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6-D-GLUCOPYRANOSYL FATTY ACID ESTERS FROM *BRASSICA NAPUS* POLLEN

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Key Word Index—*Brassica napus*; Cruciferae; rape pollen; identification; glucosyl esters; fatty acids.

Abstract—6-D-Glucopyranosyl esters of palmitic, oleic, linoleic and linolenic acids were identified in *Brassica napus* (rape) pollen. These esters are inactive as plant growth promoters in the bean second-internode bioassay.

INTRODUCTION

In previous work, fractionation of *Brassica napus* (rape) pollen gave a preparation called 'brassins' which exhibited novel plant growth-promoting activity, and which contained a series of glucosyl esters of various fatty acids [1]. When bioassayed by the bean second-internode method [2], brassin preparations cause both elongation and thickening of the internode. As the concentration of the active principle increases, swelling and curvature of the stem are enhanced and often give rise to split internodes. The esters were reported earlier [1] to be 1-glucosyl esters mainly on the basis of MS evidence obtained from a mixture. Individual esters were not separated, and there was no correlation of biological activity with any physical measurement [3]. As a continuation of this research, we report here a series of new 6-glucosyl esters from rape pollen which are distinct from the unidentified active principle. Esters identified as 6-D-glucopyranosyl linolenate, palmitate, linoleate and oleate are inactive in the bean second-internode bioassay.

RESULTS AND DISCUSSION

An *iso*-PrOH extract of H_2O washed *Brassica napus* (rape) pollen was fractionated by countercurrent distribution (CCD) and column chromatography on Si gel. The bean second-internode bioassay was used to follow fractionation. Rechromatography of those fractions exhibiting significant brassin-type activity afforded an inactive major fraction which was eluted just prior to the growth-promoting material. The IR (Nujol) spectrum of the inactive fraction showed ester absorption at 1730 cm^{-1}. Transesterification with NaOMe gave Me esters which were analyzed by GLC on the basis of equivalent chain length. The following fatty acid Me esters were characterized (percent composition given in parentheses): 14:0(0.6), 16:0(39), 18:0(0.8), 18:1(3), 18:2(11.8), 18:3(44.8). Assignments were confirmed by GC–MS which showed the appropriate M^+ for each ester. Double bond positions in unsaturated esters were established by GC–MS of TMSi derivatives of the alcohols obtained by per-hydroxylation with OsO_4 [4]. These procedures identified the unsaturated acids as oleic, linoleic and linolenic acids. The identity of D-glucose in hydrolysates of esters subsequently isolated by PLC was established by TLC, GLC and colour reaction.

Individual glucosyl esters were separated by PLC on $AgNO_3$–Si gel. Although the layers exhibited gray streaks indicating reduction of Ag ion, the reaction was only partial. Because reduction of Ag ion was not observed with synthetic 1- and 2-substituted glucosyl esters, an alternate site of fatty acid attachment in the natural material was indicated. Three bands were removed which afforded the following major glucosyl esters in order of decreasing R_f: palmitate, linoleate and linolenate. Alternatively, glucosyl esters could be separated by reversed phase HPLC on μ-Bondapak C_{18}. Esters were eluted in the order: linolenate, linoleate, palmitate and oleate (contaminated with palmitate). HPLC of a biologically active fraction that also contained glucosyl esters resulted in elution of material that exhibited brassin growth-promoting activity well before the elution of glucosyl linolenate.

GC–MS of TMSi derivatives of natural glucosyl esters (see Experimental) gave ions for M^+ minus Me and/or one and two TMSiOH groups. In addition to acylium ions (RCO^+) indicative of the fatty acid, ions corresponding to rearranged RCO_2DMSi^+ were also observed. The derivatized esters exhibited two GLC peaks of variable intensity representing the anomeric mixture; the first peak due to the β-anomer (see below) was usually less intense. The MS of the first GLC peak exhibited strong ions at *m/e* 217 (100%) and 204 (*ca* 50%) while the second peak showed these ions with the intensities reversed [*m/e* 217 (50%), 204 (100%)]. These ions represent three- and two-carbon fragments from the carbohydrate ring [5]. In comparison, MS of synthetic TMSi-1-glucopyranosyl palmitates showed the ion at *m/e* 217 three times stronger than the 204 ion.

