

Isolation and quantification of tuliposides and tulipalins in tulips (*Tulipa*) by high-performance liquid chromatography

LARS P. CHRISTENSEN¹ AND KELL KRISTIANSEN²

¹Department of Fruit, Vegetable and Food Science, ²Department of Ornamentals, Danish Institute of Agricultural Sciences, Kirstinebjergvej 10, DK-5792 Aarslev, Denmark

The content of tuliposides and tulipalins were determined in *Tulipa* species and cultivars by reversed-phase high-performance liquid chromatography (RP-HPLC), using a water:methanol gradient as mobile phase. The compounds were detected by a diode array detector employed at 208 nm. The investigation revealed, in addition to 1- and 6-tuliposide A, tuliposide D and the lactonized aglycones tulipalin A and (–)-tulipalin B, the new tuliposide F and 6-tuliposide B, the latter being a new acyl derivative of the known 1-tuliposide B. All compounds were isolated by preparative RP-HPLC and identified by NMR and mass spectroscopy. The predominant compounds were 6-tuliposide A and B present in amounts up to 1.5% and 1.3% of fresh weight, respectively. 6-Tuliposide A and tulipalin A seem to be the major allergens in tulips, although tuliposide D and F may also contribute to the allergenic properties. Tulipalin A and (–)-tulipalin B occur in intact tulips and are not only produced in response to fungal attack or after excision of the plants. A few species were found to have very low allergen content and a relatively high level of tuliposide B, indicating it should be possible to breed non-allergenic and disease-resistant tulips.

Key words: *Tulipa*; Liliaceae; tuliposides; tulipalins; allergens; allergen isolation by HPLC; biosynthesis; chemosystematics; plants; prevention; allergic contact dermatitis. © Munksgaard, 1999.

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The genus *Tulipa* (Liliaceae) comprises about 100 species distributed from Morocco through Southern Europe to central Asia (1). The genus *Tulipa* is divided into 5 sections of which *Leiostemones* and *Eriostemones* are the largest ones (2–4). Tulips were introduced to Western Europe in the middle of the 15th century and are now widely cultivated in gardens and as cut flowers. Many cultivars are interspecific hybrids often with unknown ancestral species, however, many cultivars belong to the *T. gesneriana* complex in the section *Leiostemones*.

Tulips and *Alstroemeria* are responsible for one of the best-known occupational dermatoses. The causative agents in both genera have been identified as tuliposide A and tulipalin A (5–11). Recent investigations of *Alstroemeria* revealed the presence of a further tuliposide, tuliposide D (12–14), which has also recently been isolated from *Tulipa turkestanica* together with a new tuliposide, tuliposide F (15). Although their allergenic properties have not been determined, the structural

similarities with tuliposide A suggest such activity. Tuliposide B is widely distributed in *Tulipa* and *Erythronium* (Liliaceae) but seems not to be present in *Alstroemeria* (2, 13, 14, 16, 17). According to Hausen (7) and Slob et al. (17, 18), tuliposide B is non-allergenic, although the optically active aglycone (–)-tulipalin B is able to sensitize guinea pigs and cross-react with the achiral tulipalin A (19). (–)-Tulipalin B is, however, considerably less allergenic than tulipalin A (20).

Tuliposides and tulipalins have shown to be strong antibiotics (21–23) and it has been suggested that breeding for low tuliposide A and high tuliposide B content might give non-allergenic, disease-resistant tulips (2, 17). In *Alstroemeria* the tuliposide content is controlled by additive genes, whereas no relation between parents and offspring were found for the tulipalin A content (24).

The purpose of the present study was to develop an HPLC method for isolation and quantification of tuliposides and tulipalins in tulips, in order to

Table 1. Contents of tuliposides (1-3, 5, 6) and tulipalins (7, 8) determined by analytical HPLC in leaves (L), stems (S) and flowers (F) of *Tulipa* species and cultivars^{a)}

