

# Ferulic Acid-Sucrose Esters from Immature Anthers of *Tulipa* cv. "Apeldoorn"

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Two ferulic acid conjugates have been isolated from the extract of immature anthers of *Tulipa* cv. "Apeldoorn" and their structures elucidated using chromatography, electron impact mass spectrometry, and NMR spectroscopy. These two conjugates are 6,3'-diferuloylsucrose and 6,3',4'-triferuloylsucrose. High performance liquid chromatographic analysis of the extract showed that these esters are transient major constituents of the phenylpropanoid pattern of the immature anthers and are present in the ratio of 1:2. Previous investigations showed that these components are accumulated in the loculus.

## Introduction

During development of pollen of *Tulipa* cv. "Apeldoorn" there are in the loculus of the anthers sequential synthesis of different classes of phenylpropanoids. At the end of meiosis II phenylpropanoid metabolism is initiated by the formation of hydroxycinnamic acid conjugates and this is followed by accumulation of chalcones, various flavonol glycosides and finally one anthocyanin [1, 2]. The structural identity of the chalcones, anthocyanin [1, 2] and flavonol glycosides [3] has been made, however, the hydroxycinnamic acid conjugates remained to be identified.

In the present study two major conjugates have been isolated and their structures shown to be ferulic acid esters of sucrose.

## Extraction

Freeze-dried anthers were submerged into 80% aq. methanol and treated as described in ref. [3].

## Chromatography

The crude extract was prefractionated on polyamide columns as described in ref. [4] using water, 60% aq. methanol, and methanol as eluents.

Preparative thin layer chromatography on microcrystalline cellulose (Avicel), approx. 0.5 mm on 20 × 20 cm plates, in  $SS_1$  (chloroform-gl. acetic acid, 3:2, water saturated) was used for isolation of the individual ferulic acid esters. Prior to identification procedures, the isolated compounds were rechromatographed on polyamide and chromatographed on Sephadex LH-20 (Pharmacia, Uppsala, Sweden) columns (90 × 2 cm), eluted with methanol.

Analytical chromatography of ferulic acid was done on Avicel plates in  $SS_2$  (toluene-gl. acetic acid, 2:1, water saturated) and sucrose according to ref. [5] on pre-coated silica gel G-60 plates (Merck, Darmstadt).

Ferulic acid and its esters were detected under UV light (366 nm) and sucrose was visualized by spraying with 70% aq. sulfuric acid and subsequent heating at 100 °C. As expected, the isolated ester moieties did not respond to spray reagents (e.g. anilinephthalate) commonly used for detection of reducing sugars.

## Materials and Methods

### Plant material

Anthers of *Tulipa* cv. "Apeldoorn", containing pollen at the early and middle postmeiotic developmental stages, were collected from plants cultivated in the botanical garden of Münster and lyophilized immediately after harvesting.

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HPLC was carried out using a Spectra-Physics system (Santa Clara, Calif., USA) and the applied column (250 × 4 mm) was prepacked with LiChrosorb RP-8 (5 µm) (Merck, Darmstadt). Separation was accomplished by a linear gradient elution with 20 to 70% solvent B (methanol) in solvent A (0.5% gl. acetic acid in water) + B within 25 min (1 ml/min) or alternatively as described in ref. [3]. Elution was monitored at 330 nm. For further details on HPLC see ref. [6].

### Hydrolysis

The compounds were treated with 1 N HCl for 30 min or 1 N NaOH for 5 min at 100 °C.

### Derivatization

For acetylation, samples were dissolved in (CH<sub>3</sub>CO)<sub>2</sub>O/pyridine (1:2) and kept at room temperature for 48 h.

### UV spectroscopy

Estimation of the molecular weight of the ferulic acid esters was performed by quantitative UV spectroscopic analyses using ferulic acid methylester as reference.

### Mass spectrometry

Mass spectrometry was carried out with a MAT 731 and CH7A (Varian MAT, Bremen), equipped with a total ion current controlled emitter heating device; spectra were recorded with a strip chart recorder.

### NMR spectroscopy

For <sup>1</sup>H NMR spectroscopy a Varian EM 90 CW and a Bruker WP 60 FT spectrometer were used (CD<sub>3</sub>OD as solvent and TMS as int. standard).

## Results and Discussion

From immature anthers of *Tulipa* cv. "Apeldoorn" two ferulic acid conjugates were isolated and identified as 6,3'-diferuloylsucrose (**I**) and 6,3',4'-triferuloylsucrose (**II**). Fig. 1 depicts their structures. Previous studies had shown that these esters are predominantly localized in the loculus of the anthers [1]. HPLC of the crude extract demon-

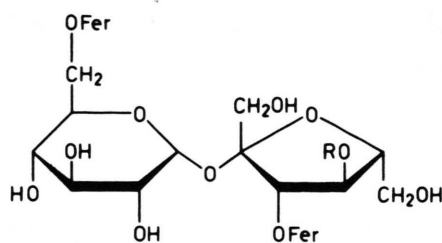


Fig. 1. Structures of compound **I** (R, H) and compound **II** (R, Fer = feruloyl).

strated that **I** and **II** are major phenolic constituents in this system and are present in the ratio of 1:2.

HPLC analysis of extracts from mature pollen showed that the two esters are still present in the same ratio, however, in much lower quantities. Since mature pollen accumulate flavonoids, we used in this case the system described in ref. [3], in order to avoid overlapping elution of the flavonoids and the ferulic acid esters. In a joining communication we will report on a detailed HPLC study of the sequential changes of the phenylpropanoid pattern in developing *Tulipa* pollen.

