Acetylation and Silylation of Piperidine Solubilized Sporopollenin from Pollen of *Typha angustifolia* L.

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Silyl and acetyl derivatives of sporopollenin from the pollen of *Typha angustifolia* L. were prepared. The derivatized products were readily soluble in piperidine-d_{11} and could be investigated employing one- and two-dimensional proton and carbon NMR (nuclear magnetic resonance) spectroscopy (^{1}H,^{1}H-COSY and ^{13}C,^{1}H-HETCOR techniques). For the first time, a two dimensional ^{13}C,^{1}H-HETCOR NMR spectrum of a sporopollenin could be obtained. The results underline the importance of derivatization techniques for obtaining two dimensional ^{13}C-NMR spectra of sporopollenins. Moreover, piperidine turns out to be a more suitable solvent for sporopollenins than 2-aminoethanol, as it allows for higher solubilities, being important for 2D-NMR investigations. From the HETCOR and COSY spectra of the silylated and the acetylated *Typha* samples the occurrence of aliphatic polyhydroxy compounds as well as phenolic OH groups became evident.

Key words: Typha angustifolia L., Sporopollenin, Solubilization in Piperidine

Introduction

The biopolymer sporopollenin is the main component of the outer walls of spores and pollen. Little is known about the definite chemical structure of sporopollenins. Its insolubility in common acids and most organic solvents has limited structural investigations of sporopollenins to techniques applicable in the solid state. For a long time, sporopollenin was even thought to be completely insoluble, until the complete solubilization of sporopollenin was demonstrated (Jungfermann *et al.*, 1997). The successful dissolution of sporopollenin from the pollen of *Typha angustifolia* L. in 2-aminoethanol in this work opened sporopollenin research to a wide variety of techniques like size exclusion chromatography (SEC), matrix assisted laser desorption ionisation/mass spectrometry (MALDI/MS) and liquid state nuclear magnetic resonance (NMR). Especially the latter technique allowed structural information of sporopollenins to be obtained (Ahlers *et al.*, 1999). According to present knowledge, sporopollenin is a polymer consisting mainly of unbranched aliphatics with a variable amount of aromatics [for review see Wiermann *et al.* (2001) and literature cited therein]. More detailed structural information on sporopollenins is anticipated from the application of liquid state ^{13}C-NMR techniques, especially in its two dimensionsional variants. The commonly achievable solubilities of 5 mg·ml^{-1} in 2-aminoethanol (Jungfermann *et al.*, 1997) limit NMR-investigations to the ^{1}H nucleus. ^{13}C NMR investigations could, however, reveal more subtle structural details of sporopollenins. In this paper, a technique is described allowing two dimensional liquid state ^{13}C nuclear magnetic resonance spectra (HETCOR spectra; Morris and Freeman, 1979) to be obtained. This technique, based on derivatization and the choice of a more suitable solvent, is applied to the structural elucidation of sporopollenin from the pollen of *Typha angustifolia* and allows new functional groups to be identified.

Experimental

Materials and methods

For the investigations sporopollenin of *Typha angustifolia* L. was used, isolation and purification were performed as described earlier (Ahlers *et al.*,

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2000). Here, the extraction procedure was applied without acetone.

Procedures for acetylation and silylation

All equipment should be cleaned with a 1:1 mixture of chloroform/methanol (both analysis grade) before usage. After drying, the glassware used was flooded with nitrogen.

For the peracetylation reaction a mixture of 1 ml of acetic acid anhydrid and 1 ml of piperidine was added to 50 mg of sporopollenin from *Typha angustifolia* L. After overnight storage at room temperature, crushed ice was added to the reaction mixture, followed by 10 min of centrifugation at 2420 × *g*. The residue was washed once with diethyl ether and several times with distilled acetone. After neutralization, the residue was freeze dried for 60 h.

For the silylation reaction a mixture of 1 ml of N-methyl-N-(trifluorosilyl)-trifluoroacetamide (MSTFA) and 0.1 ml of pyridine was added to 50 mg of sporopollenin from *Typha angustifolia L.* After overnight storage at 50 °C in the dark, crushed ice was added to the reaction mixture, followed by 10 min of centrifugation at 2420 × *g*. The residue was washed once with diethyl ether and several times with distilled acetone. After neutralization, the residue was freeze dried for 60 h.

For the preparation of NMR samples 1 ml of piperidine-d_{11} was added to 15 mg of the peracetylated resp. silylated sporopollenin and refluxed for 2 h, followed by centrifugation at 2420 × *g* for 10 min. The fluid was filled into NMR tubes in a nitrogen flooded glove box.

