Organic LETTERS

Letter

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New Metabolites of *C*-Glycosidic Ellagitannin from Japanese Oak Sapwood

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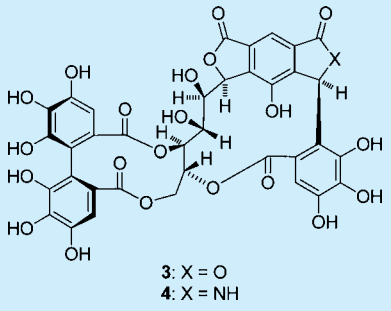
Supporting Information

ABSTRACT: Two unusual ellagitannin metabolites, quercusnins A (**3**) and B (**4**), have been isolated from the sapwood of *Quercus crispula*, and their structures determined by spectroscopic methods, as well as DFT calculations of ^{1}H and ^{13}C NMR chemical shifts of the possible four diastereomers. Treatment of the major ellagitannin species, vescalagin, with Shiitake mushroom (*Lentinula edodes*) gave **3**, which indicated that these unique ellagitannins were the fungal metabolites of ellagitannins.



Quercus species (oak trees) are woody plants typically found in the northern hemisphere and are commonly used as building materials. The high durability of this wood has been attributed to its high concentration of ellagitannins, including mainly vescalagin (**1**) and its C-1 isomer castalagin (**2**) (Figure 1), which defend the wood against fungal decay.[1] The heartwood (inner area of the wood) of plants belonging to this species is generally composed of dead cells, whereas the sapwood (outer layer of the wood) consists of living ray parenchyma cells. As the tree grows, the cells in the transition area between sapwood and heartwood die according to a programmed process, and in tandem with this, ellagitannins are biosynthesized and subsequently accumulated.[2] During the course of our recent study toward understanding the dynamic ellagitannin metabolism in wood samples belonging to the Quercus species,[3] we isolated the unusual ellagitannin metabolites **3** and **4** from the sapwood of *Quercus crispula*.

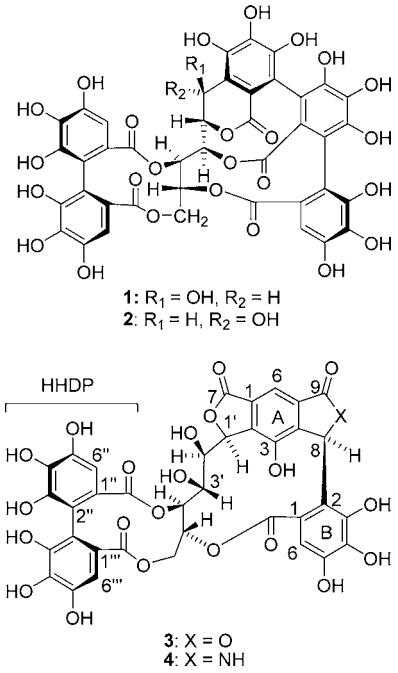


Figure 1. Structures of compounds **1**−**4**.

The dried sapwood samples were extracted with acetone−H_2O (7:3 v/v) and fractionated into 14 fractions by column chromatography over a Sephadex LH-20 column, which was eluted with H_2O containing increasing amounts of MeOH and then finally with 60% acetone. Fractions 9−14 were individually purified by column chromatography over Diaion HP20SS, Chromatorex ODS, and Sephadex LH-20 columns to yield compounds **3** (isolation yield: 0.003% from dried sapwood) and **4** (0.0004%), together with **1** (0.23%), **2** (0.25%), ellagic acid (0.0007%), 1,2,6-trigalloyl-β-D-glucopyranose (0.001%), pedunculagin (0.022%), flavogallonic acid dilactone (0.001%), dehydrocastalagin (0.0004%), mongolicain A (0.003%), roburin D (0.004%), and castaneanin A (0.0004%) (see Supporting Information for structures).

Compound **3** was obtained as a yellow amorphous powder with $[\alpha]^{20}_{D}$ −75.8 (c = 0.1, MeOH), and its molecular formula was determined to be $C_{36}H_{24}O_{22}$ by HRFABMS analysis based on its $[M + H]^+$ peak at m/z 809.0831 [calcd for $C_{36}H_{25}O_{22}$ = 809.0838], as well as its NMR spectra. The ^{13}C NMR (Table 1) and HMBC spectra of **3** indicated the presence of a hexahydroxydiphenoyl (HHDP) ester group attached to the 4,6-positions of an open-chain hexose moiety with *C*-glycosidic

