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PIGMENT FORMATION BY CALLUS OF *LAVANDULA ANGUSTIFOLIA*

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Key Word Index—*Lavandula angustifolia*; Labiatae; lavender; tissue culture; phenylpropanoid pigments; caffeic esters of enols.

Abstract—Callus cultures of *Lavandula angustifolia* accumulated and secreted the (*Z*,*E*)-2-(3,4-dihydroxyphenyl)ethenyl ester of 3-(3,4-dihydroxyphenyl)-2-propenoic acid and its (*E*,*E*)-isomer under a wide range of culture conditions. The secreted compounds formed intensely blue pigments by chelation with Fe^{2+} in the media. These unusual enol esters could not be detected in the parent plant but the (*Z*,*E*)-isomer occurred in shoots and foliage of *Plectranthus caninus*.

INTRODUCTION

Recently we have investigated terpenoid metabolism in callus cultures of *Lavandula angustifolia* Mill. Subsp. *augustifolia* (≡ *L. officinalis* Chaix ≡ *L. vera* DC) [1]. These cultures also appeared to secrete an intense blue pigment into the supporting medium under a wide variety of conditions, and we now follow up this observation. There are a number of reports of pigmentation occurring in callus cultures of *L. vera* (*sic*) but the nature of the pigment has not been investigated [2, 3; Lappin, G. and Tampion, J., personal communication]. No such coloration has been reported in other studies of cultures of the same species [4, 5].

RESULTS AND DISCUSSION

Dark blue pigment appeared to be excreted into the medium within a few days of callus tissue forming on the original explants from stems of *L. angustifolia*, and its production persisted unabated over a period of 5 years and up to 120 sub-cultures. During this period the morphology and chemical characteristics of the cultures did not alter in other respects [1]. The coloration was repeatedly observed with fresh explants using *L. angustifolia* from three sources, and persisted in varying degrees over variations in culture conditions (a) to (g) as detailed in the Experimental. Cultures maintained in the dark produced considerably more pigment (up to five-fold) than light-exposed or greened cultures.

Colorimetric measurements indicated that pigmentation was also increased (i) by increase in sucrose concentration in medium—doubling the sucrose concentration from that in the standard medium (Murashige and Skoog), leading to an approximate doubling of colour intensity; (ii) by accidental bacterial or fungal infection of cultures; and (iii) by supplantation of the amino acid in the medium with asparagine (c.f. [1]). Pigmentation was reduced by addition of coconut milk or of excess of pyridoxine hydrochloride (above that specified in the formulation) to the medium, and was completely suppressed by casein hydrolysate (1 g. l^{-1}) or by removal of Fe^{2+} from the culture medium. Others have found that secretion of pigment by *L. vera* cultures did not occur unless cysteine was added to the medium [2], but we found no such dependence on this amino acid. In suspension culture, the liquid medium (M & S) became intensely blue within 5 days of initial transfer of the disrupted callus, but after this the colour disappeared within days—perhaps having been oxidized.

The blue coloration resulted from partial secretion of yellow pigments produced within the callus: these excreted products complexed with Fe^{2+} present in the nutrient medium. Removal of metal from such complexes could only be effectively achieved by acid-treatment which decomposed the organic components: consequently identification was carried out on the pigments extracted directly from the callus. Two such yellow pigments (up to 0.2% crude wt per wet wt of callus; 5–10-fold more was secreted per 16–21 day culture period) were isolated as a difficulty resolvable mixture but were methylated to yield derivatives that could be separated and purified. Both components were enol esters and each methylated derivative yielded equivalent amounts of 3,4-dimethoxyphenylacetaldehyde and 3,4-dimethoxy-(*E*)-cinnamic acid on hydrolysis. ^{1}H NMR and MS showed the methylated derivatives to be **1** and **2**—where the numbering refers to ^{1}H NMR assignments detailed in the Experimental; and the original pigments were shown to be **3** and **4**: the (*Z*,*E*)-2-(3,4-dihydroxyphenyl)ethenyl ester of 3-(3,4-dihydroxyphenyl)-2-propenoic acid and its (*E*,*E*) isomer [N.B. (*E*)-3-(3,4-dihydroxyphenyl)-2-propenoic acid ≡ caffeic acid]. The crucial (*E*,*Z*) geometry in **1** was deduced from $J_{7,8}$ couplings of 7.2 Hz (*cis*-H in enol system) and $J_{10,11}$ = 15.9 Hz (*trans*-H in cinnamic acid: cf. $J_{H,H}$ in *E* and *Z*-cinnamic acid = 15.5 and 11.9 Hz respectively). The (*E*,*E*) geometry in **2** followed from corresponding $J_{7,8}$ and $J_{10,11}$ couplings of 12.8 and 15.9 Hz respectively. Similarly the ^{1}H NMR spectrum of

