Phytochemistry, 1976, Vol. 15, pp. 421–424. Pergamon Press. Printed in England

ISOLATION OF GIBBERELLINS A_3, A_4 AND A_7 FROM *PINUS ATTENUATA* POLLEN*

ANIELA KAMIENSKA, RICHARD C. DURLEY† and RICHARD P. PHARIS
Department of Biology, University of Calgary, Calgary, Alberta T2N 1N4, Canada

(*Received* 15 *August* 1975)

Key Word Index—*Pinus attenuata*; Pinaceae; pollen; gibberellins; isolation; GA_3; GA_4; GA_7; GC–MS.

Abstract—Three native gibberellins of *Pinus attenuata* pollen, GA_3, GA_4 and GA_7 have been characterized by GC–MS and a fourth, less polar, GA with chromatographic characteristics similar to GA_9 was shown to be present. At least two other as yet unidentified, less polar GA-like substances are also present in the dormant and/or germinating pollen. The concentration of the GA_9-like substance, and of GA_4 and GA_7, decreases during germination, while peaks of biological activity of a more polar nature increase. The most predominant of the polar peaks present 15 hr after germination was GA_3.

INTRODUCTION

Previous work [1] using dormant and germinating pollen of *Pinus attenuata*, *P. coulteri* and *P. ponderosa* showed that *P. attenuata* contained the largest amounts of gibberellin (GA)-like substances. In dormant pollen there were large amounts of "less-polar" GAs (those GAs chromatographically similar to GA_4, GA_5, GA_9 etc.) and "polar" GAs (those chromatographically similar to GA_1, GA_3, GA_8, etc.). On germination, the amounts of less-polar GAs decrease whereas the amounts of polar GAs increase markedly, reaching a maximum after *ca* 15 hr, which corresponds with the time of maximal growth of the pollen tubes [1]. It is known that a large number of higher plants, as well as the fungus *Gibberella fujikuroi*, convert less polar GAs to more polar GAs and to butanol soluble (presumably highly polar acidic or conjugated compounds [2–7]. Thus it is reasonable to suppose that in *P. attenuata* the less polar GAs present in large amount in dormant pollen are converted to more polar and possibly more active GAs during germination. This has been demonstrated for [^{3}H]-GA_4 which was metabolized to GA_1, GA_{34} and other more polar substances [8].

Since endogenous GAs are correlated with the germination and subsequent elongation of the pollen tube of *P. attenuata* [1] it is important to undertake identification of the major GAs in this system. In the present paper the identification by GC–MS of three of the major GA components of *P. attenuata* is reported.

RESULTS

Germinating pollen

GAs were extracted with MeOH 15 hr after germination and purified by PVP, Sephadex G-10, silica gel (gradient A) and TLC, as described in the Experimental. Fractions from the silica gel partition column were bioassayed and biological activity is shown in Fig. 1.

Since the amount of GA-like substance(s) located in fractions 17–19 was relatively high [i.e. up to 200 μg/kg (Fig. 1)] an attempt was undertaken to extract and isolate this substance(s) first. Additional purification was accomplished by a second silica gel partition column (gradient A) and TLC (system 1). The biologically active substance(s), which was chromatographically similar to GA_3 was examined by GLC as the MeTMSi derivative. Peaks were observed with R_t (16 min on 2% QF 1 and 16·5 min on 2% SE 30) identical with those of standard GA_3MeTMSi and further confirmation was obtained by GC–MS [9].

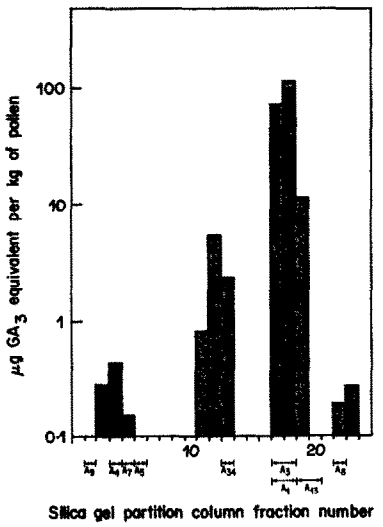

Fig. 1. Biological activity obtained from germinating (15 hr) *Pinus attenuata* pollen after initial purification and Si gel partition chromatography. Fractions 17–19 used in characterization of GA_3. Fractions 1–13 used in characterization of GA_4 and GA_7.