Received: January 15, 2014
Published: February 24, 2014

   dx.doi.org/10.1021/ol500146a | *Org. Lett.* 2014, 16, 1378−1381

Table 1. ^{1}H and ^{13}C NMR Data for Compounds 3 and 4[a]

	3		4	
position	δ_H (mult, J)	δ_C	δ_H (mult, J)	δ_C
Ring A				
1		129.6		128.2
2		140.2		138.4
3		148.1		147.1[d]
4		141.4		139.2[e]
5		133.8		139.4[e]
6	7.79 s	114.5	7.78 s	113.0
7		170.6		171.5
8	7.28 s	75.1	6.42 s	52.6
9		169.9		169.4
Ring B				
1		123.0		122.1
2		112.8		114.8
3		147.0		147.0[d]
4		137.3		137.2
5		146.2		145.1
6	7.02 s	111.5	6.95 s	111.2
7		165.5		165.4
glucose				
1′	5.87 d (4.1)	83.4	5.84 d (4.0)	83.5
2′	4.27 d (4.1)	73.2	4.29 d (4.0)	73.2
3′	2.69 s	68.3	2.81 s	68.0
4′	4.77 dd (8.2, 0.9)	74.8	4.77 dd (8.3, 1.0)	74.9
5′	5.08 dd (8.2, 2.7)	72.9	5.05 dd (8.3, 2.8)	72.7
6′	3.86 br d (12.9)	64.6	3.85[b]	64.6
	4.75 dd (12.9, 2.7)		4.73 dd (13.2, 2.8)	
HHDP (glc-4)				
1″		126.7[c]		125.6[f]
2″		115.8		115.56[g]
3″		144.2		144.2
4″		136.4		136.3
5″		145.1		145.1
6″	6.80 s	108.5	6.78 s	108.4
7″		167.6		167.7
HHDP (glc-6)				
1‴		125.7[c]		126.6[f]
2‴		115.6		115.64[g]
3‴		144.2		144.2
4‴		135.9		135.9
5‴		145.0		144.9
6‴	6.59 s	107.4	6.57 s	107.3
7‴		169.1		169.2

[a]Measured in acetone-d_6 (500 MHz for ^{1}H and 125 MHz for ^{13}C). [b]Overlapped with the H_2O signal. [c−g]May be interchanged in the same column.

linkage (δ_C 83.4, C-1′; 73.2, C-2′; 68.3, C-3′; 74.8, C-4′; 72.9, C-5′; 64.6, C-6′) (Figure 2). Furthermore, the HMBC correlation of an aromatic singlet at δ_H 7.02 revealed the presence of a 3,4,5-trihydroxybenzoyl group (ring B), which suggested that **3** was structurally related to **1** and **2**.

The remaining carbon signals were attributed to a penta-substituted benzene ring (Table 1, A1−A6, δ_H 7.79, H-A6), two carboxyl carbons (δ_C 170.6, A7; δ_C 169.9, A9), and an oxygenated methine carbon (δ_C 75.1, δ_H 7.28, A8). The HMBC spectrum of **3** revealed correlations from the A8 methine proton to the pyrogallol carbons (i.e., B1, B2, and B3), indicating that this proton was connected to the B ring at C-2. Correlations between the methine proton and the aromatic carbons at A3 (δ_C 148.1), A4 (δ_C 141.4), and A5 (δ_C 133.8) suggested that the methine carbon was also attached to the A4 aromatic carbon. The chemical shift (δ_C 148.1) of the A3 carbon indicated that it was attached to a phenolic hydroxyl group. In contrast, the A6 aromatic proton signal (δ_H 7.79, s) showed correlations to two aromatic carbons [A2 (δ_C 140.2) and A4] and two carboxyl carbons (A7 and A9). The A9 carboxyl carbon was also correlated with the A8 proton, indicating the presence of a γ-lactone ring between these two positions. The A1 (δ_C 129.6), A2, and A7 carbon signals showed correlations to the H-1′ proton of the hexose chain (δ_H 5.87, d, J = 4.1 Hz). The HMBC correlations and the chemical shift of H-1′ indicated the presence of an ester linkage between C-1′ and A-7. Taken together, these HMBC correlations enabled us to construct subunit A, containing two γ-lactone rings.

dx.doi.org/10.1021/ol500146a | Org. Lett. 2014, 16, 1378−1381

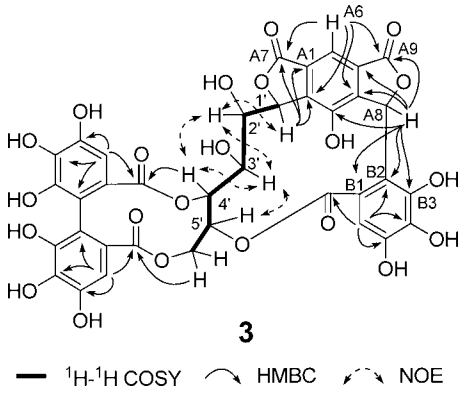


Figure 2. Selected ^{1}H−^{1}H COSY, HMBC, and NOESY correlations of **3**.

