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NOTE

Extractives of *Quercus crispula* sapwood infected by the pathogenic fungus *Raffaelea quercivora* II: isolation and identification of phenolic compounds from infected sapwood

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Abstract There has been a mass mortality of oak trees in the area along the coast of the Japan Sea. This phenomenon is caused by the ambrosia beetle *Platypus quercivorus*, which carries the ambrosia fungus *Raffaelea quercivora*. Extractives of a necrotic brownish coloration formed in the infected sapwood of *Quercus crispula* were investigated. The methanol extract of the damaged sapwood of *Q. crispula* was concentrated *in vacuo* and centrifuged to yield precipitates and the supernatant. The precipitates were subjected to Sephadex LH-20 column chromatography and preparative HPLC to describe a novel ellagic acid derivative (**1**). The 10 % methanol water-soluble part of the supernatant was subjected to medium-pressure ODS column chromatography and preparative HPLC, respectively, to analyze a known lignan (**2**). Sulfuric acid hydrolysis of (**1**) yielded an ellagic acid and a gallic acid. NMR and LC-TOF/MS indicated that an ellagic acid and a gallic acid bonded to a xylose with glycosidic and ester bonds, respectively. Compound (**1**) was identified as 4,5-dihydroxy-6-(3,7,8–trihydroxy-5,10-dihydro–chromeno[5,4,3-cde]chromen-2-yloxy)-tetrahydro-pyran-3-yl ester, and compound (**2**) was identified as (–)-lyoniresinol. The presence of (–)-lyoniresinol from damaged sapwood indicated that infection of *R. quercivora* may cause the formation of a pseudo-heartwood in the sapwood of *Q. crispula*.

Keywords *Quercus crispula* · *Raffaelea quercivora* · (–)-lyoniresinol · Infection · Tannin

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Introduction

The mass mortality of oak trees has been prevalent along the eastern coast of the Japan Sea since the late 1980s [1]. This large-scale forest mortality is caused by the ambrosia fungus, *Raffaelea quercivora*, which is a symbiotic microorganism carried by the ambrosia beetle, *Platypus quercivorus* Murayama [2]. The beetle carries the fungus in a specialized fungal storage organ called a mycangium for breeding its larva. The ambrosia beetle bores into the trunk of a dead or wilting oak tree and a brownish yellow coloration is generated around the beetle gallery in the sapwood. The colored part could contain several substances, such as repellents against the beetle, because beetles newly attacking a tree have an inclination to avoid colored parts of the trunk. However, knowledge about the chemical components and the biological function of the colored part is limited.

Sapwood coloration caused by enzymatic reactions has been reported in Douglas fir, *Pseudotsuga menziesii* [3, 4]. Compounds existing in the living cells of Douglas-fir sapwood, such as *o*-dihydroxy phenols, (–)-epicatechin, and dihydroquercetin, react with enzymes naturally occurring in the trees and form brown-colored materials in the exposed surface of lumber from Douglas fir. Bauch et al. [5] elucidated that the yellow coloration in *Quercus robur* L. and *Q. petraea* is due to the reaction products of hydrolyzable tannins caused by the fungus *Paecillomyces variotii*. The tannase of *P. variotii* [6] would play the important role of releasing the reactants from wood components for the coloration.

In our previous report, tannase and laccase activities were detected in a culture of *R. quercivora*, and they decomposed hydrolyzable tannin to gallic acid followed by oxidization to purprogallin carboxylic acid (PGCA).

Furthermore, we suggested that PGCA is one of the coloration substances or precursors of the unextractable colored substances of *Q. crispula* sapwood infected by *R. quercivora* [7].

This paper deals with the isolation and identification of a lignan and hydrolyzable tannins from yellowed *Q. crispula* sapwood infected by the pathogenic fungus *R. quercivora*.

Experimental

General

HPLC equipped with a reversed phase column (Inertsil® ODS-3 V, 4.6 mm i.d. × 250 mm, GL Science, Japan) and a diode array detector (SPD-M10A vp, SHIMADZU) was used for analysis of the components. The following solvent system was used: a linear gradient elution for 45 min from 5 to 100 % of solvent A, (solvent A: methanol; solvent B: water including 0.05 % trifluoroacetic acid). The flow rate was 1 mL/min, monitored at 280 nm.

Semi-preparative HPLC was performed on an Inertsil® ODS-3 column (10φ × 250 mm) at room temperature. The mobile phase was a mixture of solvent B and 100 % acetonitrile (solvent C) at a flow rate of 3 mL/min using a gradient elution of 0–30 % of solvent C by a linear decrease for 0–60 min. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a JEOL ECA-500 (500 MHz), and chemical shifts are given in δ (ppm) values relative to that of the solvent [methanol-d_4 (δ_H 3.35; δ_C 49.0)].