* Part 1 in the series "Endogenous Gibberellins of Pine Pollen".

† Present address: Department of Crop Science, University of Saskatchewan, Saskatoon, Saskatchewan. S7N 0W0, Canada.

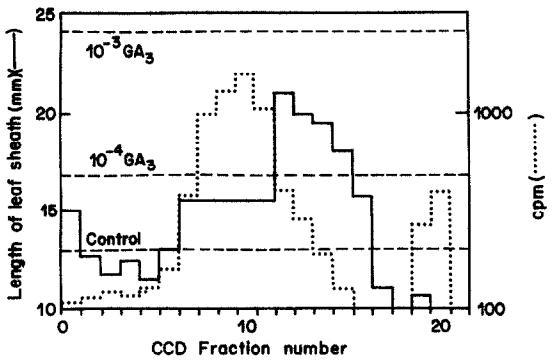

Fig. 2. Biological activity and radioactivity of less polar GA-like substances from germinating pine pollen after counter-current distribution (CCD). The distribution of activity in extract from dormant pollen was much the same.

Fractions 1–13 from the silica gel partition column (Fig. 1) which contained less polar GA-like substances were combined. Since the amount of GAs in these fractions was relatively low as determined by bioassay, a trace amount of [^{3}H]-GA_4 was added to facilitate their purification. The amount of [^{3}H]-GA_4 added was sufficient to follow purification procedures by radioactivity, but insufficient to be detected by bioassay, GLC or GC–MS. The distribution of biological activity and radioactivity after counter-current distribution (CCD) is shown in Fig. 2. It should be noted here that whereas 95 + % of [^{3}H]-GA_4 will normally be localized in the center tubes of a test CCD (i.e. w/o extract), addition of 391·1 mg plant extract to the trace amount of [^{3}H]-GA_4 caused 17% of the radioactivity to migrate with the solvent front (Fig. 2). What this implies for endogenous GAs of a less polar nature is uncertain, although one could assume that a similar situation is indeed occurring.

The biologically active tubes (7–15) from CCD were further purified by Sephadex G-25 partition chromatography [10] which separates GA_4 and GA_7 and the distribution of biological activity and radioactivity is shown in Fig. 3; GA_4 would be expected to elute in zones 56–67 and GA_7 in zones 83–97. Here again one should note that the trace amount of [^{3}H]-GA_4 cannot be detected by bioassay at dilutions of 1/150 and 1/500.

Each of the active zones was further purified by TLC, silica gel partition column chromatography (gradient B) and all biologically active compounds were examined by GLC as the MeTMSi derivatives. From 4 biologically active fractions (Fig. 3) only 2 (fractions 56–67 and 83–97) gave peaks comparable to standard GAs. From fractions 56–67 GLC peaks were observed (R_t 9·6 min on 2% QF 1, 8·7 min on 2% SE 30 and 11·3 min on 1% XE 60) which corresponded to those of GA_4MeTMSi and confirmation was obtained by GC–MS (published spectrum [9]). From fractions 83–97 small peaks were observed in the GA_7MeTMSi zone (R_t 10·8 min on 2% QF 1, 9·3 min on 2% SE 30, and 14·4 min on 1% XE 60) but the quantities were insufficient for proper identification. The amount of GA_4 exceeded by several times the amount of GA_7, but exact quantities of these GAs were not determined by GLC peak area, since extensive purification procedures considerably lowered the amount. By bioassay, GA_4 and GA_7 were estimated to be present in amounts of 0·7 μg/kg and 0·2 μg/kg fr wt germinating pollen, respectively.

