Germination Inhibitor from *Eucalyptus pulverulenta*

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A potent germination inhibitor was found in the *n*-hexane soluble fraction of the methanolic extract of *Eucalyptus pulverulenta*. This inhibitor was identified as grandinol (1) which had been isolated from *E. grandis*. On the cress germination test, 1 showed I_{50} at 10 ppm. The other eight compounds in the fraction containing 1 on silica gel chromatography were also identified. Compounds (2) and (3) are regarded as phloroglucinol metabolites characteristic of the *Eucalyptus* species. The known compound (4) may be a good taxonomical marker for *E. pulverulenta*, and (5)~(9) are possibly common metabolites of *Eucalyptus*.

Eucalyptus pulverulenta is a somewhat rare eucalypt that grows naturally in only a few localities of the central and southern tablelands of New South Wales. It is, however, widely cultivated in California and, to a less extent, in Japan: the young shoots are eagerly sought after by the cut flower trade and the tree itself is used for garden planting. The silvery-blue leaves are rounded or heart-shaped in opposite pairs, lacking a petiole and set more or less at right angles to the stem. The occurrence of plant growth regulating substances in *E. pulverulenta* has been investigated as part of a general programme of work within this gene. In particular, the existence of manipulated crosses, *E. pulverulenta*/*E. grandis* offers an opportunity for inheritance studies.

Recently, we have detected the existence, indicated by physiological studies, of a germination inhibitor in *E. pulverulenta*. We now wish to report the isolation of grandinol (1) as the active principle in the hexane extractable fraction, accompanied by two new and taxonomically important compounds (2 and 3)

in the same fraction. Other common eucalypt metabolites were also identified (4~9); with the exception of 7, these have not previously been reported as occurring in *E. pulverulenta*.

Fractionation of the methanol extract was guided by a cress seed germination test.¹⁾ Silica gel column chromatography of the hexane extract (from hexane-methanol partition), gave an active fraction (F11). Purification of this fraction (Fig. 2) gave nine compounds (Fig. 1).

The inhibitory activity of all nine compounds was checked using the cress seed germination test; at 250 ppm, 6 and a mixture of 7, 8 and 9 were found to have no activity, while 4, 5 and a mixture of 2 and 3 showed weak activity. In contrast, grandinol (1) showed 50% germination inhibition at 10 ppm.

The presence of grandinol²⁾ was confirmed using GC-MS [M^+ 252, m/z 195 ($M^+ - C_4H_9$)], the retention time and fragmentation pattern being identical with those of an authentic sample of grandinol. ¹H NMR showed the expected methyl (δ 0.98, $J=7$ Hz)

FIG. 1. Compounds Isolated from *E. pulverulenta*.

and methylene (3.02, $J=8$ Hz) doublets for the isobutyl side-chain. The aromatic methyl showed a sharp singlet at 2.02 ppm, while the aldehyde proton was observed at 10.2 ppm.

The structure of **2** (C₁₂H₁₆O₄ from high resolution MS) was elucidated as follows. The high resolution MS of **2** showed an expected isopropyl fragmentation, m/z 181 ($M^+ - C_3H_7$), together with the standard aromatic methoxyl fragmentation pattern, 166 ($181^+ - CH_3$) and 138 ($166^+ - CO$). By treating **2** with bistrimethylsilyl acetamide (BSA), the incorporation of two trimethylsilyl groups (GC-MS) indicated the presence of two OH groups. ¹H NMR showed a characteristic isopropyl doublet with aromatic methyl (δ 2.05) and *O*-methyl (3.84) groups. The 2,6-dihydroxy substitution pattern was confirmed by UV spectrophotometry (see Exp.). Riedl *et al.*³⁾ has reported that a 2,6-substitution pattern showed a 10 nm red shift with base, while a 2,4-pattern gave a 45 nm red shift with base. The structure of **2** was assigned as 2,6-dihydroxy-4-methoxy-3-methylisopropiophenone and was confirmed by comparison with an authentic sample which was synthesized from phloropropiophenone.

The GC of **2** showed a small impurity, to which the structure **3** was assigned. The GC-high resolution mass spectrum of **3**, $M^+ = 238.1208$ (C₁₃H₁₈O₄), showed a strong frag-

ment $M^+ - C_4H_9$, followed by a fragmentation pattern identical to **2**. No $M^+ - C_3H_7$ fragment was detected, thus the side-chain has a 2-butyl substitution pattern rather than an isobutyl pattern.

