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FORMATION OF THREE NEW FLAVONES BY DIFFERENTIATING CALLUS CULTURES OF *ANDROGRAPHIS PANICULATA*

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Key Word Index—*Andrographis paniculata*; Acanthaceae; new flavones; differentiating tissue cultures.

Abstract—Differentiating tissue cultures of *Andrographis paniculata* produce three new flavones, 5-hydroxy-7,8,2′-trimethoxy-, 5,2′-dihydroxy-7,8-dimethoxy- and 5-hydroxy-7,8-dimethoxy-flavones. Flavones are not synthesized by the de-differentiated callus. Closely related flavones have been isolated from intact plants of *Andrographis* species.

INTRODUCTION

One of the most remarkable characteristics of plant tissue cultures is their ability to regenerate organs or intact plants, a property that botanists descriptively term 'totipotency' [1, 2]. The term implies the notion that even a single de-differentiated cell retains the 'total potency' of the parent plant, and this must include the potential to produce the plant's characteristic secondary metabolites. Nevertheless, it is frequently found that de-differentiated tissues do not produce such metabolites and this has repeatedly frustrated attempts to exploit tissue cultures for the controlled production of commercially and particularly medicinally valuable plant products.

It is clearly of interest to establish whether the re-differentiation of callus tissues during morphogenesis is accompanied by reversion to the secondary metabolism of the parent plant, and such reversion has indeed been previously observed, particularly in alkaloid-producing plants [3–6]. We have previously commented [7, 8] on the fact that the terpenoid metabolism in *Andrographis* plants and callus cultures derived from them is entirely distinct, intact plants producing diterpenoids while the tissue cultures produce sesquiterpenoids. This

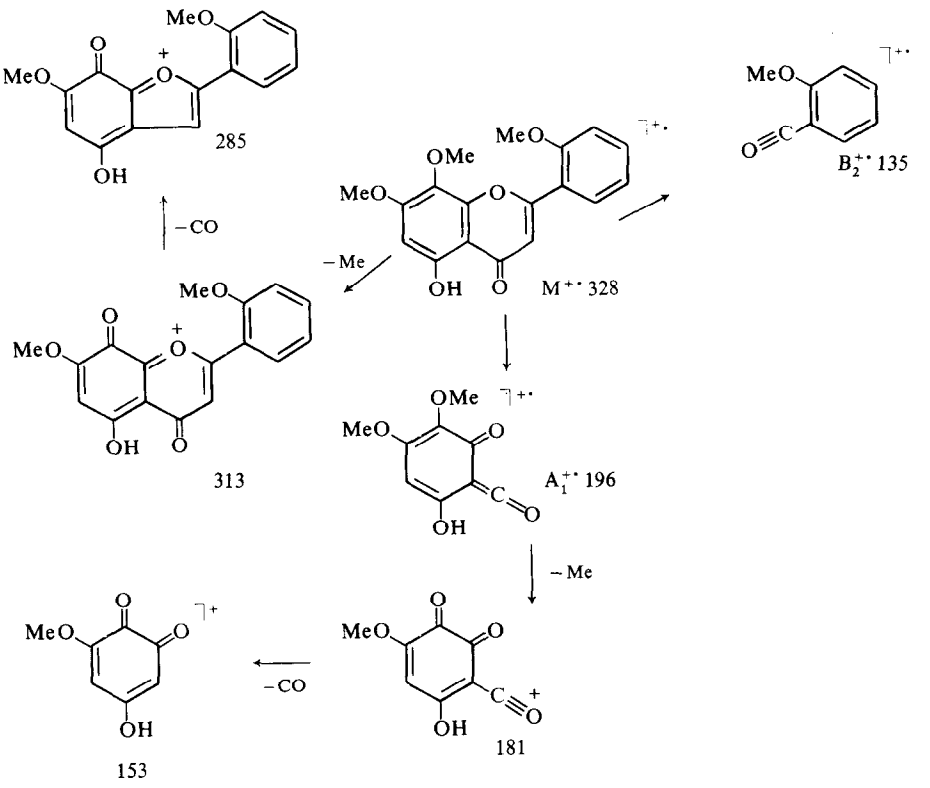
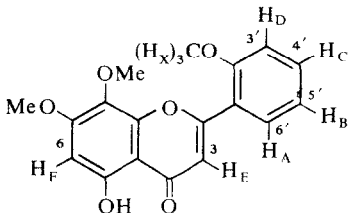


Fig. 1. Major ions in the mass spectrum of flavone A.



