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Structural characteristics of lignin in primitive pteridophytes: *Selaginella* species

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Abstract The lignin chemical structures of eight species of the *Selaginella* family, which are primitive vascular plants, were characterized by alkaline nitrobenzene oxidation, acidolysis, and ozonation. *Selaginella involvens*, *Selaginella tamariscina*, and *Selaginella remotifolia* were collected from the University Forest in Chiba, the University of Tokyo, Japan, and *Selaginella biformis*, *Selaginella pennata*, *S. involvens, Selaginella chrysorrhizos*, and unidentified *Selaginella* species (*Selaginella* sp.) were collected from northern Thailand. Lignin of all *Selaginella* species examined in this study was rich in syringyl nuclei. It was confirmed that a considerable portion of syringyl nuclei of *Selaginella* lignin formed syringylglycerol-β-aryl ether intermonomer linkages. The major diastereomer of arylglycerol-β-aryl ether intermonomer linkages of *Selaginella* lignins was the *erythro*-form exhibiting angiosperm lignin characteristics. In addition, lignins of *S. involvens*, *S. tamariscina*, and *S. remotifolia* collected from the University Forest in Chiba, the University of Tokyo, Japan, were isolated according to Björkman's procedure, and structural features of the lignins were spectrometrically analyzed. It was confirmed that lignin of *Selaginella* species, which are primitive pteridophytes, was typical guaiacyl–syringyl type as well as being similar to angiosperm lignin.

Key words *Selaginella* · Lignin of primitive vascular plant · Syringylglycerol-β-aryl ether intermonomer linkage · Acidolysis · Ozonation

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Introduction

Selaginella belongs to Lycopsida *Selaginellale*, an ancient group of fern allies composed of an estimated 750 living species. Although *Selaginella* are primitive vascular plants, some species of *Selaginella* contain vessel elements,[1–3] a characteristic of the angiosperm vascular system. In addition, a positive Mäule reaction was observed on the lignified cortex rather than the xylem in *Selaginella* in many cases, suggesting the presence of syringyl nuclei in the lignin.[4,5]

It is accepted that lignins of pteridophytes and gymnosperms are composed of guaiacyl nuclei together with *p*-hydroxyphenyl nuclei as the minor component, and lignin of angiosperms is composed of syringyl nuclei in addition to guaiacyl and *p*-hydroxyphenyl nuclei.[5–8] Towers and Gibbs[5] reported positive Mäule reactions for 3 species of *Selaginella*, and the presence of syringaldehyde in alkaline cupric oxide oxidation products of those species of *Selaginella*. White and Towers[9] reported that 11 species of *Selaginella* produced syringaldehyde and vanillin by alkaline cupric oxide oxidation, and the ratios of syringaldehyde to vanillin were generally high, while syringaldehyde was absent from *Lycopodium*. Lewis[10] and Logan and Thomas[11] also confirmed the presence of syringaldehyde in *Selaginella*. Despite these studies, there is not sufficient evidence to conclude that *Selaginella* species contain syringylguaiacyl lignin, namely angiosperm lignin because the formation of syringaldehyde does not necessarily mean that syringyl nuclei constitute lignin as a phenylpropane unit (C_6-C_3 unit).

The arylglycerol-β-aryl ether linkage is the most prominent bond type connecting phenylpropane (C_6-C_3) units of lignin.[7] Uncondensed-type phenylpropane units are broken into corresponding C_6-C_1 compounds by alkaline nitroben-

zene oxidation and cupric oxide oxidation, whereas acidolysis provides specific C_6-C_3 products, Hibbert's ketones, by the cleavage of arylglycerol-β-aryl ether linkages.[6,12,13] Therefore acidolysis has been regarded as the most reliable criterion to examine the presence of lignin. We have confirmed the presence of syringylglycerol-β-aryl ether intermonomer linkages in lignin of *Selaginella tamariscina* by acid hydrolysis.[14]