The 90 MHz PMR spectrum (C_5D_5N) of natural glucosyl palmitate showed it to be an anomeric mixture of 75% α (δ 5.88, J = 3.5 Hz): 25% β (δ 5.32, J = 7.0 Hz). In contrast, anomeric protons for synthetic 1- and 2-glucopyranosyl palmitates [6, 7] were observed as follows: 1-α, δ 6.92 (J = 3.5 Hz); 1-β, δ 6.41 (J = 6.7 Hz);

2-α:2-β (1:1 mixture), δ 6.10 (J = 3.5 Hz), δ 5.38 (J = 8 Hz). The 220 MHz PMR spectrum of natural glucosyl palmitate was compared with that of synthetic 6-α-D-glucopyranosyl palmitate [8]. Except for differences due to anomeric composition, the spectra were identical. Substitution at C-6 was verified by the observation of the low field resonances of the C-6 methylene protons at δ 4.3 (Me_2CO-d_6) in agreement with previously reported values [9]. However, a detailed analysis of the coupling constants could not be readily performed due to the complexities given by the anomeric mixture and the degeneracy of the C-6 methylene proton resonances. Heating the synthetic α-anomer in C_5D_5N–D_2O at 50° for 15 min gave a 60:40 α:β-anomeric distribution that more closely resembled the natural material. Similarly, a sample consisting of at least 90% α-anomer was converted to a 50:50 mixture after 3 days in C_5D_5N at room temperature. GC–MS of the TMSi derivative of this mixture now showed a corresponding increase in the first GLC peak representing the β-anomer and exhibiting the intense m/e 217 (100%) ion. The mutarotation of glucose in pyridine explains why 6-glucosyl palmitate was obtained as an anomeric mixture [10]. The PMR spectra of natural 6-glucosyl linoleate and linolenate likewise showed the predominant α-anomeric protons at δ 5.88 (J = 3.8 Hz) and δ 5.87 (J = 3.5 Hz) respectively. The amount of β-anomer was not determined because of proximity to the olefinic proton signals centered at δ 5.49.

These esters are new natural products, but are closely related to the steryl-β-D-(6-O-fattyacyl)-glucopyranosides which are well-known plant constituents [11]. Although we were able to separate the brassin growth-promoting activity from the reported 6-glucosyl esters, we were unable to confirm by PMR the presence of 1-glucosyl esters. The following synthetic glucosyl esters were nevertheless evaluated in the bean second-internode bioassay and all failed to exhibit brassin-type activity: 1-α- and 1-β-palmitates; 1-α-stearate; 2-α- and 2-β-palmitates; 6-α-palmitate, oleate, linoleate and linolenate.

EXPERIMENTAL

Extraction and preliminary fractionation. Bee-collected *Brassica napus* L. pollen (70–90% *B. napus* plus mixed pollens) from the McRory Apiaries, Benito, Manitoba, Canada (500 g) was washed with H_2O (1.2 l., 1 l., 1 l.) and centrifuged. The residual pollen (*ca* 250 g) was extracted twice at room temp for 1 hr with *iso*-PrOH (1400 ml, 1000 ml) The extracts were filtered through Celite and conc. under red. pres. to give 55–60 g. Countercurrent distribution was carried out in four 250 ml separatory funnels with CCl_4–MeOH–H_2O (2:1·0.15) [12]. The extract was dissolved in 200 ml of upper and lower phase in funnel 1. Three remaining funnels contained 100 ml of upper phase. Fifteen 100 ml portions of lower phase were sequentially equilibrated with upper phases. Twelve portions of lower phase were drawn off funnel 4 and discarded. Upper and lower phases in funnels 2–4 were combined and conc carefully (foams) under red. pres. to give *ca* 4 g from each 500 g of pollen

Column chromatography. Combined CCD fractions from 1 kg of pollen in MeOH–$CHCl_3$ (5:95) were applied to Si gel (150 g, 70–230 mesh). Material eluted with MeOH–$CHCl_3$ (1:9) was combined on the basis of TLC analysis ($CHCl_3$–MeOH–HOAc, 90:10:1; sprayed with 3% ceric sulfate in 3 N H_2SO_4 and heated at 120°). Fractions exhibiting significant activity in the bean second-internode bioassay were rechromatographed on Si gel with absolute EtOH–toluene. A biologically inactive major fraction was eluted with 10% EtOH (R_f 0.4 on TLC, 95% EtOH-toluene, 1:3) just prior to the active material. Combined inactive fractions from 6 kg pollen (230 mg) were rechromatographed on Si gel (23 g) to give the glucosyl esters (190 mg).