Chromatography on polyamide columns produced two fractions each containing one of the two ferulic acid esters. 60% aq. methanol eluted compound **I** and methanol alone compound **II**. The esters were isolated from these fractions after TLC in SS<sub>1</sub>. Purification was achieved by rechromatography on polyamide and chromatography (twice) on Sephadex LH-20. Elution was monitored with an UV detector (254 nm) and eluates were collected at their peak maxima.

Ferulic acid and sucrose, degradation products of alkaline hydrolyses of **I** and **II**, were found by co-chromatography with standard markers – hydroxycinnamic acids in SS<sub>2</sub> and sugars according to ref. [5].

The identity of these hydrolytic products has been substantiated by electron impact mass spectrometry and <sup>1</sup>H NMR spectroscopy. The data, discussed in detail in the following sections, also revealed the number of ferulic moieties, present in each compound, and the sites of esterification.

6-Feruloyl- $\alpha$ -D-glucopyranosyl-(1→2)- $\beta$ -D-(3'-feruloyl)-fructofuranose (**I**, MW: 694, C<sub>32</sub>H<sub>38</sub>O<sub>17</sub>)

Electron impact mass spectrometry of the un-derivatized compound **I** gave the highest fragment

at  $m/z$  338, which represents feruloylglucose ( $-H_2O$ );  $m/z$  194 corresponds to ferulic acid,  $m/z$  177 to feruloyl, and  $m/z$  150/135 to ring fragments of the acid.

The fragmentation pattern of **I** acetate exhibited the same pathway as described for 6,3'-disinapoylsucrose acetate in ref. [7], but the masses are shifted by the increment of one methoxy group, *i.e.* 30 u. The molecular ion was found as expected for the octaacetate at  $m/z$  1030. This finding is also in agreement with MS data obtained from acetyl-disinapoylsucrose ( $M^+$  1090). At  $m/z$  507 ( $C_{24}H_{27}O_{12}$ ) the ion of one hexose with four acetyl and one feruloyl groups can be seen;  $m/z$  219 ( $C_{12}H_{11}O_4$ ) represents an acetylferuloyl ion.

The  $^1H$  NMR spectrum shows signals of two ferulic acids esterified with sucrose. Their olefinic protons give rise to well separated doublets at 7.68 ppm (1 H, 16 Hz), 7.61 ppm (1 H, 16 Hz), and 6.24 ppm (2 H, 16 Hz). In addition to the unresolved multiplets between 7.2 and 6.8 ppm and 4.8 and 3.3 ppm for the aromatic ring protons of the ferulic acids and sucrose, respectively, the methoxy singlets appear at 3.89 ppm (3 H) and 3.87 ppm (3 H). A complex pattern of a signal (2 H) at 5.5 ppm is due to an overlapping of the anomeric proton of the  $\alpha$ -D-glucose (5.48 ppm, broad d  $\sim$  3 Hz) and H-3' of  $\beta$ -D-fructose (5.43 ppm) which is shifted downfield because of the esterification of the geminal alcoholic function by ferulic acid. In an ABX approximation of the protons H-3', H-4', and H-5' the H-3' forms the X part with  $J_{3'4'} + J_{3'5'} = 7$  Hz.

The molecular weight of **I**, estimated *via* quantitative UV spectroscopy, was found to be  $\sim$  680, which is consistent with the MS analysis (MW: 694).

**6-Feruloyl- $\alpha$ -D-glucopyranosyl-(1 $\rightarrow$ 2)- $\beta$ -D-(3',4'-diferuloyl)-fructofuranose**  
(**II**, MW: 870,  $C_{42}H_{46}O_{20}$ )

Electron impact mass spectrometry of the underivatized compound **II** gave the same fragments as **I**. **II** acetate gave signals at  $m/z$  507 and  $m/z$  219, corresponding to the fragments of **I** acetate.

The molecular ion of the octaacetate was found to be at  $m/z$  1206. The MW difference to that of **I** acetate corresponds to one acetylferuloyl residue, thus indicating three ferulic acids attached to sucrose. This was proven by  $^1H$  NMR spectroscopy.

The signals of three feruloyl residues are to be seen in the aromatic and olefinic region of the  $^1H$  NMR spectrum. The resonances of the olefinic protons (doublets, 16 Hz) are at 7.69, 7.65, 7.62, 6.40 (2  $\times$ ), and 6.33 ppm. The methoxy signals appear at 3.87 (2  $\times$ ) and 3.83 ppm, surrounded by the multiplets of the sugar moiety (4.5–3.3 ppm). The anomeric H-1 proton (5.52 ppm, broad d  $\sim$  3 Hz) of the glucose is superimposed by two strongly coupling hydrogens attached to carbons bearing ester functions. Irradiation at 3.53 ppm (H-5') simplified the system to a broad singlet at 5.57 ppm (2 H), indicating an almost degenerated AB system of H-3' and H-4'. Irradiation at 5.57 ppm resolved the system at 3.53 ppm (H-5') to a broad doublet ( $J_{5'6'} \sim$  7 Hz).

Further evidence of the sites of esterification can be drawn from  $^1H$  NMR of a product isolated after acidic hydrolysis. This product (glucose with one ferulic acid) exhibits in the region between 5 and 6 ppm only the H-1 at 5.13 ppm ( $\alpha$ -epimer, intensity of half a proton).

Quantitative UV spectroscopy gave an approx. MW of 850, which is in good agreement with that obtained by MS (MW: 870).

On the basis of the results, presented in this communication, we propose that immature anthers and especially the loculus with young pollen of *Tulipa* cv. "Apeldoorn" contain 6,3'-diferuloyl- and 6,3',4'-triferuloylsucrose as transient major constituents in the ratio of 1:2. It will be of great interest, to study the mechanisms of biosynthesis and degradation pathways of these complex structures.

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