PERGAMON

Phytochemistry 50 (1999) 1095–1098

PHYTOCHEMISTRY

^{1}H NMR analysis of sporopollenin from *Typha Angustifolia*

Friedhelm Ahlers[a], Ina Thom[a], Jörg Lambert[b], Rüdiger Kuckuk[b], Rolf Wiermann[a,*]

[a]*Institut für Botanik, Schloßgarten 3, D-48149 Münster, Germany*
[b]*ISAS, Institut für Spektrochemie und Angewandte Spektroskopie, Bunsen-Kirchhoff Strasse 11, D-44139 Dortmund, Germany*

Received 3 March 1998

Abstract

The first analysis of sporopollenin using ^{1}H NMR-techniques revealed the presence of four phenolic compounds in different amounts. The phenolic compounds are tri- or tetrasubstituted. The sporopollenin was isolated and purified from *Typha angustifolia* by an enzymatic procedure, followed by extraction with organic solvents.

Keywords: Typha angustifolia; Typhaceae; Sporopollenin; Phenolic compounds; ^{1}H NMR

1. Introduction

Sporopollenin is an unusual resistant biopolymer that composes the outer wall of spores and pollen. In the past, chemical analysis of sporopollenin has been limited to methods that can be applied to the solid state only. ^{13}C NMR solid-state studies indicated that sporopollenin is mainly an aliphatic polymer [1–4]. Degradation experiments also showed that phenols represented integral components of sporopollenin [5, 6]. Investigations of sporopollenin from *Pinus mugo* pollen using several methods suggested *p*-coumaric acid as the main phenolic degradation compound of sporopollenin [7]. In sporopollenin, aromatic groups were also found by pyrolysis GC [8]. The main structure of sporopollenin seemed to be a simple aliphatic polymer containing aromatic or conjugated side-chains [9]. Furthermore, from immunocytochemical experiments it was concluded that phenols are important structural components of sporopollenin [10]. Recently, the solubilisation of sporopollenin in 2-aminoethanol and the following reaggregation of a sporopollenin-like material were described [11]. With this, new possibilities were opened to apply new techniques to the research of sporopollenin. Until now it has not been possible to obtain a ^{1}H NMR spectrum from the sporopollenin of any species.

2. Results and discussion

Purified sporopollenin from the pollen of *Typha angustifolia* was dissolved in a mixture of deuterated 2-aminoethanol-water under optimised conditions. The ^{1}H NMR (400 MHz) spectrum of the polymer is shown in Fig. 1. Interpretation of the ^{1}H NMR spectrum is only possible for the aromatic range from δ 5.5 to 8.0 and the signal at δ 3.43, because in comparison with the spectrum of the pure solvent other δ ranges show similar signals. Signals between 2 and 4 ppm (a1–a4) and those at δ 8.60 and 8.67 (c) do not derive from the sporopollenin, because their lines also appear in the spectrum of the blank. The signal at δ 4.90 (b) is assigned to HDO and the exchangeable protons of 2-aminoethanol.

The broad peaks in the δ range from 0.8 to 1.6 are characteristic of polymers with different aliphatic protons. Because of the influence of contamination from

* Corresponding author. Fax: +49-(0)251-8323823 e-mail: wiermann@uni-muenster.de.

