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1,3,5-HYDROXYBENZENE STRUCTURES IN MOSSES

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Key Word Index—Moss; lignin; ^{13}C NMR; magic angle spinning.

Abstract—A number of mosses from widely different families have been studied by cross polarization solid state ^{13}C NMR spectroscopy. Although polysaccharide-type materials dominate the NMR spectra, significant amounts of aromatic carbons are observed in some mosses. Some of this material can be removed by ultrasonic bath treatment, and is lignin derived, probably from impurities from fine root material from associated higher plants. However other material is truly moss-derived and appears to be from 1,3,5-hydroxybenzene structures. This is inconsistent with lignin as being a component of mosses, and suggests a tannin or hydroxybenzofuran polymer is responsible for moss rigidity.

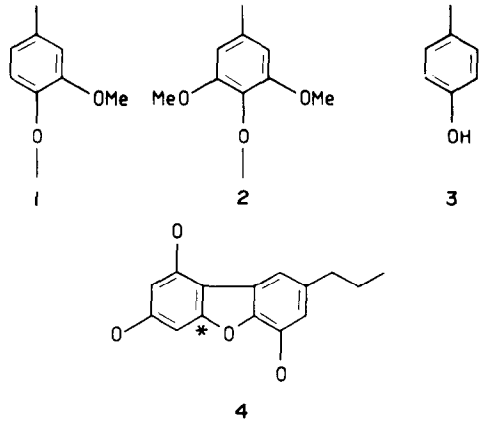
INTRODUCTION

The heteropolymer composed of guaiacyl (**1**) and/or syringyl (**2**) and *p*-hydroxyphenol (**3**) units and known as lignin is an important structural component of higher plants. Mosses are believed to be among the earliest of land plants, and hence there is considerable interest in whether they contain lignin or not [1–12].

The identification of lignin in plants has been based on a number of analytical techniques including extraction, oxidative degradation and more recently NMR spectroscopy and pyrolysis GC-MS. Attempts to isolate lignin from mosses using vibrational ball milling methods and extraction with neutral solvents produce very low yields. Nevertheless, Nilsson and Tottmar [8] suggest that *Sphagnum nemoreum* contains both *p*-hydroxyphenyl and guaiacyl units in the ratio of 2.5:1. Syringyl groups were not detected. Bland *et al.* [5] found vanillin (4-hydroxy-3-methoxybenzaldehyde) and syringaldehyde (3,5-dimethoxy-4-hydroxybenzaldehyde) after nitrobenzene oxidation. Using GC-MS, Van Smeerdijk and Boon [12] found some possible lignin derived materials in pyrolysates such as phenol, *p*-ethylphenol and *p*-vinylphenol and minor amounts of guaiacol, guaiacyethene, phenyl-2-propene, syringol and *p*-hydroxybenzaldehyde.

Erickson and Miksche [3] are exponents of the hypothesis that mosses do not contain lignin. They found no evidence for guaiacyl or syringyl groups using their degradation procedures. Nimz and Tutschek [4] used solution ^{13}C NMR to search for lignin in *Sphagnum*. They found evidence for *p*-hydroxyphenyl groups, but they were unable to identify the typical *β-O*-4 and phenylcoumaran structures of lignin in the aliphatic region of spectra of extracts. They conclude that *Sphagnum* is free of lignin.

The giant mosses of Australasia such as *Dawsonia* are also possible candidates to test for the presence of lignin because they have an erect habit not unlike higher plants. Some of the giant mosses have been reported to contain lignin [9, 10], but they could also contain another type of aromatic material. Veratric acid (3,4-dimethoxybenzoic acid) has been identified in *Dawsonia* and other members of the same order (polytrichales) [1, 11].