The arylglycerol-β-aryl ether intermonomer linkage can be either the *erythro*- or *threo*-form, and the ratio of *erythro*- to *threo*-form of arylglycerol-β-aryl ether intermonomer linkage (E/T ratio) is an important characteristic of lignin.[15,16] The *erythro*-form is the predominant stereoisomer of arylglycerol-β-aryl ether intermonomer linkage in angiosperm lignin, while gymnosperm lignin contains approximately equal amounts of *erythro*- and *threo*-forms of the linkage. The E/T ratio is strictly controlled by the aromatic ring type of phenylpropane units, and the *erythro*-form predominates in syringyl units.[15–17] The aromatic nuclei of lignin are degraded completely by ozone and side chain parts are released as simple aliphatic acids. By ozonation, erythronic and threonic acids are obtained from *erythro*- and *threo*-forms of arylglycerol-β-aryl ether intermonomer linkage, respectively, and on the basis of the yields of these two acids, the E/T ratio can be measured.[18,19]

In this study, the structural features of lignin in *Selaginella* species collected from the University Forest in Chiba, the University of Tokyo, Japan, and northern Thailand were investigated and compared with those of *Polystichum polyblepharum*. Lignin structural features were characterized by alkaline nitrobenzene oxidation, acidolysis, and ozonation methods. Björkman lignins were isolated from some species of *Selaginella*, and ultraviolet (UV), Fourier transform-infrared (FTIR), and ^{1}H nuclear magnetic resonance (NMR) spectroscopic analyses were performed to investigate the structural features of *Selaginella* lignin.

Materials and methods

Sample preparation

Selaginella involvens, *Selaginella tamariscina*, *Selaginella remotifolia*, and *Polystichum polyblepharum* were collected from the University Forest in Chiba (35°40′ N, 140°05′ W), the University of Tokyo, Japan, and *S. involvens, Selaginella pennata* (Don) Spring, *Selaginella chrysorrhizos* Spring, *Selaginella biformis* A. Br. Ex Kuhn, and *Selaginella* sp. (species not unidentified) were collected from Doi Angkha (19°48′ N, 98°58′ E) which is about 100 km north of Chiang Mai in northern Thailand. The sample was freeze-dried and then ground to pass a 420-μm sieve using a Wiley mill. The sample was ground further using a stainless steel vibratory ball mill (MM200, Retsch, Germany) for 15 min at a vibration rate of 30 s^{-1} before being subject to chemical analyses. *Fagus crenata*, which is a typical angiosperm species, was used for comparison with *Selaginella* lignin.

Determination of lignin content

Lignin content of the ground sample was determined according to the Klason procedure (Tappi Standard T222 om-88) with minor modifications. The ground sample was treated with 72% H_2SO_4 for 3 h at room temperature. The reaction mixture was diluted with water to a sulfuric acid concentration of 3%, and then digested at 121°C for 30 min. After cooling, the insoluble fraction (Klason residue) was separated by filtration using a glass filter (G4). Klason residue was measured gravimetrically after drying at 105°C overnight. Acid-soluble lignin was determined by measuring the UV absorption at 205 nm using an extinction coefficient of 110 l g^{-1} cm^{-1}.[20] Klason lignin content was determined as the total amount of Klason residue and acid-soluble lignin.

Björkman lignin isolation

S. involvens, *S. tamariscina*, *S. remotifolia*, and *P. polyblepharum* were extracted with boiling 80% (v/v) ethanol for 1 h (three times) followed by overnight extraction with water at 40°C with shaking. The residue was dried in a vacuum oven for 48 h at 40°C over P_2O_5, and then was ground finely with a vibratory ball mill VS-2 (Irie Shokai, Tokyo, Japan) for 72 h with cooling by tap water. Dispersing solvent was not used during milling.[21] Björkman lignin was extracted from the finely ground sample with dioxane–water (9:1, v/v) and purified according to the procedure of Björkman.[22] The yields of purified Björkman lignin isolated from *S. involvens*, *S. tamariscina*, *S. remotifolia*, and *P. polyblepharum* were 5.1%, 6.4%, 2.5%, and 3.5% (of finely ground sample), respectively.

