Study of pollen phenolamides by tandem mass spectrometry

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Introduction

The chemical compounds produced by the specialized metabolism of flowering plants (Angiospermae) represent a large source of potentially bioactive molecules [1]. Thanks to their pharmacological actions, these compounds can be used by humans and animals to fight diseases and parasites [2, 3]. However, only few data are available with regard to their effects on pollinators whose development is intimately associated with flowering plants.

Generally, pharmacognosy studies are focused on plant parts such as bark, roots, stems and leaves. However, recent researches indicate that flowers, and particularly pollen, may contain bioactive compounds [4, 5]. Among specialized metabolites, phenolamides (PA) are frequently found in pollen grains (Fig. 1). They consist of a polyamine backbone substituted by one or several hydroxycinnamic acids and are known to play crucial roles in plant development and protection.

Even so, their physiological impacts on insects are still unclear [6]. Yet, insects such as bees feed on floral resources throughout their life and are consequently exposed to phenolamides. Thus, it is capital to determine whether compounds of this class have a detrimental or a beneficial action towards bees. Additionally, knowing that bees can be infected by various parasites, from bacteria to protozoans or even mites, it is important to find out which easily accessible compounds could heal, or at least alleviate infection. In this prospect, phenolamides are worth taking into consideration.

Nevertheless, before conducting biological tests, phenolamides must be fully characterized, which is an area still relatively uncovered in literature [7]. In the present study, three melliferous plants were selected, namely willow (*Salix* sp. L.), common hawthorn (*Crataegus monogyna* Jacq.) and common sunflower (*Helianthus annuus* L.) (Fig. 2), and their pollen was studied, by comparison with synthesized standards. Phenolamides were analyzed by liquid chromatography (LC) coupled with mass spectrometry (MS). To characterize the different congeners, LC-MS and LC-MSMS experiments were performed to obtain collision-induced dissociation (CID) spectra.

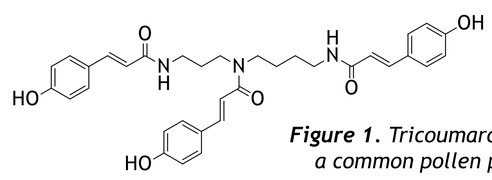

Figure 1. *Tricoumaroyl spermidine, a common pollen phenolamide*

Figure 2. *Goat willow, common hawthorn and common sunflower flowers*

Experimental section

Extraction protocol

Salix sp. pollen was purchased from Ruchers de Lorraine, *C. monogyna* pollen from Aristée and *H. annuus* pollen from INRAE (France). Each pollen load was hand-sorted and stored at -20°C. Samples were sonicated for 30 minutes in MeOH/water (70:30). After centrifugation, the supernatants were filtrated and diluted appropriately to be analyzed by HPLC-MS and HPLC-MSMS.

Synthesis protocol

In this study, four phenolamides were synthesized, namely triferuloyl spermidine, tricaffeoyl spermidine, tricoumaroyl spermidine and dicaffeoyl coumaroyl spermidine. They were produced separately.

To do so, 10 mL of a solution of dicyclohexylcarbodiimide (DCC) (250 mM) in dichloromethane were added to 40 mL of a solution of the chosen hydroxycinnamic acid (62.5 mM) and spermidine (18.5 mM). The mixture was stirred at room temperature for 24 h.

HPLC conditions

The HPLC system is a Waters Alliance 2695. The compounds were eluted on a C18 column, with a gradient of methanol and water.

Mass spectrometry analyses

The pollen extracts as well as the synthesized standards were analyzed in ESI (+) and ESI (-) with the Waters Q-ToF US mass spectrometer.