Species/cultivars	1			2			3			5			6			7			8		
	L	S	F	L	S	F	L	S	F	L	S	F	L	S	F	L	S	F	L	S	F
Section Eriostemones																					
Subsection I: Australes																					
<i>Tulipa sylvestris</i> L. ^{d)}	0.41	0.19	0.16	0.60	0.15	0.22	nd ^{b)}	tr ^{c)}	tr	tr	tr	nd	tr	nd	tr	0.01	0.56	0.34	0.26	0.28	0.15
<i>T. sylvestris</i> L. ^{e)}	1.22	0.85	0.49	0.46	0.15	0.29	nd	nd	nd	0.03	0.03	0.03	nd	tr	nd	0.02	0.04	0.07	0.06	0.03	0.07
<i>T. sylvestris</i> ssp. <i>australis</i>	1.56	0.95	0.64	0.49	0.12	0.29	nd	nd	nd	0.03	0.03	0.03	0.05	0.15	0.12	0.02	0.04	0.07	0.06	0.03	0.07
(Link) Pampanini ^{e)}																					
<i>T. sylvestris</i> ssp. <i>sylvestris</i> ^{d)}	0.60	0.91	0.15	0.67	0.25	0.22	tr	0.01	tr	0.07	0.34	nd	tr	tr	nd	0.03	0.04	0.32	0.26	0.04	0.27
<i>T. patens</i> Agardh. ex Schultes ^{d)}	1.30	0.75	0.26	0.25	0.15	0.37	0.07	0.03	tr	1.28	0.65	0.11	0.41	0.15	0.26	0.11	0.11	0.13	0.11	0.09	0.26
<i>T. urumienensis</i> Stapf ^{b)}	0.56	0.19	0.15	0.33	0.19	0.19	0.07	nd	nd	tr	tr	nd	0.18	0.02	tr	0.43	0.24	0.23	0.27	0.25	0.23
Subsection II: Saxatiles																					
<i>T. humilis</i> Herb. ^{d)}	0.39	0.45	0.30	0.75	0.58	0.28	0.01	tr	tr	0.04	0.07	0.01	0.23	0.14	0.05	0.02	0.01	0.05	0.09	0.10	0.12
(= <i>T. aucheriana</i> Baker) ^{d)}																					
<i>T. humilis</i> 'Persian Pearl' ^{d)}	0.37	0.15	0.12	0.89	1.34	0.55	tr	tr	tr	nd	nd	tr	0.10	0.10	0.13	0.01	tr	0.03	0.04	0.11	0.03
Subsection III: Biflores																					
<i>T. biflora</i> Pallas ^{d)}	0.34	0.35	0.42	0.94	0.73	0.47	tr	nd	tr	nd	0.04	tr	0.11	0.22	0.08	tr	0.03	0.03	0.14	0.12	0.13
(= <i>T. polychroma</i> Stapf)																					
<i>T. tarda</i> Stapf ^{b)}	0.46	0.40	0.11	0.50	0.44	0.51	0.06	0.04	nd	0.03	0.05	nd	0.53	0.44	0.07	0.14	0.15	0.20	0.14	0.32	0.23
<i>T. tarda</i> Stapf ^{e)}	0.51	0.43	0.15	0.21	0.20	0.15	0.01	0.01	tr	0.02	0.05	tr	0.25	0.05	0.02	0.05	0.05	0.03	0.03	0.04	0.05
<i>T. turkestanica</i> Regel ^{d)}	0.19	0.13	0.28	0.54	0.33	0.44	0.10	0.08	tr	0.01	0.09	0.10	0.52	0.53	0.30	0.03	0.09	0.05	0.28	0.21	0.21
<i>T. turkestanica</i> Regel ^{e)}	0.52	0.16	0.29	0.18	0.18	0.42	0.09	0.05	0.01	0.06	0.06	0.08	0.33	0.37	0.15	0.05	0.01	0.11	0.12	0.09	0.15
Section Leiostemones																					
Subsection I: Clusianae																					
<i>T. batatinifolia</i> Regel ^{e)}	0.01	0.34	0.42	0.75	0.44	0.49	nd	0.01	nd	nd	tr	0.03	0.29	0.08	0.10	0.01	0.01	0.02	0.06	0.02	0.09
<i>T. clusiana</i> DC. 'Cynthia' ^{d)}	0.20	0.37	0.08	0.94	0.41	0.12	nd	nd	nd	nd	nd	nd	tr	0.03	nd	tr	tr	0.02	0.11	0.05	0.02
<i>T. clusiana</i> var. <i>chrysantha</i>	0.42	0.45	0.12	0.86	0.66	0.51	nd	nd	nd	0.04	0.02	nd	0.41	0.26	0.03	0.06	0.05	0.16	0.12	0.06	0.39
(Hall) Sealy ^{d)}																					
<i>T. linifolia</i> Regel ^{e)}	0.06	0.08	0.30	0.80	0.39	0.38	0.01	0.04	0.01	0.04	tr	0.10	0.34	0.18	0.30	0.01	0.18	0.02	0.06	0.23	0.06
Subsection III: Oculus-solis																					
<i>T. agenensis</i> DC. ^{d)}	0.11	tr	0.13	1.06	0.48	0.86	nd	nd	0.02	nd	nd	0.08	tr	tr	0.36	tr	tr	0.03	0.26	0.23	0.26
<i>T. undulatifolia</i> Boiss. ^{d)}	0.09	0.07	tr	0.68	0.47	0.69	nd	0.02	tr	nd	tr	tr	tr	0.07	0.15	tr	0.02	0.02	0.22	0.20	0.15
Subsection IV: Eichleres																					
<i>T. praestans</i> Hoog ^{d)}	tr	0.05	0.04	0.62	0.32	0.39	nd	tr	nd	nd	nd	nd	nd	nd	nd	tr	0.02	tr	0.09	0.07	0.07
<i>T. kaufmanniana</i> Regel ^{d)}	tr	tr	tr	tr	0.01	0.10	nd	nd	nd	nd	nd	nd	0.04	tr	nd	0.02	tr	tr	tr	tr	0.16
Lily-flowered Group																					
<i>T. 'Red Shine'</i> ^{e)}	0.23	0.34	0.08	0.25	0.16	0.22	nd	nd	nd	nd	nd	nd	0.01	0.02	0.05	tr	0.01	tr	0.01	tr	0.04
<i>T. 'West Point'</i> ^{e)}	0.44	0.42	0.12	0.21	0.08	0.26	nd	nd	nd	nd	nd	nd	0.03	0.04	0.10	tr	0.02	0.10	0.02	0.01	0.09
Single Late Group																					
<i>T. 'Queen of Night'</i> ^{e)}	0.20	0.54	0.30	0.29	0.11	0.26	nd	nd	nd	nd	nd	nd	0.01	0.04	0.01	tr	0.01	0.01	0.02	0.01	0.05
<i>T. S' Aristocrat'</i> ^{e)}	0.03	0.15	0.15	0.31	0.10	0.37	nd	nd	nd	nd	nd	nd	nd	tr	tr	tr	tr	0.04	0.01	0.01	0.04

^{a)} Concentrations are given in % fresh weight. Values are means of three extractions of the same plant material with coefficients of variation less than 8%.
^{b)} nd, Not detectable. ^{c)} tr, Trace (less than 0.01%). ^{d)} Collected in mid-April 1998. ^{e)} Collected in mid-May 1998. ^{f)} Synonyms are given in parentheses.

select parents for breeding of non-allergenic tulips. The aim was also to establish the most likely biosynthesis of tuliposides and tulipalins and their roles as allergens and antibiotics. Furthermore, the possibility of using tuliposides and tulipalins as chemotaxonomic markers is discussed.

Materials and Methods

Plant material

Leaves, stems and flowers of tulips (Table 1) were obtained from the Botanical Garden, University of Copenhagen, and the Royal Veterinary and Agricultural University, Copenhagen. Plant material was collected from plants growing out-of-doors at time of flowering. Voucher specimens are deposited at the botanical gardens.

Chemicals

Methanol (MeOH), chloroform (CHCl₃) and diethyl ether (Et₂O, stabilized with BHT) were of HPLC grade or higher quality obtained from Aldrich (Steinheim, Germany). The water for HPLC and extraction was ultra pure, generated by an Elgastat Maxima Analytica (Elga Ltd, England) water purification system.

Instrumentation and chromatographic conditions for HPLC

Analytical HPLC was performed with a Merck Hitachi HPLC equipped with an interface module (D-7000) to control the autosampler (L-7200), column oven (L-7300), pumps (L-7100) and diode array detector (L-7450). The diode array detector with a spectral range from 195–600 nm was employed at 208 nm and UV spectra were recorded between 200–400 nm. The acquired data was transferred to a PC equipped with Hitachi D-7000 HPLC system manager software. A LiChrospher 100 RP-18 (Merck) column (5 µm; 244×4 mm i.d.) protected with a guard cartridge (LiChrosorb RP-18 (5 µm), Merck) was used. Separations were performed at 25°C, by gradient elution with solvent A (H₂O–MeOH, 90:10) and solvent B (H₂O–MeOH, 60:40). Elution profile: 0–6 min 0% B, 6–35 min gradually to 100% B, 35–40 min gradually to 0% B, 40–50 min 0% B (Figs. 2a–c). Flow: 0.8 ml/min. Injection volume: 10 µl. Mobile phases were degassed with ultrasound for 20 min and filtered through a nylon membrane (0.45 µm, Sigma) before use.