NMR measurements

All NMR spectra were measured on a JEOL GX400 NMR spectrometer operating at 400 MHz and 100 MHz for ^{1}H and ^{13}C, respectively. Solvent for all samples was piperidine-d_{11}. Frequency ranges for ^{1}H and ^{13}C measurements were 5 kHz and 24 kHz, respectively. ^{1}H pulse widths were determined on the *Typha* samples directly, whereas ^{13}C pulse widths were calibrated on a sample of 2-butanol in D_2O. The relaxation delay was 1 s for all measurements.

Two dimensional ^{1}H,^{1}H-correlation spectra (COSY) (Aue and Bartholdi, 1976) were measured for the native, the acetylated and the silylated *Typha* samples. Two dimensional ^{13}C,^{1}H-correlation spectra (HETCOR) (Morris and Freeman, 1979) were measured for the acetylated and the silylated *Typha* samples, but not for the native sample due to severe sensitivity problems. (Possibly some degree of disaggregation is required to minimize signal losses from spin-spin relaxation.) For the COSY spectra, spectral ranges were 5 kHz in both dimensions and 512 t_1 increments of 128 scans each were taken. The delay τ of the HETCOR spectra is adjusted to the one bond ^{13}C,^{1}H-coupling constant ^1J(C,H) according to the relationship $\tau = 1/(2 \cdot {}^1J(C,H))$. A t value of 3.52 ms, corresponding to a coupling constant value ^1J(C,H) = 142 Hz, is suitable for the observation of the signals of both aliphatic and aromatic structures. 128 t_1 increments were used for HETCOR experiments, frequency ranges in the F_1 and F_2 dimension were 5 kHz and 24 kHz, respectively. 2048 scans were measured for each t_1-increment. All 2D spectra were processed in the absolute value mode.

Results and Discussion

Fig. 1 displays the ^{1}H NMR spectra of the native, peracetylated and silylated sporopollenin samples of *Typha angustifolia* pollen. The aromatic regions of the spectra of the silylated and acetylated samples are in close correspondence to that of the native *Typha* sample. The aromatic ranges of the ^{1}H NMR spectra (Fig. 1) of all three samples show large signals at δ_H = 7.45 ppm (doublet, J = 13 Hz, 1H), δ_H = 6.15 ppm (doublet, J = 13 Hz, 1H) and δ_H = 7.25 ppm (doublet, J = 8 Hz, 2H) and δ_H = 6.58 ppm (doublet, J = 8 Hz, 2H), that indicate the presence of a *para*-disubstituted benzene and a *trans*-disubstituted olefin. The δ_H = 6.58 ppm signal accounts for an OH-group or an OR-group (R = alkyl) as substituent in the 4-position and increment calculations of the proton chemical shifts favour 4-methoxy-cinnamic acid or 4-hydroxy-cinnamic acid. As the signal at δ_H = 6.58 ppm shows no shift or splitting after peracetylation or silylation (Fig. 1), the substituent in the 4-position is probably no OH-group but an ether-oxygen linked to the sporopollenin.

The aromatic range of the two dimensional COSY NMR spectrum of the native *Typha* sample (Fig. 2) displays crosspeaks at δ_H/δ_H = 7.45/

