Comparative FTIR and ^{13}C CP/MAS NMR Spectroscopic Investigations on Sporopollenin of Different Systematic Origins

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Comparative phytochemical investigations were carried out on sporopollenins of the following plant species: Bryophyta – *Reboulia hemisphaerica* and *Polytrichum strictum;* Pteridophyta – *Equisetum arvense, Selaginella selaginoides* micro- and megaspores; Gymnospermae – *Cycas circinalis;* Angiospermae – *Typha angustifolia* and *Tulipa cv.* "Apeldoorn". Although the range of the analyses included species from the Bryophyta to the Angiospermae, fundamental correspondences could be detected in the ^{13}C NMR and FTIR spectra concerning the occurrence of aliphatics, aromatics, ether and carbonyl/carboxylic functions in varying degrees. While the amount of aliphatics strongly dominated in the molecular structure of all other sporopollenins investigated here, it was found in sporopollenin of *Equisetum arvense* in a much lower extent. Despite the different morphology of *Selaginella selaginoides* micro- and megaspores, their sporopollenin spectra were nearly identical.

Introduction

Sporopollenin is an extremely resistant biopolymer, which is substantially involved in the formation of the outer spore and pollen wall. This biopolymer is resistant against non-oxidative chemical, physical and biological degradation to a high extent. In the past substantial results concerning the structure of sporopollenin have been obtained [1], nevertheless fundamental aspects of the definite polymer structures and especially the biosynthetic pathways leading to it are not yet clear in detail. The results of tracer experiments [2–5] revealed that, in comparison to all other precursors, such as D,L-[2-^{14}C]mevalonate, D-[1-^{14}C]glucose, [U-^{14}C]-acetate, [2-^{14}C]malonic acid, L-[U-^{14}C]tyrosine, [4-^{14}C]isopentenylpyrophosphate and [2-^{14}C]-*p*-coumaric acid a high level of incorporation into the sporopollenin fraction could be achieved using L-[U-^{14}C]phenylalanine as a tracer. Degradation of the sporopollenin samples labelled *via* [ring-^{14}C]-phenylalanine by potash fusion resulted in the formation of *p*-hydroxybenzoic acid as the main labelled degradation product. Thus the integral incorporation of the aromatic ring system into the polymer could be clearly demonstrated. Therefore it can be assumed that the aromatic metabolism is involved in sporopollenin biosynthesis.

Only a low level of incorporation was achieved using [U-^{14}C]-acetate and [2-^{14}C]malonic acid. These results were unexpected because, according to results of ^{13}C NMR spectroscopic analyses obtained so far, sporopollenin mainly consists of long-chain aliphatics with a substantial amount of acid and/or ester groups [6, 7]. The oxygen content in ether, hydroxy, carboxylic acid, ester or ketonic carbonyl groups may vary considerably.

On the basis of ^{13}C NMR analyses, Hemsley *et al.* [8] observed that, despite far-reaching similarities, several differences occur in the spectra of fossil and modern sporopollenins. In contrast to recent material they detected fewer but broader peaks in the spectra of fossil material. These findings were interpreted as a hint of the loss of functional groups particularly of those containing oxygen.

Due to the higher complexity of IR spectra, only few applications of this method for the structural elucidation of sporopollenin can be found in the recent literature [9, 10]. The IR spectroscopy, improved by the Fourier transform technique with its fast realization and high degree of resolution offers,

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in contrast to older studies [11, 12], various possibilities for the characterization of sporopollenin structures, especially in comparative studies of sporopollenins from different origin and in combination with the results of ^{13}C NMR spectroscopy. To our knowledge such phytochemical investigations do not exist until now. Therefore, comparative FTIR and ^{13}C NMR spectroscopic studies were carried out on a broad basis. It was the aim to obtain more knowledge about the structure and molecular composition of sporopollenin and to characterize the correspondences and differences in the composition of sporopollenin from various systematic origin.

Materials and Methods

Sporopollenin was isolated from pollen or spores of the following plant species: Bryophyta – *Reboulia hemisphaerica* and *Polytrichum strictum;* Pteridophyta – *Equisetum arvense, Selaginella selaginoides* micro- and megaspores; Gymnospermae – *Cycas circinalis;* Angiospermae – *Typha angustifolia* and *Tulipa cv.* "Apeldoorn". To eliminate soluble phenolic, lipoid or terpenoid components being accumulated in and/or on the structures of the exine/exospor, the pollen and spore material was extracted exhaustively for several weeks with different solvents. The sporopollenin fraction was isolated in a conventional way by treatment with phosphoric acid (80%, 50 °C, 10 d) followed by a further solvent extraction procedure of the residue. The obtained material was lyophilized and stored in a desiccator until examination.

FTIR analyses were carried out at the Institut für Organische Chemie der Westfälischen Wilhelms-Universität Münster on a Nicolet 5 DXC spectrometer. FTIR spectra of the samples (KBr pellets) were obtained between 4000 cm^{-1} and 400 cm^{-1}.