4 was identified as methyl eudesmate. No depression in the melting point was observed when mixed with an authentic sample⁴⁾ (synthesized from eudesmic acid). The authenticity of the methyl ester grouping was confirmed by isolating **4**, using acetone instead of methanol as an extraction solvent.

Globulol (**5**) was identified from data (mp, IR, ¹H-NMR) reported by Blumann *et al.*⁵⁾ **7**, **8**, and **9** were respectively identified as *n*-tritriacontan-16,18-dione, *n*-hentriacontan-14,16-dione, and *n*-nonacosan-12,14-dione by comparison with data (GC-MS, UV, ¹H-NMR) reported by Osawa *et al.*⁶⁾ and Horn *et al.*⁷⁾ The structure of **6** was assigned as 11,12-dehydrourosolic lactone acetate by comparing with available data (mp, IR, ¹H-NMR) reported by Horn *et al.*⁸⁾

Various kinds of simple acylphloroglucinols are commonly found as constituents of eucalypts,⁹⁾ as are compounds of related origin. The ability of a species to C- and/or O-alkylate specific positions of the phloroglucinol nucleus may be of some taxonomic significance and the isolation of these derivatives from a wide range of eucalypts may help to solve the question of subgenetic classification.

Inheritance studies between *E. grandis*/*E. pulverulenta* crosses (now being conducted) require genetic markers. The G-regulators are good *E. grandis* markers.¹⁰⁾ The high concentration and easy detection (UV/TLC) of methyl eudesmate make it an ideal *E. pulverulenta* marker.

The existence of germination inhibitors in the leaves of *E. grandis* led to the isolation of grandinol by Crow *et al.*²⁾ We have now reported grandinol in *E. pulverulenta*, and early results show that grandinol is present in some other eucalypts. The biological significance of grandinol is not yet clear, but it appears to be a common constituent of some eucalypts. Further work in this area will be

reported shortly.¹¹⁾

EXPERIMENTAL

Fresh leaves and stems (400 g) were ground in methanol (5 liters) for two hours and left to soak overnight. The methanol extract was then reduced in volume (1 liter) and water (2 liters) was added. The solution was extracted with hexane (5 × 1.5 liters). The hexane was removed to give a green gum (10 g) which was introduced onto a silica gel column (300 g) and eluted with hexane (2 liters), hexane-ethyl acetate (9:1, 1 liter), hexane-ethyl acetate (8:2, 1 liter) and hexane-ethyl acetate (7:3, 1 liter). Twenty 250 ml fractions were collected. Fraction eleven (F11) showed strong inhibition of cress seed germination at 1000 ppm. The GC of F11 showed nine major peaks (OV-1, 2 m, 150~300°C).

Treatment of F11 (Fig. 2) with cold methanol gave a colorless solid mixture of **7**, **8** and **9** (12 mg). The methanol-soluble fraction was treated with cold hexane to give a colorless solid **6** (25 mg). Preparative TLC of the residue (silica, hexane-ethyl acetate 50:50) gave two bands. That of *R_f* 0.3~0.35 gave a red oil which recrystallised from methanol to give **5** (50 mg), while that of *R_f* 0.4~0.6 showed three components on HPLC (Nucleosil 7-OH, hexane-isopropanol 96:4). The components were separated using HPLC to give **4** (30 mg), **2** and **3** (1.4 mg), and **1** (10 mg).

Grandinol (1). mp 131~133°C (lit. mp 130~132°C).²⁾ GC-MS (OV-1, 2 m, 150°C)¹²⁾ *m/z*: 252 (*M*⁺, int. 35%), 237 (*M*⁺ - CH₃, 15), 209 (*M*⁺ - C₃H₇, 12), 195 (*M*⁺ - C₄H₉, 100), 167 (*M*⁺ - C₅H₉O, 18). ¹H NMR (TMS, CDCl₃)¹²⁾ δ ppm: 0.98 (d, *J* = 7 Hz, CH₃ × 2), 2.02 (s, ArCH₃), 2.30 (m, *J* = 7 and 8 Hz, 1H), 3.02 (d, *J* = 8 Hz, -CH₂-CO-), 10.20 (s, aldehyde H). Other data are consistent with the literature.²⁾