0031-9422/99 $ - see front matter
PII: S0031-9422(98)00225-8

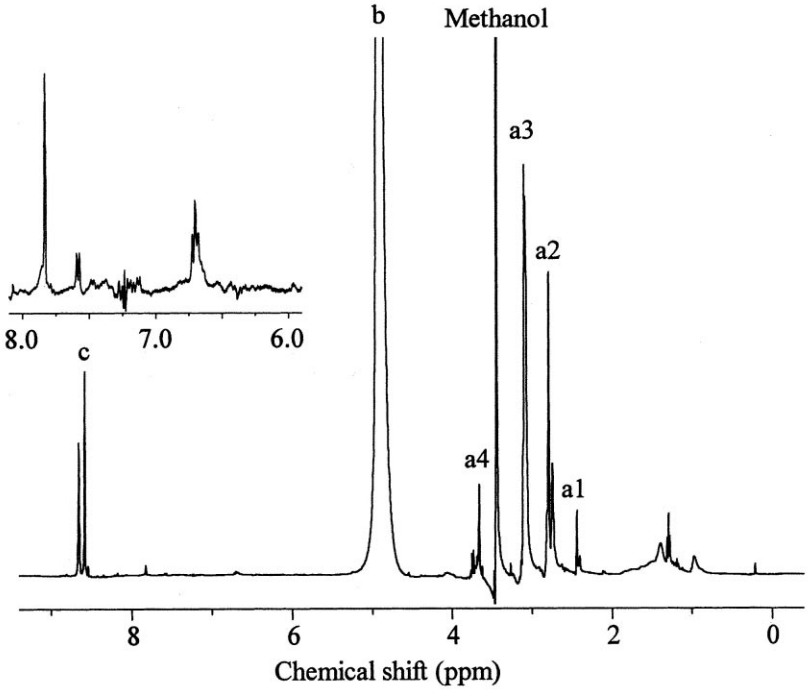

Fig. 1. ^{1}H NMR spectrum of sporopollenin.

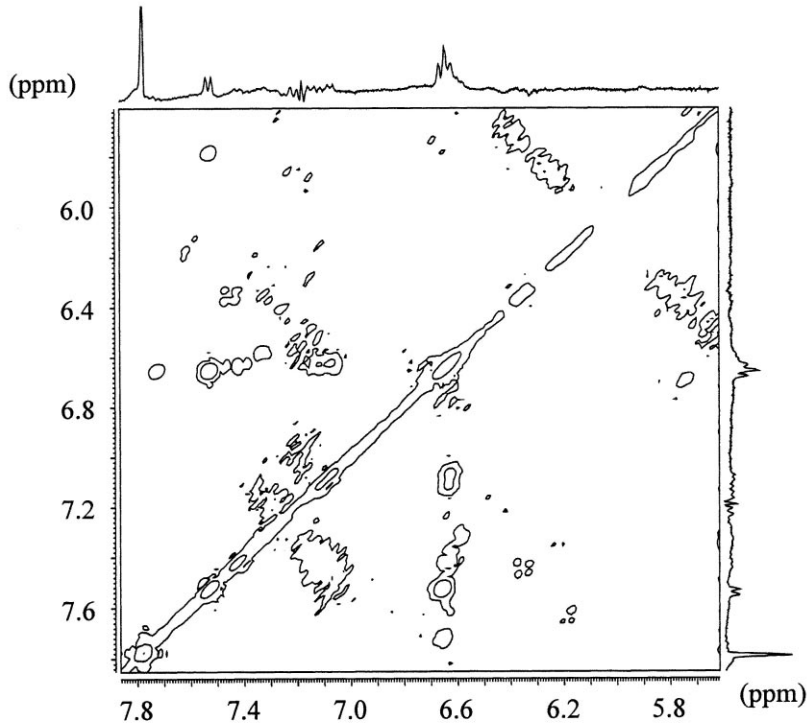

Fig. 2. *1*H–^{1}H COSY NMR spectrum of sporopollenin.

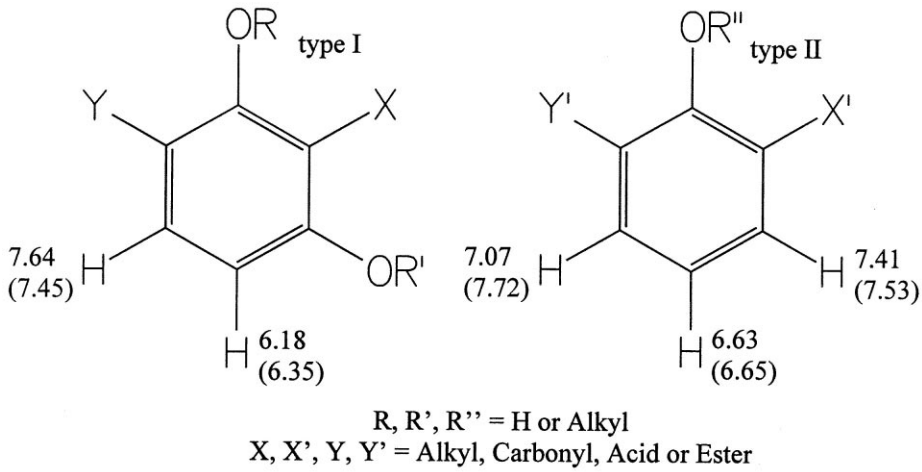

Fig. 3. ^{1}H chemical shifts of the two types of phenolic compounds (ratio 1:20, type I:II).