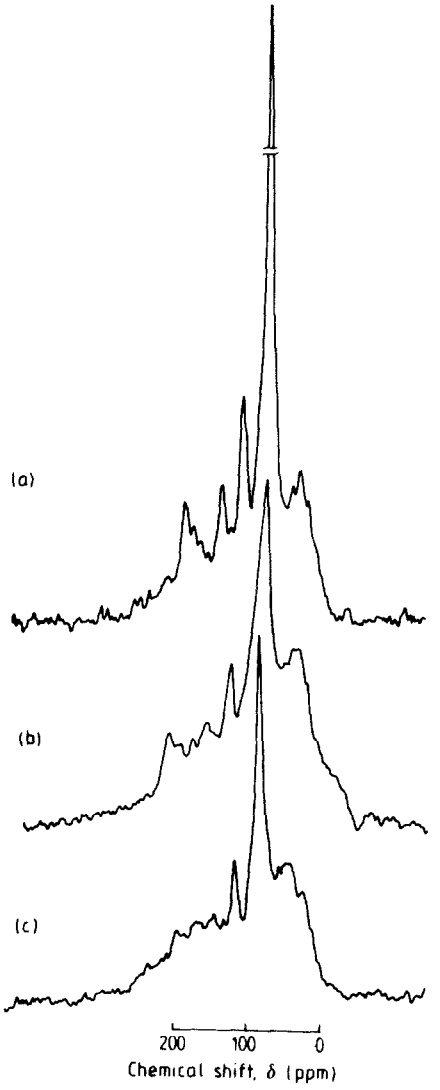
Both GC-MS and solution NMR are not ideal for analysis of plant material. Often only small amounts of the mass of the total material are observed by GC-MS so it is difficult to evaluate the significance of the observation of a small amount of possible lignin markers. Likewise, not all organic material is solubilized by pre-extraction for observation by solution NMR. Solid state ^{13}C NMR is a technique which circumvents these problems, but has not been previously applied to the analysis of mosses. In this paper we examine the structure of a range of mosses (Table 1) including *Sphagnum* and *Dawsonia* by high resolution solid state NMR in order to look for and possibly identify aromatic materials including lignin.

RESULTS AND DISCUSSION

Effect of ultrasonic cleaning

Typical ^{13}C CP/MAS spectra of as received samples are shown in Fig. 1. A number of these samples contain

resonances from aromatic carbon (110–160 ppm) as well as carboxylic (~175 ppm), alcoholic (~73 ppm) dioxygenated (~105 ppm) and alkyl carbon (<50 ppm). Although these samples contain no visible ground mass material, careful cleaning and purification using an ultrasonic bath reduced the aromatic content of *Rhizogonium* and *Sphagnum* species (compare Fig. 1b, c with Fig. 2b, c). As already noted, Bland *et al.* [5] and Van Smeerdijk and Boon [12] found lignin markers in mosses. Sarkanen and Hergert [16] state that it is premature to identify polyphenolic material isolated from *Sphagnum* as lignin, but they do not exclude the possibility that minor amounts of lignin might be contained in these materials. Van Smeerdijk and Boon raise the possibility [12] that peat mosses can be contaminated with rootlets because raised bogs, even when they appear to be exclusively peat mosses, are inhabited by different species of heather and it is difficult to separate the fine root material from the abundant mass of peat moss. This fine root material can contain abundant lignin markers in studies using analytical pyrolysis.



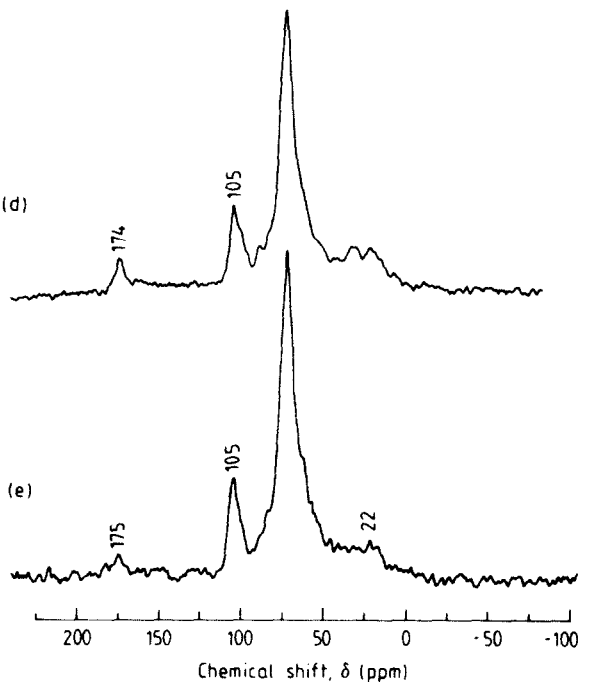


Fig. 1. ^{13}C CP/MAS NMR spectra of as received mosses. (a) *Thamnobryum pandum*; (b) *Rhizogonium parramatense*; (c) *Sphagnum cristatum*; (d) *Dawsonia superba*; (e) *Leucobryum candidum*.

