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Isolation and Structure of Tulip Flower Factors which Stimulate the Germination of Chrysanthemum Pollen

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Four aliphatic esters capable of stimulating pollen germination of *Chrysanthemum leucanthemum*, isolated from the flowers of *Tulipa gesneriana*, have been characterized as methyl 9, 18-dihydroxystearate, methyl 10, 18-dihydroxystearate and a mixture of α-glyceryl linoleate and α-glyceryl linolenate.

INTRODUCTION

A very low percentage of pollen germination of a chrysanthemum species has been found to occur in aqueous medium consisting of an oligosaccharide such as sucrose, lactose or raffinose and an inorganic ion of boron or calcium.[1] Previously, substances capable of stimulating pollen germination in related species have been shown to exist in a variety of plant tissues.[2] The partial purification of the factors with similar activity has also been found in the onion bulb.[3]

In the course of a continuing search for pollen germinating stimulants of plant origin, we have found that methanol extracts of *Tulipa gesneriana* L. show significant stimulating activity in the germination of chrysanthemum pollen. We report herein the isolation and characterization of stimulating factors, in the form of α-glyceryl linoleate, α-glyceryl linolenate, methyl 9,18-dihydroxystearate and methyl 10,18-dihydroxystearate, from *T. gesneriana*.

RESULT AND DISCUSSION

Isolation of Factors A, B and C. The extraction and purification of the stimulating factors were controlled by pollen germination bioassay using fresh pollen grains of *Chrysanthemum leucanthemum* as described previously.[4] Methanol extracts obtained from 79 kg of tulip flowers were concentrated and the aqueous concentrate was extracted successively with benzene, ethyl acetate and then *n*-butanol. The stimulating activity was found in both fractions of benzene and ethyl acetate, but not in the *n*-butanol. The benzene fraction, which was the most active, was purified through several steps by adsorption chromatography on silicic acid and Florisil to give the stimulators,

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tentatively named as factors A, B and C.

Characterization of Factor A. Factor A was obtained as a colorless oil in yield of 70 mg and oxydized with air. The factor showed a single spot on thin-layer chromatograms (TLC) using various developing solvent systems, although it was found to be a mixture of two compounds as described below. Further separation into each of the compounds was unsuccessful.

The i.r. spectrum ($CHCl_3$) of factor A showed absorption bands at 3580, 1725, 1610, 1180 and 990 cm^{-1}, and the u.v. spectrum* only end absorption, indicative the presence of hydroxyl, ester carbonyl and vinyl groups. In its p.m.r. spectrum*, the chemical shifts and splitting pattern of all signals in the regions at 0.8–3.1 and 5.0–5.8 were in good agreement with those of methyl linolenate[5], except the discrepancy of the relative intensities between the observed signals. A broad signal at 2.90 disappeared by the addition of deuterium oxide, and the signals at 3.5–4.0 were deshielded to 3.9–4.5 and 5.28 together with the appearence of a sharp singlet at 2.10 ascribable to acetoxyl groups on acetylation. These facts substantiated the presence of primary and secondary hydroxyl groups in factor A. The spectrum of the acetates was also superimposable to that of triacetin in the region at 3.9–4.5.[6] Therefore, it can be inferred that the factor is a mixture of α-monoglycerides.

Factor A on alkaline hydrolysis afforded fatty acids and an alcohol. The alcohol, after purification by paper chromatography, was acetylated to give triacetin, which was confirmed by the p.m.r., i.r. and co-TLC with an authentic sample.

The fatty acids were converted by treatment with diazomethane into methyl esters, which were directly examined by gas chromatography (GLC) on a column of 5% PEG 20 M at 170°. The GLC trace showed two peaks at the retention time 9.4 and 11.4 min. When scanned by combined gas chromatography-mass spectrometry (GLC-MS), the two peaks of shorter and longer retention time gave identical mass spectra with those of methyl linoleate and methyl linolenate, respectively. By measurement of the peak areas from standard injections, the two fatty acid esters were shown to be a 1:1 mixture of them.

From the results described above factor A was characterized as a mixture of approximately equal amounts of α-glyceryl linoleate and α-glyceryl linolenate.

Characterization of Factor B. Factor B crystallized from *n*-hexane-benzene in yield of 18 mg as colorless plates, m.p. 62–63°. It exhibited i.r. (3580, 1725 and 1080 cm^{-1}, Fig. 1) and u.v. spectra (200 nm, ε 300) characteristic for hydroxyl and ester carbonyl groups. Its p.m.r. spectrum showed signals ascribable to methylene group (1.2–1.7, *ca.* 28 H, broad s.), $-CH_2-CH_2-COO-$(2.31, 2H, perturbed t., $J=7$) and a carbomethoxyl group (3.67, 3H, s.). The disappearance of signals equivalent to two protons by addition of deuterium oxide showed the presence of two hydroxyl groups in the factor.

