fraction eluted with 50% EtOAc–petrol gave an oil which was further purified by PLC to afford 8β-hydroxyfruticolone (0.5 g) as an oil which was homogeneous by TLC. It showed $\nu_{max}$ 3450, 1735 (*br*), 875 $cm^{-1}$; δ 1.28 (3H, *s*, 20-$H_3$), 1.46 (3H, *s*, 17-$H_3$), 2.01 (3H, *s*, OAc), 2.55 and 3.22 (each 1H, *d*, *J*-15 Hz, 7-$H_2$), 2.25 and 3.37 (each 1H, *d*, *J* = 5 Hz, 18-$H_2$), 2.62 (1H, *m*, 10-H), 4.41 (1H, 27 Hz, 1-H), 4.94 and 5.47 (each 1H, *d*, *J* = 13 Hz, 19-$H_2$), 6.25, 7.18 and 7.30 (each 1H, *m*, 14, 15, and 16-H), MS 406 ($M^+$, $C_{22}H_{30}O_7$), 388, 373, 328, 315, 297, 234, 215, 203, 175, 159, 135, 121, 109, 95, 81.

*Oxidation with chromium trioxide.* 8β-Hydroxyfruticolone (200 mg) in dry Py (3 ml) was treated with $CrO_3$ (200 mg) at room temp. 18 hr. The soln was diluted with EtOAc and washed with dil. HCl, $H_2O$ and dried. The solvent was evapd and the residue chromatographed on $SiO_2$. Elution with 30% EtOAc–petrol gave (**6**) as an oil, δ 1.48 (3H, *s*, 20-$H_3$), 1.97 (3H, *s*, 17-$H_3$), 1.99 (3H, *s*, OAc), 2.85 and 3.25 (each 1H, *d*, *J* = 4 Hz, 18-$H_2$), 3.28 (1H, *s*, H-10), 3.92 and 4.19 (each 1H, *d*, *J* = 11 Hz, 19-$H_2$), 5.92 (1H, *s*, 7-H), 6.20, 7.17 and 7.24 (each 1H, *m*, 14, 15, 16-H), MS 386 ($M^+$), 371, 313, 292, 261, 231, 219, 201, 159, 109, 95, 81. Elution with 50% EtOAc–petrol gave the diketone (**5**) which crystallized from EtOAc–petrol as needles, mp 155° (Found: C, 65.5; H, 6.9 $C_{22}H_{28}O_7$ requires C, 65.3, H, 6.9%), $\nu_{max}$ 3400, 1760, 1700, 1250, 875 $cm^{-1}$, δ 1.38 (3H, *s*, 20-$H_3$), 1.49 (3H, *s*, 17-$H_3$), 2.10 (3H, *s*, OAc), 2.79 and 3.21 (each 1H, *d*, 4 Hz, 18-$H_2$), 2.30 and 3.16 (each 1H, *d*, *J* = 15 Hz, 7-$H_2$), 3.81 (1H, *s*, H-10), 4.42 (2H, *dd*, *J* = 11.5 Hz), 19-$H_2$), 6.26, 7.20 and 7.33 (each 1H, *m*, 14, 15 and 16-H), MS 404 ($M^+$), 386, 313, 292, 159, 109, 95, and 81. The diketone (5) (50 mg) in EtOAc (5 ml) was shaken with 6N HCl (5 ml) for 10 min. The organic phase was dried and the solvent evapd to afford a gum, δ 1.31 and 1.34 (each 3H, *s*, 17 and 20-$H_3$), 1.98 (3H, *s*, OAc), 2.25 and 3.05 (each 1H, *d*, *J* = 15 Hz, 7-$H_2$), 3.50 (1H, *s*, 10-H), 3.90 and 4.15 (each 1H, *d*, *J* = 13 Hz, 19-$H_2$), 4.51 (2H, *s*, 18-$H_2$), 6.22, 7.15 and 7.30 (each 1H, *s*, 14, 15 and 16-H).