A Merck L-6200 intelligent pump and a Merck L-4200 UV-VIS detector were used for preparative HPLC performed on a Develosil ODS-HG-5 (RP-18, 250×20 mm i.d., Nomura Chemical Co., Ja-

pan) column protected with a guard cartridge (50×20 mm i.d.) packed with the same material as the column. Compounds to be separated were dissolved in 30 ml water (injection volume) and pumped into the column via a suction filter (25×10 mm). Separations were performed at 25°C (column placed in a thermostated waterbath) with a flow rate of 7 ml/min and compounds were detected at 208 nm. Mobile phases were degassed with ultrasound and filtered as described above.

Preparation of samples for analytical HPLC

As no significant differences in the contents of compounds 1–3 and 5–8 were found between fresh and frozen plant material, and as 24-h extraction gave a slightly higher yield of each compound than 5 min, frozen plant material and 24-h extraction were chosen. Further, the content of tuliposides and tulipalins in frozen plant material did not change significantly during 1 year and extracts did not change their composition within the 1st week when stored in the dark at 4°C.

Freshly frozen flowers, stems and leaves (fresh weight from 1–2 g) of *Tulipa* species and cultivars were ground in water (20 ml) with an Ultra-Turrax T25 and stored in the dark at 4°C for 24 h. After filtering, the extracts were analysed by TLC and HPLC. The pH of the extracts were in general below 5.5. Samples (2 ml) were pressure-filtered through cellulose acetate membranes (0.2 µm, Sigma) before analysis and each compound quantified by using pure standards of 1–3, 5–8 (Fig. 1) for calibration. Detection limits for tuliposides and tulipalins were approximately 0.2 µg/ml and 0.1 µg/ml, respectively.

Extraction and isolation of tuliposides and tulipalins

Freshly frozen aerial parts (150 g) of *T. tarda* (harvested in mid-April 1998) were extracted 2× with 1.5 l water. The combined extracts were filtered and evaporated under reduced pressure at 25°C to give 15.4 g brownish syrup which was, in part (10.2 g), chromatographed on a water cooled column (85×5 cm) packed with silica gel (silica gel 60, 70–230 mesh, Merck), using a CHCl₃–MeOH–H₂O gradient (400 ml 65:35:2; 200 ml 30:20:1; 200 ml 25:25:1; 200 ml 45:55:2; 200 ml 35:65:2; 200 ml 25:75:2 and 460 ml 5:45:1) as eluent, to give 46 fractions of 40 ml each, that were checked by TLC and analytical HPLC. Fractions 6–13 contained compound 7 and 8, fractions 14–25 a mixture of 1, 3, 5 and 6, fractions 26–38 a mixture of D-glucose, D-fructose, xylose, sucrose, 1–3 and 6 and fractions 39–46 contained only saccharides. The crude fractions 6–38 were applied to preparative

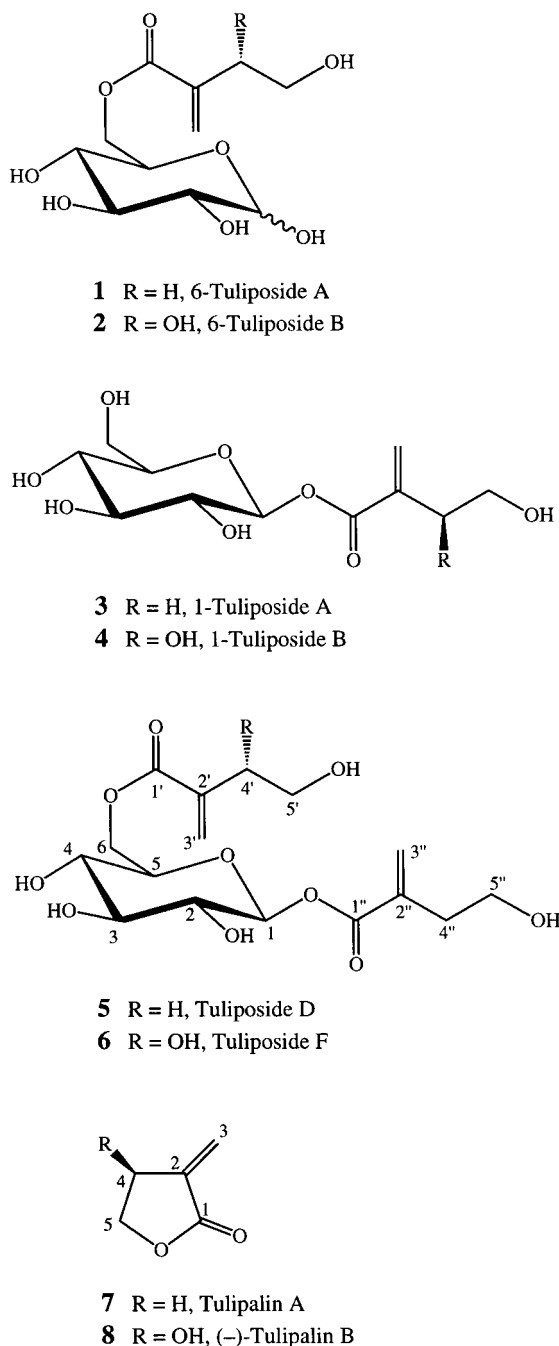


Fig. 1. Chemical structures of tuliposides (**1–6**) and tulipalins (**7, 8**) identified as natural products in tulips. Compounds **1–3** and **5–8** were found in the present investigation.

HPLC for further purification. Fractions 6–13 were separated by isocratic elution with H₂O to afford **7** (93 mg, oily liquid, R_t =85–95 min) and **8** (115 mg, oil, R_t =25–30 min), whereas fractions 14–25 and 26–38 were separated by a H₂O–MeOH gradient (200 ml H₂O; 150 ml 95:5; 100 ml 90:10 and 200 ml 50:50) as eluent. Fractions 14–25 afforded **1** (208 mg, syrup, R_t =40–65 min), **3** (15 mg,

syrup, R_t =31–35 min), **5** (30 mg, syrup, R_t =125–135 min) and **6** (250 mg, syrup, R_t =88–100 min). Fractions 26–38 afforded D-glucose, D-fructose, sucrose, xylose (450 mg, R_t =10–15 min), **1** (120 mg), **2** (330 mg, syrup, R_t =18–25 min), **3** (8 mg) and **6** (66 mg).