^{13}C CP/MAS NMR analyses were carried out with a Bruker MSL 200 or MSL 500 spectrometer with the following set-ups: C-13 frequency: 50.3 or 125.7 MHz; sample rotation: 5 kHz round an axis tilted 54.4° against the main magnetic field axis; contact time for cross polarization (CP): 1 ms; aquisition period: 5–10 ms according to field intensity; 90° pulse length: 4 µs; spectral width: 50–60 kHz according to field intensity; repetitions: 800–16,000; pulse repetition time: 3 s. Chemical shifts are expressed in parts per million (ppm) and are relative to external tetramethylsilane.

Results and Discussion

The ^{13}C NMR and FTIR spectra of the analyzed sporopollenin samples are shown in Figs. 1 and 2. The ^{13}C NMR spectra reveal that long-chain aliphatics represent a high amount of the sporopollenin structure, because all NMR spectra are dominated by the large signal No. 7 except for *Cycas circinalis* and *Equisetum arvense.* The IR spectra confirm the occurrence of aliphatics by the bands C, H, I and P (Table II). The presence of aromatic compounds is indicated by the NMR peaks 3, 4, 5 (Table I) and the IR signals B, G and O.

The NMR signal 6 and IR bands L, M, N are characteristic of ether groups, whereas the NMR bands 1, 2 and the IR signals D, E, F are attributed to carbonyl and carboxyl functions.

A comparative examination of the sporopollenin spectra from Bryophyta, Pteridophyta, Gymnospermae and Angiospermae reveals a good accord-

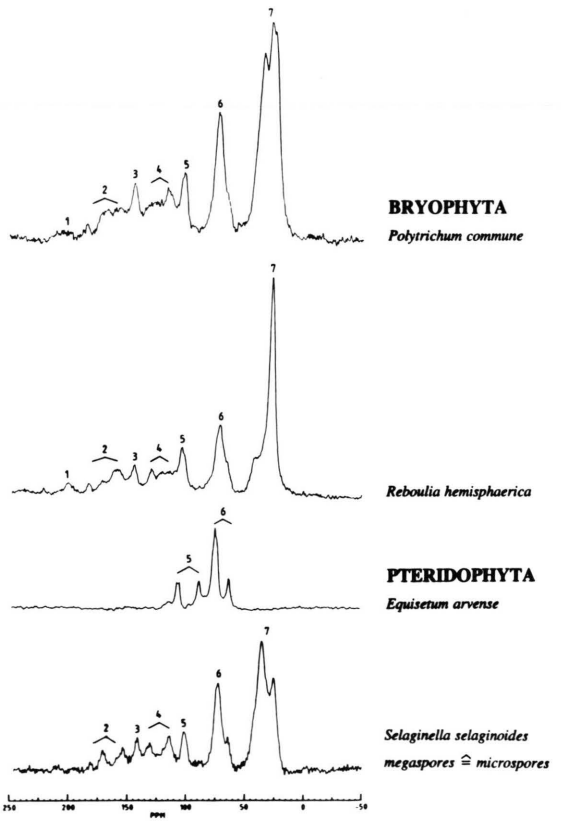

Fig. 1a. ^{13}C CP/MAS NMR spectra of sporopollenin isolated from different plant taxa; for interpretation see Table I.

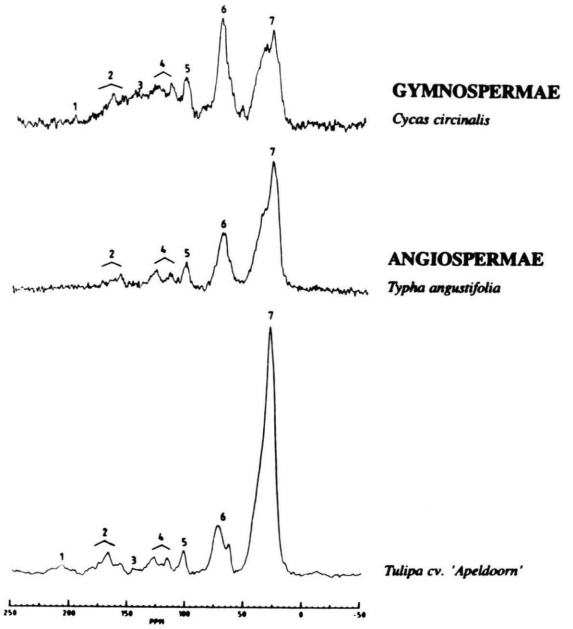

Fig. 1b. ^{13}C CP/MAS NMR spectra of sporopollenin isolated from different plant taxa; for interpretation see Table I.

ance in the type and position of the detected peaks. On the other hand there is a great diversity concerning the peak intensities; *e.g.* among the species presented here, *Cycas* exhibits the highest relative content of aromatic structures, whereas sporopollenin from *Tulipa* pollen is characterized by its high content of aliphatics.

Sporopollenin from spores of *Equisetum arvense* seems to be extraordinary different since the aliphatic signals in its spectra are strongly reduced (Fig. 2) or completely missing (Fig. 1). As to the existence of this structural element sporopollenin of *Equisetum arvense* probably differs from other pteridophytic spores. A further peculiarity of the *Equisetum* spores is for example its sporodermal permeability of water [13].