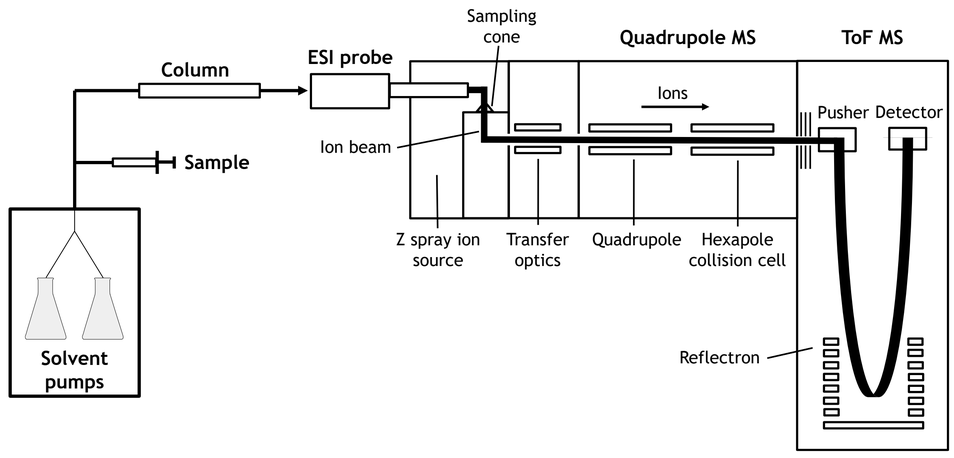

Figure 3. *HPLC connected to the Waters Q-ToF US mass spectrometer*

Fragmentation of phenolamides in positive mode

This fragmentation study was focused on trisubstituted spermidines, as they were the most abundant phenolamides in the selected pollens. Spermidine-derived phenolamides in positive mode were already studied in literature. Briefly, the main fragmentation pathway is the amide bond cleavage, yielding ions of a characteristic *m/z* ratio depending on the leaving hydroxycinnamic substituent [7]. Moreover, a loss of methanol (32 amu) is observed in the case of a methoxylated hydroxycinnamic acid (Table 1).

Unfortunately, a complete characterization is not possible using only the positive mode. Indeed, the protonated ions are prone to undergo transamidation reactions, which cause the substituents to switch from one nitrogen to another (Fig. 4).

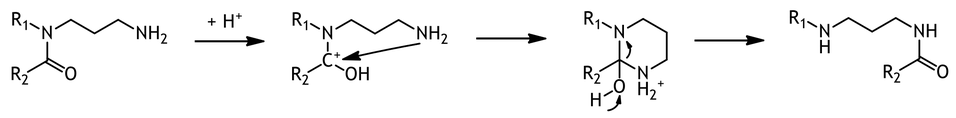

Figure 4. *Transamidation reaction mechanism*

Fragmentation of phenolamides in negative mode

The CID spectrum analysis of the synthesized dicaffeoyl coumaroyl spermidine isomers allowed us to propose two fragmentation pathways for spermidine-derived phenolamides (Fig. 5 and 6).