Thin-layer chromatography (TLC)

Silica gel plates (20×20 cm, silica gel 60, 0.25 mm, Merck) were used for TLC. Tuliposides and saccharides were detected as blue spots after spraying with a solution of aniline and diphenylamine in acidified acetone (Sigma No. A 8142) followed by heating with a flameless heat gun. Tulipalins were visualized as yellow spots by spraying with 1 M KMnO₄. TLC was performed with CHCl₃–MeOH–H₂O (15:10:2) for separation of tuliposides. In crude extracts of *T. tarda* were the following R_f values recorded: 0.32 (D-glucose, D-fructose, xylose and sucrose); 0.42 (6-tuliposide B, **2**); 0.55 (6-tuliposide A, **1**; 1-tuliposide A, **3**); 0.57 (tuliposide F, **6**); 0.70 (tuliposide D, **5**). TLC in Et₂O of the crude extracts gave two spots at R_f =0.41 and 0.61 corresponding to (–)-tulipalin B (**7**) and tulipalin A (**8**), respectively.

Hydrolysis of tuliposides

A solution of compound **1** (200 mg) in 2 M HCl (10 ml) was heated at 100°C for 2 h. After cooling, the solution was saturated with NaCl and extracted 6 × with CHCl₃ (20 ml) and the combined CHCl₃ phases were dried over anhydrous Na₂SO₄. After removal of CHCl₃ under reduced pressure, compound **7** (62 mg) was obtained. Acid hydrolysis of **5** (30 mg) gave **7** (12 mg) and acid hydrolysis of **2** (185 mg) gave **8** (63 mg), whereas acid hydrolysis of **6** (220 mg) afforded **7** (46 mg) and **8** (56 mg). The aqueous phases consisted of D-glucose as shown by TLC and NMR. Partial hydrolysis of **1–3, 5** and **6** was performed by heating the compounds in water for 5 min at 100°C. Partial hydrolysis of e.g. **5** afforded **1, 3** and **7** whereas **6** gave **2, 3, 7** and **8** as shown by HPLC.

Correlations

Pearson's coefficients of correlation (R) between allergens and the different plant parts were determined.

Results

Identification of tuliposides and tulipalins

The spectral data (UV, NMR and FAB-MS) of compound **1** was in accordance with those pre-

viously reported for 6-tuliposide A from *Alstroemeria* (11, 12, 16) and *Tulipa* (15, 21). Acid hydrolysis of **1** gave tulipalin A (15, 21) in accordance with **1** being 6-tuliposide A. Acid hydrolysis of compound **2** gave the optically active tulipalin B (**8**), whose NMR and EI-MS data was in accordance with literature values (15, 21). The absolute configuration of the chiral center in tulipalin B was determined to be *S* by comparing its optical rotation ($[\alpha]_D = -84^\circ$ ($c=1.0$, CHCl_3)) with literature values (21), indicating the presence of a (*S*)-3,4-dihydroxy-2-methylenebutanoate moiety in **2** (15, 21). The NMR data of **2** was very similar to those of **1** (11, 12, 15), the main differences being the absence of the signals ascribable to a CH_2 group (δ_{H} 2.52 and δ_{C} 34.9) and the occurrence of signals from an oxygenated CH group (δ_{H} 4.60 and δ_{C} 71.2) clearly showing **2** to be a mixture of α and β 6-((*S*)-3,4-dihydroxy-2-methylenebutanoate)-D-glucopyranose (6-tuliposide B); a new acyl derivative of the known 1-tuliposide B (**4**) (21). The FAB-mass spectrum of **2** gave a molecular ion peak at m/z 295 $[\text{M}+\text{H}]^+$ ($\text{C}_{11}\text{H}_{19}\text{O}_9$) in accordance with **2** being 6-tuliposide B. The equilibria between the α - and β -anomers of both **1** and **2** were determined to approximately 2:3 by NMR and HPLC (Figs. 2a–c). Although the isomers of **1** and **2** are easily separated by HPLC the equilibrium is readily adjusted and no attempt was therefore made to isolate them separately.

Spectral data (UV, NMR and FAB-MS) of **3** and **5** were in accordance with those previously reported for 1-tuliposide A (15, 21) and tuliposide D (12, 13, 15), respectively. Partial hydrolysis of **5** gave **1**, **3** and **7**, whereas **3** gave **7**, in accordance with **5** and **3** being tuliposide D and 1-tuliposide A, respectively.

Acid hydrolysis of compound **6** yielded **7** and **8** in approximately the ratio 1:1, whereas partial hydrolysis gave **2**, **3**, **7** and **8**, indicating the presence of a 4-hydroxy-2-methylenebutanoate and a (*S*)-3,4-dihydroxy-2-methylenebutanoate moiety in **6** linked in position 1 and 6 of β -D-glucose, respectively. This was further confirmed by the ^1H and ^{13}C NMR and the FAB-mass spectrum which showed a molecular ion peak at m/z 393 $[\text{M}+\text{H}]^+$ ($\text{C}_{16}\text{H}_{25}\text{O}_{11}$). The β -nature of **6** was confirmed by the signal at δ 5.59 with a coupling constant of 7.6 Hz, thus **6** is 1-(4-hydroxy-2-methylenebutanoate)-6-((*S*)-3,4-dihydroxy-2-methylenebutanoate)- β -D-glucopyranose (tuliposide F).

6-Tuliposide B (**2**)

^1H -NMR (D_2O ; 300 MHz; dioxane int. std): δ 3.52 (1H, *dd*, $J=6.5$, 11.5 Hz, H-5'), 3.69 (1H, *dd*, $J=3.4$, 11.5 Hz, H-5'), 3.2–4.0 (4H, *m*, H-2–H-5), 4.60