The heterosporous Lycopsida *Selaginella selaginoides* was the other member of the Pteridophyta investigated. So far studies on the walls of micro- and megaspores mainly concerned their ultrastructure and fluorescence behaviour. On the basis of such results Willemse [14] showed that sporopollenin is not only a component of the microspore wall but also of the wall surrounding the female gametophyte of *Cycas revoluta.*

Fig. 2a. FTIR spectra of sporopollenin isolated from different plant taxa; for interpretation see Table II.

Here, for the first time sporopollenin of micro- and megaspores have been comparatively analyzed by FTIR and ^{13}C NMR spectroscopy. Both spectroscopic methods led to almost identical spectra (for this reason only one of them is presented), revealing a high degree of consistence in the chemical composition of the micro- and megaspore sporopollenin of this system. Considering the strongly different morphology of *Selaginella selaginoides* micro- and megaspores these results are remarkable. Whereas the sporodermal sporopollenin of the smaller microspores is located in the para-exospore and exospore, in the ten times larger megaspores it occurs in an bilayered exospore, whose voluminous external layer consists of a spongy structure which does not occur in any other recent pteridophytic spore [15].

In the obvious presence of aliphatic signals *Selaginella* sporopollenin seriously differs from *Equise-*

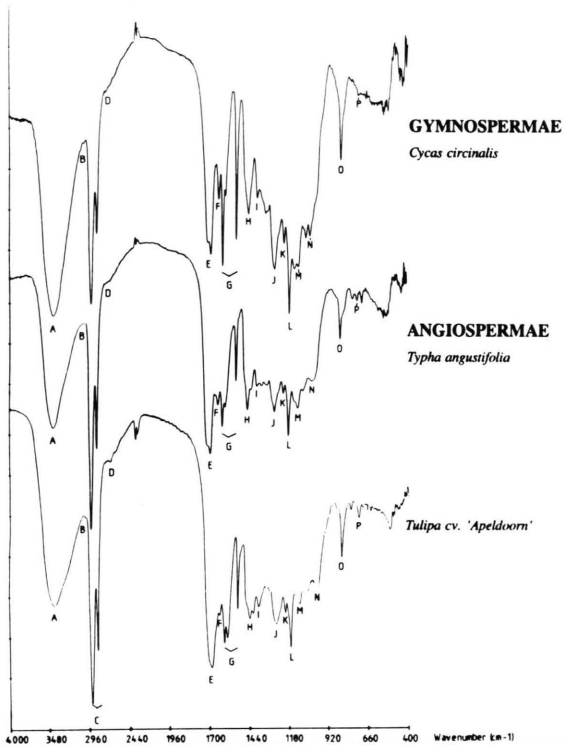

Fig. 2b. FTIR spectra of sporopollenin isolated from different plant taxa; for interpretation see Table II.

Table I. ^{13}C NMR assignments for the analyzed sporopollenins.

No. in Fig. 1	Carbon type	Chemical shift [ppm]
1	ketonic carbonyl	200–210
2	carboxylic acid	170–180
	carboxylic ester	160–170
3	non-protonated aromatic carbon	145
4	aromatic	130
5	alkene and/or aromatic	105
6	alcohol and ether	60–75
7	CH_2 in polymethylenic chains and CH_3	30 15

tum sporopollenin, the other pteridophytic species investigated.

In summary it can be concluded from the results of FTIR and NMR spectroscopy that the analyzed sporopollenins show principle correspondences in the occurrence of aliphatics, aromatics and ether, carbonyl/carboxylic groups but also significant differences. Consequently, sporopollenin cannot be considered as a uniform macromolecule; it rather represents a type of closely related biopolymers with different chemical groups occurring in varying amounts.

Table II. Assignment of the FTIR signals marked in Fig. 2; ν, stretching; δ, deformation.

Letter in Fig. 2	Band origin, short comment		Wave number [cm^{-1}] of the absorption minimum
A	ν(O–H)	polymeric hydroxyls, broad band	3420
B	ν(C–H)	aromatics	3050–3030
C	ν(C–H)	in methyl and methylene groups	2930/2860
D	ν(O–H)	in hydrogen bonds of carboxylic acids	3000–2500
E	ν(C=O)	carbonyl	1700–1680
F	ν(C=O)	in conjugated carbonyls	1640
	ν(C=C)	or in alkenes	
G	ν(C=C)	aromatic skeletal vibrations	1610, 1580, 1517
H	δ(C–H)	in methyl and methylene groups	1440
I	δ(C–H)	in methyl groups	1380–1350
J	ν(C–O)	in arylalkyl ethers or	1270
	δ(OH)	in alcohols	
K	ν(C–O)	in ester groups	1200
L	ν(C–O)	in methyl esters of aliphatic acids or in ethers	1165
M	ν(C–O)	in aliphatic ethers or secondary alcohols	1110
N	ν(C–O)	in ethers	1010
O	δ(C–H)	in aromatics with two neighbouring H	830
P	δ(CH_2)	in aliphatic chains with $n > 4$	720

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