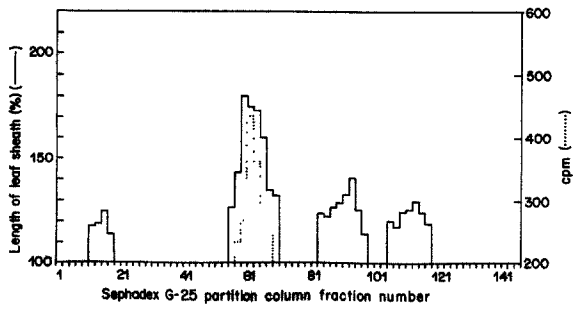

Fig. 3. Biological activity and radioactivity obtained after partition chromatography of active CCD fractions 7–15 from germinating pine pollen (see Fig. 2) on Sephadex G-25.

The less polar biologically active substance(s) from fractions 11–17 (Fig. 3) were purified by TLC using system 2 (Fig. 4). The substance(s) at R_f 0·9 corresponded to GA_9. When chromatographed on a silica gel partition column (gradient B) it was localized in fractions 4–6, the same as standard GA_9. However on GLC the esterified derivative gave peaks corresponding to GA_9MeTMSi on 2% QF 1 and 1% XE 60, but not on 2% SE 30 column. When this substance was chromatographed by TLC in solvent systems 3–10 it gave identical R_f values to those of standard GA_9 only in system 10. We were therefore unable to identify this GA-like substance as GA_9. As the MeTMSi derivative the sample did not give distinct peaks corresponding to other GAs (1 → 42).

Fractions 105–119 (Fig. 2), when subjected to similar procedures as described above, gave biologically active peaks (Fig. 5) which separated into at least 4 substances of GA-like biological activity. Insufficient quantities, however, precluded GLC analysis.

Dormant pollen

Similar procedures were used for dormant pollen. Biological activity obtained after silica gel partition column chromatography is shown in Fig. 6. Active fractions (7–15) from CCD were combined and purified further by Sephadex G-25 partition chromatography (Fig. 7). This gave 3 biologically active zones in fractions 12–17,

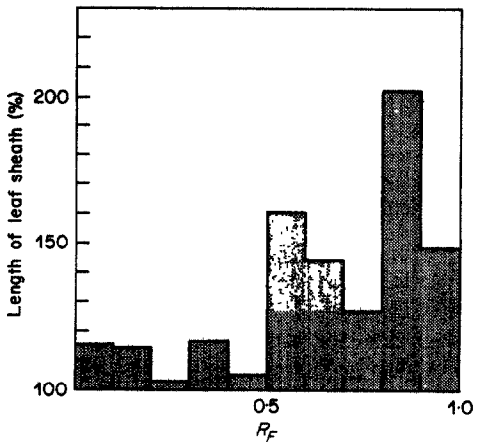

Fig. 4. Biological activity of GA_9-like zone from Sephadex G-25 partition column (fraction 11–17) from germinating pollen after TLC in solvent system 2.

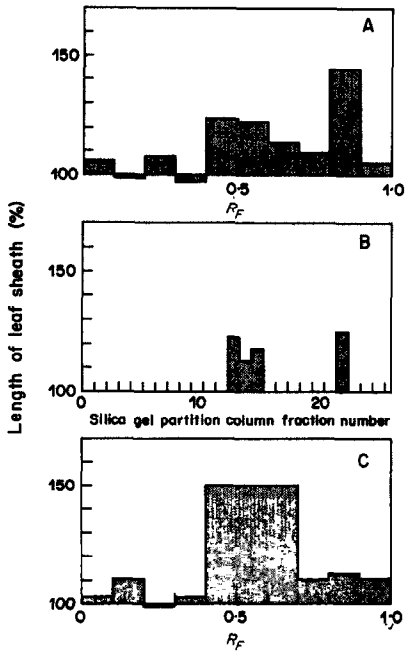

Fig. 5. Biological activity of fractions 105–119 from Sephadex G-25 partition column (see Fig. 3) from extract of germinating pollen after TLC in solvent system 2(A). Active zone R_f 0·8–0·9 after Si gel column chromatography in gradient B separated into 2 biologically active fractions (B). Fractions 13–15 were then subjected to TLC in solvent system 1(C).

62–85 and 104–115. Overlapping peaks in fractions 62–85 correspond to 2 peaks (fractions 56–67 and 83–97) from the earlier Sephadex G-25 column for germinating pollen. Due to indefinite separation of the compounds they were treated as one sample.