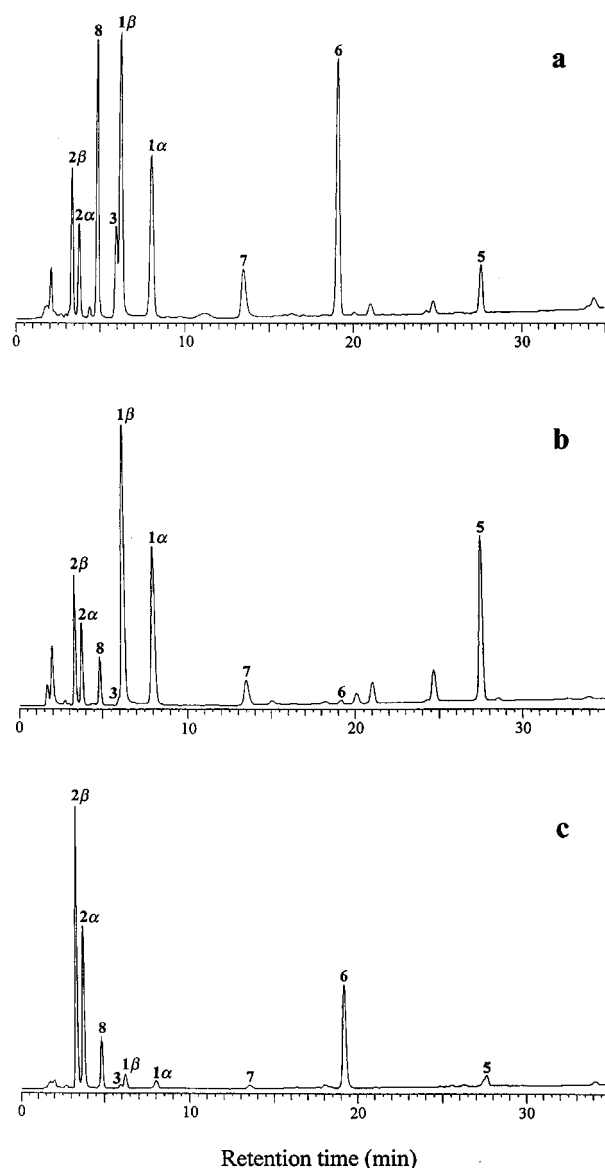


Fig. 2. Selected HPLC profiles at 208 nm of aqueous extracts of (a) *Tulipa turkestanica* leaves (collected in mid May 1998), (b) *T. sylvestris* ssp. *sylvestris* stems and (c) *T. linifolia* leaves. Identified peaks: **1** β , 6-tuliposide A (β -anomer); **1** α , 6-tuliposide A (α -anomer); **2** β , 6-tuliposide B (β -anomer); **2** α , 6-tuliposide B (α -anomer); **3**, 1-tuliposide A; **5**, tuliposide D; **6**, tuliposide F; **7**, tulipalin A; **8**, (–)-tulipalin B.

(1H, *m*, H-4'), 4.3–4.5 (2H, *m*, H-6), 4.61 (*d*, $J=7.9$ Hz, H-1(β)), 5.16 (*d*, $J=3.7$ Hz, H-1(α)), 5.97 (1H, *s*, H-3'), 6.39 (1H, *br s*, H-3'). The ratio α (H-1): β (H-1) was ca. 2:3 at room temperature. ^{13}C -NMR (D_2O ; 75 MHz; dioxane int. std): δ 65.2 (*t*, C-5'), 71.2 (*d*, C-4'), 129.1 (*t*, C-3'(α)), 129.2 (*t*, C-3'(β)), 139.8 (*s*, C-2'), 167.9 (*s*, C-1'): α -D-glucose: 93.0 (*d*, C-1), 72.2 (*d*, C-2), 73.4 (*d*, C-3), 70.0 (*d*, C-4), 70.6 (*d*, C-5), 64.5 (*t*, C-6). β -D-glucose: 96.9 (*d*, C-1), 74.9 (*d*, C-2), 76.4 (*d*, C-3), 70.4 (*d*, C-4), 74.2 (*d*, C-5), 64.6 (*t*, C-6).

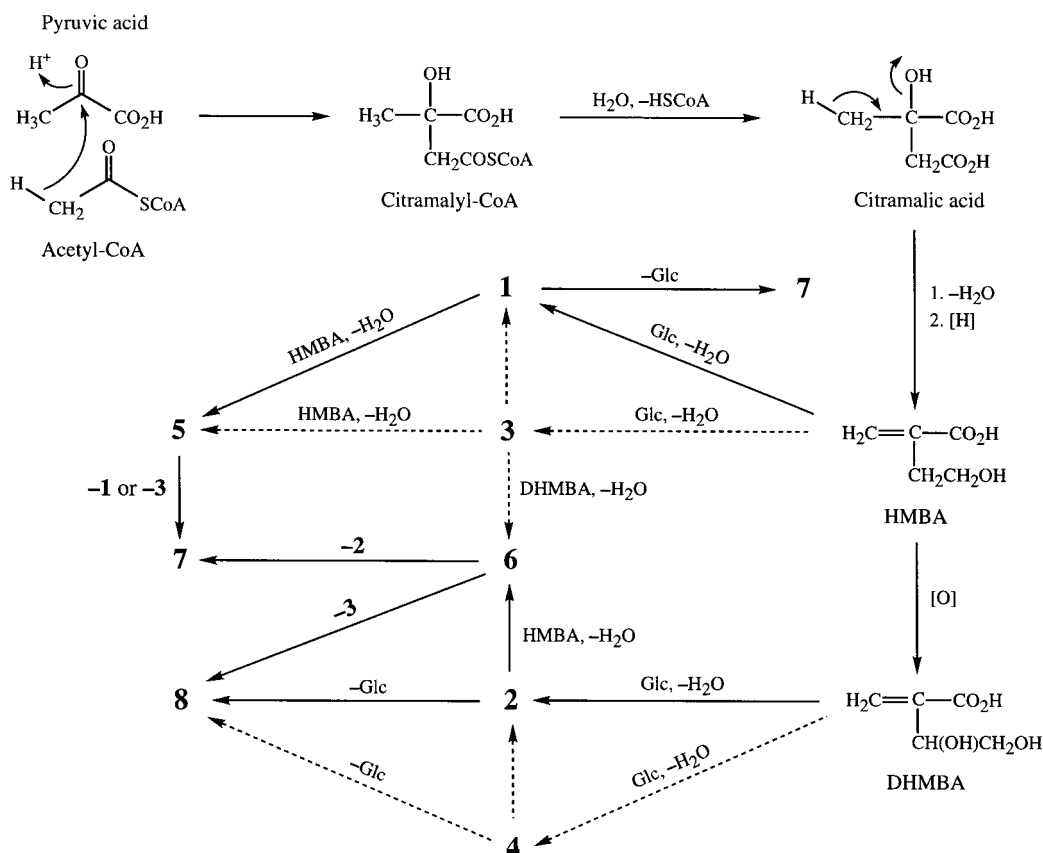


Fig. 3. The possible biosynthesis of compounds 1–8 from D-glucose (Glc) and 4-hydroxy-2-methylenebutanoic acid (HMBA) and/or (S)-3,4-dihydroxy-2-methylenebutanoic acid (DHMBA). Dotted arrows indicate the pathway to be of minor importance.