the solvent, no interpretation is possible. The signal at δ 3.43 is due to residual methanol from the sporopollenin purification process. The proton-proton coupling network was determined by two dimensional ^{1}H–^{1}H-COSY NMR spectroscopy at 400 MHz. The resulting contour plot is shown in Fig. 2. The aromatic region of the COSY spectrum of sporopollenin reveals a set of crosspeaks that are indicative of two distinct aromatic structures (Fig. 3). The crosspeak δ 6.18–δ 7.64 shows splitting due to J-coupling that is of the size of an ^{3}J-(*ortho*)-coupling and is therefore to be assigned to two hydrogens bound to neighbouring aromatic carbons. The shifts of δ 6.18 and δ 7.64 are not involved in any further crosspeaks, i.e. there are no further hydrogens in the aromatic region, as these would give rise to crosspeaks based on a ^{3}J-(*ortho*)- or a ^{4}J-(*meta*)-coupling. Hence, this crosspeak is indicative of either a 1,4-disubstituted or a 1,2,3,4-tetrasubstituted benzene system. The shift value of δ 6.18 indicates the presence of either two OH(OR)-substituents (R = alkyl) in the two *ortho*-positions or OH(OR)-substituents in both the *ortho* and *para*-positions of the proton under investigation. Both aforementioned substitution patterns are clearly not in accordance with a proton framed by two *ortho* substituents. On the other hand, *ortho*/*para* disubstitution with respect to one of the aromatic protons is only compatible with a 1,2,3,4-tetrasubstituted benzene ring and obviously not with a 1,4-disubstituted one. Increment calculations [12] (see Fig. 4) of the aromatic proton chemical shift favour alkyl or carbonyl/carboxyl as the remaining two substituents. The resulting structure is therefore best characterized as "type I" given in Fig. 3. The same arguments as outlined before hold for the crosspeak δ 6.35–δ 7.45, also indicating a "type I" structure (Fig. 3).

The crosspeaks δ 7.07–δ 6.63 and δ 6.63–δ 7.41 show splittings due to J-couplings that are of the size of ^{3}J-(*ortho*)-couplings and are therefore to be assigned to two pairs of hydrogens each bound to neighbouring aromatic carbons. Furthermore, as both crosspeaks

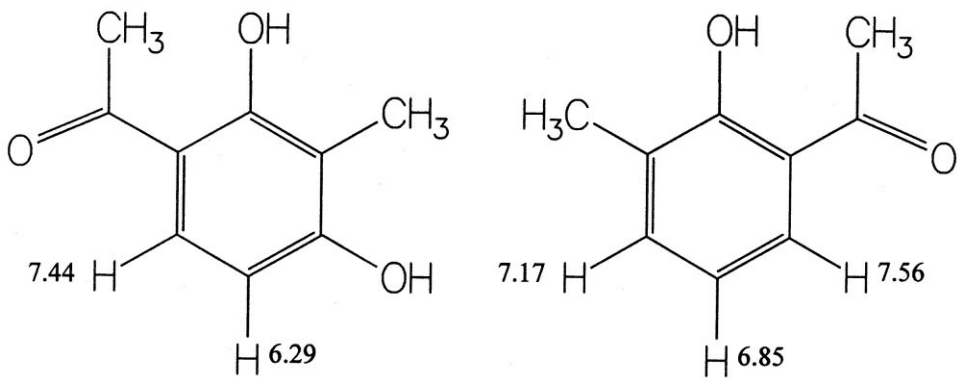

Fig. 4. ^{1}H chemical shifts, obtained from increment calculations, of two model compounds.

have a common coupling partner (δ 6.63), the crosspeak pair resembles three protons bound to neighbouring aromatic carbons with the δ 6.63 proton being the central proton. All the above mentioned shifts are not involved in any further crosspeaks, i.e. there are no further hydrogens in the aromatic region, as these would give rise to crosspeaks based on a ^{3}J-(*ortho*)- or a ^{4}J-(*meta*)-coupling. Hence, this crosspeak pair is indicative of a 1,2,3-trisubstituted benzene system. A shift value of δ 6.63 indicates the presence of a OH(OR)-substituent (R = alkyl) in the *para*-position of the δ 6.63 proton. Increment calculations (see Fig. 4) of the aromatic proton chemical shift favour alkyl or carbonyl/carboxyl as the remaining two substituents. The resulting structure is therefore best characterized as "type II" given in Fig. 3. The same arguments as outlined before also hold for the pair of crosspeaks δ 7.53–δ 6.65 and δ 6.65–δ 7.72, indicating a "type II" structure (Fig. 3).