On acetylation the factor yielded a diacetate. In the p.m.r. spectrum of the acetate,

* Unless otherwise stated, the u.v. spectra were taken in ethanol and the p.m.r. spectra in deuteriochloroform at 60 MHz. Chemical shifts were expressed as δ values (ppm) from tetramethylsilane as an internal standard and coupling constant in Hz. Singlet, triplet and multiplet are abbreviated to s., t. and m., respectively.

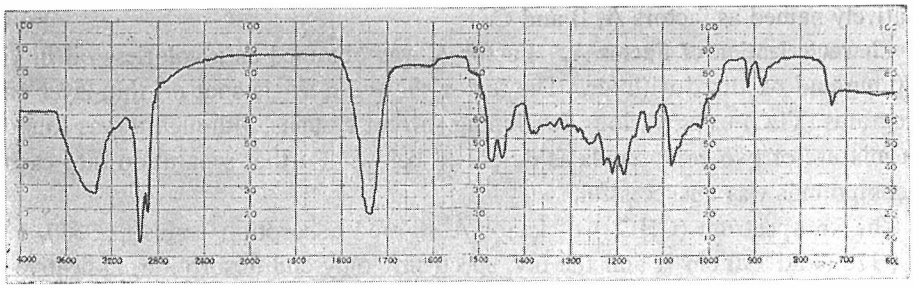

Fig. 1. The i.r. spectrum of factor B (KBr pellet).

the three-proton multiplets at 3.61 in the spectrum of factor B were displaced to 4.05 (2H, t., J=6.5, $-CH_2-CH_2-OAc$) and 4.87 (1H, broad m., $-CH_2-CHOAc-CH_2-$). This observation indicated the presence of a primary and secondary hydroxyl group in the factor. Above spectral data suggested that the factor is a normal long-chain fatty acid ester depicted by a general formula I.

The mass spectrum of factor B in the high-mass range is reproduced in Fig. 2,

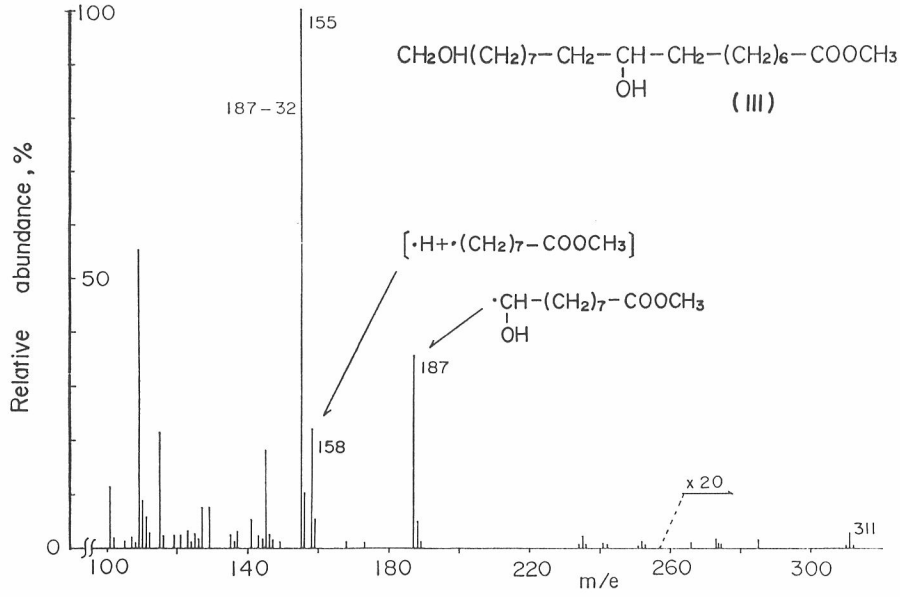

Fig. 2. The mass spectrum of factor B in the high-mass range.

and a molecular ion peak is not seen. It exhibited the highest peak at m/e 311, corresponding to an oxonium ion as illustrated in the type II (m+n=13). The acetate of

$$HO-CH_2(CH_2)_mCH_2-CHOH-CH_2(CH_2)_nCOOCH_3 \qquad (I)$$

$$\left.\begin{array}{l} HO^+=CH(CH_2)_mCH_2-CH=CH(CH_2)_nCOOCH_3 \\ \qquad\qquad\qquad\qquad \text{or} \\ // \qquad\qquad -CH=CH-CH_2- \qquad // \end{array}\right\} \quad (II,\ M\text{-}19)$$

the factor showed also no peak at m/e=M, and the peaks of the highest mass number was found at m/e 294 (M-2×CH_3COOH). The molecular formula $C_{19}H_{38}O_4$ (corresponding to M=m/e 330) was, thus, assigned to the original factor (I, m+n=13).