2,6-Dihydroxy-4-methoxy-3-methyl-isopropiophenone (2). mp 141°C. UV λ_{max}^{MeOH} nm (log ε)¹²⁾: 283 (3.3) and 204 (3.4); with the addition of HCl, 286 (2.9) and 204 (3.2); with the addition of NaOH, 296 (3.3) and 202 (3.3). IR ν_{max}^{nujol} cm⁻¹¹²⁾: 3350 (free OH), 2500 (H-bounded OH), 1637 (C=O), 1585 (Ar), 1235 (COC). High resolution MS¹²⁾ *m/z*: *M*⁺ = 224.1030 (int. 91%, 224.1047 calcd. for C₁₂H₁₆O₄), 181 (*M*⁺ - C₃H₇, 100), 166 (181⁺ - CH₃, 12), 138 (166⁺ - CO, 12), 149 (181⁺ - CH₃OH, 18). The addition of BSA to the MS sample showed *M*⁺ = 368 (TMS × 2).

2,6-Dihydroxy-2',3-dimethyl-4-methoxy-butyrophenone (3). This was detected as a small impurity of **2** by GC-MS. High resolution MS, *m/z*: *M*⁺ = 238.1208 (int. 87%, 238.1205 calcd. for C₁₃H₁₈O₄), 181 (*M*⁺ - C₄H₉, 100), 166 (181⁺ - CH₃, 15), 138 (166⁺ - CO, 12), 149 (181⁺ - CH₃OH). The addition of BSA to the MS sample showed *M*⁺ = 382 (TMS × 2).

Methyl eudesmate (4). mp 82°C (lit. mp 82°C).⁴⁾ UV λ_{max}^{MeOH} nm (log ε): 265 (4.2) and 214 (3.7). IR ν_{max}^{nujol} cm⁻¹: 1715 (C=O), 1595 (Ar), 1340, 1230, 750, 740, 720. ¹H NMR (TMS, CDCl₃) δ ppm: 3.92 (s, CH₃O × 4) and 7.23 (s, Ar-H × 2). ¹³C NMR (TMS, CDCl₃) δ ppm: 166.5 (C-7), 152.9 (C-3 and 5), 142.2 (C-4), 106.9 (C-2 and 6), 60.7 (C-10), 56.1 (C-9 and 11), 51.0 (C-8). High resolution MS, *m/z*: *M*⁺ = 226.0853 (int. 35%, 226.0840 calcd. for C₁₁H₁₄O₅), 211 (*M*⁺ - CH₃, 7), 195 (*M*⁺ - CH₃O, 100), 183 (211⁺ - CO, 5), 167 (*M*⁺ - CO₂CH₃, 14).

Globulol (5). mp 86.5~87°C (lit. mp 87°C).⁵⁾ IR ν_{max}^{film} cm⁻¹: 3400 (OH), 2950, 1450, 1380. ¹H NMR (TMS, CDCl₃) δ ppm: 0.98 (d, *J* = 8 Hz, 3H), 0.99 (s, 3H), 1.02 (s, 3H), 1.12 (s, 3H). ¹³C NMR (TMS, CDCl₃) δ ppm: 74.5 (s), 57.1 (d), 45.2 (t), 40.0 (d), 36.8 (t), 34.9 (s), 29.0 (d), 28.9 (d), 26.7 (d), 26.0 (t), 20.7 (q × 2), 19.5 (t), 16.6 (q), 16.4 (q).

* Denotes active for germination inhibition.

FIG. 2. Isolation of Active Components from F 11.