Clearly, our results show the importance of ultrasonic cleaning in the preparation of mosses for analysis, and we note that previous studies have not used this technique. Our NMR studies on ultrasonically cleaned and uncleaned samples show that this treatment reduces the amount of aromatics present. We were also able to show by pyrolysis GC-MS that it reduces the amount of lignin markers, which include guaiacol, 4-methylguaiacol, C_2-guaiacol, 4-vinylguaiacol, 2,6-dimethoxyphenol, 4-methyl-2,6-dimethoxyphenol, 4-vinyl-2,6-dimethoxyphenol and acetoguaiacone in pyrolysates, to trace or undetectable quantities.

CP/MAS spectra of cleaned samples

The spectra from the five mosses (Fig. 2) all show intense resonances from carbohydrates at 72–73 ppm (CH_2OH) and 105 ppm (CHO_2). It is clear that the dominant components of mosses are the carbohydrates. The resonance at 105 ppm appears to overlap with another resonance at ~104 ppm. The origin of this resonance will be discussed later. All mosses also show a broad aliphatic resonance extending down to about 10 ppm. There appears to be distinct resonances at 30–33 and 22 ppm in spectra of *Thamnobryum*, *Rhizogonium* and *Dawsonia* species. The former is from polymethylene carbon and the latter from acetyl units of hemicellulose. All spectra also show strong resonances from carboxylic acids at 174–175 ppm. This suggests that uronic acids are contributors although amino acids may be responsible. Aliphatic carbons of protein (CHNH) also resonate between 51–56 ppm. The carbohydrate resonance broadens at low chemical shift values due to the C_6 resonance of cellulose at or about 64 ppm and there is a small compo-