Analysis of sporopollenin by ^{1}H NMR spectroscopy is the starting-point of further investigations using different analytical techniques which the solubilisation of this substance now allows.

3. Experimental

3.1. NMR.

^{1}H NMR spectra were recorded with a JEOL GX-400 NMR spectrometer at room temp. in a mixt. of 2-aminoethanol-d_4 and D_2O (see below) with external ref. to a sample of sodium-3-trimethylsilyl-2,2,3,3-tetradeuteriopropionate in D_2O. A control sample of 2-aminoethanol-d_4 and D_2O without sporopollenin was measured under the same experimental conditions (including 2D expts) as the sporopollenin sample in order to identify signals from solvents and their impurities. Pulse repetition times were 1.7 s for ^{1}H NMR-expts. 2D–^{1}H–^{1}H-COSY was measured in absolute value mode using standard pulse programs.

3.2. Isolation and purification of sporopollenin.

Pollen from *Typha angustifolia* L. 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 H_2O for 24 hr and washing by filtration through nylon meshes (20 μm). Intine was degraded by enzymatic hydrolysis using Mazerozym R10 and Cellulase Onozuka R10 (Yakult, Nishinomiya, Japan), each 1% dissolved in 0.1 M Na acetate buffer, pH 4.5, at 30°C, for 3 days. The resulting material was extracted using solvents of increasing polarity ($CHCl_3-MeOH$, 1:1, Et_2O, Me_2CO, MeOH, ethylene glycol monoethyl ether and H_2O), until purification was obtained (monitored by TLC analysis). After extraction, the material was lyophilised for 48 hr and stored in a desiccator until examinaed. For ^{1}H NMR, sporopollenin was dissolved in a mixt. of hot $NH_2CD_2CD_2OH$(98%)-D_2O (2:5); the conc. of sporopollenin was 5 mg ml^{-1}.

Acknowledgements

The authors thank the "Deutsche Forschungsgemeinschaft" and the "Fonds der Chemischen Industrie" for financial support.

References

Guilford, W. J., Schneider, D. M., Labowitz, J., & Opella, S. J. (1988). *Plant Physiology, 86,* 134.

Espelie, K. E., Loewus, F. A., Pugmire, R. J., Woolfenden, W. R., Baldi, B. G., & Given, P. H. (1989). *Phytochemistry, 28,* 751.

Hemsley, A. R., Barrie, P. J., Chaloner, W. G., & Scott, A. C. (1993). *Grana, Suppl.1,* 2.

Wilmesmeier, S., Steuernagel, S., & Wiermann, R. (1993). *Zeitschrift für Naturforschung C: Biosience, 48,* 697.

Schulze Osthoff, K., & Wiermann, R. (1987). *Journal of Plant Physiology, 131,* 5.

Herminghaus, S., Arendt, S., Gubatz, S., Rittscher, M. and Wiermann, R., in *Sexual Reproduction in Higher Plants*, ed. M. Cresti, P. Gori and E. Pacini. Springer, New York, 1988, p. 169–174.

Wehling, K., Niester, Ch., Boon, J. J., Willemse, M. T. M., & Wiermann, R. (1989). *Planta, 179,* 376.

Van Bergen, P. F., Collinson, M. E., & De Leeuw, J. W. (1993). *Grana, Suppl.1,* 18.

Kawase, M., & Takahashi, M. (1995). *Grana, 34,* 242.

Niester-Nyveld, C., Haubrich, A., Kampendonk, H., Gubatz, S., Tenberge, K. B., Rittscher, M., Wilmesmeier, S., & Wiermann, R. (1997). *Protoplasma, 197,* 148.

Jungfermann, C., Ahlers, F., Grote, M., Gubatz, S., Steuernagel, S., Thom, I., Wetzels, G., & Wiermann, R. (1997). *Journal of Plant Physiology, 151,* 513.

Hesse, M., Meier, H., Zeeh, B., *Spektroskopische Methoden in der organischen Chemie*, Thieme, Stuttgart, 1979, p. 188.