Fatty acid analysis. A soln of glucosyl esters (40 mg) in CH_2Cl_2–MeOH was treated with 0.5 M NaOMe (2 ml) at 60° for 20 min. The soln was cooled, acidfied with 4 N HCl, diluted with H_2O and extracted with hexane to give the Me esters: IR (CCl_4) 1740 cm^{-1}, no *trans* unsaturation. GLC analysis was at 180° on a 3 m × 4 mm column packed with 5% LAC-2-R446. GLC separation of TMSi derivatives of the alcohols obtained from OsO_4 per-hydroxylation of unsaturated Me esters [4] was on a 1.2 m × 4 mm column packed with 3% OV-1 programmed from 150 to 250° at 4°/min.

Sugar identification. Samples of hexose esters from PLC (0.6 mg) in 2 N HCl (1 ml) were heated under reflux for 1 hr, cooled, washed with hexane and evap. to dryness under red. pres. The residues were dissolved in H_2O (30 μl) and applied to Lilly Tes-tape^R urine sugar analysis paper to give an immediate positive reaction for D-glucose. The sugar migrated with D-glucose on TLC using 0.1 N H_3BO_3–Si gel G (*n*-BuOH–Me_2CO–H_2O; 4:5:1). GLC analysis of the TMSi derivative on a 1.2 m × 2 mm column packed with 3% Dexsil 300 and programmed from 100 to 200° at 3°/min confirmed the presence of D-glucose

Separation of glucosyl esters. PLC: Solns of esters in MeOH–$CHCl_3$ (1:9) were applied to 0.5 mm thick layers of $AgNO_3$–Si gel G (5:95) which were developed with EtOH–toluene (1:3). The plates were dried in a stream of N_2 and 1 cm of each side was sprayed with 0.2% 2′,7′-dichlorofluorescein in EtOH. Three bands observed under long UV light were removed and extracted with MeOH–$CHCl_3$ (1:9). Each extract was rechromatographed on a Si gel column (1 g) with EtOH–toluene (1:9). Glucosyl linolenate and linoleate were obtained as oils. Combined samples of glucosyl palmitate were subjected to PLC on Si gel G. Material migrating 5.5–9 cm from the origin was extracted and crystallized from EtOH: mp 129–132°; mmp with synthetic 6-α-D-glucopyranosyl palmitate was not depressed HPLC: A Waters Associates ALC-201 instrument equipped with two 0.33 m × 4 mm ID μ-Bondapak C_{18} columns and a refractive index detector was used. The solvent was MeOH–H_2O (3:1) at 200 kg/cm^2 and 1 ml/min. Typically, glucosyl esters were eluted with the following solvent vols: 18:3 (26–31 ml), 18:2 (37–40 ml), 16:0 (44–52 ml), 16:0 plus 18:1 (53–60 ml).

Synthesis of 6-α-D-glucopyranosyl palmitate. Dry β-D-glucose was allowed to react with palmitoyl chloride [8]. The MeOH–$CHCl_3$ (1:9) soluble portion of the reaction mixture was chromatographed on Si gel with EtOH–toluene to remove some 1-β-D-glucopyranosyl palmitate which eluted prior to the 6-isomer. The major 6-α-ester was crystallized from absolute EtOH: mp 133–135° (lit mp 134–136°). Other 6-glucosyl ester standards were similarly prepared.

