bottom to store massive amounts of carbohydrates and water. Because of its shade- and drought-tolerant nature, *P. aquatica* is now popular in Japan as an indoor ornamental tree.[1)] In the course of a chemical study on antifungal agents of this plant, we have isolated and identified isohemigossypolone (**1**) and 2-*O*-methylisohemigossypolone (**2**) as major fungitoxic compounds from the underground part of the swollen trunk. Topical accumulation of compounds **1** and **2** has been found in the outer bark of the swollen trunk, and gas-chromatographic quantification of **1** has revealed its concentration to be 1,700–2,100 μg/g f.w.[2)] However, only trace amounts of **1** have been detected in the inner bark and the heartwood.[2)] The fibrous roots, leaves and green stems also contained only trace amounts of **1** and **2** (Shibatani, unpublished data). We therefore concluded that antifungal compounds **1** and **2** constitutively accumulated in the outer bark were typical prohibitins of *P. aquatica* to protect the storage tissues.

The question then arose as to whether *P. aquatica* would be able to exert any phytochemical defensive response when this chemical barrier had been broken by mechanical damage. We found some diseased *P. aquatica* individuals with canker-like symptoms in the swollen trunks whose inner tissues were soft rot-like and turned a dark brown color. The present chemical investigation of the diseased inner tissues led to the detection of some antifungal substances characteristically apparent in the infected inner tissues. Accordingly, three major antifungal compounds, two of which were identical to **1** and **2**, were isolated. Because both **1** and **2** had been present in only trace amounts in the non-infected inner tissues, their phytochemical behavior was like that of phytoalexins. In this report, we describe the identification and structural elucidation of the antifungal compounds found in diseased inner tissues of *P. aquatica* trunks, and further discuss their significance in chemical defense.

Materials and Methods

General. ^{1}H- and ^{13}C-NMR spectra were measured by a JEOL EX 270 instrument at 270 and 68 MHz or by a Bruker AMX 500 instrument (for HMBC and HMQC) at 500 and 125 MHz, respectively, using TMS as an internal standard. EI-MS spectra were measured by a JEOL DX-500 spectrometer, and gas-chromatography was conducted with a Hitachi G 5000 instrument equipped with TC-1 glass-capillary column (GL Science, 30 m × 0.32 mm i.d., corresponding to OV-1).

Plant materials. Pachira aquatica plants used in this study were purchased from a plant shop in Sapporo. Individuals of 20–30 cm in height and 15–20 cm in girth of the swollen trunks were conditioned for a few days in the laboratory. Diseased individuals whose inner tissues (41.3 g) had partially turned a dark brown color (Fig. 1)

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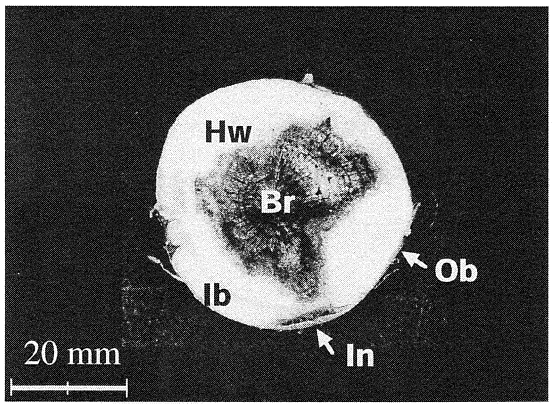


Ob, outer bark; **Ib**, inner bark; **Hw**, heartwood; **Br**, browned tissues; **In**, area in which the occurrence of initial infection is speculated.

Fig. 1. Cross-Sectioned Swollen Trunk of *P. aquatica* with Canker-like Symptoms.

were chopped and extracted with EtOAc.

Antifungal assay. The inner tissues from the diseased and healthy individuals were separately chopped and then extracted with excess EtOAc for three days. The solvent from each extract was evaporated to dryness *in vacuo*, and re-dissolved in EtOAc at a concentration of 50 mg of solute/ml. A 25 μl aliquot of each solution was then charged on TLC plates (0.25 mm thick, Kieselgel 60 F_{254} Merck; the amount of the charged solute was 1.25 mg each), and the TLC plates were developed in *n*-hexane-EtOAc=2:1. A spore suspension of *Cladosporium herbarum* AHU 9262 was sprayed over the developed TLC plates, which were then incubated at 25°C under highly humid conditions for three days.[3]