Alkaline nitrobenzene oxidation

The aromaticity of lignin of the ground samples and the Björkman lignins was examined by alkaline nitrobenzene oxidation.[23] The reaction products were trimethylsilylated with *N*,*O*-bis(trimethylsilyl)acetamide. The products were analyzed by a Shimadzu GC-17A gas chromatograph (Shimadzu, Japan) using NB1 capillary column (25 m × 0.25 mm i.d.) equipped with a flame ionization detector (FID). Both injector and detector temperatures were 280°C. The column temperature was kept at 150°C for 10 min, and then programmed to rise to 250°C at 5°C min^{-1}. 3-Ethoxy-4-hydroxybenzaldehyde (ethylvanillin) was used as an internal standard.

Acidolysis

Acidolysis of the ground sample was performed according to the procedure of Lapierre et al.[13] using a mixture of dioxane and 2M aqueous hydrochloric acid (9:1, v/v). The suspension was refluxed for 4 h. Acidolysis products were trimethylsilylated with *N*,*O*-bis(trimethylsilyl)acetamide

and identified by gas chromatography-mass spectroscopy (GC-MS) (Shimadzu GC-17A coupled with a Shimadzu QP-5000 mass spectrometer). The NB1 capillary column (25 m × 0.25 mm i.d.) was used. The injector temperature was 280°C. The column temperature was kept at 150°C for 10 min, and then programmed to rise to 250°C at 5°C min^{-1}. 3-Ethoxy-4-hydroxybenzaldehyde was used as an internal standard.

Ozonation

Ozonation analysis was carried out according to the scheme of Akiyama et al.[19] for the ground samples and Björkman lignins. Erythronic and threonic acids are produced from the *erythro*- and *threo*-form of arylglycerol-β-aryl ether intermonomer linkage, respectively, by ozonation. The ozonation products were converted to ammonium salts using a cation exchanger (Dowex-50W-X4, NH_4^+ form), trimethylsilylated with dimethylsulfoxide, hexamethyldisilazane, and hexamethyldisilazane, and then analyzed by GC (Shimadzu GC-17A gas chromatograph using NB1 capillary column, 25 m × 0.25 mm i.d.) using flame ionization detection. The injector and detector temperatures were 280°C. The column temperature was kept at 120°C for 5 min, and then programmed to rise to 170°C at 4°C min^{-1} and then to 280°C at 10°C min^{-1}. Erythritol was used as an internal standard. All chemical analyses were performed in triplicate.

Spectroscopic analyses

The Björkman lignin was acetylated with acetic anhydride together with a catalytic amount of pyridine (0.1 ml) at room temperature overnight with stirring. The acetylated sample was dissolved in chloroform-*d* and the ^{1}H NMR spectrum was recorded using a Jeol JNM-A 500 spectrometer (Jeol, Japan). FTIR spectra were recorded by KBr disc method on an FT/IR-615 spectrometer (Jasco, Japan), using 64 scans and a resolution of 4 cm^{-1}. UV spectra were recorded on a Hitachi U-3010 spectrophotometer (Hitachi, Japan) using mixture of methylcellosolve and water (9:1, v/v) as solvent.

Results and discussion

Lignin content and aromatic composition of *Selaginella* lignin

Lignin content of *Selaginella* species, which was calculated as the combined amounts of Klason residue and acid-soluble lignin, ranged from 21% to 40% of oven-dry ground sample, and that of *P. polyblepharum* was 31% (Table 1). The level of acid-soluble lignin of *Selaginella* species was 3%–5% of oven-dry sample, which is similar to the value for angiosperm woods. The huge distribution of values of lignin content would be due to extractives and composition of different tissues.

Previous observations that *Selaginella* produces syringaldehyde by nitrobenzene oxidation or cupric oxidation,[5,9–11,24,25] were strikingly revealed in this study. The total yield of alkaline nitrobenzene oxidation products of *Selaginella* species based on the lignin content ranged from 90 to 281 mmol·per 200 g lignin. Syringaldehyde was detected from *Selaginella* species together with vanillin by alkaline nitrobenzene oxidation, while syringaldehyde was absent in *P. polyblepharum* (Table 1). The molar ratios of syringaldehyde to vanillin (S/V) of ground samples of *Selaginella* species ranged from 1.3 to 4.7, and several *Selaginella* species gave even higher S/V ratios than *F. crenata* (S/V ratio was 2.7), which is a typical angiosperm species (Table 1).