*Dehydration of 8β-hydroxyfruticolone.* The diterpenoid (100 mg) was allowed to stand in $CHCl_3$ (2 ml) for 3 days. The solvent was evapd and the product purified by PLC to afford the unsaturated ketone (4), $\nu_{max}$ 1735, 1680, 875 $cm^{-1}$, δ 1.46 (3H, *s*, 20-$H_3$), 1.93 (3H, *d*, *J* = 1 Hz, 17-$H_3$) 1.98 (3H, *s*, OAc), 2.82 and 3.30 (each 1H, *d*, *J* = 4 Hz, 18-$H_2$) 3.30 (1H, *m*, 10-H), 3.92 and 4.20 (each 1H, *d*, *J* = 11 Hz, 19-$H_2$), 5.93 (1H, *s*, 7-H), 6.23, 7.20 and 7.37 (each 1H, *s*, 14, 15 and 16-H), MS 388 ($M^+$), 373, 313, 292, 232, 219, 201, 189, 173, 159, 95, 81.

*Acknowledgements*—We thank the National Research Council (C.N.R.), Roma, and N.A.T.O. for financial support.

### REFERENCES

1. Brieskorn, C. H. and Pfeufer, T. (1967) *Chem. Ber.* **100**, 1998
2. Fujita, E., Uchida, I. and Fujita, T. (1974) *J. Chem. Soc. Perkin I* 1547.
3. Popa, D. P. and Reinbol'd, A. M. *Khim. Prirod. Soedin.* 324, 589.
4. Savona, G., S. Passannanti, Paternostro, M. P., Piozzi, F., Hanson, J. R., Hitchcock, P. B. and Siverns, M. (1977) *J. Chem. Soc. Perkin I* in press.

*Phytochemistry*, 1978, Vol 17, pp 322–323 Pergamon Press. Printed in England

# ABSCISIC ACID FROM *PINUS DENSIFLORA* POLLEN*

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(*Revised received* 20 *August* 1977)

**Key Word Index**—*Pinus densiflora*; Pinaceae; pollen; abscisic acid; germination inhibitor.

**Abstract**—(+)-Abscisic acid was isolated as the methyl ester from *Pinus densiflora* pollen and identified spectroscopically.

## INTRODUCTION

The pollen of *Pinus attenuata* contains growth-inhibitors and growth-stimulators [1, 2]; that of *P. densiflora* contains two ether soluble acidic substances which inhibit the growth of the pollen tubes of this plant, *Tradescantia* spp. and *Impatiens balsamina* [3], and the germination of *Brassica* seeds [4]. Since one of these growth-inhibitors occurs in the so-called β-inhibitor zone, it was suggested that ABA or a closely related compound was present [5]. In this paper the first identification of an inhibitor from the pollen of *P. densiflora* is reported.

## RESULTS AND DISCUSSION

The plant material was extracted with $CH_2Cl_2$ and the extract was fractionated as described in the Experimental. Attempts to isolate the inhibitor by gel filtration through Sephadex LH-20, employing MeOH as eluent, were unsuccessful because of poor resolution from the

*Part 1 in the series 'Constituents of Pine Pollen'.

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large amounts of contaminants. Further isolation, therefore, was carried out on methylated material, since the methyl ester of ABA retains its activity in the bioassay used.

The purified inhibitor from HPLC was examined by GLC and one peak was observed with $R_t$ (22.0 min on 2% QF-1 and 17.3 min on 2% OV-17) identical with that of standard MeABA and further confirmation was obtained by mass and other spectral data (IR, UV, ORD and PMR) as described in the Experimental. Its spectral data agreed with those reported for (+)-MeABA [6–11] except that the extinction coefficient and the specific rotation of our material were a little smaller. Thus, the natural inhibitor in the pollen of *P. densiflora* was identified as (+)-ABA. This is the first isolation of ABA from pollen.

One of two other esters, isolated by HPLC on μ-Porasil, was identified as *trans*-MeABA on the basis of chromatographic behaviour and mass spectrometry. However, whether the *trans*-isomer occurs naturally or was formed during isolation was not investigated. The other compound is a diterpene and its structure will be described elsewhere.

## EXPERIMENTAL

*Chromatography.* GLC was carried out on an instrument with FID using He as a carrier gas at 20 ml/min. Two different phases (QF-1 and OV-17, 2% on Gaschrom Q) were used in stainless steel columns (1.5 m × 3 mm i.d.) at 150 or 180°. For GC–MS, a glass column (1 m × 3 mm i.d.) packed with 1.5% OV-17 on Chromosorb W AW DMCS using He at 23 ml/min. HPLC was carried out on a Waters Model 200 using Hitachi gel 3040, 80 cm × 9.4 mm i.d., and 1.2% EtOH in $CHCl_3$ at 5 ml/min; μ-Porasil, 2(30 cm × 4 mm i.d.), and 0.8% EtOH in $CHCl_3$ at 1 ml/min.