Tuliposide F (6)

¹H-NMR (D₂O; 300 MHz; dioxane int. std): δ 2.52 (2H, *t*, $J=6.5$ Hz, H-4''), 3.4–3.9 (4H, *m*, H-2–H-5), 3.52 (1H, *m*, H-5'), 3.70 (1H, *dd*, $J=3.4, 11.5$ Hz, H-5'), 3.69 (2H, *t*, $J=6.5$ Hz, H-5''), 4.32 (1H, *dd*, $J=5.6, 12.2$ Hz, H-6), 4.50 (1H, *dd*, $J=2.0, 12.2$ Hz, H-6), 4.58 (1H, *br dd*, $J=3.4, 6.5$ Hz, H-4'), 5.59 (1H, *d*, $J=7.6$ Hz, H-1(β)), 5.86 (1H, *s*, H-3''), 5.98 (1H, *s*, H-3'), 6.38 (1H, *s*, H-3''), 6.39 (1H, *s*, H-3'). ¹³C-NMR (D₂O; 75 MHz; dioxane int. std): δ 34.7 (*t*, C-4''), 60.7 (*t*, C-5''), 65.2 (*t*, C-5'), 71.2 (*d*, C-4'), 129.2 (*t*, C-3'), 131.5 (*t*, C-3''), 136.4 (*s*, C-2''), 139.8 (*s*, C-2'), 167.7 (*s*, C-1''), 167.9 (*s*, C-1'): β -D-glucose: 95.1 (*d*, C-1), 72.7 (*d*, C-2), 76.1 (*d*, C-3), 70.3 (*d*, C-4), 75.2 (*d*, C-5), 64.1 (*t*, C-6).

Determination of tuliposides and tulipalins in tulips by analytical HPLC

The major tuliposides 6-tuliposide A (1) and B (2) were found in all tulips investigated. Concentrations ranged from trace amounts in leaves of *T. kaufmanniana* to more than 1.5% of 1 and 1.3% of 2 of fresh weight (Table 1). The content of 1 and 2

varied much between leaves, stems and flowers, with no correlation between 1 and 2 within each plant part ($R < 0.21$). Tulips belonging to the section *Leiostemones* contained larger amounts of 2 than of 1 whereas species in *Eriostemones* had relatively large amounts of both 1 and 2 with no consistent interrelationship between these 2 tuliposides. In the section *Eriostemones* most species had a higher concentration of 1 and 2 in leaves as compared to stems and flowers, whereas in *Leiostemones* this was only true for compound 2. 1-Tuliposide A (3) exceeded 0.1% of fresh weight only in leaves of *T. turkestanica* and was not detected in 9 taxa. Neither of the cultivars in the present investigation contained compounds 3 or 5 (Table 1).

In *T. patens* leaves, compound 5 exceeded 1.2% of fresh weight but was otherwise only found in concentrations above 0.1% in 1 of the 3 investigated *T. sylvestris* taxa. Significant correlations between the content of 5 and its precursor 1 were found in leaves and stems ($R \approx 0.5$). In 7 taxa 5 was not detected and in further 4 only in trace amounts (Table 1). Compound 6 is more widely distributed in the genus, with concentrations above 0.5% in *T.*

tarda and *T. turkestanica*. A large variation between the different plant parts was also found for **6**, most often with the lowest concentration in the flowers (Table 1). Only in the flowers was a significant correlation found between the content of **6** and its precursor **2** ($R=0.63$).

Tulipalin A (**7**) and (–)-tulipalin B (**8**) were found in all genotypes and plant parts up to 0.56% and 0.39% of fresh weight, respectively (Table 1). No consistency was found for the distribution of tulipalins in the different plant parts and no correlations between the contents of tulipalins and tuliposides were present ($R<0.38$).

Plants with a high content of tuliposides and tulipalins in leaves most often also had a high content in stems ($R>0.7$), whereas the coefficients between leaves and flowers as well as between stems and flowers were smaller and often not significant. Despite the significant correlations mentioned, a large variation between individual plants in content and distributional patterns was found.

Discussion

Contact dermatitis from tulips and *Alstroemeria* is one of the best-known occupational dermatoses and is caused by exposure to recently damaged plant parts, e.g., during handling of flower bulbs or cut flowers. It normally gives rise to a severe dermatitis of the hands, a condition known as 'tulip fingers' (5–11). The causative agents have so far been identified as tuliposide A and its lactonized aglycone tulipalin A (5–11). Tuliposide A was first isolated from *T. gesneriana* by Tschesche et al. (21), together with the non-allergenic tuliposide B, and identified as 1-tuliposide A (**3**) and B (**4**), respectively. Although both compounds readily rearrange by an acyl migration into the more stable 6-acyl derivatives (21), tuliposide A and B have often been associated with the 1-acyl derivatives when found in other plants, even though the position of the acyl group was not determined (2, 6, 10, 17, 18). The present investigation of several *Tulipa* species and cultivars revealed, in addition to 1- and 6-tuliposide A (**1**, **3**), tuliposide D (**5**), tulipalin A (**7**) and (–)-tulipalin B (**8**), the new tuliposide F (**6**) and 6-tuliposide B (**2**) but not 1-tuliposide B (**4**) (Fig. 1). Since no significant differences in the content of **1–3** were found between fresh and frozen plant material and the composition did not vary at different extraction times, compounds **1** and **2** are not artefacts produced from the 1-acyl derivatives (**3**, **4**). Tuliposides are relatively stable at pH below 5.5 whereas above pH 6.5 they release 4-hydroxy-2-methylenebutanoic acid and/or (*S*)-3,4-dihydroxy-2-methylenebutanoic acid which lactonize spontaneously to **7** and **8**, respectively (18, 21, 22,

25, 26). In the present investigation, pH of the extracts was in general below 5.5, explaining the relatively high stability of the isolated tuliposides. The tulipalins **7** and **8** are therefore not artefacts. This is also supported by the fact that the levels of **7** and **8** remained unchanged during the extraction procedure.

For the analysis of tuliposides and tulipalins in tulips, we established an RP-HPLC method using a methanol:water solvent gradient, allowing separation of the tuliposides **1**, **2**, **5**, **6**, the α - and β -anomers of compounds **1** and **2**, as well as the tulipalins **7** and **8**, and to some extent separation of **3** from the β -anomer of **1** (Figs. 2a–c). 1-Tuliposide B (**4**) is not supposed to be separable from the β -anomer of **2** by the HPLC method used (Figs. 2a–c). However, since the ratios between the α - and β -anomers of **2** were approximately 2:3 in all extracts and the NMR spectra of **2** showed no sign of **4**, this compound was not present in the tulips investigated (Table 1).

The predominant compounds were 6-tuliposide A (**1**) and B (**2**), whereas 1-tuliposide A (**3**) was detected in only 13 taxa and always in minute amounts (Table 1). Tuliposide D (**5**) was found in large amounts in *T. patens*, but was not detected or only found in trace amounts in 10 taxa. Tuliposide F (**6**) appears more widely distributed in the genus, being present in nearly all tulips investigated (Table 1). Tulipalin A (**7**) and (–)-tulipalin B (**8**) were found in all tulips investigated but was not correlated with the content of tuliposides. This lack of correlation was also found in *Alstroemeria* (24), indicating that the production of tulipalins is controlled mainly by environmental influences. Slob & Varekamp (2) have previously investigated a large number of *Tulipa* species for their contents of tuliposide A and B (position of the acyl group not identified), and their results differ to some extent from the present. For example, they most often found the highest content in the flowers, which is not the case with our plant material (Table 1). The differences may be due to seasonal variation or genotypic differences.

