Structural Elements of Sporopollenin from the Pollen of *Torreya californica* Torr. (Gymnospermae): Using the ^{1}H-NMR Technique

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Sporopollenin was isolated and purified from pollen of the gymnospermae *Torreya californica* using an enzymatic treatment followed by extraction with organic solvents. ^{1}H-NMR (1D and 2D) was used for analysis of this biopolymer and reveals the presence of three phenolic compounds. Comparison with the analysis of sporopollenin from the angiospermae *Typha angustifolia* L. shows high similarity between the aromatic compounds.

Introduction

Sporopollenin is an unusually resistant biopolymer which is the main component of the exine which forms the outer pollen wall. It is insoluble in common solvents and acids, and therefore, former investigations have been limited to methods that can be applied to the polymer in its solid state only (Guilford *et al.,* 1988; Espelie *et al.,* 1989; Hemsley *et al.,* 1993, 1996; Wilmesmeier *et al.,* 1993; Kawase and Takahashi, 1995). Additional investigations in which different methods were used, indicate the presence of aromatic fragments in sporopollenin (Schulze Osthoff and Wiermann, 1987; Herminghaus *et al.,* 1988; Wehling *et al.,* 1989; Van Bergen *et al.,* 1993).

The solubilisation of sporopollenin in 2-aminoethanol (Jungfermann *et al.,* 1997; Thom *et al.,* 1998) opens a wide range of possibilities for further investigations, one of which is ^{1}H-NMR (Ahlers *et al.,* 1999). Experiments carried out with sporopollenin from *Typha* pollen reveal the presence of several tri- or tetrasubstituted aromatic structures in varying amounts. *Typha angustifolia* L. belongs to the angiospermae.

Until now the solubilisation and ^{1}H-NMR analysis of sporopollenin derived from plants belonging to lower phylogenetic categories has not been attempted; the experiment described in this report investigate this aspect of sporopollenin research.

Materials and Methods

Isolation and purification of sporopollenin

Pollen from *Torreya california* Torr. was collected at the shedding stage from plants growing in the Botanical Garden, Münster, Germany. The exine material was obtained by stirring the pollen in water for 24 h and washing by filtration through nylon-meshes (mesh 20 µm). The intine was degraded by enzymatic hydrolysis using Mazerozym R10 and Cellulase Onozuka R10 (Yakult, Nishinomiya, Japan), each 1% dissolved in 0.1 M sodium acetate buffer, pH 4.5, 30 °C, 3 d). The resulting material was extracted using solvents with increasing polarity ($CHCl_3$/MeOH 1:1 v/v; diethyl ether, acetone, MeOH, ethylene glycol monoethyl ether, H_2O), until absolute purification was gained (controlled by TLC analysis). The purification procedure reached to several times. TLC plates silicagel 60 F_{254}, 20×20 cm, layer thickness 0.2 mm were obtained from Merck (Darmstadt, Germany). Solvent for development was toluene. The solvent free layer was sprayed with solution of bromothymol blue from Merck (40 mg in 100 ml 0.01 M sodium hydroxide) for detection. After the extraction procedure the material was lyophilised for 48 h and stored in a desiccator until examination. For ^{1}H-NMR analysis sporopollenin was dissolved in a mixture of hot $NH_2CD_2CD_2OH$(98%)–D_2O (2:5 v/v), until a final concentration of 5 mg/ml.

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NMR measurements

^{1}H-NMR spectra were measured with a JEOL GX-400 NMR spectrometer in a solvent mixture of 2-aminoethanol-d_4 and D_2O at ambient temperature. The reference was applied from an external sample of sodium-3-trimethylsilyl-2,2,3,3-tetradeuteriopropionate in D_2O. In order to identify signals from the solvents and from impurities, all NMR spectra (including the 2D COSY) were measured from a control sample of 2-aminoethanol-d_4 and D_2O without sporopollenin under the same experimental conditions. The pulse repetition times were 1.7 s for all NMR experiments. The 2D-^{1}H–^{1}H-COSY was measured in absolute value mode using a standard pulse program.

Results and Discussion

The sporopollenin of *Torreya californica* pollen is soluble in 2-aminoethanol at a scale that allows ^{1}H-NMR-analysis. The ^{1}H-NMR spectrum of the purified sporopollenin is shown in Fig. 1.