GC–MS of glucosyl esters. The esters were converted to TMSi esters by treatment with excess HMDS and TMCS in C_5H_5N for 1 hr at room temp. GLC separation was on a 1 m × 2 mm column packed with 3% OV-1 and programmed from 200 to 350° at 4°/min. Effluent from the GLC was directed to a Dupont (CEC) 21-492-1 MS through a jet-type separator with a transfer line temp. of 300°. Spectra were obtained with an ionizing energy of 70 eV and a source temp. of 250°. MS m/e (rel. int.): 6-α-D-glucopyranosyl linolenate 728 [M^+] (0.3), 713 (0.2), 638 (2), 623 (0.3), 548 (1), 533 (0.3), 335 [RCO_2DMSi^+] (4), 261 [RCO^+] (4), 217 (46), 204 (100), 191 (34). 6-β-D-glucopyranosyl linolenate· 548 (0.7), 533 (0.2), 335 (2), 261 (3), 217 (100), 204 (33), 191 (12).

6-α-D-glucopyranosyl linoleate: 730 [M^+] (0.2), 715 (0.2), 640 (1), 625 (0.3), 550 (1), 535 (0.3), 337 (3), 263 (3), 217 (49), 204 (100), 191 (50). 6-β-D-glucopyranosyl linoleate: 640 (0.6), 625 (0.2), 550 (0.8), 535 (0.3), 337 (3), 263 (6), 217 (100), 204 (46), 191 (23).

6-α-D-glucopyranosyl oleate· 717 [M^+-Me] (0.1), 642 (2), 627 (0.2), 552 (1), 537 (0.2), 339 (3), 265 (4), 217 (51), 204 (100), 191

(52). 6-β-D-glucopyranosyl oleate: 642 (0.3), 627 (0.1), 552 (1), 537 (0.2), 339 (2), 265 (4), 217 (100), 204 (43), 191 (18).

6-α-D-glucopyranosyl palmitate: 691 [M^+-Me] (1), 616 (0.7), 601 (1), 526 (1), 511 (1), 313 [RCO_2DMSi^+] (12), 239 [RCO^+] (8), 217 (54), 204 (100), 191 (44). 6-β-D-glucopyranosyl palmitate: 691 (1), 616 (1), 526 (1), 511 (1), 313 (21), 239 (17), 217 (100), 204 (55), 191 (39).

1-α-D-glucopyranosyl palmitate: 691 (0.6), 616 (0.3), 601 (2), 511 (3), 313 (10), 239 (18), 217 (99), 204 (30), 191 (71). 1-β-D-glucopyranosyl palmitate: 691 (0.3), 601 (0.4), 511 (0.6), 313 (6), 239 (11), 217 (100), 204 (35), 191 (25).

2-α:2-β-D-glucopyranosyl palmitate: 691 (0.7), 616 (0.3), 601 (1), 511 (1), 313 (9), 239 (18), 217 (93), 204 (39), 191 (71).

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REFERENCES

1. Mandava, N. and Mitchell, J. W. (1972) *Chem. Ind.* 930.
2. Mitchell, J. W. and Livingston, G. A. (1968) *Methods of Studying Plant Hormones and Growth-Regulating Substances*, p. 26. Agriculture Handbook No. 336, U.S. Government Printing Office, Washington, D.C.
3. Milborrow, B. V. and Pryce, R. J. (1973) *Nature* **243**, 46.
4. Murawski, U. and Egge, H. (1975) *J. Chromotogr. Sci.* **13**, 497.
5. DeJongh, D. C., Radford, T., Hribar, J. H., Hanessian, S., Bieber, M., Dawson, G. and Sweeley, C. C. (1969) *J. Amer. Chem. Soc.* **91**, 1728.
6. Pfeffer, P. E., Rothman, E. S. and Moore, G. G. (1976) *J. Org. Chem.* **41**, 2925.
7. Pfeffer, P. E., Moore, G. G., Hoagland, P. D. and Rothman, E. S. (1977) *Synthetic Methods for Carbohydrates* (El Khadem, H. S., ed.), p. 155, ACS Symposium Series, Vol. 39. American Chemical Society, Washington, D.C.
8. Reinefeld, E. and Korn, H. F. (1968) *Staerke* **20**, 181.
9. Moyer, B. G., Pfeffer, P. E., Moniot, J. L., Shamma, M. and Rustine, D. L. (1977) *Phytochemistry* **16**, 375.
10. Hill, A. S. and Shallenberger, R. S. (1969) *Carbohydr. Res.* **11**, 541.
11. Aneja, R. and Harries, P. C. (1974) *Chem. Phys. Lipids* **12**, 351
12. Hecker, E. and Schmidt, R. (1974) *Fortschr. Chem. Org. Naturst.* **31**, 377.