A methylene group in α position is important for the allergenic activity of tulipalin A (**7**) and tuliposide A (**1**, **3**) (11). This is also in accordance with chemical considerations, because the methylene group is activated by the electron-withdrawing carbonyl group, making it suitable for nucleophilic attacks by skin proteins, generating sensitizing antigens. For example, the reaction that can occur between **7** and a protein nucleophile is one of the most common haptenation reactions found with naturally occurring sensitizers (27). Although the allergenic properties of **5** and **6** have not been investigated, their structural relationship to the aller-

genic tuliposides clearly suggest that they are allergens and most likely cross-react with these compounds. However, should future investigations show that **5** and **6** are not allergenic, their presence still cannot be ignored, since they are converted into the allergens **1**, **3** and **7** in the plants and/or after deposition on the skin.

Biological function

Tulipalin A (**7**) is not only sensitive to nucleophilic reagents but is also very reactive in Diels-Alder type reactions (27, 28). The tulipalins may therefore react with double bonds in unsaturated fatty acids or with nucleophilic thiol and amino groups in proteins and amino acids if they occur free in the cytoplasm. This leads to severe damage to the cells and hindrance of their biological functions (26). Consequently, it has been proposed that the antibiotic tulipalins are stored in separate compartments of the cells, e.g., in vesicles (26). It has also been stated that tulipalins do not occur in healthy plants but are produced from tuliposides only in response to, e.g., fungal attack and/or mechanical damage (2, 6, 10, 17, 22, 25). However, the present investigations have shown that tulipalins are not artefacts but occur naturally, a conclusion also supported by the lack of correlation between the contents of tulipalins and tuliposides and the different amounts of tulipalins detected in different plant parts (see Results and Table 1). Tuliposides may be storage products for tulipalins, which act as antifungal agents in order to prevent penetration of fungi (26). High concentrations of tulipalins, however, do not necessarily implicate fungal attack, but may also depend on factors such as genotype, growth conditions, season and developmental stage (24).

Biosynthesis

Tuliposides are most likely biosynthesized from D-glucose and 4-hydroxy-2-methylenebutanoic acid (HMBA) and/or (*S*)-3,4-dihydroxy-2-methylenebutanoic acid (DHMBA) (Fig. 3), with HMBA and its oxidation product DHMBA being biosynthesized from pyruvate and acetate (29). 6-Tuliposide A (**1**) is probably formed by a condensation of β -D-glucose and HMBA with the loss of water, whereas a condensation between β -D-glucose and DHMBA leads to 6-tuliposide B (**2**). The total absence of 1-tuliposide B (**4**) and other tuliposides having a (*S*)-3,4-dihydroxy-2-methylenebutanoate moiety in position 1 of β -D-glucose, the absence or low amounts of **3** and the high amounts of **1** and **2** in the tulips investigated (Table 1) exclude that the 6-acyl derivatives arise from the 1-acyl derivatives. Also the fact that **3** was stable in the extracts and that the levels of tuliposid-

es remained the same under the extraction procedure support this conclusion. Further condensation between HMBA and **1** and **2** leads to tuliposide D (**5**) and F(**6**), respectively. Compounds **5** and **6** may also be biosynthesized via a condensation between **3** and HMBA, and **3** and DHMBA, respectively, but this biosynthetic route seems to be of minor importance since the 6-acyl derivatives are the predominant compounds (Table 1). Degradation of **5** and **6** can lead to **1**, **3** and **2**, **3**, respectively, but this pathway also seems to be of minor importance due to the lack of consistent correlations between **5** and **6** and their degradation products. Although **3** may also be formed by a condensation between β -D-glucose and HMBA, the small amounts of **3** indicates that the degradation of **5** and **6** is the major biosynthetic route for this compound. Tulipalins are produced by enzymatic hydrolysis of the different tuliposides (Fig. 3).

Breeding potentials

To reduce the risk of contact dermatitis in the tulip industry, the stems and bulbs particularly should have a low allergen content, as a consequence of the handling procedures. Selection should therefore concern all possible allergens, i.e., compounds **1**, **3**, **5**–**7**. In order to obtain disease-resistant tulips, a high content of the non-allergenic 6-tuliposide B (**2**) is desirable. The lack of correlation between the allergenic tuliposides and **2** clearly indicate that it should be possible to produce low-allergenic resistant cultivars. Factors such as plant age, season and growing conditions must be taken into account during selection, since they influence the content of tuliposides and tulipalins (2, 24). This may explain the differences in the content of tuliposides and tulipalins between 2 collections, with ca. 30 days in between, of *T. sylvestris*, *T. tarda* and *T. turkestanica*, respectively (Table 1). Even at 5 days interval, Slob & Varekamp (2) found very large differences. Potential parents for crossings should therefore be screened for their allergen content several times. Among the tulips investigated, *T. kaufmanniana*, *T. praestans* and *T. undulatifolia* are potential parents in a breeding program as they have a low allergen content (Table 1).

Chemotaxonomy

Slob & Varekamp (2) suggested tuliposides to be useful in a natural classification of the genus. In the present investigation, 6-tuliposide A (**1**) and B (**2**) most often separated the sections *Leiostemones* and *Eriostemones*, as species of the former had relatively low amounts of **1** as compared to **2**, whereas species belonging to the latter section con-

tained relatively high concentrations of both **1** and **2** (Table 1; Figs. 2a–c). These 2 sections also have different geographical origin. *Leiostemones* is mainly found in Central Asia, whereas *Eriostemones* originate from the Caucasus and westwards into Western Europe (3, 4). Within each section, compounds **1** and **2** cannot discriminate between sub-sections. Also with respect to other characteristics, a clear discrimination between sub-sections is difficult (3, 4). Neither tuliposide D (**5**) nor F (**6**) seem useful as taxonomic markers since in both sections they are found in variable amounts (Table 1). However, if further investigations show that in the Liliaceae compounds **5** and **6** are restricted to *Tulipa*, they may be useful in separating this genus from other genera within the Liliaceae.