*Spectral analysis.* GC–MS was carried out on a Shimadzu-LKB-9000 at 70 eV. IR spectra were measured in $CCl_4$, PMR spectra in $CDCl_3$ at 60 MHz using TMS as an int. stand.

*Plant material.* Pollen of *Pinus densiflora*, collected in the Kitakami area of Japan in May of 1975, was kindly supplied by Dr. K. Hata, Jujo Paper Co..

*Isolation and identification.* The pollen (1640 g) was Soxhlet extracted with $CH_2Cl_2$ for 24 hr. The extract was evapd under red. pres. at 30°. After MeOH-insoluble material, the urea adduct, and excess urea had been removed, the resulting brown oil (24.0 g) was dissolved in EtOAc (500 ml) and extracted with satd $NaHCO_3$ soln (3 × 150 ml). The combined $NaHCO_3$ fractions were washed with EtOAc, then acidified to pH 2 with 6 N HCl, and extracted with EtOAc (3 × 150 ml). These EtOAc fractions were washed with brine, dried and evapd to dryness *in vacuo* yielding a brown oil (2 g), a 1 mg/ml soln of which showed inhibitory activity. This oil (1.6 g) was chromatographed on Sephadex LH-20 (150 cm long, 3 cm i.d.) in MeOH. Ten fractions (25 ml) were collected and bioassayed. The 5th fraction gave strong inhibitory activity. The residue (367 mg) was methylated with ethereal diazomethane. It retained inhibitory activity and was chromatographed on Merck 70–230 mesh Si gel (40 g) using *n*-hexane–$Et_2O$ (2:3) as eluent. A 59 mg fraction was subjected to HPLC on Hitachi gel and μ-Porasil and gave 1.245 mg of an oily inhibitor, a 15 μg/ml soln of which inhibited the germination of lettuce seeds completely. This methylated inhibitor exhibited a very intense Cotton effect $[\alpha]_{290}$ +21 200°, $[\alpha]_{248}$ −42 800° (MeOH, $c$ = 0.0108). This inhibitor showed the following spectral data: UV $\lambda_{max}^{MeOH}$ nm (log ε): 265, 237 *sh* (4.11, 3.99); and IR, PMR and MS as reported previously for methyl abscisate [11].

*Bioassay.* The lettuce seed germination test (50 seeds of *Lactica sativa* L. cv Wayahead in 0.5 ml of 100 ppm aq. Tween-80 containing a test material for 48 hr at 22° in the dark) was used as an indicator of biological activity. It was established that the both ABA and its methyl ester are active in concn 1–10 μg/ml in this bioassay.

*Acknowledgements*—The authors are grateful to Dr. K. Hata, Jujo Paper Co., for generous gift of *Pinus densiflora* pollen. They also wish to thank Professor Dr. K. Tanaka, Department of Biology, Hirosaki University, for useful suggestions and Dr. T. Koyama, Chemical Research Institute of Non-Aqueous Solutions, Tohoku University, for recording GC–MS.

## REFERENCES

1. Kamienska, A., Dupley, R. C. and Pharis, R. P. (1976) *Phytochemistry* **15**, 421.
2. Michalski, L. (1967) *Acta Soc. Bot. Pol.* **36**, 475 and references cited therein.
3. Tanaka, K. (1958) *Sci. Rep. Tohoku Univ. 4th Ser.* **24**, 45.
4. Tanaka, K. (1964) *Sci. Rep. Tohoku Univ. 4th Ser.* **30**, 21.
5. Milborrow, B. V. (1968) in *Biochemistry and Physiology of Plant Growth Substances* (Wightman, F. and Setterfield, G. eds) p. 1531. Runge Press, Ottawa.
6. Cornforth, J. W., Milborrow, B. V. and Ryback, G. (1965) *Nature* **205**, 1269.
7. Ohkuma, K., Addicott, F. T. and Smith, O. E. and Thiesen, W. E. (1965) *Tetrahedron Letters* 2529.
8. Cornforth, J. W., Milborrow, B. V. and Ryback, G. (1966) *Nature* **210**, 627.
9. Jenkins, P. A. and Shepherd, K. R. (1972) *New Phytol.* **71**, 501.
10. Gray, R. T., Mallaby, R., Ryback, G. and Williams, V. P. (1974) *J. Chem. Soc., Perkin Trans. 2* 919.
11. Milborrow, B. V. (1974) *Ann. Rev. Plant Physiol.* **25**, 259.