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MINOR HYDROXYCINNAMIC ACID SPERMIDINES FROM POLLEN OF *QUERCUS DENTATA*

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Key Word Index—*Quercus dentata*; Fagaceae; hydroxycinnamic acid amides; caffeic acid; *p*-coumaric acid; ferulic acid; structural elucidation; electrospray MS/MS.

Abstract—Three minor hydroxycinnamic acid spermidines were isolated from pollen of *Quercus dentata*, and identified as N^1-feruloyl-N^5,N^{10}-dicaffeoylspermidine, N^1-*p*-coumaroyl-N^5-caffeoyl-N^{10}-feruloylspermidine, and as N^1,N^5-di-*p*-coumaroyl-N^{10}-caffeoylspermidine. Copyright © 1996 Published by Elsevier Science Ltd

INTRODUCTION

We recently reported the structures of three novel and one known triacylated spermidine conjugates, that occur as major constituents in pollen of probably all oak species [1, 2]. The pollen of other species in the Higher Hamamelidae (Betulaceae, Juglandaceae) are characterized by the accumulation of diacylated spermidine conjugates such as N^1,N^5-di-*p*-coumaroylspermidine in *Pterocarya fraxinifolia*, N^5,N^{10}-diferuloylspermidine in *Betula verrucosa*, and N^1-caffeoyl-N^{10}-feruloylspermidine in *Corylus avellana* [3, 4]. Trisubstituted spermidines have been reported from the Rosaceae [5], and may indicate a much closer relationship of the Higher Hamamelidae to the Rosaceae *sensu strictu* than previously assumed. This is supported by chloroplast DNA restriction-site analysis and morphological characters [6–8]. We report here the structures of three minor triacylated hydroxycinnamoyl spermidines that were identified from pollen of *Quercus dentata* Thunb.

RESULTS

All compounds were obtained from the same extract that was used for the structural elucidation of the four major tri-cinnamoylspermidines reported by Bokern *et al.* [1]. Minor constituents were purified by preparative HPLC from a methanolic extract, and the structures were elucidated by electrospray mass spectrometry–mass spectrometry (MS/MS), supported by strong alkaline hydrolysis. Because the fragmentation pathway of triacylated spermidine conjugates has already been described in detail by Bokern *et al.* [1], only the diagnostic fragments are discussed here. The three compounds were present only in mixtures and in very small quantities, and so no NMR experiments were performed.

A minor compound eluting between the major compounds, N^1-*p*-coumaroyl-N^5,N^{10}-dicaffeoylspermidine and the N^1,N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine [1] during semipreparative HPLC separation was subjected to electrospray mass spectrometry. In the negative ion mode, this fraction yielded an intense deprotonated $[M]^-$ at m/z 614, indicative of a *p*-coumaroyldicaffeoylspermidine, accompanied by weaker signals at m/z 644, 628, 642 and 658. The signals at m/z 642 and 628 suggest the presence of monomethylated or dimethylated derivatives of *p*-coumaroyl-dicaffeoylspermidine containing one or two ferulic acid residues instead of caffeic acid, and/or a sinapic acid residue. The signals at m/z 644 and 658 are generated by a mono- or di-methylated derivative of tricaffeoylspermidine (M_r 631, [1]). For structural elucidation, however, positive-mode ESI-MS/MS had to be used, because only the protonated molecular ions yielded relevant structural information. Unfortunately, the relative intensities of the protonated $[M]^+$ signals were much lower in the positive ion mode, thus limiting MS/MS experiments to the most abundant derivatives. These ions yield relevant structural information when subjected to collision-induced dissociation (CID) [1]. Fragments indicative for the substitution pattern of the molecular ion at m/z 616 showed the presence of N^1-*p*-coumaroyl-N^5,N^{10}-dicaffeoylspermidine previously described by Bokern *et al.* [1]. MS/MS of the $[M]^+$ at m/z 646 yielded fragment ions at