The prominent peak at m/e 187 in the spectrum of factor B seemed to be caused by ions resulting from the simple α-cleavage on the primary alcohol side with respect to the secondary hydroxyl function (rupture of the 9,10-bond* in III) on the chain. In addition, the fairly high peak at m/e 158 seemed to be due to ions formed by the α-cleavage on the ester side (rupture of the 8,9-bond in III) with rearrangement of one hydrogen atom. The base peak at m/e 155, then, corresponded with ions formed by 9,10-cleavage with simultaneous loss of the elements of methanol. These characteristics were in excellent agreement with those obtained for methyl esters of normal chain hydroxy-acids.[7,8] Thus most probable structural representation for factor B is methyl 9,18-dihydroxystearate (III; I, m=7, n=6).

Characterization of Factor C. Factor C was obtained in yield of 24 mg as colorless needles (*n*-hexane-benzene), m.p. 72–74°.

The i.r. (Fig. 3) and p.m.r. (see experimental) spectra of factor C resemble those

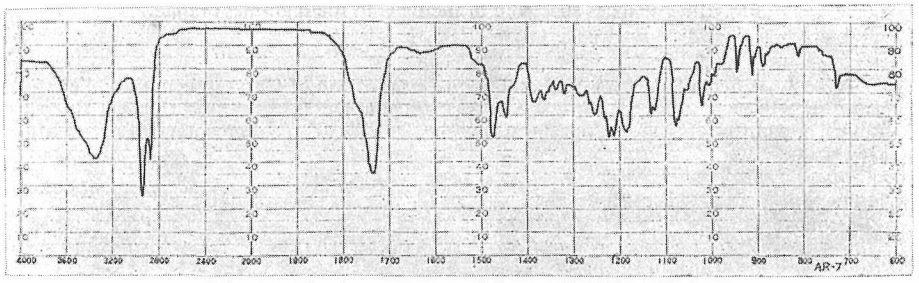

Fig. 3. The i.r. spectrum of factor C (KBr pellet).

of factor B. In the mass spectrum (Fig. 4), the highest peak at m/e 311 corresponding to the ion II (m+n=13) suggested the molecular formula $C_{19}H_{38}O_4$ (M=m/e 330) for factor C. The factor, therefore, seemed to be an isomer of factor B. The characteristic peaks at m/e 169 (the base peak), 172 and 201 in Fig. 4 are exactly similar to those in the case of factor B (Fig. 2) excepting that the peaks are shifted to higher mass number by 14 mass units indicating the presence of a secondary hydroxyl group at C-10 on the carbon skeleton of methyl ω-hydroxystearate.

Based on the above evidences, factor C was assigned the structure IV, methyl 10, 18-dihydroxystearate.

Biological Activity. Factors A, B and C were tested on the stimulating activities of chrysanthemum pollen germination at concentrations of 1, 10 and 100 ppm in a basal medium containing 10 ppm boric acid in 25% sucrose solution. Results are summarized in Table 1.

Factors A and C were strongly active. The degree of their activities was almost the same at a concentration of 10 ppm. Significant differences in activity were observed

* The carboxyl carbon atom is numbered one.

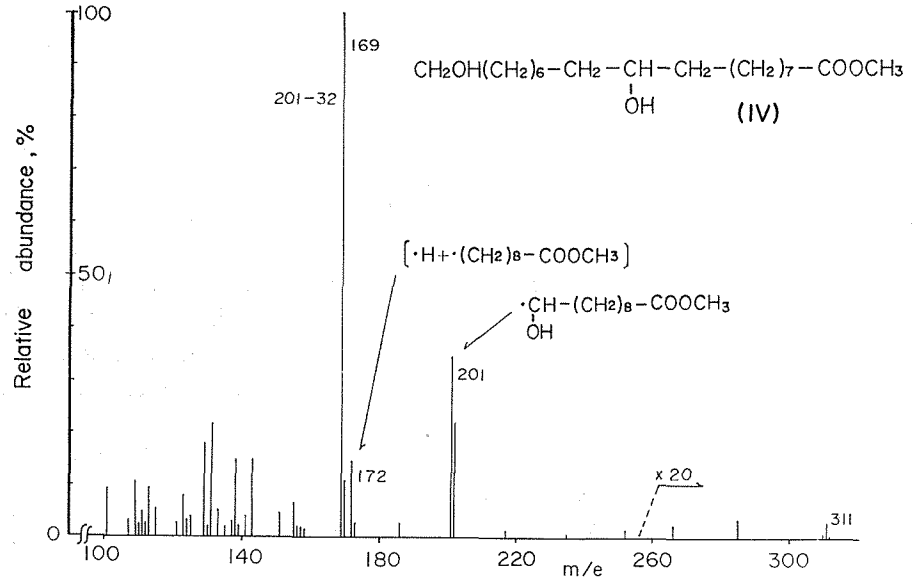

Fig. 4. The mass spectrum of factor C in the high-mass range.