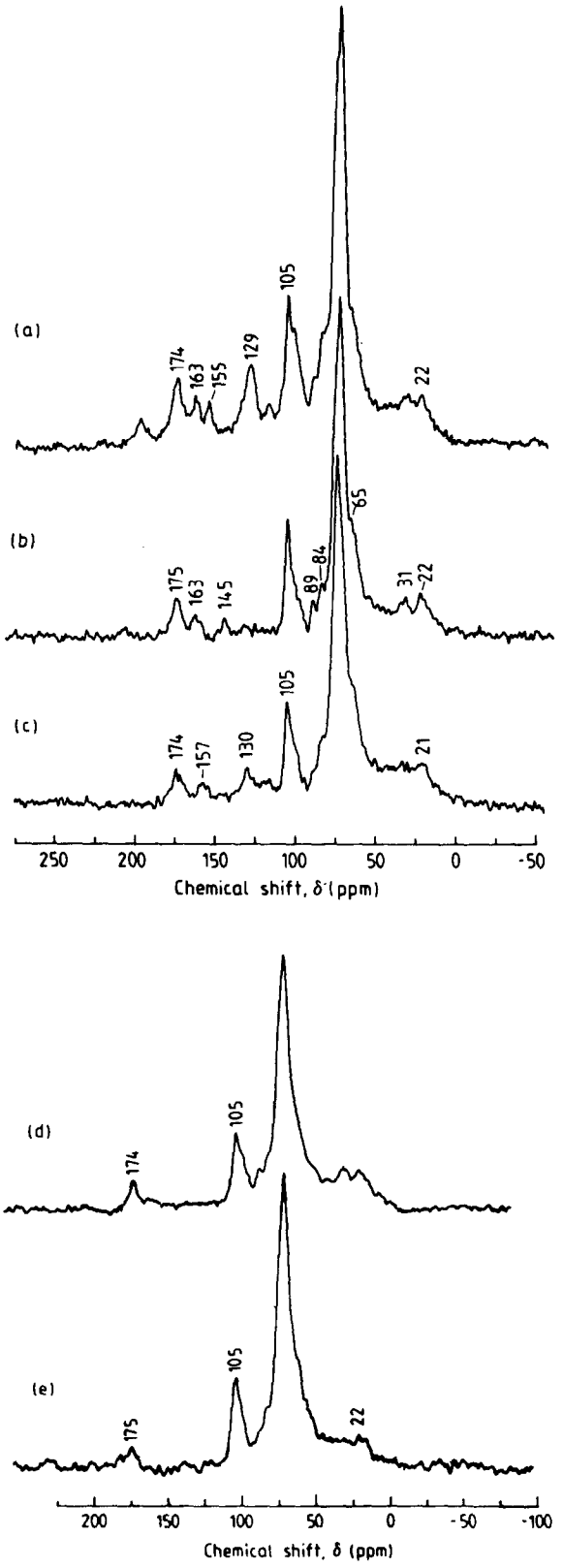


Fig. 2. ^{13}C CP/MAS NMR spectra of ultrasonically treated mosses. (a) *Thamnobryum pandum*; (b) *Rhizogonium parramatense*; (c) *Sphagnum cristatum*; (d) *Dawsonia superba*; (e) *Leucobryum candidum*.

nent at even lower chemical shift (~ 50 ppm) which possibly could arise from amino carbon. Thus a contribution of amide carbons of proteins to the resonance at 175 ppm cannot be ruled out. There appears to be some ketone or aldehyde carbon in the spectrum of *Thamnobryum* at 197 ppm.

The most interesting features of the spectra are in the aromatic region. There are aromatic resonances in all spectra but these are very weak in the spectra of *Dawsonia* and *Leucobryum* spectra. Nevertheless when concentrates of *Dawsonia* stems were examined (Fig. 3) aromatic resonances were observed at 163 and 144 ppm. The resonances at 129–130 ppm in *Thamnobryum* and *Sphagnum* are mainly from alkyl substituted aromatic carbon (C-Ar) although some protonated carbons may contribute. The resonances at 144–145 ppm in spectra of *Dawsonia* stems (Fig. 3) and *Rhizogonium* (Fig. 2b) arise from dioxygenated phenolic structures such as catechol (1,2-dihydroxybenzene) or guaiacol (2-methoxyphenol) [14].

The phenolic carbon resonances at 163–165 ppm in *Dawsonia* stems, *Thamnobryum* and *Rhizogonium* species are at an unusually high chemical shift for most phenolic substances, but peaks at 163–165 ppm have been observed for the C_{8a} carbons in the solid state spectra of tannins [17]. These resonances are characteristic for the trihydroxybenzene structures typical of tannins [18–21]. A NMR spectrum for quercetin, a substance that is similar to structural units in tannin, is shown in Fig. 4. Note that a peak is observed for the C_{8a}-carbon at 164 ppm. Erickson and Miksche [3] propose dibenzofuran structures (structure **4**) to be present in some moss species, and our data is also consistent with this proposal since these structures should also show chemical shifts at 163–165 ppm for C* carbons.

Relaxation data

Two types of solid state relaxation measurements have been carried out on the mosses. The first is the measurement of the spin lattice relaxation time in the rotating frame of protons through carbon ($T_{1\rho}H$). This measurement is made to check on the quantitative nature of the CP/MAS experiments. A full explanation is available elsewhere [14].

A typical plot of signal intensity versus contact time for *Thamnobryum* is shown in Fig. 5 and $T_{1\rho}H$ relaxation data for all the mosses are listed in Table 2. $T_{1\rho}H$'s for carbohydrate carbons at ~ 72–73 ppm are surprisingly similar for all mosses and of the order of 6.0 msec. These values are similar to values obtained for carbohydrates in soils and woods and shows no unusual effects due to the presence of paramagnetics. Cross polarization appears to take a little longer for the aromatic-carbon peaks at 164 and 155 ppm because build up of signal intensity does not reach a maximum until 2 msec, however, for other carbons the signal intensities peak at $\leqslant 1$ msec. This reflects the fact that the aromatic carbons resonating at chemical shifts of 164 and 155 ppm are distant from protons. That is, they are non-protonated.

This result is confirmed by dipolar dephasing experiments, which measure apparent spin–spin relaxation times (T_2's) in the presence of dipolar coupling. They are measured by turning the decoupler off for a period before data is acquired by the spectrometer computer. During this period signal is lost from different carbon types at different rates. It suffices to say that non-protonated