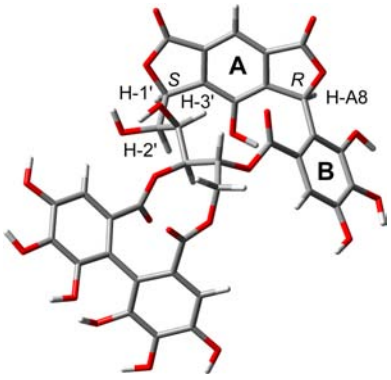


Figure 3. Structure of the lowest energy conformer of **3** calculated at the B3LYP-SCRF/6-31G(d,p) level in acetone with the PCM model.

The HMBC spectrum of **3** revealed that there were no correlations between the H-2′ and H-3′ protons of the hexose chain and the ester carbonyl carbons, indicating that these positions contained free hydroxyl groups. This was supported by a deuterium-induced differential isotope shift ^{13}C NMR measurement in acetone-d_6 + D_2O (95:5) and acetone-d_6 + H_2O (95:5), which showed an isotopic shift of C-2′ ($\Delta\delta$ 0.11) and C-3′ ($\Delta\delta$ 0.11). The result confirmed the presence of hydroxyl protons exchangeable with deuterium atoms at these positions. Although the H-5′ proton did not show an HMBC correlation with any of the ester carbonyl carbons, the chemical shift (δ_H 5.08) of this methine proton indicated that it was part of an ester moiety connected to the 3,4,5-trihydroxybenzoyl group (ring B).

The atropisomerism of the HHDP group was determined to be *S* by CD spectroscopy (positive and negative Cotton effects at 230 and 263 nm, respectively).[4]

Based on the assumption that **3** was produced from **1**, DFT calculations of the ^{1}H and ^{13}C NMR chemical shifts of the four possible diastereomers (i.e., 1′*S*,A8*S*; 1′*S*,A8*R*; 1′*R*,A8*S*; and 1′*R*,A8*R*) were performed using the GIAO method at the mPW1PW91-SCRF/6-311+G(2d,p) level in acetone with the PCM model, and the results were compared with those from the experimental NMR spectra.[5] The calculated ^{1}H and ^{13}C NMR chemical shifts of the 1′*S*,A8*R* structure showed the best correlation of the four diastereomers with the experimental data of **3** (see Supporting Information). The DP4 probability analysis was also performed based on the calculated and experimental ^{1}H and ^{13}C NMR chemical shifts of **3**.[6] The DP4 analysis of the four possible structures gave 100.0% probability for the 1′*S*,A8*R* structure. Thus, the configuration of **3** was assigned to be 1′*S*,A8*R*. The most stable conformation of the structure is shown in Figure 3. It is noteworthy that the NOEs between the aliphatic protons observed in the NOESY spectrum were consistent with the stereostructure. Furthermore, the calculated ^{1}H−^{1}H coupling constants of the 1′*S*,A8*R* structure also showed the best correlation with the experimental data of **3**, which supported the configuration of hexose moiety, open-chain glucose (see Supporting Information).

The unusual upfield shift of H-3′ (δ_H 2.69) could be understood in terms of the anisotropic effect of the aromatic ring in subunit A. The downfield shift of the methine proton at A8 (δ_H 7.28) suggested that there was an ester at this position together with a strong deshielding effect from an aromatic ring. This result also supported the absolute structure of **3**. In addition, further support for the absolute structure of **3** was obtained by TDDFT calculation of the ECD spectrum performed at the CAM-B3LYP-SCRF/6-31G(d,p) level in MeOH with the PCM model. The calculated ECD spectrum of **3** showed good agreement with experimental ECD spectrum (Figure 4).