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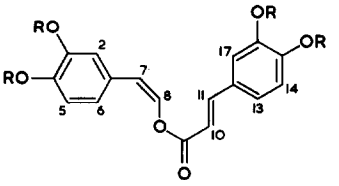

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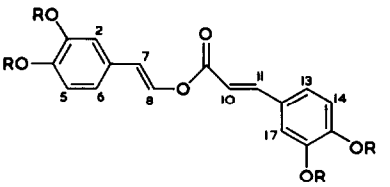
3 R = H



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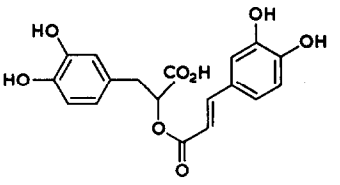

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the unresolved mixture of 3 and 4 could be analysed to give $J_{7,8} = 7.2$ and $J_{10,11} = 16.0$ Hz [3 ≡ (*Z*,*E*) isomer], and $J_{7,8} = 12.7$, $J_{10,11} = 15.9$ Hz [4 ≡ (*E*,*E*) isomer]. The amounts of 3 and 4 varied from 5:1 to 1.7:1 (as estimated by ^{1}H NMR) in different cultures. The ratio remained constant during purification and derivation and any fluctuations seem unlikely to be artefacts of handling; both pigments were thus presumably biosynthesized in the callus.

Extensive screening of the foliage, stem and flowerheads of *L. angustifolia* and related cultivars (with different coloured petals) revealed the anthocyanidin aglycones delphinidin, malvidin, the flavone chrysoeriol and coumarin and 7-methoxycoumarin, but no 3 or 4 could be detected. Anthocyanins have been reported as accumulating in plant cultures [6], but enol esters such as 3 and 4 are almost unique in any situation. The only reported occurrence of such a type is that of 3 from the leaf pigments of *Plectranthus caninus* Roth (≡ *Coleus spicatis*; Labiatae) [7]. We have confirmed that our compound 3 is indeed identical to this pigment. The nearest recorded relative to 3 and 4 is rosmarinic acid 5 which occurs in many Labiatae [8] and is produced by tissue cultures of *Coleus blumei* [9].

The formation of secondary metabolites by tissue cultures is not common and has often been thought to be associated with cytodifferentiation. However, flavonoids and related pigments may be produced in relatively undifferentiated tissue [10] and our cultures certainly showed little signs of differentiation [1]. Also accumulation of the other pigments usually occurred in slow-growing or senescent cultures whereas our cultures were extremely fast growing. Secretion of pigment increased when the cultures were kept in the dark and greened cultures maintained under normal light intensities (for such cultures) secreted much less pigment. It is noteworthy that pigment formation as in *L. angustifolia* did not occur in cultures of some 40 species of higher plants that we have established—many of these being Labiatae and being maintained under conditions very similar to that for the *Lavandula* species. It is also noteworthy that pigment production and secretion occurred from the onset of callus formation from the explant and was not apparently a somatic adaption. Excretion of various compounds (often phenylpropanoids) from plant roots is common [11] and these compounds often chelate metals, especially Fe^{2+} and Al^{3+} [12]. Compound 3 and 4 were shown to chelate both the latter metal ions, but not Mn^{2+}, Mg^{2+} or Ca^{2+}. It is tempting to assign roles as metal carriers to these compounds: this aspect is being investigated.

EXPERIMENTAL

Culture methods. Lavandula angustifolia was obtained from the UCL Botanical garden, the Royal Botanic Gardens, Kew and from the Norfolk Lavender Farm, Heacham, Norfolk. Callus was initiated from stem material on Murashige and Skoog's medium [13] modified by the addition of asparagine (100 mg l^{-1}) and by replacement of IAA by NAA (2 mg l^{-1}) supported on 0.8–1.2% w/w agar (Oxoid: conc. varied to ensure medium from different batches allowed callus to 'bed-down') containing coconut milk (10% v/v). The last was omitted after the first subculture, and subsequent subcultures were carried out at 2–3 week intervals over up to 5 yr. Some 10% of the original explants formed callus and throughout subculture a cream-coloured friable material was obtained which showed little tendency to brown pigmentation even at high light intensities. The following light-temperature regimes were used and growth indexes *G* were obtained [$G = (W_t - W_0)/W_t \cdot t$ where W_0 and W_t are weights of callus at start and end of sub-culture periods of time *t* days]. (a) 28°, natural illumination *ca* 400 lux max.; $G = 0.40$; (b) 28°, constant white light (Thorn 'White' tube λ_{max} 580 nm), *ca* 600 lux: $G = 0.11$; (c) Periodic cycle, 30° white light *ca* 1500 lux for 16 hr, 25° dark for 8 hr: $G = 0.31$; (d) 28°, constant Thorn 'Growlux' tube λ_{max} 660 nm, *ca* 600 lux: $G = 0.26$; (e) 37°, natural illumination *ca* 400 lux max.: $G = 0.37$; (f) natural room temp. and light cycle 10–20°, 1500–2500 lux: $G = 0.08$; (g) 28°, total darkness: *G*