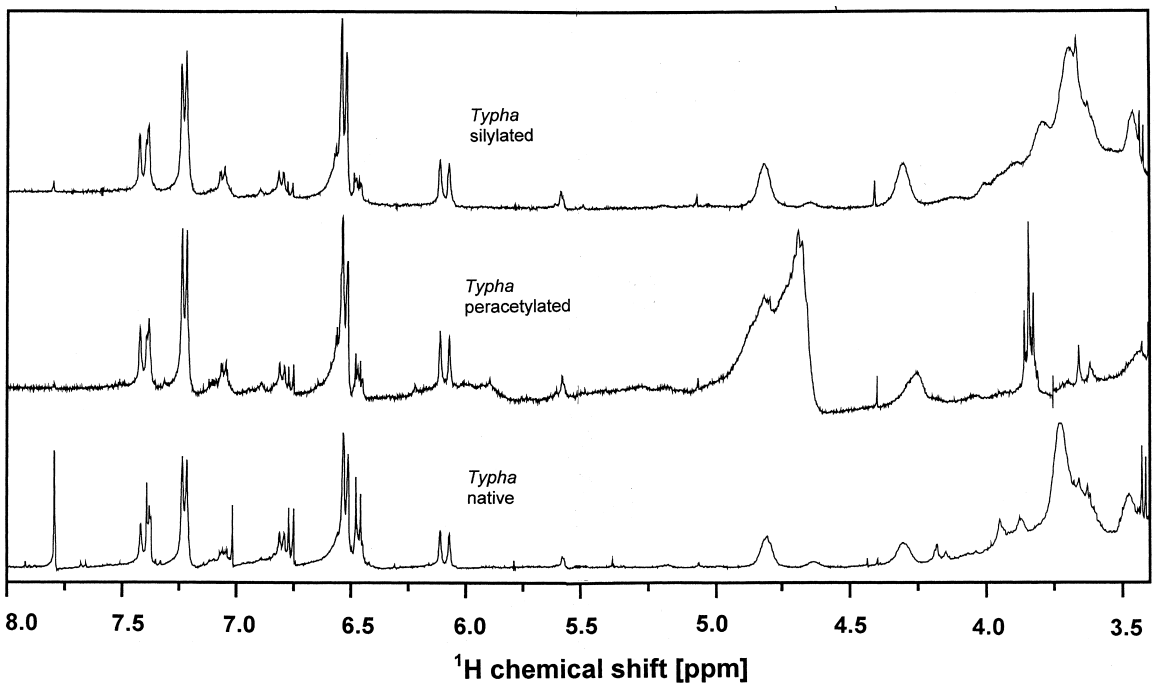

Fig. 1. ^{1}H NMR spectra of different sporopollenin preparations from *Typha angustifolia* pollen.

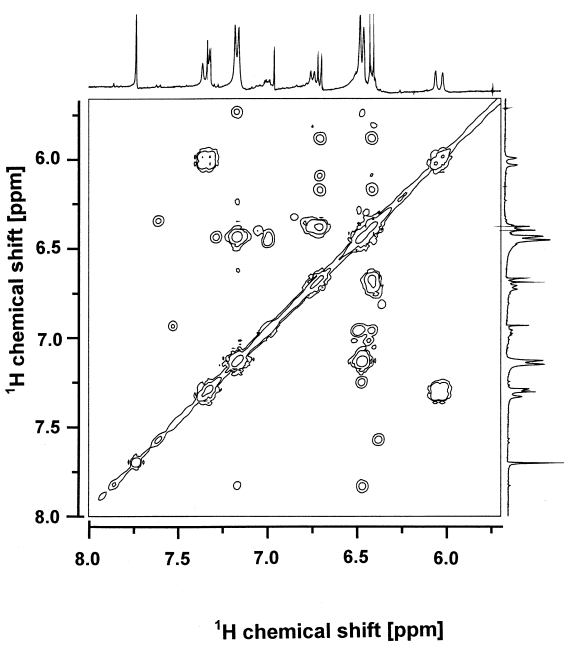

Fig. 2. Two dimensional ^{1}H,^{1}H-COSY NMR spectrum (aromatic range) of native sporopollenin from *Typha angustifolia* pollen.

6.15 ppm and δ_H/δ_H = 7.25/6.58 ppm that support the aforementioned assignment. Another intense doublet signal at δ_H = 6.48 ppm (J = 8 Hz) in the ^{1}H NMR spectrum of the native *Typha* sample (Fig. 1) is reduced in intensity in the corresponding spectra of peracetylated and silylated samples due to further line splittings. As the chemical shift of δ_H = 6.48 ppm most probably accounts for a proton in the *ortho*-position to an OH-group, the splitting is the result of a partial acetylation resp. silylation of the OH-group. Hence the observation of a second signal set and the identification of 4-hydroxy-cinnamic acid clearly indicate the presence of aromatic hydroxy groups in sporopollenin of *Typha angustifolia* pollen. The two dimensional COSY spectrum of the native *Typha* sample (Fig. 2) shows that the doublet signal (J = 8 Hz) at δ_H = 6.48 ppm is coupled to another doublet signal (J = 8 Hz) at δ_H = 6.77 ppm. As both these signals have equal integrals and show a splitting due to an aromatic *ortho*-coupling, they are part of a 1,4-disubstituted phenol.