The interpretation of the spectrum is limited to the aromatic region from δ 5.8 to 8.2 (see insert in Fig. 1), because the spectrum of the pure solvent shows similar signals in the other δ ranges. As these lines also appear in the spectrum of the reference, all signals between 2 and 4 ppm (a1–a4) and 8.60/8.67 ppm (c) cannot originate from the sporopollenin. The signal at δ 4.90 (b) is caused by HDO and the exchangeable protons of 2-aminoethanol. The broad signals between 0.8 and 1.6 ppm are assigned polymers with different aliphatic protons. Due possible contamination with the solvent, these signals cannot be interpreted. The signal at 3.43 ppm derives from residual methanol from the sporopollenin purification process. The proton–proton coupling network can be traced out by two dimensional ^{1}H–^{1}H-COSY NMR spectroscopy at 400 MHz. The COSY contour plot is displayed in Fig. 2.

In the aromatic region of the COSY spectrum of the sporopollenin a set of crosspeaks indicates the aromatic structures displayed in Fig. 3A. The following structural details can be extracted from the 2D-spectrum:

Crosspeak δ 6.41–δ 7.51: Because of its splitting due to a *J* coupling that is of the size of an ^3J-(*ortho*)-coupling this crosspeak is indicative of two hydrogens bound to neighbour aromatic carbons. There are no further hydrogens in the aromatic region as these would give rise to crosspeaks based on a ^3J-(*ortho*)- or a ^4J-(*meta*)-coupling and the shifts of δ 6.41 ppm and δ 7.51 ppm are not involved in any further crosspeaks. Thus, this crosspeak indicates either a 1,4-disubstituted or a 1,2,3,4-tetrasubstituted benzene system.

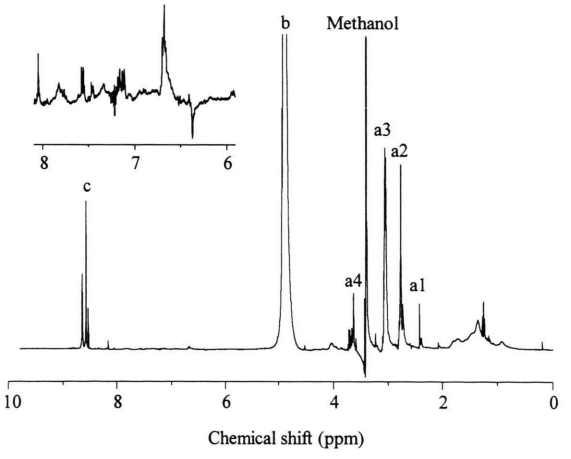

Fig. 1. ^{1}H-NMR spectrum of sporopollenin from *Torreya californica* pollen.

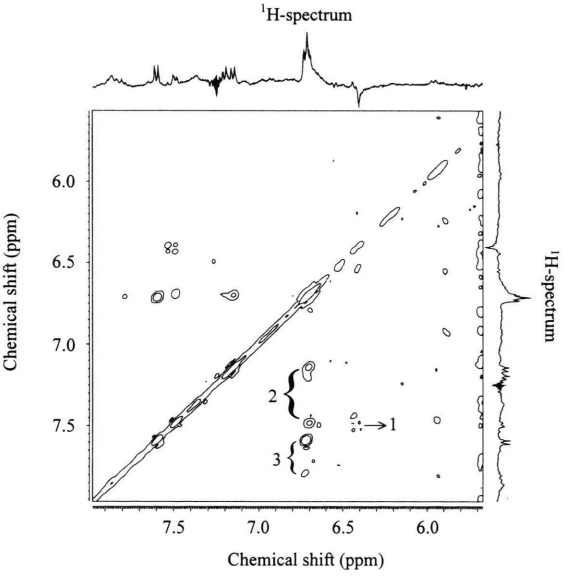

Fig. 2. ^{1}H–^{1}H COSY NMR spectrum of sporopollenin from *Torreya californica* pollen; *Crosspeak 1 δ 6.41–δ 7.51, Crosspeaks 2 δ 7.13–δ 6.70 and δ 6.70–δ 7.48, Crosspeaks 3 δ 7.59–δ 6.72 and δ 6.72–δ 7.79.*

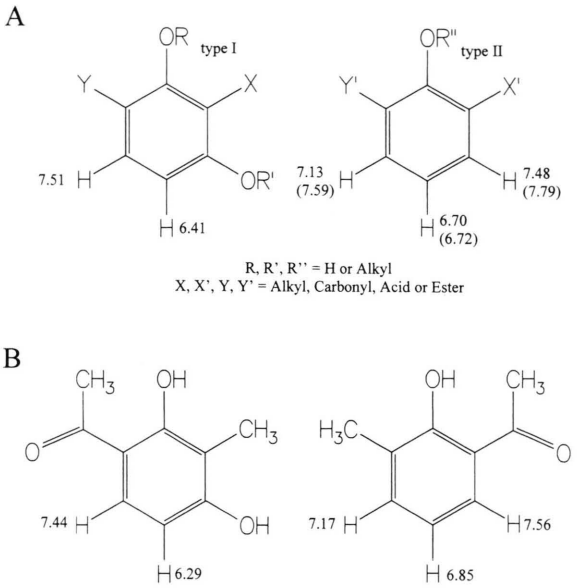

Fig. 3. A: ^{1}H chemical shifts of the two types of phenolic compounds (ratio 1:14 w/w, type I:II); B: ^{1}H chemical shifts, obtained from increment calculations, of two model compound.