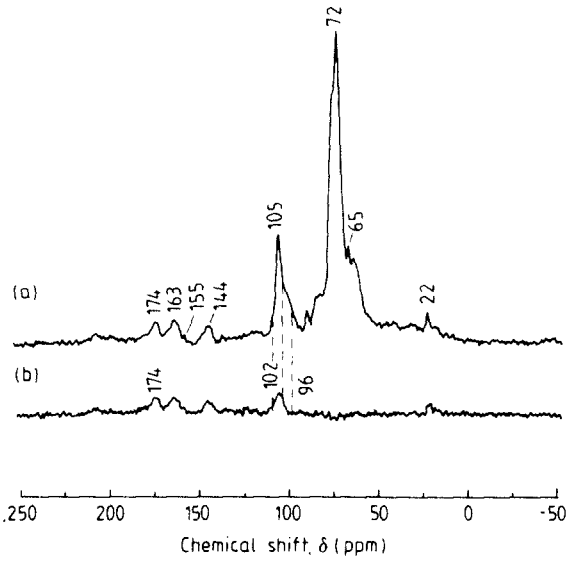


Fig. 3. ^{13}C CP/MAS NMR spectra of *Dawsonia* stems. (a) Conventional spectrum; (b) 50 μsec dipolar dephased spectrum.

carbons loose signal intensity more slowly than protonated carbons.

Typical dipolar dephasing data for mosses are shown in Fig. 6 and tabulated in Table 2. Loss of signal intensity following the rate law:

$$I = I^0 \exp(-t/T'_2) \quad (1)$$

(where I = signal intensity and t = dephasing time) for all carbons except protonated carbons other than methyl carbons. This sort of behaviour is that expected for non-protonated aromatic carbon or carboxylic carbon and is that predicted for the assignments of the 175, 164 and 156 ppm resonances. Decay of the resonance at 73 ppm follows the rate law

$$I = I^0 \exp(-t^2/2T'^2_2) \quad (2)$$

expected for protonated carbons [14]. Nonprotonated carbons usually have T'_2 values >100 μs and the data in Table 2 are consistent with the assignment of the peaks at 175, 164 and 156 ppm as being those of nonprotonated carbons. Likewise, carbohydrate carbons usually have T'_2 values of between 15 and 32 μsec. Thus the values of T'_2 observed for the 73 ppm resonance in the mosses are consistent, except that perhaps the *Sphagnum* value is a little high. We note that the only measureable value for alkyl carbon (33 ppm) is also high. This probably reflects the fact that the resonance contains a contribution from CH, CH_2, CH_3 and non-protonated carbons. Methyl carbons usually have T'_2 values around 55 μsec, (although they can be higher [14]) and non-protonated aliphatic carbon T'_2s are long (>100 μsec).

As already noted the 105 ppm resonance appears to tail to 104 ppm indicating an overlapping resonance at $\sim$104 ppm. The fact that the decay of signal from 105 ppm resonance does not follow a simple rate law confirms that at least two types of carbons resonate in this region. Moreover, if the areas under the 72 and 105 ppm resonances are compared, the ratio of the two areas average 3.5:1 for the different mosses (R, Table 2). This is lower than the 5:1 expected for normal polysaccharides.

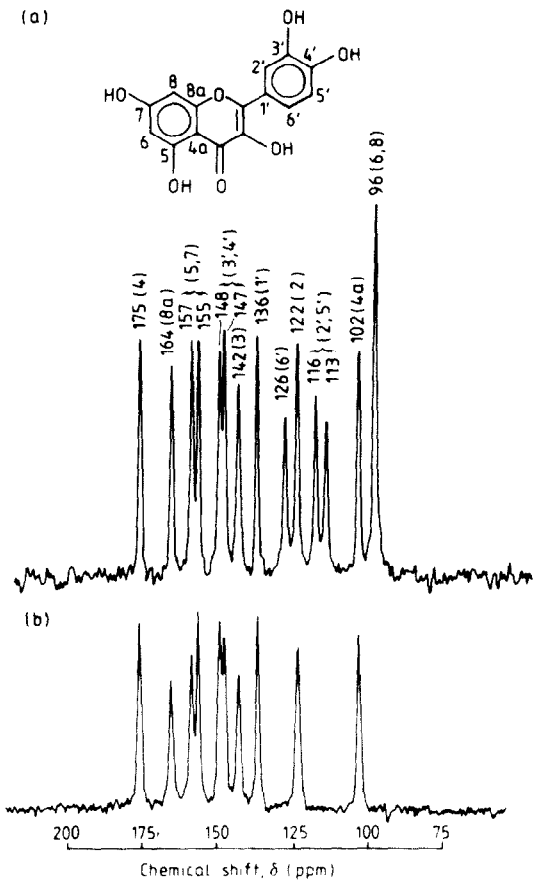


Fig. 4. ^{13}C CP/MAS spectra of quercetin. (a) Conventional spectrum; (b) 50 μsec dipolar dephased spectrum.