FAB-MS analysis was performed with a JMS-700/GI (JEOL) spectrometer operated in positive ion mode with 3-Nitrobenzyl alcohol (NBA) as the matrix. LC-TOF/MS analysis was performed with a Waters® Xevo™ QTof MS spectrometer operated in negative ion mode with a spray source type ES, capillary voltage of 3.3 kV, cone voltage of 30.0 V, collision energy of 21.0 V, and calibration temperature of 500 °C. The mass range was 50–1000 Da. Optical rotations were measured using a JASCO P-2300 system.

Plant material

Quercus crispula infected by *R. quercivora* was collected in Toyama Forest and Forestry Products Research Center in November 2007.

Extraction and isolation

Air-dried plant material was separated into bark, unaffected sapwood, affected sapwood and heartwood. The affected sapwood was ground by a ultra centrifugal mill and the powder (725.6 g) was extracted with 70 % aqueous acetone (11.0 L). The aliquot of the extract was analyzed using HPLC. The decoction was concentrated in vacuo and centrifuged (3500 rpm for 10 min at 4 °C) to yield a precipitate (5.25 g) and supernatant (15.4 g). The ethanol soluble part of the precipitate was pre-fractionated by Sephadex LH-20 column chromatography (CC) (φ2.4 cm × 20 cm) eluting with ethanol (500 mL), 50 % aqueous ethanol (200 mL), 50 % aqueous methanol (200 mL), and 70 % aqueous acetone (200 mL) to give 8 fractions. Fraction 5 (eluted with 50 % aqueous ethanol) was purified by preparative HPLC to give (**1**) (5.5 mg).

The 10 % methanol soluble part of the supernatant was subjected to medium-pressure CC with ODS (C18 120A 20/40 μm) eluting with 10, 20, 30, 35, 40, and 50 % methanol to give 6 fractions. The 35 % methanol eluate was subjected to preparative HPLC to give (**2**) (106.7 mg).

4,5-dihydroxy-6-(3,7,8-trihydroxy-5,10-dihydro-chromeno[5,4,3-cde]chromen-2-yloxy)-tetrahydro-pyran-3-yl ester (**1**)

Brown oil; 5.5 mg; LC-TOF/MS: m/z 584.9888 [M − H]$^-$, (calcd. for $C_{26}H_{18}O_{16}$), m/z 300.9526 [M-ellagic acid]$^{-\cdot}$$C_{12}H_{13}O_9$; ^{1}H NMR (500 MHz, CD_3OD) δ 7.79, 7.49 (2H in total, each s, ellagic acid unit protons), 7.09 (2H, s, galloyl-H), 3.62, 3.73, 3.88, 4.22, 5.00, 5.11 (6H in total, sugar protons), see Table 1; ^{13}C NMR (150 MHz, CD_3OD) δ166.4 (galloyl C-7), 159.8 (ellagic acid unit C-7,7′), 148.8 (ellagic acid unit C-4′), 146.9 (ellagic acid unit C-4), 145.2 (galloyl C-3,5), 141.4 (ellagic acid unit C-3), 139.6 (ellagic acid unit C-3′), 138.7 (galloyl C-4), 136.7 (ellagic acid unit C-2′), 136.4 (ellagic acid unit C-2), 119.7 (galloyl C-1), 115.4 (ellagic acid unit C-1), 112.4 (ellagic acid unit C-5), 112.0 (ellagic acid unit C-1′), 110.6 (ellagic acid unit C-5′), 109.0 (galloyl C-2,6), 108.8 (ellagic acid unit C-6′), 108.0 (ellagic acid unit C-6), 102.9 (xylose C-1), 73.3 (xylose C-2), 73.1 (xylose C-3), 71.5 (xylose C-4), 62.5 (xylose C-5).

(–)-lyoniresinol (**2**)

White amorphous crystal; FAB-MS (positive mode) m/z 421.0 [M + H]$^+$ ($C_{22}H_{28}O_8$); ^{1}H NMR (CD_3OD, 600 MHz) δ 1.60–1.61 (1H, m, H-8′), 1.94–1.96 (1H, m, H-8), 2.55 (1H, dd, J = 11.5 and 15.0 H-7′) 2.67 (1H, dd, J = 4.8 and 15.1 H-7′), 3.35 (3H, s, 3′-OMe), 3.47 (1H, m, H-9′), 3.57 (1H, dd, J = 4.8 and 10.3 H-9′), 3.48 (2H, d, J = 2.76, H-9), 3.71 (6H, s, 3-OMe and 5-OMe), 3.82 (3H, s, 5′-OMe), 4.29 (1H, d, J = 5.52, H-7), 6.36 (2H, s, H-2,6), 6.56 (1H, s, H-2′); ^{13}C NMR (CD_3OD, 150 MHz): δ 32.2 (C7′), 39.5 (C8′), 41.0 (C-7), 47.7 (C-8), 55.3 (3′-OMe), 55.5 (5-OMe, 3-OMe), 58.8 (5′-OMe), 62.9 (C-9),