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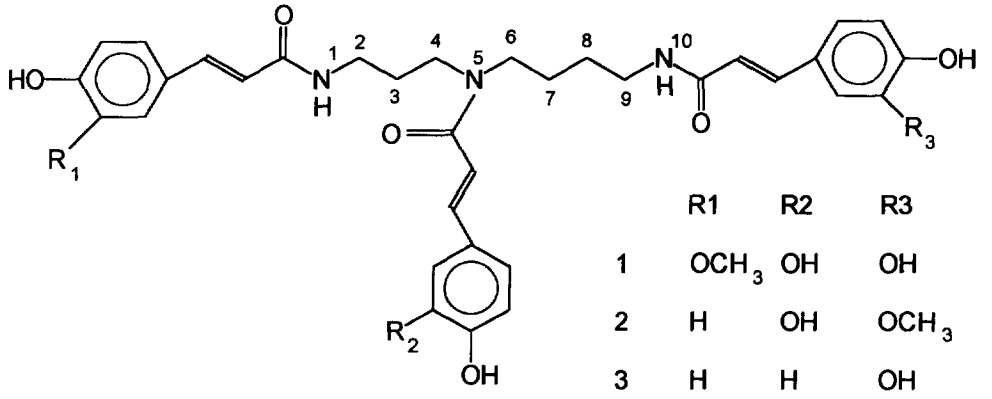
m/z 163 and 177 confirming the presence of both caffeoyl- and feruloyl-residues. The intense signal at *m/z* = 234 comprising the cinnamoyl-residue linked to N^1 of the C_3 chain of the spermidine residue indicates substitution with ferulic acid at this position. The preferential loss of the cinnamoyl residue linked to N^5 (caffeic acid) is shown by an intense fragment at *m/z* 484, accompanied by signals of equal intensity at *m/z* 322 and 308 generated by the loss of the second caffeoyl residue (*m/z* 322) or the feruloyl residue (*m/z* 308). This suggests the structure of N^1-feruloyl-N^5,N^{10}-dicaffeoylspermidine (**1**) as the dominant isomer. No other protonated molecular ions were detected; the structure of other minor constituents could, therefore, not be elucidated. The presence of ferulic acid in this fraction was confirmed by strong alkaline hydrolysis yielding three cinnamic acids, which were separated after trimethylsilylation by GC-MS. The retention times and fragmentation pattern was identical to an authentic sample of ferulic acid.

A minor compound eluting between the N^1,N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine and the N^1,N^5,N^{10}-tri-*p*-coumaroylspermidine was also investigated by electrospray mass spectrometry. This fraction yielded deprotonated (-ESI) $[M]^-$ signals at *m/z* 598, 628 and weaker ones at *m/z* 612 and 642. The strong signals suggest the presence of a di-*p*-coumaroyl-caffeoylspermidine (M_r 599 [1]) and a monomethylated derivative, possibly coumaroyl-caffeoyl-feruloylspermidine (*m/z* 628). The weaker signals indicate the presence of small amounts of a dimethylated derivative, possibly *p*-coumaroyl-diferuloylspermidine (*m/z* 642). Positive ion MS/MS was successful only for the major compound present, and yielded a signal at *m/z* 630 (*p*-coumaroyl-caffeoyl-feruloylspermidine) affording fragments at *m/z* 147, 163, and 177. These fragments confirm the presence of three different cinnamoyl moieties. The diagnostic fragments at *m/z* 204 showed the substitution of N^1 by *p*-coumaric acid, whereas as the signal at *m/z* 248 can be explained by a fragment comprising the C_4 chain, including a ferulic acid residue linked to N^{10}. The position N^5 must, therefore, be substituted by caffeic acid, as confirmed by the preferential loss of this residue. This is indicated by a fragment at *m/z* 468 and signals of equal intensities at *m/z* 322 and 292 generated by the additional loss of a coumaroyl or feruloyl residue, respectively. Thus, the dominant compound has the structure N^1-*p*-coumaroyl-N^5-caffeoyl-N^{10}-feruloylspermidine (**2**). The weaker signals of the deprotonated molecular ions indicate the presence of other monomethylated derivatives: *m/z* 612 (598 + Me) and *m/z* 642 (628 + Me). Structural elucidation of these minor compounds was not possible, because no signals were obtained in the positive ion mode. The presence of *p*-coumaric, caffeic and ferulic acid moieties in this fraction was again confirmed by strong alkaline hydrolysis and GC–mass spectrometry as outlined above.

