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Lignan Biosynthesis in Liverworts *Jamesoniella autumnalis* and *Lophocolea heterophylla*

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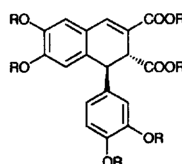
Abstract: The formation of the lignans in liverworts, *Jamesoniella autumnalis* and *Lophocolea heterophylla* was investigated. Feeding experiment of [8-^{2}H] caffeic acid (**4**) on axenic cultures of *J. autumnalis* has shown that jamesopyrone (**2**) and scapaniapyrone (**3**) were derived from the coupling of two intact caffeic molecules. Studies in a cell-free system of *J. autumnalis* and *L. heterophylla* have shown the formation of (±)-**1** from **4**. © 1998 Elsevier Science Ltd. All rights reserved.

Lignans are widely distributed secondary metabolites, which consist of two phenylpropane units, linked by 8,8' bonds.[1] Their biosyntheses in vascular plants are generally originated from monolignols represented by coniferyl alcohol. Recently, 78-kilodalton protein named 'dirigent protein' has been isolated from *Forsythia suspensa*, which effects stereoselective bimolecular phenoxy radical coupling in the presence of an oxidase and one electron oxidant.[2] Dirigent proteins inherently lack a catalytically active site, and are thought to involve capture of *E*-coniferyl alcohol-derived free-radical intermediates for enantioselective formation of (+)-pinoresinol. On the other hand, a lignan as an unusual naphthalene derivative, 1-(3,4-dihydroxyphenyl)-6,7-dihydroxy-1,2-dihydro-2,3-naphthalenedicarboxylic acid (**1**) has been isolated from gametophytes of liverwort *Pellia epiphylla*.[3] A further oxygenated lignan, 2,3,6'-tricarboxy-6,7-dihydroxy-1(3')-2'-pyranonyl-1,2-dihydronaphthalene (**2**) has also been isolated from *Jamesoniella autumnalis*,[4] which is referred to as jamesopyrone in this paper. They were optically active and considered to be biosynthesized from two caffeic molecules, a common phenolic unit in liverworts. It suggests that the stereochemical mechanism of lignan biosynthesis in liverworts is very similar to that in vascular plants in spite of the differences in the precursor. Thus, we have been intrested in the biosynthesis of lignans in liverworts. In this paper, we report their biosynthesis in liverworts *J. autumnalis* and *Lophocolea heterophylla* by a feeding experiment of



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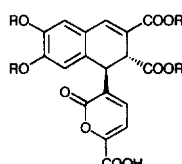
5 : R = Me



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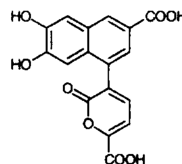

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[8-^{2}H]caffeic acid (4), and a formation of (±)-1 from 4 in their cell-free extracts with H_2O_2.

An intact plant of *J. autumnalis* was collected in Germany and identified by Dr. R. Mues (University of Saarlandes, Germany). The cultures were grown in 300-ml flasks with 50 ml of a modified B5 agar medium (pH 5.75) containing 5 g/l of sucrose. The flasks were kept under continuous light of 2000 lux at 22°C and subcultured at intervals of 2 months. The incorporation of [8-^{2}H] 4[5] (50 atom% ^{2}H) was carried out by feeding the caffeic acid (5 mM) to the gametophytes in 5 x 30 ml of the B5 liquid medium. The gametophytes were grown for 28 days in the medium with [8-^{2}H] 4 (1.5 g dw), then harvested by filtration, air-dried and pulverized. The powdered materials were extracted with MeOH (100 ml x 2), each MeOH solution then being concentrated *in vacuo* to dryness (121 mg). The MeOH extracts were separated by chromatography on a Sephadex LH-20 column (25 mm i.d. x 150 cm) with MeOH as an eluent, and then HPLC with 0.5 % HCOOH 20% AcCN/H_2O to give (+)-jamesopyrone (2) (0.7 mg, $[\alpha]_D$ = +51.0° *c* 0.07) and scapaniapyrone (3) (0.3 mg). Purified (+)-2 and 3 were identified by direct comparison of the ^{1}H NMR spectra with an authentic sample. 3 was first isolated from the liverwort *Scapania undulata*.[6] Additional chiral HPLC analysis of methylated lignan 6 from (+)-2 with CH_2N_2, indicated that the labeled (+)-2 was enantiomerically pure. The signals at δ 4.09 and 7.82 were observed in the ^{2}H-NMR spectra of (+)-2 and 3, respectively. The presumed locations of deuterium at the H-2 in (+)-2 and 3 show that they originate from the H-8 of 4.