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NEUE EUDESMAN-DERIVATE AUS *PLUCHEA FOETIDA**

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Key Word Index—*Pluchea foetida*; Compositae; new eudesmane derivatives.

Die bisher untersuchten *Pluchea*-Arten (Tribus Inuleae) enthalten neben Thiophenacetylenverbindungen vor allem Eudesman-Derivate mit charakteristischem Substitutionsmuster [1]. Auch die Wurzeln von *Pluchea foetida* (L.) DC. enthalten **1**, **2** und **3**, während die oberirdischen Teile neben **2**, **3**, und **9** die ebenfalls bekannten Sesquiterpene **4** und **5** enthalten. Daneben isoliert man zwei weitere Verbindungen, bei denen es sich nach den spektroskopischen Daten um **6** und **7** handelt (s. Tabelle 1). **6** ist bereits durch Verseifung des Cuauhtemondiesters **5** erhalten worden [2]. Die Konstitution von **6** wird weiterhin durch das ^{1}H-NMR-Spektrum des daraus erhaltenen Acetonids **8** gesichert. Die vorliegende Untersuchung zeigt erneut, daß für die Gattung *Pluchea* offenbar Verbindungen vom Typ **1** und **2** bzw. **3**–**7** charakteristisch sind.

EXPERIMENTELLES

IR. Beckman IR 9; ^{1}H-NMR. Bruker WH 270, $CDCl_3$, δ-Werte, TMS als innerer Standard; MS. Varian MAT 711, 70 eV, Direkteinlaß; Optische Rotation: Perkin–Elmer-Polarimeter, $CHCl_3$. Die lufttrockenen zerkleinerten Pflanzenteile (Herbar Nr. R.M.K. 230) extrahierte man mit Et_2O–Petrol (1:2) und trennte die erhaltenen Extrakte zunächst grob durch SC (Si gel, Akt.-St. II) und weiter durch mehrfache DC (Si gel, GF 254). 50 g Wurzeln ergaben 13 mg **1** und **2** (*ca* 1:1) und 8 mg **3**, während 500 g oberirdische Teile 50 mg **9**, Spuren von **2**,

Tabelle 1 ^{1}H-NMR-Daten von **6**, **7** und **8** (270 MHz, δ-Werte)

	6	**7**	**8**
3β-H	*dd* 3.67	*dd* 5.91	*dd* 3.94
6α-H	*dd* 2.95	*d* 6.94	*dd* 2.87
6β-H	*dd* 2.10		*dd* 2.07
9α-H	*d* 2.20	*d* 2.32	*s* 2.20
9β-H	*d* 2.27	*d* 2.41	
12-H	*d* 2.01	*s* 1.58	*s*(*br*) 1.99
13-H	*s*(*br*) 1.84	*s* 1.45	*s*(*br*) 1.83
14-H	*s* 1.22	*s* 1.45	*s* 1.26
15-H	*s* 0.93	*s* 1.01	*s* 0.89
OCOR	—	*q* 3.06	—
		d 1.32	
		s 1.56	
OAc	—	*s* 2.02	—

J (Hz) **6**: 2α,3β = 2β,3β = 3; 5α,6α = 4; 5α,6β = 13; 6α,6β = 15; 6α,12 = 2; 9α,9β = 15; **7**: 2α,3β = 2β,3β = 3; 5α,6 = 2.5; 9α,9β = 17; 17,18 = 6. **8**: 2α,3β = 2β,3β = 3; 5α,6α = 3; 6α,6β = 13 (Acetonid *s* 1.49 und 1.38).

* 146. Mitteil. in der Serie 'Natürlich vorkommende Terpen-Derivate'; 145. Mitteil. Bohlmann, F. und Le Van, N. (1978) *Phytochemistry* **17**, 1173.