Table 1. Effect of Factors A, B and C on Germination of Chrysanthemum Pollen.

Substance added to medium	Concentration, ppm	Percentage of germination of *Chrysanthemum leucanthemum* pollen
Factor A	100	36.1
	10	57.2
	1	4.2
Factor B	100	31.2
	10	21.0
	1	5.0
Factor C	100	16.4
	10	54.8
	1	28.1
Control (basal medium)		1.2

at the lowest concentration. Factor B was less active than factor C. On the other hand, an isomer of factors B and C, methyl *erythro*-9,10-dihydroxystearate, which was prepared from methyl oleate was completely inactive.

Structurally speaking, the factors might have surface active properties. However, the inactivity of surface active agents, i.e. Tween 80, in the bioassay suggested that stimulation is not due to physicochemical properties of the factors in their capacity as surface active agents. The active principle in factor A may be either α-glyceryl linoleate or α-glyceryl linolenate, or pherhaps both.

EXPERIMENTAL

General. M.ps are uncorrected. U.v. spectra were determined on a Hitachi EPS-3T spectrophotometer and i.r. spectra on a Shimazu AR 275 instrument. P.m.r. spectra were determined at 60 MHz with TMS as an internal standard and mass spectra were taken on a Hitachi RMU-6L spectrometer. Gas chromatography was performed on a Hitachi K-53 instrument with a T.C.D. detector. Stainless steel columns (1 m×3 mm i.d.) were packed with 5% PEG 20 M on 80–100 mesh Chromosorb W. AW. HMPS. Esters were chromatographed on the columns at 190° with a helium flow rate of 17 ml/min. In combined gas chromatography-mass spectrometry, using a Hitachi RMS-4 instrument, peaks were scanned for m/e 10–600 in 30 sec. at a source temp. of 150°. TLC was carried out on silica gel G plates using the following solvent systems (v/v): (a) $CHCl_3$-acetone (3:2), (b) benzene-EtOAc-EtOH (30:20:1). The spots were developed with a 5% soln. of vanillin in conc. H_2SO_4 or with a soln. of ammonium metavanadate (2.0 g) in 50% H_2SO_4 (50 ml). Paper chromatography was performed on Whatman No. 1 paper using *i*-propyl alcohol-AcOH-H_2O (3:1:1 v/v) as a solvent system. Detection of the compounds on paper chromatograms was accomplished with a 0.5% aq. soln. of $KMnO_4$.

Bioassay. The pollen germination activity of the fractions and compounds was measured by the ability to stimulate germination of *Chrysanthemum leucanthemum* pollen as described previously.[4] Samples and fractions to be tested were added at the various concentrations in the basal medium (pH 5.3), which was a 25% sucrose solution containing 10 ppm boric acid. The fresh pollen grains were sown on a drop of the basal medium on a microscopic coverglass placed on a Van Tieghem's cell and incubated at 20° for 2 hrs.

Extraction. Flowers (79 kg) of *Tulipa gesneriana* in full bloom were steeped in MeOH (160 l.) for several weeks at room temp. and filtered. This extraction was repeated with another fresh portion of MeOH. The extracts were combined and their volume was reduced to 8 l. The aqueous concentrate was extracted successively with benzene (3×4.8 l.), ethyl acetate (3×4.8 l.) and *n*-butanol (3×4 l.). The benzene, ethyl acetate and *n*-butanol extracts were each concentrated to give tarry residues (28.4 g, 10.6 g and 71 g, respectively).