A third compound was detected by HPLC as a minor constituent in a fraction eluting before the known N^1,N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine [1]. Although the fraction contained predominantly the known substance, and negative ion ESI-MS yielded only one deprotonated molecular ion signal at *m/z* 598 [1], MS/MS allowed tentative identification of an isomer present in this fraction. The daughter ion spectrum obtained after CID of the protonated $[M]^+$ at *m/z* 600 showed some differences from the spectrum of the known N^1,N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine. A major fragment was detected at *m/z* 454 corresponding to the loss of a coumaroyl moiety from the parent ion. Based on our recent observation that the cinnamoyl moiety located at the N^5 position is preferentially lost during CID, the high intensity of this fragment ion suggests a N^5-linked coumaroyl residue. The signal at *m/z* 234 comprising a caffeoyl substituent linked to the C_4-chain of the spermidine moiety, which was barely detectable in the spectrum of N^1,N^{10}-di-*p*-coumaroyl-N^5-caffeoylspermidine [1] was clearly visible, and the corresponding signal at *m/z* 204 comprising the C_3-chain linked to a coumaroyl residue was much more intense than the signal at *m/z* 220, indicating a caffeoyl residue at N^1. Therefore, N^1,N^5-di-*p*-coumaroyl-N^{10}-caffeoylspermidine is the dominant additional isomer present (**3**).

In conclusion, several yet unknown minor triacylated spermidine derivatives occur in pollen of *Q. dentata*. Two of these minor compounds were elucidated and shown to contain ferulic acid, and the presence of additional isomers can be assumed. The compound



	R1	R2	R3
1	OCH_3	OH	OH
2	H	OH	OCH_3
3	H	H	OH

shown to be N^1-*p*-coumaroyl-N^5-caffeoyl-N^{10}-feruloyl spermidine is the first cinnamoyl amide known to contain three different cinnamoyl moieties. Such diversity of different cinnamoyl substituents is known only from anthocyanidins, such as lobelin B, a 3-coumaroylrutinosyl-5-malonylglucosyl-3′-caffeoyl-glucosyl-5′-feruloylglucosyldelphinidin [9].

EXPERIMENTAL

Isolation. Pollen from *Q. dentata* were collected from a tree growing on the grounds of the New York Botanical Garden in spring 1992 [1]. Pollen (5 g) was extracted ×5 with 40 ml H_2O, and ×5 with 40 ml 80% aq. MeOH. The MeOH extractions were combined and subjected to prep. HPLC (in 2 ml portions) on a C-18 Nucleosil column (2.1 × 25 cm, 10 μm, Phenomenex) using a linear gradient from 80% solvent A in B to 100% solvent B in 80 min, and continued isocratically at 100% B for 30 min at 10 ml/min^{-1}. Solvent A was 4% HCOOH in H_2O, and solvent B was 4% HCOOH, 46% H_2O, and 50% MeOH. Elution was monitored by photodiode array detection at 220–400 nm. Frs corresponding to each peak apex were collected. The frs were evapd to dryness under the repeated addition of H_2O to remove the HCOOH.

Alkaline hydrolysis. Alkaline hydrolyses were carried out in a final concn of 4 N NaOH under the addition of $NaBH_4$ in a helium saturated, sealed tube for 2 to 4 hr at 100°. After acidification, the hydrolysis assays were partitioned against EtOAc. The EtOAc phases were analysed for hydroxycinnamic acids by GC/MS.

Mass spectrometry. A Finnigan MAT TSQ 700 triple quadrupol mass spectrometer equipped with Finnigan electrospray ion source was used for electrospray mass spectrometry. The spermidine derivatives were dissolved in MeOH containing 2% of formic acid and injected at 2 μl/min^{-1} into the electrospray chamber. A voltage of 4.5 kV was applied to the electrospray needle. For CID experiments, parent ions were selectively transmitted by the first mass analyser and directed into the collision cell (collision gas Ar) with a kinetic energy set at −35 eV.

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