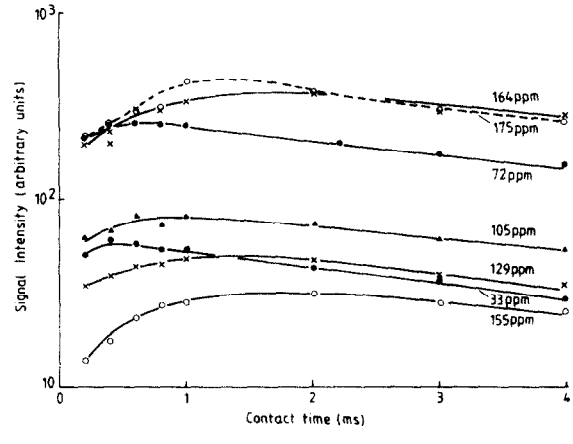


Fig. 5. Plot of signal height for carbons of different chemical shifts in *Thamnobryum* against contact time (chemical shifts are accurate to ± 2 ppm).

Thus an additional contributing resonance is present. The dipolar dephasing behaviour for the resonance at 105 ppm can be fitted to the equation.

$$I = I^0_A \exp(-t^2/2T'^2_A) + I^0_B \exp(-t/T'_{2B})$$

where t is the dipolar dephasing time. Similar decay behaviour is found for coals. coalified woods, humic substances and soils [14]. As T'_{2B} is much larger than

T'_{2A}, then I_B^0 can be found by extrapolation (Fig. 6) and hence I_A^0 calculated as at $t=0$, $I_A^0+I_B^0=I$.

These calculations show that 62% of the carbon is due to a rapidly relaxing protonated components, i.e. acetal carbons in polysaccharides, and the remainder is from some other source. If the magnitude of the acetal resonance, or the protonated component, is compared with that of the resonance at 72 ppm, the relative ratio of signal intensities is 5:1. This agrees with the proposal that polysaccharides are present.

The other types of carbon must be non-protonated. Resonances from non-protonated aromatic carbons are rarely observed at such low chemical shifts and are indicative of the C_{4a} carbons such as those in tannins or equivalent carbons of *meta* trihydroxyphenols in hydroxybenzofurans. Close examination also indicates that the resonances of C_6 and C_8 carbons in tannin-like materials may also be accounted for as part of the protonated part of the 105 ppm resonances, although this would mean that we should expect the ratio of signal from protonated carbon at 105 to that at 72 ppm resonance to be greater than five. Probably these differences observed reflect experimental error.

The appropriateness of this analysis is illustrated nicely by the 50 μsec dipolar dephased spectrum of Dawsonia stems (Fig. 3b). Here all protonated carbon has been removed from the spectrum and non-protonated carbon is left at a reduced intensity according to T'_2. It is clear that the mean chemical shift of the 105 ppm resonance reduces to 102 ppm. There is also some loss of signal intensity at lower chemical shift which could be assigned to C_8, C_6 carbons in tannins or equivalent in hydroxybenzofurans.

We are not able to specifically distinguish between hydroxybenzofuran or tannin like structures in *Dawsonia* stems and *Thamnobryum* species, but there is also evidence for resonances from the catechol ring of tannin being present at 155 and 115 ppm particularly for *Tham-*

Table 1. Mosses studied by CP/MAS ^{13}C NMR

Name	Habit and Locality
Thamnobryum pandum	Mt Glorious, Queensland. Maiala National Park. Greene's Falls Walking Track, 3.5 km along track. Dendritic habit forming loose mats on boulders and earth.
Dawsonia superba	Springbrook, Queensland. On road side between Best of All Look Out and Gwongorella National Park Moss. Moss is 15 cm tall on steep cutting beside the road.
Sphagnum cristatum	3 km from Coolum Beach, Queensland, freshwater swamp.
Leucobryum candidum	Mt Glorious, Queensland. Maiala National Park, Green's Falls Walking Track, 2.5 km along track. Collected from dense mats on rotting logs in Rain Forest.
Rhizogonium parramatense	As above 3 km along track.