Zones 62–85 were further purified by TLC (system 1) and silica gel partition column chromatography (gradient B). Biologically active substances were extracted and a sample was examined by GLC as the MeTMSi derivatives. Peaks were observed which corresponded to GA_4MeTMSi (R_t 9·6 min on 2% QF 1, 8·7 min on 2% SE 30 and 11·3 min on 1% XE 60) and GA_7MeTMSi (R_t 10·8 min on 2% QF 1, 9·3 min on 2% SE 30 and

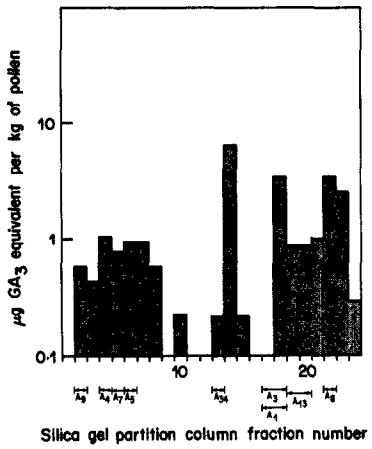

Fig. 6. Biological activity obtained from dormant pollen after purification on Si gel partition column chromatography. Fractions 1–13 used in characterization of GA_4 and GA_7.

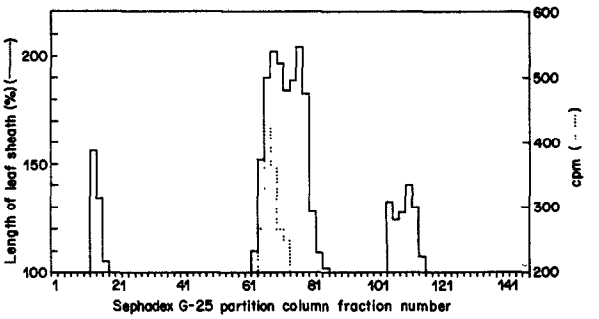

Fig. 7. Biological activity and radioactivity obtained from dormant pollen after partition chromatography on Sephadex G-25.

14·4 min on 1% XE 60). The peaks corresponding to GA_7MeTMSi were very clear but the peaks corresponding to GA_4MeTMSi were sometimes distorted by other larger peaks of unknown substances. The sample was further examined by GC–MS using a 2% QF 1 column and a scan of the 10·7 min peak gave a "mixed" MS with ions at *m/e* 418 (M^+) and fragment ions at *m/e* 224, 225, 284 and 289 characteristic of GA_4MeTMSi [9]. Other ions observed in the MS were of an unknown impurity and could be observed in the spectrum at the correct relative intensities which co-chromatographed with GA_4MeTMSi. A scan of the peak at 12·2 min gave a MS identical with that of the published spectrum [9] of GA_7MeTMSi. GA_7 was present in a larger amount than GA_4 but again exact quantities of these GAs were not determined by GLC, since the extensive purification procedures considerably lowered the actual amounts. According to bioassay, determined after TLC and prior to GLC, there was 1·1 μg/kg dormant pollen of GA_4 and 1·3 μg/kg of GA_7.

Biologically active zones 12–17 and 104–115 (Fig. 7) were examined in a similar manner to fractions 11–17 and 105–119 from the corresponding extract of germinating pollen. Again, the active substance in fractions 12–17 was similar to but distinct from GA_9. The active substances from fractions 104–115 were chromatographically similar to the substances found in fractions 105–119 from germinating pollen (Fig. 5).

EXPERIMENTAL

Plant material. Pollen of *Pinus attenuata* was used for analysis 15 hr after germination (1200 g) or in the dormant state (900 g). Conditions for storing and germination were given previously [1].

Large scale extraction of GAs. Pollen, frozen in liquid N_2 was extracted 2× in 80% MeOH and once in 0·5 M phosphate buffer at pH 8. The aq residue after evaporating the MeOH *in vacuo* was combined with the buffer extract and washed ×3 with Et_2O at pH 9 to remove neutral substances. The buffer fraction was then partitioned with EtOAc at pH 3 and the EtOAc fractions after evaporation *in vacuo*, were then purified on Sephadex G-10 [11] and PVP [12] columns followed by chromatography on a Si gel partition column using solvent gradient A (see below). 25 fractions were collected and bioassayed [13].