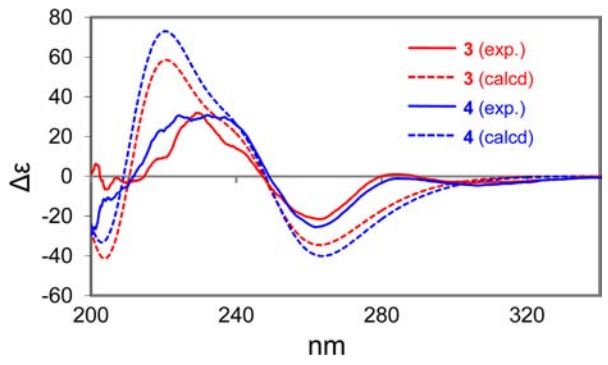


Figure 4. Experimental and calculated ECD spectra of **3** and **4**. Experimental ECD spectra were measured in MeOH. Calculation of ECD spectra was performed at the TD-CAM-B3LYP-SCRF/6-31G(d,p) level in MeOH with the PCM model. Calculated spectra were red-shifted by 10 nm.

Taken together, these data suggested that the structure of **3** was as shown in Figure 1, and the compound was named quercusnin A. The subunit A was unusual from the perspective of ellagitannin biogenesis, where ellagitannins are typically produced via the oxidative metabolism of 3,4,5-trihydroxybenzoic acid. The ^{1}H NMR spectrum of **3** revealed another unusual feature, with the H-3′ proton of the hexose moiety resonating at an unusually highfield position (δ_H 2.69). This indicates that the subunit A exerts a strong shielding effect on H-3′.

The ^{1}H and ^{13}C NMR spectra of **4** were closely related to those of **3**, and the HMBC correlations also indicated that **4** was an analogue of **3**. The only significant differences observed between the two compounds were the chemical shifts of the aliphatic methine proton at A8 (δ_H 6.42, s) and the carbon at A8 (δ_C 52.6), which were shifted upfield compared with those of **3** (δ_H 7.28 and δ_C 75.1). These chemical shift values suggested that the oxygen atom at A8 of **3** had been replaced by a nitrogen atom in **4**. The presence of the nitrogen atom in the molecule was confirmed by the HRFABMS [*m*/*z* 808.1004 [M + H]$^+$ (calcd for $C_{36}H_{26}NO_{21}$, 808.0997)]. DFT calculations of the ^{1}H and ^{13}C NMR chemical shifts of four diastereomers of **4**

dx.doi.org/10.1021/ol500146a | *Org. Lett.* 2014, 16, 1378−1381

were performed in a manner similar to that used for **3**, and the results suggested that the stereochemistry of **4** was the same as that of **3** (see Supporting Information). Based on these results, the structure of **4** was determined to be as shown in Figure 1. We have named the compound quercusnin B. This compound represents the first reported example of an ellagitannin containing a nitrogen atom in its skeleton to be reported in the literature.

The unusual structures of **3** and **4**, especially subunit A, implied that these compounds were not produced via a normal plant biosynthetic pathway and that they were more likely produced as a consequence of metabolic processes in the living wood involving endophytes or degradation by fungi during the lumbering and drying processes. To determine which of these scenarios was the most likely, we conducted an experiment involving the degradation of **1** using microorganisms. Briefly, compound **1** was placed in a medium containing malt extract, and the resulting mixture was treated with the mycelia of Shiitake mushroom (*Lentinula edodes*), which is typically cultivated using ellagitannin-rich wood belonging to the Quercus species, such as *Q. acutissima*. Following a cultivation period of 6 days, the mixture was filtered and the filtrate was purified by column chromatography over a Sephadex LH-20 column (using an increasing amount of MeOH in H_2O as the eluent, followed by 60% acetone). The fraction eluted with 60% acetone was further purified by column chromatography over a Diaion HP20SS column to give **3** (0.15% from **1**) with $[\alpha]^{20}_{D}$ −81.9 (c = 0.12, MeOH), which was identified by comparison of its HPLC, UV, and ^{1}H NMR spectroscopic data with those of our initial sample. These results also confirmed the absolute structure of **3** including that the sugar moiety of **3** was derived from D-glucose. Compound **4** was most likely generated by the incorporation of ammonia during the production of **3**. Several unstable byproducts were also detected during the production process which may be related to the production of **3**. Further studies are currently underway in our laboratory to determine the mechanism responsible for the production of the unique structures of **3** and **4**.

It is well-known that wood-decaying fungi possess oxidative enzymes, such as laccase, lignin peroxidase, and manganese peroxidase that they use for the degradation of phenolic compounds, which can accumulate in the wood to provide some form of chemical defense mechanism. The degradation of these materials by microorganisms is important from the perspective of chemical ecology, phytochemistry, and agricultural chemistry.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details, 1D and 2D NMR, and computational results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was supported in part by the Japan Society for the Promotion of Science KAKENHI (Grant No. 25870532).

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