An intact plant of *L. heterophylla* was collected in Japan and identified by Dr. T. Furuki (Natural History Museum & Institute, Chiba, Japan). The cultures of dedifferentiated cells were grown in 30-ml test tubes with 8 ml of a modified MSK agar medium (pH 5.7) containing 20 g/l of glucose. The tubes were kept under continuous light of 2000 lux at 25°C and subcultured at intervals of six weeks. The incorporation of [8-^{2}H] 4 was carried out in the same manner. The MeOH extracts from the plant were separated by HPLC with 0.5 % HCOOH 20% AcCN/H_2O to give 1 (1.3 mg) which was identified by direct comparison of the ^{1}H NMR spectra with an authentic sample. Additional chiral HPLC analysis of methylated lignan 5 (0.5 mg, $[\alpha]_D$ = -137.3° *c* 0.05) from 1 with CH_2N_2, indicated that the labeled 1 was (-)-isomer and enantiomerically pure. The signal at δ 4.00 was observed in the ^{2}H-NMR spectra of (-)-1. The presumed locations of deuterium at the H-2 in (-)-1 show that it originates from the H-8 of 4.

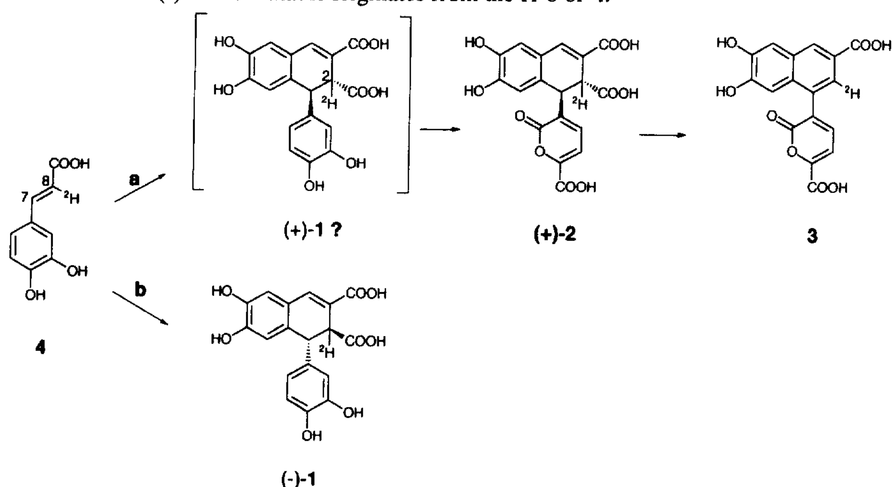

Figure 1 Formation of lignans from [8-^{2}H] caffeic acid (4) in cultured liverworts *J. autumnalis* (a) and *L. heterophylla* (b)

On the basis of these results, the biosynthetic pathway shown in Figure 1 was proposed for the lignans in cultured gametophytes of *J. autumnalis* and cells of *L. heterophylla*. Lignan (+)-**1** is formed from **4** and then oxidation of the dihydroxyphenyl moiety of (+)-**1** affords (+)-**2** and further decarboxylation of (+)-**2** gives **3** in *J. autumnalis*. The difficulty of the isolation of **1** from *J. autumnalis* with feeding of [8-^{2}H] **4** was considered to be rapid oxidation of **1** to (+)-**2** with a change in the culturing condition. On the other hand, lignan (-)-**1** is formed from **4** in *L. heterophylla*.

To reveal the direct formation of **1** from **4** in liverworts, the cell-free extracts were prepared from axenic cultured gametophytes of *J. autumnalis* and cells of *L. heterophylla.*[7] The incubation of **4** with both cell-free extracts in the presence of H_2O_2,yielded **1**, which was confirmed by HPLC analyses of the incubated solution (*J. autumnalis*; 25.7 nmol/mg protein h, *L. heterophylla*; 137.4 nmol/mg protein h).[8,9] There was no formation of **1** without H_2O_2. These results clearly showed that **1** was biosynthesized *de novo* in the gametophytes of *J. autumnalis* and the cells of *L. heterophylla* and peroxidase might be the enzyme responsible for formation of **1**. While **1** and its derivatives have been found in various plants,[10] their biosyntheses have not been clarified. Our result is not only the first example of the lignan biosynthesis in liverworts, but also a demonstration of the direct formation of phenyl dihydronaphthalene **1** from **4**, whereas, interestingly, the formation of the furofuran lignan pinoresinol from coniferyl alcohol is the first step in the vascular plant *Forsythia intermedia.*[11] The enantiomeric composition of **1** formed enzymatically from **4** was further analyzed by chiral HPLC.[12] Figure 2 shows the chiral HPLC chromatograms of the racemic and enzymatically formed **1**, and clearly indicates that (±)-**1** was formed from **4** in both cell-free extracts of *J. autumnalis* and *L. heterophylla*. The reason for the presence of enantiomerically pure **1** and **2** in both liverworts is not clear. The mechanism of the formation of **1** and its oxygenated derivatives **2** and **3**, including enantioselectivity requires to be elucidated by further enzymatic studies.