Table 1 ^{1}H and ^{13}CNMR spectral data and key HMBC correlations of (**1**)

Moiety	Position	δ_C (ppm)	δ_H (ppm)		HMBC
Ellagic acid group	1	115.4			
	2	136.4			
	3	141.4			
	4	146.9			
	5	112.4	7.79	1H, s	C1, C2, C3, C4, C5, C6, C7
	6	108.0			
	7	159.8			
	1′	112.0			
	2′	136.7			
	3′	139.6			
	4′	148.8			
	5′	110.6	7.49	1H, s	C1′, C2′, C3′, C4′, C6′, C7′
	6′	108.8			
	7′	159.8			
Xylose	1″	102.9	5.11	1H, d, $J = 6.9$	C4
	2″	73.3	3.73	1H, dd, $J = 6.85, 8.60$	
	3″	73.1	3.88	1H, t, $J = 9.15$	
	4″	71.5	5.00	1H, m	C7‴
	5″	62.5	3.62	1H, dd, $J = 9.75, 11.45$	
			4.22	1H, dd, $J = 5.70, 12.00$	
Gallic acid group	1‴	119.7			
	2‴, 6‴	109.0	7.09	2H, s	
	3‴, 5‴	145.2			
	4‴	138.7			
	7‴	166.4			

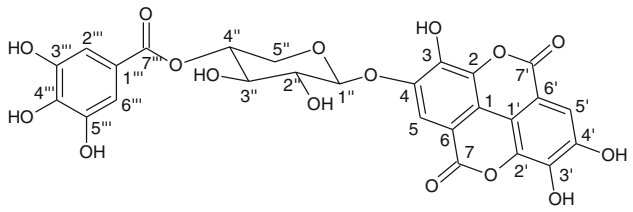
65.5 (C-9′), 105.6 (C-2, 6), 106.5 (C-2′), 124.9 (C-1′), 128.9 (C-6′), 133.2 (C-4), 137.5 (C-4′), 138.0 (C-1), 146.4 (C-5′), 147.3 (C-3′), 147.7 (C-3,C-5); $[\alpha]_D^{23.3} = -9.5°$ (c = 0.21, MeOH).

Results and discussion

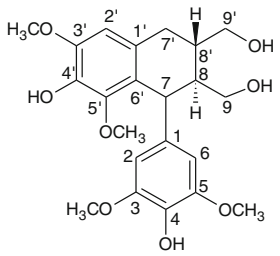
A concentrated 70 % aqueous acetone decoction was obtained from the dried sapwood of *Q. crispula* infected by *Raffaelea quercivora*. When the concentrated solution was sitting at room temperature, a precipitate was observed. After having been centrifuged, the precipitate (brownish-red colored, 5.25 g) and supernatant (khaki colored, 15.4 g) were separated. The precipitate was subjected to Sephadex LH-20 column chromatography and preparative HPLC to yield (**1**). The chemical shifts and coupling constants of ^{1}H and ^{13}C NMR spectra of (**1**) are shown in Table 1. Acid hydrolysis of (**1**) gave an ellagic acid and a gallic acid, indicating that it possesses these phenolic acids with ester binding and/or glycosidic linkage as shown in ellagitannins such as tellimagrandin II [8]. The negative ion mode of the LC-TOF/MS for (**1**) by ESI gave 2 main peaks at m/z 584.9888 and m/z 300.9526 with an intensity of 80 and 100 %, respectively. In our previous paper, ester bonds of gallic acid and hexahydroxy diphenoic acid (HHDP) to a sugar of tercheblin gave only molecular ions without any fragments using the conditions described in this paper for the negative ion mode of the LC-TOF/MS [9]. In addition, pentagalloylglucose gave 2 main peaks for the molecular ion and fragment ion corresponding to $[M - \text{galloyl}]^+$ (data not shown). These results mean that an ester binding in hydrolyzable tannins is not cleaved, while a glycosidic linkage is easily cleaved by the ESI mode of the LC-TOF/MS. If the fragment ion m/z 300.9526 could be obtained by loosing 1 mol of ellagic acid from molecular ion m/z 584.9888, an ellagic acid should be connected at the C1 position of the sugar moiety as a glycosidic linkage. Thus, a mole of gallic acid could be connected at another position of the sugar as an ester bond, which appears at m/z 300.9526. According to the mass number of the molecular ion and the fragment ion peaks, the sugar moiety of (1) is considered to be xylose.