Isolation of the antifungal compounds from diseased inner tissues. Whole inner tissues of the swollen trunks of a diseased *P. aquatica* specimen (41.3 g f.w.) were extracted with EtOAc (300 ml) for three days, and the resulting extract (1.01 g) was applied to a silica gel column (80 g of Wakogel C-60 in $CHCl_3$) and eluted with $CHCl_3$-MeOH mixtures. Fractions (100 ml each) were obtained as follows: $CHCl_3$ only (frs. 1–4), 1% MeOH/$CHCl_3$ (frs. 5–8), 2% (frs. 9–12) and 4% (frs. 13–16). 2-*O*-Methylisohemigossypolone (**2**) and isohemigossypolone (**1**) were eluted in frs. 1–2 (8.5 mg) and frs. 3–4 (7.4 mg), respectively, as single constituents, whereas 11-nor-2-*O*-methylisohemigossypolone (**3**) was contained in frs. 10–12 (43.4 mg) as one of the elutes. The latter fractions (frs. 10–12) were further applied to another silica gel column (4 g of Wakogel C-60 in 1% MeOH/$CHCl_3$) and eluted with 1% MeOH/$CHCl_3$ (ffrs. 1–12, 5 ml each). Ffrs. 10–12 eluting **3** were combined and concentrated (4.8 mg), and pure **3** (2.5 mg) was eventually obtained by preparative TLC with *n*-hexane-EtOAc=2:1.

Physicochemical properties of isolated compounds.

Isohemigossypolone (**1**) A yellow syrup. R_f 0.71 in *n*-hexane-EtOAc=2:1. FD-MS (*m/z*, %): 274 (M^+, 100). ^{1}H-NMR δ (500 MHz, in $CDCl_3$): 12.45 (s, 7-OH), 10.83 (s, H-11), 7.33 (s, H-6), 7.08 (s, 2-OH), 4.32 (sept., J=6.8 Hz, H-12), 2.08 (3H, s, H_3-15), and 1.28 (6H, d, J=6.8 Hz, H_3-13 and H_3-14).

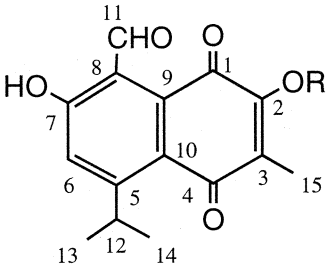
2-O-Methylisohemigossypolone (**2**) A yellow syrup. R_f 0.87 in *n*-hexane-EtOAc=2:1. FD-MS (*m/z*, %): 288 (M^+, 100). ^{1}H- and ^{13}C-NMR data (500 MHz, in $CDCl_3$) are shown in Table 1.

11-Nor-2-O-methylisohemigossypolone (**3**) A yellow syrup. R_f 0.71 in *n*-hexane-EtOAc=2:1. FD-MS (*m/z*, %): 260 (M^+, 100). EI-MS (*m/z*, %): 260 (M^+, 100), 245 (M-Me^+, 59), 229 (23), 217 (26), and 200 (24). EI-HR-MS: found, 260.1090 ($C_{15}H_{16}O_4$; calcd., 260.1049). ^{1}H- and ^{13}C-NMR data (500 MHz, in $CDCl_3$) are shown in Table 1.

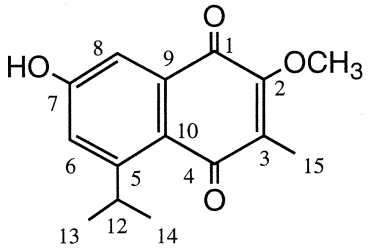
Quantification of **1** *in the browned inner tissue.* The method for quantitatively analyzing **1** by gas-chromatography has been described in our previous paper.[2] The diseased inner tissues (0.40 g) were carefully separated by a sterile blade and tweezers, and were then extracted with EtOAc (20 ml) for three days. The extract was dried over Na_2SO_4, concentrated *in vacuo*, re-dissolved into a small volume of $CHCl_3$, and applied to a Sep-Pak® silica cartridge (Waters, conditioned in $CHCl_3$) for elution with $CHCl_3$ (6 ml). After compound **1** had been eluted thoroughly, 10 μg of methyl palmitate in 100 μl of EtOAc was added as an internal standard, and the mixture was evaporated to dryness and re-dissolved in 50 μl of EtOAc. The resulting sample was applied to a gas-chromatographic analysis.