The total yields of alkaline nitrobenzene oxidation products calculated based on lignin of *Selaginella* ground samples were considerably lower than those of normal angiosperm woods (Table 1). Angiosperm lignin gives 35%–

Table 1. Lignin content and yield of alkaline nitrobenzene oxidation products of ground sample

Species	Lignin content (%)[a]	Alkaline nitrobenzene oxidation products (mmol·per 200 g lignin)							S/V
		H	HA	V	VA	S	SA	Total	
Selaginella involvens[b]	30.7	13	7	24	2	72	6	124	3.0
Selaginella tamariscina[b]	40.5	18	26	24	9	54	5	156	2.2
Selaginella remotifolia[b]	24.8	16	8	34	6	43	6	113	1.3
Selaginella involvens[c]	31.1	12	7	52	3	197	11	281	3.8
Selaginella pennata[c]	21.0	15	45	25	2	73	6	125	2.9
Selaginella chrysorrhizos[c]	25.5	17	6	42	3	88	7	164	2.1
Selaginella biformis[c]	31.4	11	4	12	2	57	4	90	4.7
Selaginella unidentified[c,d]	21.4	9	3	46	3	58	4	122	1.3
Polystichum polyblepharum	31.2	10	3	155	9	ND	ND	177	–

Total yield and S/V ratio of *Fagus crenata* were 545 mmol·per 200 g lignin and 2.7, respectively
H, *p*-Hydroxybenzaldehyde; HA, *p*-hydroxybenzoic acid; V, vanillin; VA, vanillic acid; S, syringaldehyde; SA, syringic acid; S/V, molar ratio of syringyl nuclei (S) to guaiacyl nuclei (V); ND, not determined
[a] Percentage of oven-dry sample
[b] Collected from Chiba in Japan
[c] Collected from northern Thailand
[d] This plant was identified as a species of *Selaginella* family, but could not be specified

Table 2. Yields of alkaline nitrobenzene oxidation products and ozonation products of Björkman lignin

Species	Alkaline nitrobenzene oxidation products (mmol·per 200 g Björkman lignin)							Ozonation products (mmol·per 200 g Björkman lignin)				
	H	HA	V	VA	S	SA	Total	S/V	E	T	Total	E/T
Selaginella involvens	9	6	41	2	140	9	207	3.4	52	33	85	1.6
Selaginella tamariscina	12	13	57	5	132	16	235	2.3	61	36	97	1.7
Selaginella remotifolia	12	3	62	3	101	4	185	1.6	60	43	103	1.4
Polystichum polyblepharum	16	6	205	13	ND	ND	240	–	62	72	134	0.9

E, Erythronic acid originating from *erythro*-form of arylglycerol-*β*-aryl ether intermonomer linkage; T, threonic acid originating from *threo*-form of arylglycerol-*β*-aryl ether intermonomer linkage; E/T, molar ratio of erythronic acid to threonic acid

55% based on lignin as total yield of vanillin and syringaldehyde, and gymnosperm lignin produces vanillin in the yield of 24%–28%.[6,8] The low yield of alkaline nitrobenzene oxidation products from *Selaginella* species would be due to the high lignin content determined by the Klason procedure. Although the Klason procedure is the standard method for lignin determination of wood samples, the method usually overestimates the lignin content of herbaceous plants due to contamination of protein and so on.[26] The total yields of alkaline nitrobenzene oxidation products of *Selaginella* Björkman lignins were also lower than that for wood Björkman lignin (Table 2), probably due to the domination of highly condensed lignin in *Selaginella* species caused by the sampling of young tissues. Contamination in *Selaginella* Björkman lignin would not be expected as a major reason, based on ^{1}H NMR spectra discussed later.