Germinating pollen. Fractions 17–19 (Fig. 1) were combined and the residues purified on a second Si gel partition column (gradient A) and TLC using solvent system 2. Biologically active substances eluted from zones R_f 0·3–0·4, were methylated and silylated to give MeTMSi derivatives and chromatographed on GLC using 2% QF 1 and 2% SE 30, and finally

examined by GC–MS. Fractions 1–12 from the extract of germinating pollen were further purified by CCD [11,14]. Trace of radioactive GA_4 was added prior to CCD. Biologically active fractions (7–15) were then combined and chromatographed on Sephadex G-25 partition column [10]. This gave 4 biologically active zones I—fractions 11–17, II—fractions 56–67 (radioactive) III—fractions 83–97, and IV—fractions 105–119. Each of these zones was further purified by TLC (solvent system 1 and 2).

I. Fractions 11–17 after TLC (solvent system 1) gave two active zones, R_f 0·5–0·7 and R_f 0·8–0·9. Extracts from each of these active zones were further purified on a Si gel partition column using solvent B. Substances located at R_f 0·5–0·7 from TLC separated, after this procedure, into a substance located at fractions 12–13 and another one at 22–24. Substance(s) located at R_f 0·8–0·9 from TLC gave one biologically active peak in fractions 4–6 after Si gel partition column chromatography. Active fractions were eluted, combined, and examined by TLC in systems 3–10. Active substance(s) were converted to MeTMSi derivatives and analyzed by GLC.

II. Fractions 56–67 after TLC (solvent system 2) showed activity localized at R_f 0·3–0·6. These R_f zones were eluted and active substance, as a MeTMSi derivative, analyzed by GLC and GC–MS.

III. Fractions 83–97 were subjected to identical procedures as fractions 56–67.

IV. Fractions 105–119 were combined and analyzed by TLC (solvent system 1) giving 2 active R_f zones, 0·4–0·6 and 0·8–0·9. They were further purified on a Si gel partition column (gradient B) and substances localized at R_f 0·4–0·6 gave an active zone in fractions 10–12. Substances localized at R_f 0·8–0·9 separated into 2 active compounds localized at fractions 13–15 and 22. Fractions 13–15 were eluted and run on TLC (solvent system 2) showing biological activity at R_f 0·4–0·7. All these active compounds were converted to MeTMSi derivatives and analyzed by GLC.

Dormant pollen. Identical procedures to those described above were used.

Use of trace amount of [^{3}H]-GA_4. In order to more accurately follow the fate of the less polar GAs in the extract, a trace amount of [^{3}H]-GA_4 [16 nCi, 1·87 Ci/mmol sp. act. (equivalent to 2·75 ng GA_4)] was added to the pooled Si gel partition column fractions prior to CCD. The amount of [^{3}H]-GA_4 added was below the lowest level of rice bioassay sensitivity (13·7 pg/seedling) when assayed in serial dilution.

Si gel partition column chromatography. Columns 18 cm × 13 or 18 mm id packed with Si gel for partition (Woelm) were used with the following solvent gradients. A. Chamber 1–4: 65%, 40%, 100% and 100% EtOAc in hexane (w/w), respectively (initial separation of GA-like substances). B. Chamber 1–4: 35%, 50%, 65%, 100% EtOAc in hexane (w/w), respectively (resolution of less polar GA-like substances). Procedures employed were the same as those described previously [15].

Countercurrent distribution (CCD). A 20 tube 200 ml manual extractor was used at an appropriate pH with 0·5 M Pi buffer and EtOAc so that GA_4 was localized in the centre tubes [11,14]. Since large changes in mobility of the less polar GAs are caused by relatively minor changes in pH, the use of [^{3}H]-GA_4 simplified the pin-pointing of 7·24 as the final pH. Under these conditions [^{3}H]-GA_4 and GA_7 peaked in tubes 9–13 (as determined by liquid scintillation spectrometry and examining the fluorescence of aliquots sprayed with H_2SO_4 under UV light).