Isolation of microorganisms from the browned tissues. To isolate the pathogenic microorganisms from the diseased *P. aquatica* specimen, the browned tissues (0.40 g) were dipped into 100 ml of sterile water and shaken gently. After standing for a few minutes, the tissue washings were plated on a potato-dextrose agar medium (100 μl per plate) and incubated at 25°C for 2 days. Two distinguishable bacteria were respectively separated and purified on nutrient-broth agar plates.

Pathogenicity of the Isolated Bacteria to P. aquatica. The two isolated bacteria were separately examined for their phytopathogenicity by an inoculation assay on disks of *P. aquatica* trunks. For the assay, we first cultured each isolated bacterium in a potato-dextrose medium (200 ml) for three days. Cultured cells of each were collected by centrifugation, washed several times with deionized water, and finally suspended in 100 ml of sterile water. Inner tissue disks (*ca.* 2–4 mm thickness) were prepared from the swollen trunk of a healthy *P. aquatica* specimen by cross-sectioning. The disks were put into a plastic box spread with a wet paper towel over the bottom. A resting cell suspension (100 μl) of each bacterium was applied to the disks and incubated at 23°C in the dark. As a control, sterile water alone was applied.



1: R=H isohemigossypolone
2: R=Me 2-*O*-methylisohemigossypolone



3: 11-nor-2-*O*-methylisohemigossypolone

Fig. 2. Cadinane Aldehydes and 11-Norcadinane from Diseased Inner Tissues of the Swollen Trunk of *P. aquatica*.

Results and Discussion

At the time of processing the swollen trunk tissues of *P. aquatica* into pieces, we encountered some diseased individuals whose inner tissues had turned a dark brown color. The photograph in Fig. 1 is the cross section of a swollen trunk of a diseased *P. aquatica* specimen. The inner bark and heartwood, originally with a whitish color, showed severe browning. The browned area was fused to outer bark only in one region, suggesting the origin of the infection. The pathogens seemed to have penetrated deeply and dispersed widely into the inner tissues, but had not invaded the tissues adjacent to the outer bark.

It was thought that the individuals showing brownish inner tissues were systemically affected by the infection, so the whole inner tissues, including undamaged material with a yellowish white color, were chopped and extracted to screen the antifungal compounds in the infected tissues. TLC-bioautography (in $CHCl_3$-MeOH=50:1), using *C. herbarum* as the test fungus, revealed some antifungal spots as those specific to the infected inner tissue, focusing on three major antifungal compounds at R_f 0.94, 0.72 and 0.48. Each compound was isolated by silica gel column chromatography (see the Materials and Methods section). FD-MS and ^{1}H-NMR analyses enabled two compounds giving top and middle spots on thin-layer plates to be identified as 2-*O*-methylisohemigossypolone (**2**) and isohemigossypolone (**1**), respectively, both of which have topically accumulated in the outer bark of *P. aquatica*.[2] The quantitative analysis of **1** in the browned tissues (0.40 g) by gas-chromatography revealed that a significant amount of **1** had accumulated (780 μg/g f.w.). The calculated concentration of **1** in the browned tissue was at approximately the same level as that in the outer bark of healthy *P. aquatica*.[2]

The third compound (**3**) eluted in the latter column fractions and finally purified by preparative TLC gave its parent ion at m/z 260 by FD-MS. The EI-MS data of **3** showed characteristic peaks only at m/z 260 ($[M]^+$) and 245 ($[M-Me]^+$), similar to those of **1** and **2**, and EI-HR-MS elucidated its molecular formula to be $C_{15}H_{16}O_4$. The sesquiterpene nature of **3** was apparent in the ^{1}H-NMR spectrum, which involved signals assignable to allylic methyl (δ_H 2.08) and an isopropyl [δ_H 1.25 (6H, d, J=6.9 Hz) and 4.34 (1H, sept., J=6.9 Hz)] groups. However, obviously one carbon of the sesquiterpene skeleton was missing in **3**, because of the presence of a methoxy group (δ_H 4.03, s, 3H) for the 15th carbon in **3**. In fact, signals attributable to an aldehyde proton and to an isolated aromatic proton on C-6 in **1** and **2** were both invisible, and instead of those, signals of a pair of *meta*-coupled aromatic protons were observed at δ_H 7.43 and 7.14 (each 1H, d, J=2.7 Hz), suggesting its 11-norcadinane naphthoquinone structure. HMBC correlations enabled compound **3** to be firmly elucidated as the new norcadinane sesquiterpene, 11-nor-2-*O*-methylisohemigossypolone. Similar 11-norcadinane sesquiterpenes have been found as constituents of the heartwood of *Hibiscus elatus* and *H. tiliaceus*.[4,5]