Arylglycerol-*β*-aryl ether intermonomer linkage of *Selaginella* lignin

The presence of arylglycerol-*β*-aryl ether intermonomer linkage is essential to distinguish lignin from other polyphenols.[7] The linkage is cleaved by acidolysis to give several types of arylpropanones (Hibbert's ketones) as major products together with other minor monomers. The lignin of angiosperms gives both 4-hydroxyl-3-methoxyphenylpropanones (G) and 4-hydroxy-3,5-dimethoxyphenylpropanones (S), whereas gymnosperms produce only 4-hydroxyl-3-methoxyphenylpropanones (G).[6,12,13] The yields of acidolysis products from lignin are usually lower than those of nitrobenzene oxidation products. However, the advantage of acidolysis over nitrobenzene oxidation is that acidolysis can give C_6-C_3 monomeric structures that are specifically produced from arylglycerol-*β*-aryl ether intermonomer linkages in lignin. Namely, if syringyl-type arylpropanones are produced from a sample by acidolysis, it can be concluded with certainty that syringyl nuclei are incorporated in the lignin macromolecule. Such concrete evidence can never be given by C_6-C_1 type structures such as syringaldehyde found in nitrobenzene or cupric oxide oxidation products.

The acidolysis products of ground samples were identified by GC-MS as trimethylsilyl derivatives. Two kinds of syringyl-type arylpropanones (*m*/*z* 370) together with two kinds of guaiacyl-type arylpropanones (*m*/*z* 340) were identified in the acidolysis products from the ground *Selaginella* samples

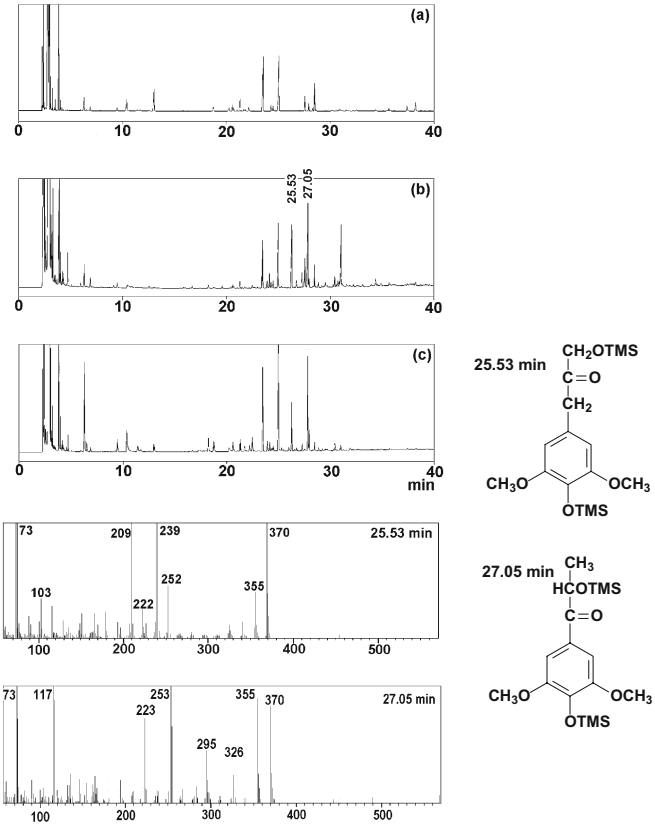

Fig. 1a–c. Gas chromatograms and mass spectra of acidolysis products of ground samples of **a** *P. polyblepharum*, **b** *S. tamariscina*, and **c** *F. crenata*. Mass spectra of retention times at 25.53 and 27.05 mm of acid hydrolysis products of *S. tamariscina* are also shown

and Björkman lignins. These four products were identical with the case of *F. crenata,* which is a typical angiosperm species (Fig. 1). The ratios of syringyl-type arylpropanones to guaiacyl-type arylpropanones (S/G) ranged from 0.31 to 1.86 for the ground *Selaginella* samples and that of *F. crenata* was 1.11 (Table 3). Syringyl-type arylpropanones were absent from the acidolysis products of *P. polyblepharum*.