Conclusions

From the present investigation, it is clear that 6-tuliposide A (**1**) and not 1-tuliposide A (**3**) is the major allergen in tulips and that 6-tuliposide B (**2**) is the acyl derivative of the non-allergenic tuliposide B. Furthermore, the present results indicate that tuliposides are storage products for tulipalins, which also occur in healthy tulips and are not only produced as a response to fungal attack and mechanical damage of the plants. Tulipalins can therefore be characterized as pre-infectional compounds undergoing post-infectional increase (inhibitins) in order to hamper fungal penetration in the plant tissue (22, 23, 30). From a dermatologic point of view the situation in tulips appears more complicated than first anticipated, due to the regular occurrence of tuliposides D (**5**) and F (**6**). These compounds, or their degradation products, are also likely allergens. Compounds **5** and **6** should therefore be included when investigating the allergenic and antibiotic properties of tulips, and selection of tulips for breeding of non-allergenic resistant cultivars should also include these compounds. Some tulips already have a low allergen content and it is therefore possible to breed non-allergenic and resistant tulips.

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References

1. Hoog M H. On the origin of *Tulipa*. *Lilies and Related Plants* 1973: 47–64.
2. Slob A, Varekamp H Q. Tuliposide contents of Tulip (*Tulipa*) species and cultivars during the flowering stage. *Proc Koninklijke Nederlandse Akad Wetensch Ser C Biol Med Sci* 1977; 80: 201–211.
3. Van Raamsdonk L W D, de Vries T. Biosystematic studies in *Tulipa* sect. *Eriostemones* (Liliaceae). *Plant Syst Evol* 1992; 179: 27–41.
4. Van Raamsdonk L W D, de Vries T. Species relationships and taxonomy in *Tulipa* subg. *Tulipa* (Liliaceae). *Plant Syst Evol* 1995; 195: 13–44.
5. Verspyck Mijnsen G A W. Pathogenesis and causative agent of "tulip finger". *Br J Dermatology* 1969; 81: 737–745.
6. Hausen B M. Airborne contact dermatitis caused by tulip bulbs. *J Am Acad Dermatology* 1982; 7: 500–503.
7. Hausen B M. Allergiepflanzen – Pflanzenallergene: *Handbuch u. Atlas d. allergie-induzierenden Wild- und Kulturpflanzen – Kontaktallergene*. Landsberg/München: Ecomed Verlagsgesellschaft mbH, 1988: 67–69 and 227–229.
8. Gette M T, Marks J E. Tulip fingers. *Arch Dermatology* 1990; 126: 203–205.
9. Thiboutot D M, Hamory B H, Marks J G. Dermatoses among floral shop workers. *J Am Acad Dermatology* 1990; 22: 54–58.
10. Hausen B M, Prater E, Schubert H. The sensitizing capacity of *Alstroemeria* cultivars in man and guinea pig. *Contact Dermatitis* 1983; 9: 46–54.
11. Santucci B, Picardo M, Iavarone C, Trogolo C. Contact dermatitis to *Alstroemeria*. *Contact Dermatitis* 1985; 12: 215–219.
12. Christensen L P. Tuliposides from *Alstroemeria revoluta*. *Phytochemistry* 1995; 38: 1371–1373.
13. Christensen L P, Kristiansen K. Isolation and quantification of a new tuliposide (tuliposide D) by HPLC in *Alstroemeria*. *Contact Dermatitis* 1995; 33: 188–192.
14. Christensen L P, Kristiansen K. Identification and quantification of the allergens (tuliposides and tulipalin A) in *Alstroemeria*. *Acta Horti* 1995; 420: 140–143.
15. Christensen L P. Tuliposides from *Tulipa sylvestris* and *T. turkestanica*. *Phytochemistry* 1999; in press.
16. Christensen L P, Kristiansen K. A simple HPLC method for the isolation and quantification of the allergens tuliposide A and tulipalin A in *Alstroemeria*. *Contact Dermatitis* 1995; 32: 199–203.
17. Slob A, Jekel B, de Jong B, Schlatmann E. On the occurrence of tuliposides in the Liliiflorae. *Phytochemistry* 1975; 14: 1997–2005.
18. Slob A. Tulip allergens in *Alstroemeria* and some other Liliiflorae. *Phytochemistry* 1973; 12: 811–815.
19. Barbier P, Benezra C. Allergenic α -methylene- γ -butyrolactones. Study of the capacity of β -acetoxy- and β -hydroxy- α -methylene- γ -butyrolactones to induce allergic contact dermatitis in guinea pigs. *J Med Chem* 1986; 29: 868–871.
20. Papageorgiou C, Stampf J-L, Benezra C. Allergic contact dermatitis to tulips: An example of enantiospecificity. *Arch Dermatol Res* 1988; 280: 5–7.
21. Tschesche R, Kämmerer F-J, Wulff G. Über die struktur der antibiotisch aktiven Substanzen der Tulpe (*Tulipa gesneriana* L.). *Chemische Berichte* 1969; 102: 2057–2071.
22. Schönbeck F, Schroeder C. Role of antimicrobial substances (tuliposides) in tulips attacked by *Botrytis* spp. *Phytopathol Plant Pathol* 1972; 2: 91–99.
23. Bergman B H H, Beijersbergen J C M, Overeem J C, Sijpesteijn A K. Isolation and identification of α -methylenebutyrolactone, a fungitoxic substance from tulips. *Receuil des Travaux Chimiques des Pays-Bas* 1967; 86: 709–714.
24. Kristiansen K, Christensen L P. Allergen contents in *Alstroemeria* can be reduced by breeding. *Euphytica* 1998; 101: 367–375.

25. Beijersbergen J C M, Lemmers C B G. Enzymatic and non-enzymatic liberation of tulipalin A (α -methylene butyrolactone) in extracts of tulips. *Physiol Plant Pathol* 1972; 2: 265–270.
26. Van Rossum M W P C, Alberda M, van der Plas L H W. Tulipaline and tuliposide in cultured explants of tulip bulb scales. *Phytochemistry* 1998; 49: 723–729.
27. Roberts D W, Lepoittevin J-P. Hapten-protein interactions. In: Lepoittevin J-P, Basketter D A, Goossens A, Karlberg A-T (eds): *Allergic contact dermatitis. The molecular basis*. Berlin, Heidelberg: Springer-Verlag, 1998: 81–111.
28. Danishefsky S, Kitahara T, Yan C F, Morris J. Diels-Alder reactions of *trans*-1-methoxy-3-trimethylsilyloxy-1,3-butadiene. *J Am Chem Soc* 1979; 101: 6996–7000.
29. Hutchinson C R, Leete E. Biosynthesis of α -methylene- γ -butyrolactone, the cyclized aglycone of tuliposide A. *Chemical Communications* 1970: 1189–1190.
30. Ingham J L. Disease resistance in higher plants. The concept of pre-infectional and post-infectional resistance. *Phytopath Z* 1973; 78: 314–335.

Address:

Lars P. Christensen
Department of Fruit, Vegetable and Food Science
Danish Institute of Agricultural Sciences
Kirstinebjergvej 10
DK-5792 Aarslev
Denmark