In this study, the major fungitoxic compound **1** and related compounds **2** and **3** were detected in the diseased inner tissue that showed severe browning. This fact suggested that the fungitoxins of naphthoquinone-type cadinane sesquiterpenoids were induced in the infecting inner bark to function as a dynamic chemical defense by *P. aquatica*. Some plants of family Malvaceae, also a

Table 1. ^{1}H- and ^{13}C-NMR Chemical Shifts of **2** and **3**

C-No	**2**[a]		**3**		
	Proton[b]	Carbon	Proton[c]	Carbon	HMBC (H→C)
1	—	183.2	—	181.5(C)	
2	—	156.5	—	156.0(C)	
3	—	131.7	—	134.1(C)	
4	—	186.2	—	186.9(C)	
5	—	161.0	—	155.6(C)	
6	7.26	123.5	7.14	118.6(CH)	159.2, 122.6, 111.5
7	—	164.9	—	159.2(C)	
8	—	116.2	7.43	111.5(CH)	181.5, 122.6, 118.6
9	—	136.5	—	135.5(C)	
10	—	121.1	—	122.6(C)	
11	10.47	197.0			
12	4.29	29.7	4.34	28.9(CH)	155.6, 118.6
13, 14	1.20	23.4	1.25	23.5(CH_3)	155.6
15	2.06	9.5	2.07	9.8(CH_3)	186.9, 156.0, 134.1
2-OMe	4.05	60.4	4.03	60.7(CH_3)	156.0
7-OH	12.34	—	ND[d]	—	

[a] The ^{13}C-NMR spectrum of compound **2** was measured for pure material that had been isolated from the outer bark of healthy *P. aquatica*.
[b] Coupling constants: 12-H, J=6.9 Hz (sept.) and 13- & 14-H_3, J=6.9 Hz (d).
[c] Coupling constants: 6-H, J=2.7 Hz (d), 8-H, J=2.7 Hz (d), 12-H, J=6.9 Hz (sept.) and 13- & 14-H_3, J=6.9 Hz (d).
[d] Not detected, because C-7-OH did not afford any intra-molecular hydrogen bonding on compound **3** which had lost the C-11 formyl group.

member of order Malvales, constitutively contain naphthoquinone and/or naphthol-type cadinane sesquiterpenoids in pigment glands being dense in the carpel walls and young leaves,[6,7] and mature seed embryo and roots of 7-day-old seedlings[8] as prohibitins. Some of the Malvaceae plants are further able to induce those cadinane sesquiterpenes in gland-free tissues (*e.g.* mature leaves) as phytoalexins.[9-11]

In the search for pathogenic microorganisms from browned inner tissue, two distinguishable species of bacterial colonies tentatively named P-B1 and P-B2 (24 whitish and 20 colorless colonies from four agar plates of 9 cm i.d., respectively) appeared on the screening plate, but no fungus was apparent. These two bacteria, which had respectively been separated and purified on nutrient-broth agar plates, were inoculated into healthy trunk disks. After three days, the disks inoculated with P-B2 showed characteristic tissue browning, suggesting P-B2 to be the phytopathogen. P-B2, a rod-shaped and gram-negative bacterium, produced a yellow-green fluorescent pigment, pyoverdins, on pseudomonas agar (Difco Lab.) and King's B agar media, both of which are used in screening *Pseudomonas* bacteria by using the pigment production as a marker. Moreover, it accumulated indole in cultured potato-dextrose broth (Difco Lab.) and nutrient broth (Nissui) media. Thus, P-B2 was tentatively identified as a *Pseudomonas* species.[12] *P. syringae* pv. *syringae* is known as a cause of bacterial canker in trees.[13]

Since we obtained preliminary results that the healthy inner bark tissues of *P. aquatica* infected with the isolated bacterium induced some sesquiterpenes, including **1** and **2** (Shibatani *et al.*, unpublished results), further study on the phytoalexin production of this plant is in progress. Compound **3** was also indicative of the dynamic metabolism of the cadinane sesquiterpenes in infected tissues of *P. aquatica.*

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