Results of acidolysis proved that some portions of syringyl nuclei in lignin of *Selaginella* species are incorporated into lignin, however, the extent of syringyl nuclei in lignin as a phenylpropane unit (C_6-C_3 unit) is still uncertain. In order to estimate this, the molar ratio of syringyl-type acidolysis products to the corresponding alkaline nitrobenzene oxidation products (S_{ac}/S_{bz}) was calculated. The results

Table 3. Molar ratio of syringaldehyde to vanillin (S/V) of alkaline nitrobenzene oxidation products, molar ratio of syringylpropanones to guaiacylpropanones (S/G) of acid hydrolysis products, and the proportion of syringylpropanones in acidolysis products to syringaldehyde in alkaline nitrobenzene oxidation products (S_{ac}/S_{bz})

Species	S/V	S/G	S_{ac}/S_{bz}
Selaginella involvens[a]	3.0	1.18	0.20
Selaginella tamariscina[a]	2.2	1.30	0.22
Selaginella remotifolia[a]	1.3	0.31	0.12
Selaginella involvens[b]	3.8	1.86	0.13
Selaginella pennata[b]	2.9	0.66	0.05
Selaginella chrysorrhizos[b]	2.1	0.74	0.11
Selaginella biformis[b]	4.7	1.06	0.07
Selaginella unidentified[b,c]	1.3	0.57	0.13
Fagus crenata	2.70	1.11	0.17

[a] Collected from Chiba Japan
[b] Collected from northern Thailand
[c] This plant was identified as a species of *Selaginella* family, but could not be specified

showed that *Selaginella* species gave a higher or similar S_{ac}/S_{bz} ratio when compared with that of *F. crenata* (Table 3). The logical conclusion was that participation of syringyl nuclei of *Selaginella* lignin as syringylglycerol-β-aryl ether intermonomer linkages in the lignin macromolecule is comparative with that in angiosperm lignin.

Stereoisomeric features of arylglycerol-β-aryl ether intermonomer linkage of *Selaginella* lignin

In the case of wood lignins, the ratio of *erythro*- to *threo*-form of arylglycerol-β-aryl ether intermonomer linkage (E/T ratio) is positively correlated to the ratio of aromatic composition (S/V ratio).[16] Therefore, if the relationship between E/T ratio and S/V ratio of *Selaginella* lignin coincides with that for wood lignin, it would be strong evidence that *Selaginella* lignin shows characteristics similar to those of angiosperm wood lignin. Erythronic and threonic acids are produced from the *erythro*- and *threo*-form of arylglycerol-β-aryl ether structures by ozonation, respectively.[18,19,27] The molar ratios of erythronic acid to threonic acids (E/T ratio) ranged from 1.6 to 2.3 for the ground samples of *Selaginella*, while the E/T ratio of *P. polyblepharum* was 1.1. Of note, the *erythro*-form was the predominant diastereomer of arylglycerol-β-aryl ether intermonomer linkage in *Selaginella* lignin, indicating strikingly that the diastereomer of arylglycerol-β-aryl ether intermonomer linkages of *Selaginella* lignin has characteristics of angiosperm lignin.

The total yields of erythronic and threonic acids in ozonation products ranged from 37 to 135 mmol·per 200 g lignin for the ground samples of *Selaginella*, and that of *F. crenata* was 332 mmol·per 200 g lignin (Table 4). Akiyama et al.[16] reported that the total yields of erythronic and threonic acids from wood meals ranged from 220 to 370 mmol·per 200 g lignin. The low yields of ozonation products from *Selaginella* species would be due to the high value of lignin content determined by the Klason procedure discussed as above. However, Björkman lignins of *Selaginella* species and *P. polyblepharum* also gave yields of ozonation products that were somewhat lower (from 85 to 134 mmol·per 200 g lignin) (Table 2) than angiosperm wood lignin.[16] These results also supported the above suggestion of the dominance of highly condensed lignin in *Selaginella* species due to the sampling of young tissues, based on total yields of the alkaline nitrobenzene oxidation products.