Table 2. Spin lattice relaxation times in the rotating frame ($T_{1\rho}$H) and spin–spin relaxation times (T'_2) for whole ultrasonically-treated mosses.

						$T_{1\rho}$H (ms)*		
		COOH		Aryl		RO_2	CH_xOH	Alkyl
Moss structure	R	175 ppm	164	156	133	105	73	33
Leucobryum	(3.2)	2.5	—	—	—	6.0	5.8	5.8
Dawsonia	(3.9)	4.3	—	—	—	4.5	6.2	6.2
Thamnobryum	(3.2)	6.9	7.2	10.0	7.9	7.6	6.2	5.6
Sphagnum	(3.5)	—	—	—	—	7.1	6.0	—
Rhizogonium	(2.9)	6.4	—	—	6.8	6.2	6.2	—
			T_2 (μs)*					
Leucobryum				—	—	44	22†	—
Dawsonia		140	—	—	—	44	22†	—
Thamnobryum		149	113	107	62	15/91‡	18†	45
Sphagnum		—	—	—	—	45	33†	—
Rhizogonium		—	—	—	—	36	21†	—

*From log of signal intensity ($\ln I$) versus contact time or dephasing time (t) plot since $I=I^0\exp(-t/T)$, $T=T_{1\rho}$H or T'_2.

†From $\ln I$ versus t^2 plot since $I=I^0\exp(-t^2/2T'^2_2)$.

‡From $\ln I$ versus t plot and $\ln[I-I_B^0\exp -t/T'_{2B}]$ versus t^2 plots since $I=I_A^0\exp-(t^2/2T'^2_{2A})+I_B^0\exp(-t/T_{2B})$. Values, quoted are for T'_{2A} and T'_{2B} respectively.

R = Ratio of 73 to 105 ppm peak area.

Chemical shifts are accurate to ±2 ppm.

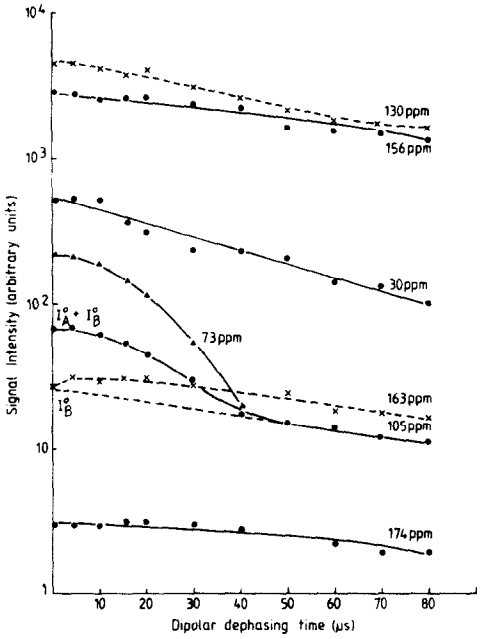


Fig. 6. Dipolar dephasing data for *Thamnobryum*. The 105 ppm resonance is divided into two parts with fast and slowly decaying signals. The contribution of the slowly decaying signal is determined by extrapolation to I_B^0 (chemical shifts are accurate to ± 2 ppm).

nobryum. This supports a tannin like composition for the aromatics. It is clear that the 1,3,5-trihydroxybenzene structure and not guaiacyl is the building block of the 'aromatic' mosses. These substances cannot therefore be described as lignin.

EXPERIMENTAL

Descriptions of the plant materials are given in Table 1. They were carefully separated from any visible unrelated tissue and ground to ~ 100 μm. Samples were also washed with H_2O and sepd from detritus and other foreign matter using an ultrasonic bath and dried. High resolution solid state NMR spectroscopy was performed on a Bruker CXP100 instrument equipped with a 2.11T magnet operating at 22.5 MHz for ^{13}C or a Chemagnetics 100S/200L spectrometer operating at 25.1 MHz for ^{13}C. Spectra were obtained in 0.5 or 1 K of data points and zero filled to 4 K before Fourier transformation using 50 Hz line broadening. Between 5 and 50 K scans were collected. The cross polarization technique with magic angle spinning (CP/MAS) was employed. Contact time was varied but was normally 1 msec. Recycle time was 1 sec and the 90° pulse for protons was 7 μsec. In some experiments the dipolar dephasing technique was employed [14] to aid in distinguishing different carbon types. Some preliminary GC-MS was also performed as described elsewhere [15].

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