= 0.38. Small amounts of chlorophyll accumulated under conditions (c), (d) and (f). Such amounts were less than 5% that in parent stem. Suspension cultures were maintained on M and S liquid medium with the same additions as to the solid medium (see above) at 27°, with continuous light (Thorn 'White' tube; 450 lux) in an orbital incubator (120 rpm).

Isolation and characterization of pigments **3** *and* **4**. The blue pigment could be recovered from the agar medium by addition of water, heating and extraction with *n*-BuOH. However, removal of metal by acid-treatment led to decomposition of the organic components, and all subsequent work was carried out on the yellow pigments stored within the cultured cells. Callus that had been cultured for one passage as a cell suspension in M and S liquid medium was homogenized with EtOAc in a blender. Such product (1 kg) yielded a yellow solid (*ca* 2.0 g). Alternatively, callus (40 g) was pulverized in liquid N_2 and extracted (Soxhlet) with Et_2O (200 cm^3; 24 hr). The resulting yellow-brown solid (20 mg) mainly comprised the yellow pigments **3** and **4** that were unresolved (R_f 0.45) by TLC on silica gel H with CH_2Cl_2–MeOH (17:3). Combined material from such extractions (150 mg) was suspended in *n*-hexane (50 cm^3) for 18 hr, the solid (120 mg) filtered, washed with *n*-hexane and chromatographed on a column of silica gel 40 (15 × 1 cm) with CH_2Cl_2 and a MeOH (0–10%) gradient. The pigments were recovered as a bright yellow oil (50–60 mg) which was not resolved by TLC (silica gel H; MeOH, CH_2Cl_2 various proportions). Separation could be achieved by HPLC on Spherisorb 10 ODS (reverse phase; 25 × 0.5 cm) with MeOH–H_2O (1:1) and 0.1% propionic acid as the mobile phase and with UV detection; but considerable decomposition here occurred on the column. Consequently, further separation was performed using **1** and **2**, the tetramethylated derivatives of **3** and **4**.

A mixture of **3** and **4** (22 mg) and K_2CO_3 (100 mg) in dry Me_2CO (5 cm^3) was treated dropwise with Me_2SO_4 (0.07 cm^3) in Me_2CO (2 cm^3) and refluxed (2 hr). Work-up and column chromatography on Silica Gel 40 with toluene and an EtOAc gradient (0–10% v/v) gave a mixture of **1** and **2** (13 mg) that ran as one spot (R_f 0.34) on TLC on silica gel H with EtOAc–toluene (1:3). This mixture (13 mg) was separated into **1** (9 mg) and **2** (3.5 mg) by HPLC on Portisil 5 Silica (25 × 0.6 cm) with *t*-butylmethyl ether–petrol bp 40–60° (3:2). Each compound was homogeneous on TLC (silica gel H; variety of solvents).

Compound **1**, a yellow oil, had $[M]^+$ = 370.1399, ($C_{21}H_{22}O_6$); *m/z* 370 (3.2%), 191 (100%; $C_{11}H_{11}O_3$), 179 (2.2; $C_{10}H_{11}O_3$), 163 (14.3) and 151 (5.6). ^{1}H NMR (200 MHz; 0.5% w/v in Me_2CO-d_6); δ values refer to hydrogens assigned as in **1**: δ3.83, 3.88, 3.89, 3.91 (all *s*, 3H, OMe); 5.80 (*d*, *J* = 7.2 Hz, 1H, H-7); 6.68 (*d*, *J* = 15.9 Hz, 1H, H-10); 6.96 (*d*, *J* = 8.3 Hz, 1H, H-5); 7.05 (*d*, *J* = 8.3 Hz, 1H, H-14); 7.23 (*dd*, *J* = 8.4, 2.0 Hz, 1H, H-6); 7.32 (*dd*, *J* = 8.3, 2.0 Hz, 1H, H-13); 7.34 (*d*, *J* = 7.3 Hz, 1H, H-8); 7.41 (*d*, *J* = 2.0 Hz, 1H, H-17 or H-2); 7.42 (*d*, *J* = 2.0 Hz, 1H, H-17 or H-2); 7.86 (*d*, *J* = 15.9 Hz, 1H, H-11).