Sephadex G-25 partition column chromatography. A column 100 cm long, 1·8 cm id eluted with C_6H_6–petrol (60°–80°)–HOAc–H_2O, 6:2:5:3 [10] was used and radio- and biological activities monitored.

TLC. Si gel HR with the following solvent systems was used. 1. EtOAc–$CHCl_3$–HCO_2H, 45:55:1; 2. EtOAc–$CHCl_3$–HCO_2H, 50:50:1; 3. EtOAc–$CHCl_3$–HOAc, 8:12:0·5; 4. EtOAc–$CHCl_3$–HOAc, 10:10:0·5; 5. EtOAc–$CHCl_3$–HOAc, 5:15:0·3; 6. EtOAc–C_6H_6–HOAc, 8:12:0·4; 7. *iso*-Pr_2O–HOAc, 100:3; 8. *iso*-Pr_2O–HOAc, 100:0·5; 9. *n*-BuOH–NH_4OH, 5:1; 10. *iso*-PrOH–H_2O, 4:1.

GLC. Columns used were 1·8 m × 3 mm packed with either 2% QF 1 (203°) or 2% SE 30 (202°) or 1% XE 60 (206°) using N_2 at 45 ml/min. Injector and FID detector temps. were both maintained at 250°. Preparation of MeTMSi derivatives was as described previously [16].

GC–MS. This was carried out on an instrument using a double stage Biemann-Watson type molecular separator. Columns were 1·8 m × 2 mm packed with 2% QF 1 and were either temp programmed from 170–120° at 4°/min or maintained at 195°, using He at 18 ml/min.

Bioassay. The sheath length of dwarf rice cv. Tan-ginbozu [13] was used as an indicator of biological activity. Extracts were applied in 1/2 μl of 95% EtOH in serial dilutions ranging from 1/150 to 1/10000.

Acknowledgements—The authors wish to acknowledge the gift of *Pinus attenuata* pollen, kindly supplied by Dr. S. Krugman, Institute of Forest Service, Pacific Southwest Forest and Range Experiment Station, Placerville, California. Financial support was supplied by Canadian Forestry Service Extramural Grant, NRC Grant No. A-2585 and a Weyerhaeuser Grant to R.P.P.

REFERENCES

1. Kamienska, A. and Pharis, R. P. (1975) *Plant Physiol.* **56,** 655.
2. Pitel, D. W., Vining, L. C. and Arsenault, G. P. (1971) *Can. J. Biochem.* **49,** 194.
3. Durley, R. C. and Pharis, R. P. (1973) *Planta* **109,** 357.
4. Durley, R. C., Railton, I. D. and Pharis, R. P. (1973) *Phytochemistry* **12,** 1609.
5. Railton, I. D., Durley, R. C. and Pharis, R. P. (1974) *Plant Physiol.* **54,** 6.
6. Durley, R. C., Railton, I. D. and Pharis, R. P. (1974) *Phytochemistry* **13,** 547.
7. Railton, I. D., Murofushi, N., Durley, R. C. and Pharis, R. P. (1974) *Phytochemistry* **13,** 793.
8. Kamienska, A., Durley, R. C. and Pharis, R. P. (1976) *Plant Physiol.* submitted.
9. Binks, R., MacMillan, J. and Pryce, R. J. (1969) *Phytochemistry* **8,** 271.
10. Pitel, D. W., Vining, L. C. and Arsenault, G. P. (1971) *Can. J. Biochem.* **49,** 185.
11. Crozier, A., Aoki, H. and Pharis, R. P. (1969) *J. Exp. Botany* **20,** 786.
12. Glenn, J. L., Kuo, C. C., Durley, R. C. and Pharis, R. P. (1972) *Phytochemistry* **11,** 345.
13. Murakami, Y. (1968) *Bot. Mag.* **81,** 33.
14. Durley, R. C. and Pharis, R. P. (1972) *Phytochemistry* **11,** 317.
15. Durley, R. C., Crozier, A., Pharis, R. P. and McLaughlin, G. E. (1972) *Phytochemistry* **11,** 3029.
16. Cavell, B. D., MacMillan, J., Pryce, R. J. and Sheppard, A. C. (1967) *Phytochemistry* **6,** 867.