Purification and Isolation. (i) *Factor A*: The residue (9 g) from benzene extract was chromatographed on silicic acid (150 g)-Celite (300 g) with benzene containing increasing amounts of EtOAc (0, 5, 10, 20, 30, 50, 70 and 100%). The column chromatography of the same scale was performed three times and the stimulating activity was found in the fractions eluted with 20 and 30% EtOAc in benzene. The 20% EtOAc eluates were combined and evaporated to give a residue (2.5 g), which was then rechromatographed on Florisil (250 g). Elution with 25% EtOAc in benzene gave an oily residue (136 mg). The active residue was further chromatographed on silicic acid (14 g) impregnated with 5% $AgNO_3$, and the chromatograms were controlled by TLC and the bioassay of the individual fractions. EtOAc subsequently eluted the biologically active fractions which gave a violet spot with the vanillin-H_2SO_4 spray reagent at R_f

0.7 in solvent (a) on chromatoplate. These fractions were combined and evaporated to give factor A (70 mg) as a colorless oil. The factor consisted of two components, α-glyceryl linoleate and α-glyceryl linolenate, which did not separate on further purification. Treatment of factor A (20 mg) with acetic anhydride and pyridine gave an oily mixture (18 mg) of the diacetates.

(ii) *Factors B and C*: The another active fraction (700 mg) eluted with 30% EtOAc in (i) was chromatographed on Florisil (7 g).

Factor B (III) was obtained by eluting with 28% EtOAc in benzene and recrystallization from *n*-hexane-benzene as colorless plates (18 mg); m.p. 62–63°; $[\alpha]_D^{20}$ −53.1 (*c* 1.16 EtOH); R_f 0.43 on TLC in solvent (b); δ ($CDCl_3$) 1.2–1.7 (*ca.* 28H, broad s., methylene group), 1.77 (2H, broad s., –O*H*), 2.31 (2H, t., J=7 Hz, –CH_2–COOCH_3), 3.67 (3H, s., –COOCH_3) and 3.45–3.78 (3H, m., –CH_2–C*H*(OH)–CH_2– and –CH_2OH). Factor B formed an oily diacetate; δ($CDCl_3$) 1.2–1.7 (*ca.* 28H, broad s., methylene group), 2.03 (6H, s., –OAc), 2.30 (2H, t., J=6.8 Hz, –CH_2–COOCH_3), 3.67 (3H, s., –COOCH_3), 4.05 (2H, t., J=6.5 Hz, –CH_2OAc) and 4.87 (1H, m., –C*H*(OAc)–); the highest peak in MS, m/e 294 (M–120).

Factor C (IV) was eluted immediately after III with 29% EtOAc in benzene from the column and recrystallized from *n*-hexane-benzene as colorless needles (24 mg); m.p. 72–74°; $[\alpha]_D^{20}$ −62.0° (*c* 1.01 EtOH); R_f 0.39 on TLC in solvent (b); δ($CDCl_3$) 1.2–1.7 (*ca.* 28H, broad s., methylene group), 1.75 (2H, broad s., –O*H*), 2.30 (2H, t., J=7 Hz, –CH_2–COOCH_3), 3.67 (3H, s., –COOCH_3) and 3.45–3.78 (3H, m., –C*H*(OH)– and –CH_2OH). Factor C formed an oily diacetate; δ($CDCl_3$) 1.2–1.7 (*ca.* 28H, broad s., methylene group), 2.03 (6H, s., 2 × –OAc), 2.30 (2H, t., J=6.8 Hz, –CH_2–COOCH_3), 3.67 (3H, s., –COOCH_3), 4.05 (2H, t., J=6.5 Hz, –CH_2OAc) and 4.86 (1H, m., –C*H*(OAc)–); the highest peak in MS, m/e 294 (M–120).

Alkaline hydrolysis of factor A. To a soln. of factor A (30 mg) in MeOH (3 ml) was added 50% aq. soln. of KOH (0.3 ml). After 12 hrs. at room temp. (20°), the reaction mixture was worked up in the usual manner by $(Et)_2O$ extraction and separation to give acidic products. The aqueous mother liquor remained, after adjustment to pH 7, was evaporated to dryness. Extraction of the residue with abs. MeOH yielded a combined extract which was filtered and concentrated to a small volume. The concentrate was purified on Whatman No. 1 paper to yield a crude alcohol (3.9 mg). When the alcohol was acetylated with acetic anhydride-pyridine and chromatographed over silicic acid, it yielded an oily acetate (5 mg) and identified as triacetin by comparison (p.m.r., i.r. and co-TLC) with an authentic sample.

The acidic products in a drop of MeOH were esterified with excess ethereal diazomethane. The total ester mixture, isolated after 12 hrs, was directly examined by GLC and GC-MS, and the two esters were identified as α-methyl linoleate and α-methyl linolenate by comparison with authentic samples.

Preparation of methyl erythro-9,10-dihydroxystearate. The *erythro*-diol was prepared from oleic acid according to the method of Coleman *et al.*,[9] and satisfactory analyses were obtained for the compound characterized by its constitutional formula.

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