Compound **2**, similarly a yellow oil, had $[M]^+$ 370.1389 ($C_{21}H_{22}O_6$); *m/z* 370 (2.5%), 191 (100.0%; $C_{11}H_{11}O_3$), 179 (2.8; $C_{10}H_{11}O_3$), 163 (13.8) and 151 (7.8). ^{1}H NMR (200 MHz): δ3.81, 3.86, 3.89, 3.92 (all *s*, 3H, OMe); 6.47 (*d*, *J* = 12.8 Hz, 1H, H-7); 6.56 (*d*, *J* = 15.9 Hz, 1H, H-10); 6.88–7.00 (*m*, 2H, H-5 and H-6); 7.04 (*d*, *J* = 8.4 Hz, 1H, H-14); 7.12 (*d*, *J* = 1.3 Hz, 1H, H-2); 7.30 (*dd*, *J* = 8.3, 1.9 Hz, 1H, H-13); 7.43 (*d*, *J* = 1.9 Hz, 1H, H-17); 7.79 (*d*, *J* = 16.1 Hz, 1H, H-11); 7.95 (*d*, *J* = 12.8 Hz, 1H, H-8).

The multiplicity of the signal at δ6.88–7.00 was confirmed by spin decoupling. Irradiation at the frequency of the δ7.12 doublet caused collapse of the multiplet to two perturbed doublets (*J* = 8.3 Hz). Irradiation at the frequency of the multiplet caused collapse of the doublet (δ7.12) to a singlet. In addition, comparison of the data for **1** and **2** indicates that the doublet at δ7.04 is due to H-14 and the doublet of doublets (δ7.30) is due to H-13, as these are comparable to the signals at δ7.05 and 7.32 respectively in **1** and are therefore in the aromatic ring of the ester moiety. Since the signals at δ7.12 and δ6.88–7.00 are mutually coupled, the assignments can all be made as above with the sole ambiguity for the *m*-coupled protons in **1**.

The MS of the purified but unresolved mixture of **3** and **4** was: $[M]^+$ = 314 ($C_{17}H_{14}O_6$); *m/z* 314 (3.7%); 180 (39.6); 163 (67.4), 136 (61.8) and 123 (100.0). The ^{1}H NMR spectrum of the mixture was essentially (differences < δ0.1) a superimposition of the spectra of **1** and **2** with the absence of signals in the range δ3.80–3.92 and a broad signal δ8.10–8.58 due to phenolic hydroxyls.

A mixture of **1** and **2** (10 mg) was hydrolysed by refluxing with 0.5 M NaOH (10 cm^3, 1 hr). After work-up, equimolar amounts of 3,4-dimethoxyphenylacetaldehyde and 3,4-dimethoxycinnamic acid were isolated and characterized by MS and ^{1}H NMR.

Composition of L. angustifolia. Foliage, stem and flowerheads (100 g) of several cultivars and subspecies of *L. angustifolia* (including those producing different flower and petal pigments) were extracted with Et_2O, aq. MeOH and *n*-C_6H_{14} following a procedure shown to recover **3** from *P. caninus*. No trace (TLC or ^{1}H NMR) of **3** was found. Stem and foliage yielded coumarin and 7-methoxycoumarin as the main components and flowers yielded delphinidin, malvidin and chryseriol. The first two of these pigments were isolated, for comparison purposes, from *Solanum melongena* (eggplant) and *Malva sylvestris*, respectively. Chrysoeriol was characterized by its reported R_f values on TLC systems.

Studies on P. caninus. Leaves (70 g) were macerated and extracted with Et_2O (300 cm^3, 1 hr) at 25° in the dark. The resulting yellow soln was decanted, evaporated to dryness, and the residue partitioned between *n*-C_6H_{14}–toluene (1:1) and EtOH–H_2O (1:1). The non-aqueous layer was extracted with EtOH–H_2O (1:1; × 2) and the combined aq. EtOH extracts were concd to half their volume and extracted with EtOAc (50 cm^3; × 3), after adding NaCl to control emulsification. The extract was concd to give an orange oily solid (80 mg) that was chromatographed on a column of Silica Gel 40 (10 × 0.5 cm) with MeOH–CH_2Cl_2 (0–10%) as eluant. Compound **3** was obtained as one of the fractions (16 mg; 0.02% wt/wet wt.) and was identified by mass and ^{1}H NMR spectroscopy. No **4** was detected.

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