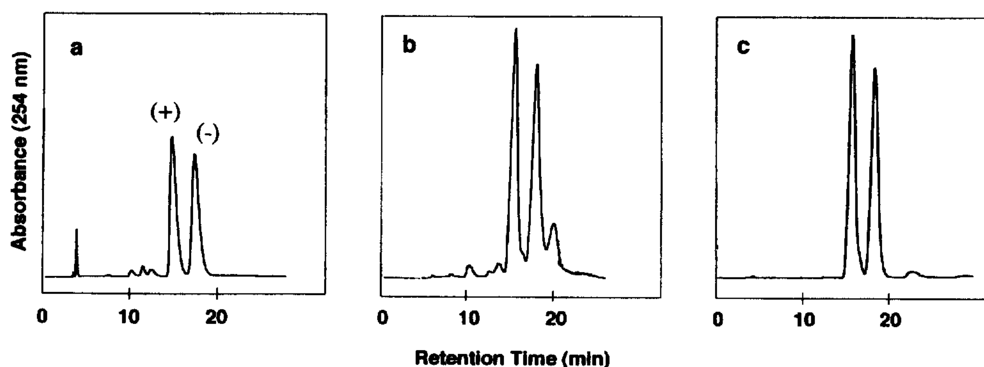

Figure 2 Chiral HPLC Chromatograms of 5s.
(a) Synthetic (±)-5, (b) methylated 1 formed from 4 with cell-free extracts of *J. autumnalis*, (c) methylated 1 formed from 4 with cell-free extracts of *L. heterophylla*

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7. Preparation of cell-free extracts. Axenic cultured gametophytes of *J. autumnalis* *L. heterophylla* were collected at 40 days after subculturing. The plants (5 g fw) were successively washed with tap and distilled water, and ground for 5 min with polyclar AT (2g), and 50mM HEPES-NaOH buffer (pH 7.5, 10 ml) containing 1mM dithiothreitol. The slurry was centrifuged (25,000 x g, 20 min, 4°C), and the supernatant was used as cell-free extracts.

8. Assay for formation of **1** by cell-free extracts. The reaction mixture contained 75 µl of 25 mM **4** in MeOH, 37.5 µl of 10 mM H_2O_2 in distilled water, and the cell-free extracts from *J.autumnalis* and *L. heterophylla* (40 ml, with 2 ml of the above supernatants). The reaction was initiated by adding H_2O_2. After incubating for 1 hr at 30° C, the reaction mixture was acidified to pH3.5 with 1N HCl, and subjected to HPLC (DAISO SP-120-5-ODS-BP, 6 x 250 mm, 0.1% HCOOH in 20% AcCN/H_2O) analysis for measuring the amounts of **1**. For measuring the enantiomeric composition of **1**, the reaction mixture was extracted with EtOAc (50 ml x 3). The EtOAc extracts were evapd. *in vacuo* and then methylated with CH_2N_2/Et_2O. The methylated extracts were subjected to HPLC (SiO_2, *n*-hexane-EtOAc 1:1) to afford **5** which was analyzed by chiral HPLC.[12]

9. Preparation of authentic **1** and **5**. 0.8 g of **4** was dissolved in hot water (100 ml) and then cooled to room temp. 40 ml of 0.1 M KMnO4 aq. was dropped into the stirred solution for 30 min. After standing for 30 min, the solution was acidified with the addition of 0.5 ml of TFA and then washed by Et_2O (80 ml x 3) followed by extraction with EtOAc (80 ml x 3). EtOAc extracts were evaporated *in vacuo* to give a brown gummy substance (380 mg) and further separated by Sephadex LH-20 column (10 x 500 mm) using MeOH as an eluant to afford **1** (147 mg, 18.5 % yield). To a soln of **1** (50mg) in Me_2CO (50ml), MeI (5ml) and K_2CO_3 (500mg) were added, and the mixture was refluxed for 12 hr. After cooling, the solvent was evapd, and the residue passed through a column of SiO_2 (2 g) with *n*-hexane-Et_2O (4 : 1) as eluant to afford **5** (37 mg, 59.9 %).

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12. The enantiomeric compositions of **5** were determined by HPLC, using chiral column (Waters Opti-Pak XC, 300 x 3.9 mm) and a UV detector at 254 nm. The mobile phase was *n*-hexane-EtOH (9 : 1) at a flow of 1 ml min^{-1} to separate (+)-**5** (Rt 15.3 min) and (-)-**5** (Rt 18.0 min) which were identified by comparison of CD spectra with those of authentic data.[10] The average ratio of the (+)-isomer to the (-)-isomer was estimated on the basis of their relative peak areas with consideration of the relative peak area of 1 : 1 mixture of the (+)- and (-)-isomers.