Table 4. Yield and E/T ratio of ozonation products of ground sample

Species	Ozonation products (mmol·per 200 g lignin)			
	E	T	Total	E/T
Selaginella involvens[a]	34	18	52	1.9
Selaginella tamariscina[a]	36	16	52	2.2
Selaginella remotifolia[a]	37	23	60	1.7
Selaginella involvens[b]	26	11	37	2.3
Selaginella pennata[b]	52	23	74	2.3
Selaginella chrysorrhizos[b]	29	18	47	1.6
Selaginella biformis[b]	92	43	135	2.1
Selaginella unidentified[b,c]	39	25	64	1.6
Polystichum polyblepharum	57	52	109	1.1

Total yield and E/T ratio of *F. crenata* were 332 (mmol·per 200 g lignin) and 2.5, respectively
[a] Collected from Chiba in Japan
[b] Collected from northern Thailand
[c] This plant was identified as a species of *Selaginella* family, but could not be specified

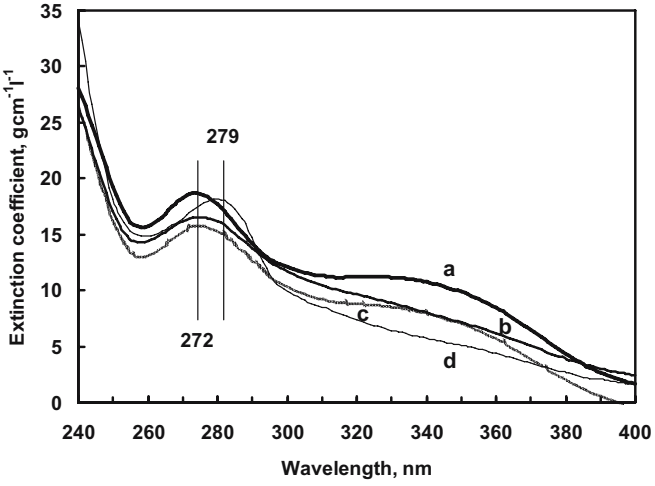

Fig. 2. Ultraviolet absorption spectra of Björkman lignins isolated from *S. involvens* (*a*), *S. tamariscina* (*b*), *S. remotifolia* (*c*), and *P. polyblepharum* (*d*)

Spectroscopic characteristics of *Selaginella* Björkman lignin

Generally, the maximum UV absorption of gymnosperm lignin is observed at 279–281 nm, whereas that of angiosperm lignin appears at 272–273 nm.[28] The maximum absorbance of *Selaginella* lignins was at 272 nm and that of *P. polyblepharum* lignin at 279 nm (Fig. 2). The results of UV analysis again show that *Selaginella* lignins possess angiosperm lignin characteristics.

In the FTIR spectra, the region from 1000 to 800 cm^{-1} is closely related to the aromatic structure of lignin. Angio-

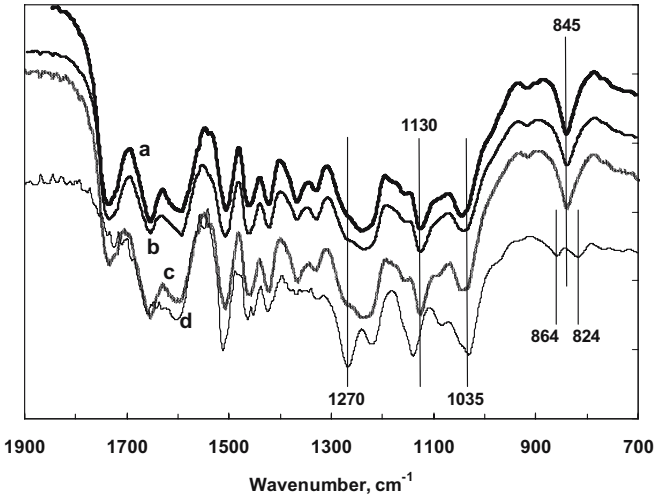

Fig. 3. Fourier transform-infrared spectra of Björkman lignins isolated from *S. involvens* (*a*), *S. tamariscina* (*b*), *S. remotifolia* (*c*), and *P. polyblepharum* (*d*)