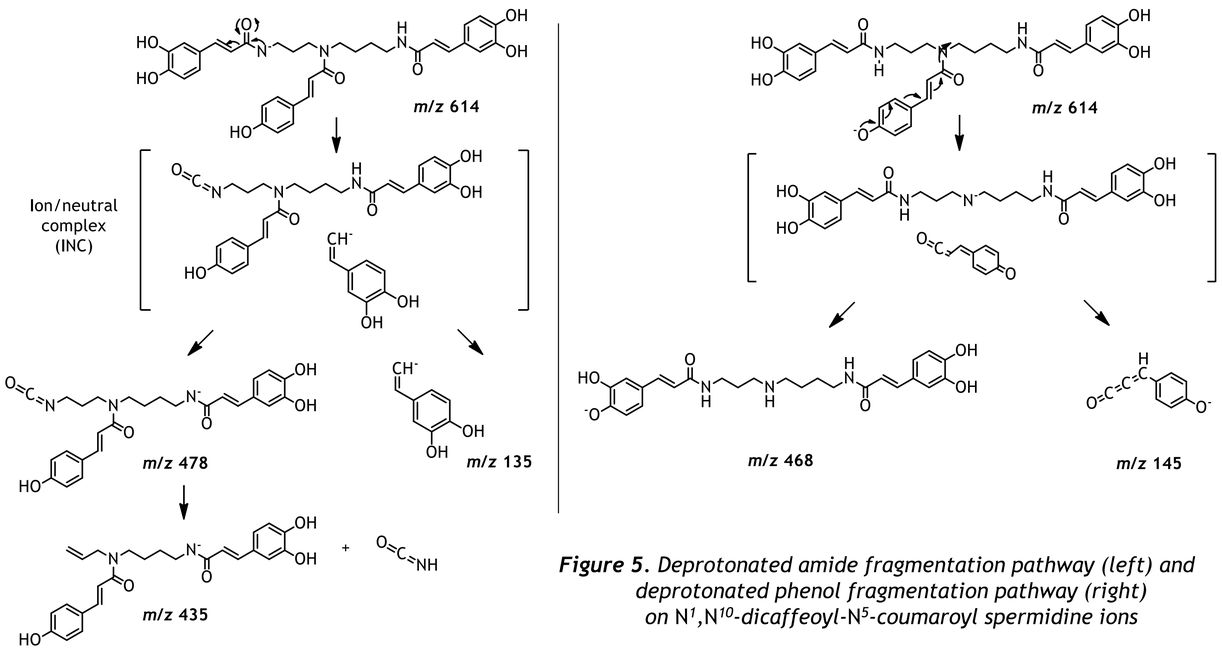

Figure 5. *Deprotonated amide fragmentation pathway (left) and deprotonated phenol fragmentation pathway (right) on N¹,N¹⁰-dicaffeoyl-N⁵-coumaroyl spermidine ions*

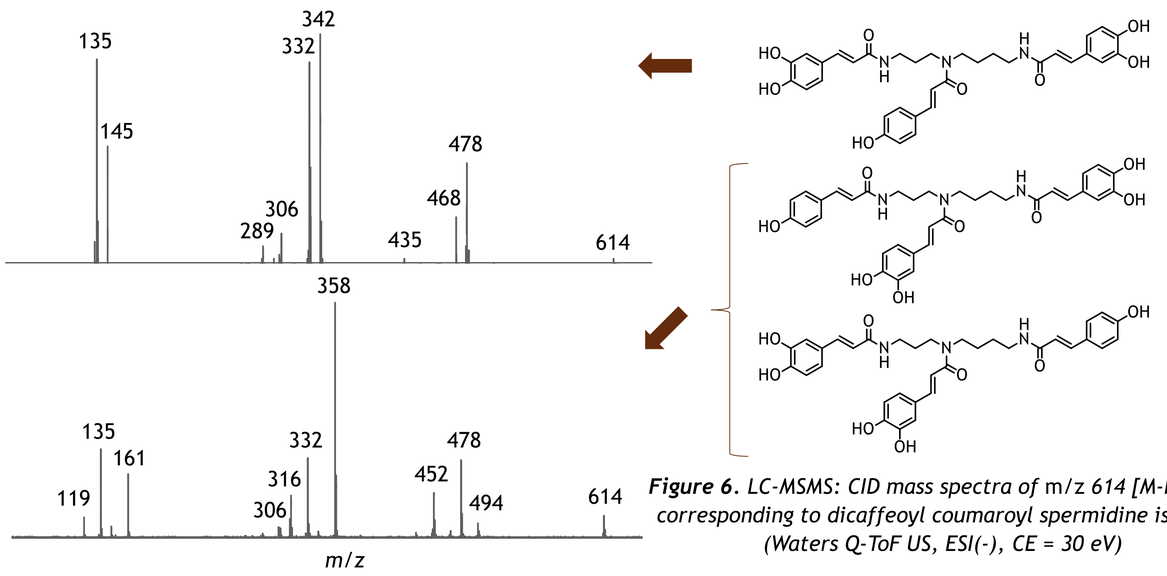

Figure 6. *LC-MSMS: CID mass spectra of* m/z *614 [M-H]⁻ ions corresponding to dicaffeoyl coumaroyl spermidine isomers (Waters Q-ToF US, ESI(-), CE = 30 eV)*