While the aliphatic signal ranges in the ^{1}H NMR spectra of the native and the silylated *Typha* samples show some similarities, the aliphatic region of

the peracetylated sample is substantially different (Fig. 1). The broad signal around δ_H = 3.7 ppm in the ^{1}H NMR spectra of the native and the silylated *Typha* samples is shifted to the δ_H = 4.5–5 ppm signal range in the ^{1}H NMR spectrum of the peracetylated sample. The observation of a signal group around δ_H = 3.7 ppm in the spectrum of the native sample that is shifted downfield by approx. 1 ppm upon peracetylation clearly indicates the presence of polyhydroxy compounds in sporopollenin of *Typha angustifolia* pollen. This result is confirmed from XPS-investigations (Ahlers *et al.*, 2000) as well as from ^{13}C,^{1}H-shift correlated 2D-NMR (HETCOR) measurements of the silylated (Fig. 3a) and the peracetylated *Typha* sample (Fig. 3b). Fig. 3a displays a group of crosspeaks with proton shifts in the signal range δ_H = 3.2–3.8 ppm that correlate with carbon shifts in the range δ_C = 68–75 ppm, that are typical of polyhydroxy compounds. The HETCOR spectrum of the peracetylated sample (Fig. 3b) shows the same set of crosspeaks shifted to approximately δ_H = 4.7 ppm in the proton dimension, in accordance with the results obtained from the one dimensional proton NMR spectra of Fig. 1.

The identification of 4-hydroxy-cinnamic acid confirms the results of our ^{1}H NMR investigations on sporopollenins of *Typha angustifolia* (Ahlers *et al.*, 1999a) and *Torreya californica* pollen (Ahlers *et al.*, 1999b). These studies revealed the presence of substituted aromatic substructures, whereas the nature of the substituents could not be determined in detail due to the low solubility of the sporopollenins in 2-amino-ethanol. The considerably higher solubility of the sporopollenins in piperidine, resulting in a much better signal to noise ratio of the spectra, now allows the identification of the aromatic substituents in this study.

To conclude, the results underline the importance of derivatization techniques for the characterization of sporopollenins. Without derivatization no ^{13}C,^{1}H-HETCOR NMR spectrum would have been obtained, as some degree of disaggregation is required to minimize the signal losses due to spin-spin relaxation. The comparison of the ^{13}C,^{1}H-HETCOR NMR spectra of the silylated and the acetylated *Typha* samples clearly indicates the occurrence of aliphatic polyhydroxy compounds as well as phenolic OH-groups in the investigated sporopollenin. This finding is in good agreement with results obtained after degradation of sporopollenin by potash fusion (Schulze-Osthoff and Wiermann, 1987), by using tracer experiments (Gubatz *et al.*, 1993) and immunocytochemical experiments (Niester-Nyveld *et al.*, 1997).

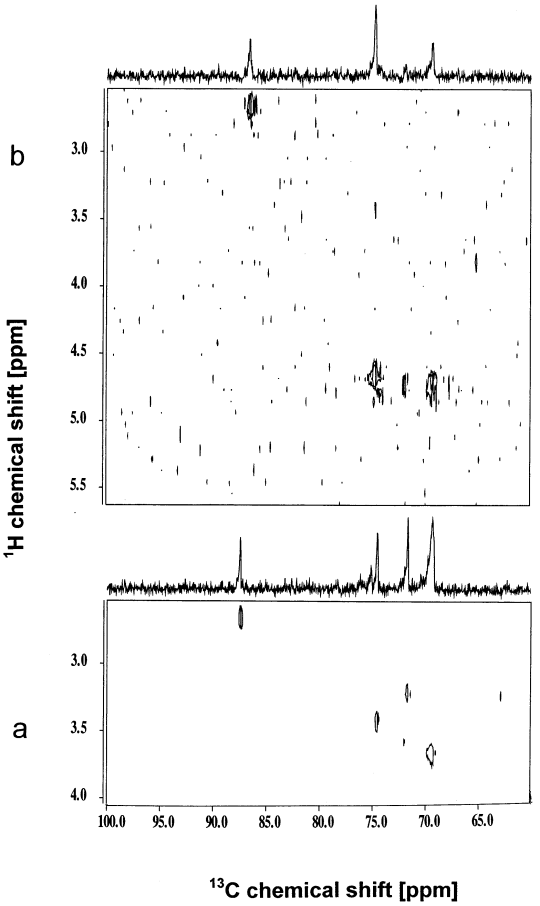

Fig. 3: Two dimensional ^{13}C,^{1}H-correlated (HETCOR) NMR spectra (aliphatic range) of derivatized sporopollenin from *Typha angustifolia* pollen; a) silylated sporopollenin; b) acetylated sporopollenin.

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