The glycoside was determined to be xylose through the chemical shifts and coupling patterns of its proton signals

(6 protons, δ 3.62–5.11), which were connected by a ^{1}H-^{1}H-COSY spectrum. HMBC (evolution time (Δ) = 200 ms) shows that the H-1″ proton on xylose (δ 5.11 ppm) was correlated with the C-4 of the ellagic acid moiety. From here onwards, the presence of the connection of the C-4 of ellagic acid to the C-1″ of xylose was indicated. Furthermore, the chemical shift of the H-4″ proton of the xylose residue appeared downfield compared to xylose, indicating a connection of the C-7‴ of gallic acid to the C-4″ of xylose. On the basis of these data, the structure of (**1**) was identified as 4,5-dihydroxy-6-(3,7,8-trihydroxy-5,10-dihydro-chromeno[5,4,3-cde] chromen-2-yloxy)-tetrahydro-pyran-3-yl ester (Fig. 1), a novel ellagic acid derivative. A paper suggested that hydrolyzable tannins such as pedunculgin, 1(β)-*O*-galloylpedunculagin, casuariin, and casuarinin in the wood of *Platycarya strobilacea* were decreased by the charring which causes radical oxidation. However, the concentration of 3′-*O*-methyl-(**1**) in the wood was increased by charring [10]. Interestingly, (**1**) was newly isolated in the yellowed sapwood of *Q. crispula*, so a similar phenomenon may occur in infected sapwood oxidized by the tannase and laccase in the pathogenic fungus *R. quercivora.* These studies indicated that radical oxidation by charring or infection may change the hydrolyzable tannins into unique compounds like (**1**).



1: 4,5-dihydroxy-6-(3,7,8-trihydroxy-5,10-dihydro-chromeno [5,4,3-cde]chromen-2-yloxy)-tetrahydro- pyran-3-yl ester



2: (-)-lyoniresinol

Fig. 1 Structures of **1** and **2**

The 10 % methanol soluble part of the supernatant was subjected to Sephadex LH-20 CC and preparative HPLC, respectively, to afford (**2**) as a known lignan. The molecular formula of (**2**) was found to be $C_{22}H_{28}O_8$ by FAB-MS (positive mode) m/z 421.0 $[M + H]^+$ ($C_{22}H_{28}O_8$). The results of ^{1}H and ^{13}C NMR spectra were shown in Table 2. Two-dimensional NMR, HMQC, HMBC, and COSY were performed to determine the structure of (**2**). The results identified it as (–)-lyoniresinol showing −9.5° of optical rotation (Fig. 1).

Table 2 ^{1}H and ^{13}CNMR spectral data and key COSY and HMBC correlations of (**2**)

Position	δ_C (ppm)	δ_H (ppm)	COSY	HMBC	
1	138.0				
2 and 6	105.6	6.36	2H, s		C1, C3, C4, C5, C7
3 and 5	147.7				
4	133.2				
7	41.0	4.29	1H, d, J = 5.52	8	C1, C2, C8, C9, C1′, C5′, C6′, C8′
8	47.7	1.94–1.96	1H, m	7, 9, 8′	C9, C7′, C8′, C9′
9	62.9	3.48	2H, d, J = 2.76		
1′	124.9				
2′	106.5	6.56	1H, s		C1′, C3′, C4′, C7′
3′	147.3				
4′	137.5				
5′	146.4				
6′	128.9				
7′	32.2	2.55, 2.67	1H, dd, J = 11.5, 15.0, 1H, dd, J = 4.8, 15.0	7′,8′	C8, C1′, C2′, C6′, C8′, C9′
8′	39.5	1.60–1.61	1H, m	8, 7′, 9′	C8, C9, C9′
9′	65.5	3.47, 3.57	1H, m, 1H, dd, J = 4.8, 10.3		C8, C7′
5′-OMe	58.8	3.82	3H, s		C5′
3-OMe, 5-OMe	55.5	3.71	3H × 2, s		C3, C5
3′-OMe	55.3	3.35	3H, s		C3′

Several papers have been published on the isolation of lyoniresinol, including on the mixture of (+)- and (–)-lyoniresinol [11], its glycoside [12–14], (+)-lyoniresinol from the heartwood of *Q. crispula* [15], and (–)-lyoniresinol from the root of *Kirkia acuminata* [16]. Interestingly, (–)-lyoniresinol was isolated from the sapwood of *Q. crispula* infected by *R. quercivora* in the present study, in spite of the fact that lyoniresinol was not contained in the non-infected sapwood of *Q. crispula*. This experimental result might show the formation of pseudo-heartwood from sapwood by *R. quercivora* infection.

On the other hand, resulting coloration parts in sapwood infected by *R. quercivora* would be observed as a repellent part against the ambrosia beetle *Platypus quercivorus* as mentioned in the Introduction. Therefore, the substances, including two compounds isolated in this paper, consisted in the colored sapwood may have repellent properties against the beetle. In the next paper, the evidence would be made clear to solve the mystery of the mass mortality of oak tree.

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