A shift value of 6.41 ppm reveals either two OH(OR)-substituents (R = alkyl) in both *ortho* positions or OH(OR)-substituents in both the *ortho* and *para* positions of the investigated proton. These substitution patterns are clearly not in accordance with a proton framed by two *ortho* substituents. *Ortho/para* disubstitution with respect to one of the aromatic protons is in accordance with a 1,2,3,4-tetrasubstituted benzene ring, but not with a 1,4-disubstituted one. Increment calculations (Hesse *et al.*, 1995) (see Fig. 3B) of the aromatic proton chemical shift indicates that the remaining two substituents are alkyl or carbonyl/carboxyl groups. The structure suggested by NMR can therefore be described as the 'type I' of Fig. 3A.

Crosspeaks δ 7.13–δ 6.70 and δ 6.70–δ 7.48: The splittings due to *J* couplings shown by these crosspeaks are of the size of ^3J-(*ortho*)-couplings and are therefore indicative of two couples of hydrogens each bound to neighbouring aromatic carbons. Because both crosspeaks have a common coupling partner (δ 6.70), three protons bound to neighbouring aromatic carbons with the δ 6.70 proton as the central proton give rise to this crosspeak. These shifts are not involved in any further crosspeaks as there are no further hydrogens in the aromatic region which would give rise to crosspeaks based on a ^3J-(*ortho*)- or a ^4J-(*meta*)-coupling. This crosspeak pair therefore is caused by a 1,2,3-trisubstituted benzene system.

A shift value of δ 6.70 reveals a OH(OR)-substituent (R = alkyl) in the *para* position of the δ 6.70 proton. Again, from increment calculations (see Fig. 3B) of the aromatic proton chemical shift the remaining two substituents are an alkyl-group and a carbonyl- or a carboxyl-group. The structure is therefore best characterized as 'type II' shown in Fig. 3A.

Crosspeaks δ 7.59–δ 6.72 and δ 6.72–δ 7.79: Following the same line of argument for this pair of crosspeaks, the NMR indicates a 'type II' structure (Fig. 3A).

In order to explore the correlations between the structure of sporopollenin from the angiospermae (*Typha angustifolia*) and the gymnospermae (*Torreya californica*) a comparison of the ^{1}H-NMR spectra in the range of 5.9 to 7.8 ppm is shown in Fig. 4. The spectra of both samples are very similar in the aromatic region. The interpretation of the two dimensional ^{1}H–^{1}H-COSY spectrum from *Typha angustifolia* shows two different types of phenolic compounds in two variations (Ahlers *et al.*, 1999). Type I is an 1,2,3,4-tetrasubstituted and type II is an 1,2,3-trisubstituted benzene system. The substitution pattern of the two types are also found in *Torreya californica*, although type II is found in two variations and type I is unvaried. Furthermore, the chemical shift of the aromatic signals differs in the two samples only by a value

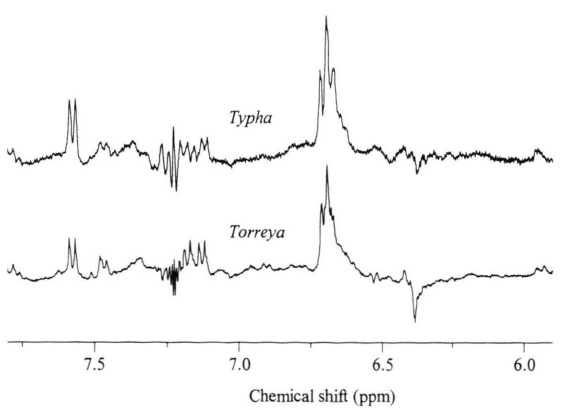

Fig. 4. ^{1}H-NMR spectra of sporopollenin from different systematic origins in the region of δ 5.8 to 8.2.

of about 0.07 ppm and is caused by the applied method. This indicates that the aromatic systems in the biopolymer of *Typha angustifolia* and *Torreya californica* have the same or equal substituents. In conclusion, the interpretation of the spectral data implies a high degree of similarity in the molecular structure of sporopollenin from both the angiospermae and the gymnospermae.

Acknowledgements

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