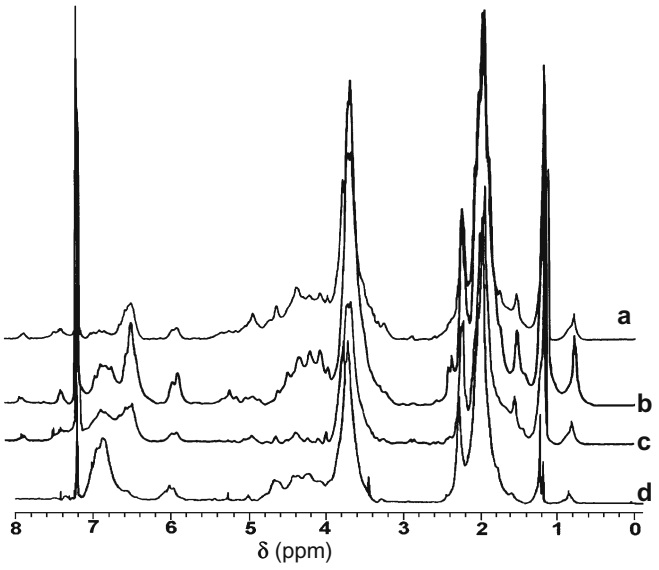

Fig. 4. ^{1}H Nuclear magnetic resonance spectra of Björkman lignin isolated from *S. involvens* (*a*), *S. tamariscina* (*b*), *S. remotifolia* (*c*), and *P. polyblepharum* (*d*)

sperm lignin gives a strong absorption at 845 cm^{-1} and a weak absorption at 915 cm^{-1}. On the other hand, gymnosperm lignin shows two bands at 864 and 824 cm^{-1}, and no absorption at 915 cm^{-1}.[29,30] The FTIR spectra of *Selaginella* Björkman lignins exhibited these characteristics of angiosperm lignin, while that of *P. polyblepharum* Björkman lignin was similar to gymnosperm lignin (Fig. 3). Intensive absorptions in *P. polyblepharum* lignin were shown at 1030 cm^{-1} and 1270 cm^{-1}, which were assigned as guaiacyl nuclei. On the contrary, intense absorptions in the FTIR spectra of *Selaginella* lignin appeared at 1130 cm^{-1} and 1230 cm^{-1}, suggesting the presence of syringyl nuclei in *Selaginella* lignin.[29,30] In the ^{1}H NMR spectra of gymnosperm lignin, signals assigned to aromatic protons appear at δ 6.90 (guaiacyl protons), while those in angiosperm lignin occur at δ 6.65 (syringyl protons) in addition to δ 6.90.[31] Acetylated Björkman lignins of *S. involvens*, *S. tamariscina*, and *S. remotifolia* exhibited two strong signals corresponding to aromatic protons of guaiacyl and syringyl nuclei in the ^{1}H NMR spectra (Fig. 4). The relative strength of these two signals suggested that syringyl nuclei predominate in *Selaginella* Björkman lignins and this was in agreement with high S/V ratio of *Selaginella* species on alkaline nitrobenzene oxidation (Table 2). *P. polyblepharum* gave only one strong signal at δ 6.90 corresponding to guaiacyl protons.

The signals at δ 6.01 and 6.06 of acetylated lignins in the ^{1}H NMR spectra are assigned to H_{α} in the *erythro*- and *threo*-form of arylglycerol-β-aryl ether intermonomer linkages, respectively.[32] Spectra of acetylated Björkman lignins of *S. involvens*, *S. tamariscina*, and *S. remotifolia* exhibited stronger signals at δ 6.01 than those at δ 6.06, indicating that the *erythro*-form was the predominant diastereomer of arylglycerol-β-aryl ether intermonomer linkages (Fig. 4). Björkman lignin of *P. polyblepharum* also gave signals at δ 6.01 and 6.06, while the intensity pattern was the opposite of that of *Selaginella* lignins. These results were in agreement with high E/T ratios of *Selaginella* species found by ozonation (Table 2).

Conclusions

The major findings of this study are summarized as follows:

1. Lignins of ground *Selaginella* samples gave syringaldehyde and vanillin as the major products of alkaline nitrobenzene oxidation, and the ratio of syringaldehyde to vanillin ranged from 1.3 to 4.7.
2. The presence of syringylglycerol-β-aryl ether together with guaiacylglycerol-β-aryl ether intermonomer linkages of *Selaginella* was verified as a part of the lignin macromolecule.
3. The *erythro*-form was the predominant diastereomer of arylglycerol-β-aryl ether intermonomer linkages in *Selaginella* lignin.
4. All results clearly indicated that the characteristics of *Selaginella* lignin are comparable with those of angiosperm lignins.

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