11,12-Dehydroursoleic lactone acetate (6). mp 261.8~262.2°C (lit. 262°C)⁸⁾ IR $\nu_{\max}^{\text{nujol}}$ cm^{-1} : 1750 (γ -lactone), 1722 (acetate), 1235. High resolution MS, m/z : $M^+ = 496.3552$ (int. 95%, 496.3540 calcd. for $\text{C}_{32}\text{H}_{48}\text{O}_4$), 452 ($M^+ - \text{CO}_2$, 100), 437 ($M^+ - \text{CH}_3\text{CO}_2$, 21). ^1H NMR (TMS, CDCl_3) δ ppm: 0.86 (s, $\text{CH}_3 \times 2$), 0.94 (broad s, 7.5H), 1.04 (broad s, 4.5H), 1.16 (s, CH_3), 2.05 (s, CH_3CO), 4.52 (t, 1H), 5.52 (d-d, $J = 10$ and 2 Hz, 1H), 5.96 (d, $J = 10$ Hz, 1H). ^{13}C NMR (TMS, CDCl_3) δ ppm: 179.66, 170.83, 133.21, 128.94, 89.51, 80.56, 60.55, 54.82, 52.92, 45.05, 41.95, 41.71, 40.25, 38.09, 38.00, 37.85, 36.27, 31.35, 31.18, 30.83, 27.73, 25.51, 23.28, 22.82, 21.24, 19.13, 18.90, 17.96, 17.84, 17.55, 16.03.

A mixture of n-tritriacontan-16,18-dione (7), *n-hentriacontan-14,16-dione* (8) and *n-nonacosan-12,14-dione* (9). mp 63~65°C (lit. for 7, mp 67~68°C).^{6,7)} IR $\nu_{\max}^{\text{film}}$ cm^{-1} : 2950, 1610, 1465, 1450 and 1370. UV $\lambda_{\max}^{\text{CHCl}_3}$ nm (log ϵ): 274 (3.90). High resolution GC-MS for 7, m/z : $M^+ = 492.4921$ (492.4906 calcd. for $\text{C}_{33}\text{H}_{64}\text{O}_2$), 281 ($M^+ - \text{C}_{15}\text{H}_{31}$), 239 ($M^+ - \text{C}_{15}\text{H}_{31}\text{COCH}_2$). High resolution GC-MS for 8, m/z : $M^+ = 464.4623$ (464.4592 calcd. for $\text{C}_{31}\text{H}_{60}\text{O}_2$), 281 ($M^+ - \text{C}_{13}\text{H}_{27}$), 239 ($M^+ - \text{C}_{13}\text{H}_{27}\text{COCH}_2$). High resolution GC-MS for 9, m/z : $M^+ = 436.4264$ (436.4280 calcd. for $\text{C}_{29}\text{H}_{56}\text{O}_2$), 281 ($M^+ - \text{C}_{11}\text{H}_{23}$), 239 ($M^+ - \text{C}_{11}\text{H}_{23}\text{COCH}_2$). The latter two compounds, 8 and 9, were detected as small impurities (less than 5% each) of 7 by the GC-MS (OV 1, 2 m, 260°C).

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REFERENCES

- 1) H. F. Taylor and R. S. Burden, *Proc. R. Soc. London, Ser. B*, **180**, 317 (1972).
- 2) W. D. Crow, T. Osawa, D. M. Paton and R. R. Willing, *Tetrahedron Lett.*, 1073 (1977).
- 3) W. Riedl and R. Mitteldrof, *Chem. Ber.*, **89**, 2589 (1956).
- 4) H. G. Smith, *J. Roy. Soc. N.S.W.*, **34**, 72 (1900).
- 5) A. Blumann, A. H. R. Cole, K. J. L. Theiberg and D. E. White, *Chemistry and Industry*, 1426 (1959).
- 6) T. Osawa and M. Namiki, *Agric. Biol. Chem.*, **45**, 735 (1981).
- 7) D. H. S. Horn, Z. H. Kranz and J. A. Lamberton, *Aust. J. Chem.*, **17**, 464 (1964).
- 8) D. H. S. Horn and J. A. Lamberton, *Aust. J. Chem.*, **17**, 477 (1964).
- 9) R. O. Hellyer, *Aust. J. Chem.*, **21**, 2825 (1968).
- 10) M. L. Bolte, W. D. Crow, T. Osawa, D. M. Paton and S. Yoshida, *Proceedings IUPAC Fifth International Congress of Pesticide Chemistry*, Vol. 2, 1983, p. 91.
- 11) Report in preparation.
- 12) IR spectra were recorded on a Jasco A-300 IR spectrophotometer. UV spectra were measured with a Hitachi 200-20 spectrophotometer. ^1H and ^{13}C NMR were recorded on a Jeol FX-100 system. High and low resolution mass spectra were obtained by a Jeol DX-300 system or a Hitachi M80-A system.