Discussion about negative mode

Fragmentations in negative mode will essentially lead to the loss of hydroxycinnamic groups. In the case of a deprotonated amide, it is the bond between the amide and the alpha carbon that will be broken. Two kinds of ions will be produced, thanks to an ion/neutral complex. After the loss of the hydroxycinnamic moiety, a loss of 43 amu is still possible, in the form of an isocyanic acid loss. This mechanism only takes place at the N^1 and N^{10} positions, as the N^5 nitrogen is involved in a tertiary amide.

In the case of a deprotonated phenol, the amide bond will be broken between the carbon and the nitrogen. This mechanism can take place at each position, but is largely favored at the N^5 position. After a first loss by either pathways, ions will undergo a second loss. Interestingly, deprotonated ions will not undergo transamidation reactions, simplifying their characterization.

Thus, as the neutral losses are different between the two pathways, it is easy to determine the nature of the substituent linked on the N^5 position. Nevertheless, CID spectra showed that methoxylated hydroxycinnamic substituents can yield additional ions by switching methyl or methoxy groups between substituents (Fig. 7), mimicking other losses. The Table 1 summarizes information available to phenolamide characterization.

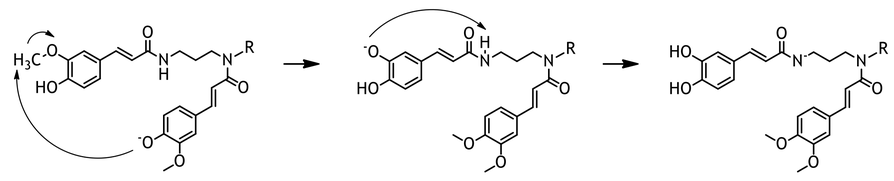

Figure 7. *Example of methyl switch on a methoxylated phenolamide*

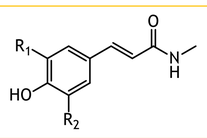	Amide cleavage (ESI +)	Additional fragmentation (ESI +)	Deprotonated phenol (ESI -)	Deprotonated amide (ESI -)	Additional fragmentation (ESI -)
***p*-Coumaroyl** (R_1 = R_2 = H)	-146 *m/z* 147	/	-146 *m/z* 145	-120 *m/z* 119	/
Caffeoyl (R_1 = OH; R_2 = H)	-162 *m/z* 163	/	-162 *m/z* 161	-136 *m/z* 135	/
Feruloyl (R_1 = OCH_3; R_2 = H)	-176 *m/z* 177	m/z 145	-176 *m/z* 175	-150 *m/z* 149	-136 / *m/z* 135
Hydroxyferuloyl (R_1 = OCH_3; R_2 = OH)	-192 *m/z* 193	m/z 161	-192 *m/z* 191	-166 *m/z* 165	-136 / *m/z* 135 -152 / *m/z* 151
Sinapoyl (R_1 = OCH_3; R_2 = OCH_3)	-206 m/z 207	m/z 175	-206 m/z 205	-180 m/z 179	-136 / *m/z* 135 -152 / *m/z* 151 -166 / *m/z* 165

Table 1. *Summary of the characteristic fragment ions depending on the leaving group, in ESI + and ESI -*

Perspectives

By investigating the behavior of deprotonated phenolamide ions, we could highlight a way to differentiate N^1 and N^{10} substituents from N^5 substituents. Unfortunately, even the association of data gathered from positive and negative mode does not allow a complete characterization of spermidine-derived phenolamides.

Therefore, there is a need to use other analytical techniques to refine the results, such as ion mobility coupled to mass spectrometry. Our findings will also be used to study other pollen-sourced phenolamides derived from aliphatic amines, such as putrescine and spermine derivatives.

References

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