	δ (ppm)		J (Hz)
H_A	7.98	AB	1.60
H_B	7.50	AC	7.85
H_C	7.14	AD	0.20
H_D	7.06	BC	7.50
H_E	7.05	BD	8.45
		CD	0.95
		DX	0.30

Fig. 2. Chemical shifts (δ) and coupling constants (J) in the proton NMR spectrum of flavone (A).

paper is concerned with the emergence of flavonoid metabolism that is totally lacking in the cultures before differentiation. The changes in terpenoid metabolism will form the subject of a separate paper.

RESULTS

The development of root primordia was induced [9] in hypocotyl cultures of *Andrographis paniculata* that had been serially cultured on a solid modified White's medium for seven months, by transferring them to a solid medium containing additionally a low concentration (0.2 mg/ml) of NAA. Three weeks later these calluses, which then clearly showed root development, were inoculated into liquid medium which still contained the NAA, and growth was maintained for three weeks more.

The tissues were extracted with methanol–water (4:1) and the portion of the extractive soluble in ethyl acetate was separated by preparative TLC into the new flavones A, B and C. These compounds were absent from the de-differentiated cultures prior to their growth on NAA-containing medium.

Flavone A (**1**) crystallized from ethyl acetate in yellow needles, mp 190–191°. Its molecular formula, $C_{15}H_6O_2$-$(OCH_3)_3OH$, suggested that it was a hydroxy-trimethoxy-flavone. This was borne out by its UV (λ_{max} 273 and 330 nm) [10, 11] and IR [ν_{max} 1660 (C = O) and 1615 (methoxylated aromatic ring)] spectra. A C-5 hydroxyl group was indicated by a bathochromic shift in the presence of $AlCl_3$ and stable to HCl, for each of the UV bands and a negative Gibbs test [12] suggested C-8 was substituted.

The mass spectrum (Fig. 1) clearly indicated that of the three methoxyls two were located in ring A and one in ring B. The proton NMR spectrum served to establish the substitution pattern of ring B, as shown in Fig. 2. Since an appropriate model compound had not been recorded in the literature, a calculated spectrum, based on the observable chemical shifts and coupling constants, was derived using a spin simulation programme [13]. Fig. 3 shows

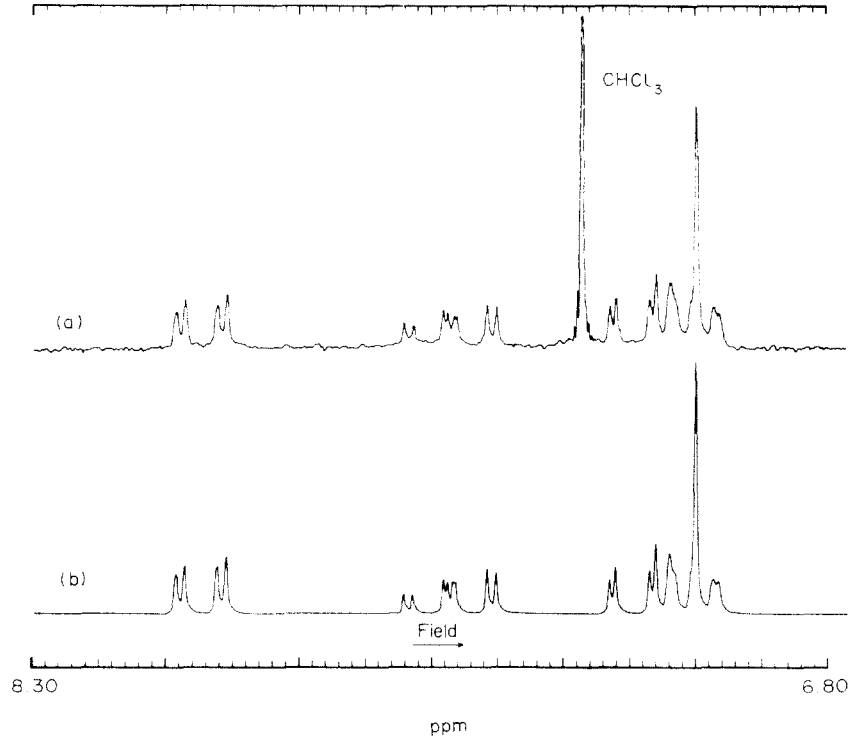
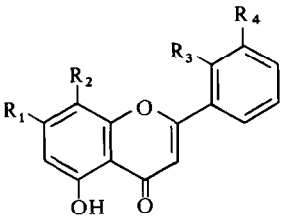


Fig. 3. (a) Part of the pulsed Fourier transform ^1H NMR spectrum of flavone A ($CDCl_3$); (b) Simulated spectrum using parameters listed in Fig. 2.



(1) $R_1 = R_2 = R_3 = OMe$; $R_4 = H$
(2) $R_1 = R_2 = OMe$; $R_3 = OH$; $R_4 = H$
(3) $R_1 = R_2 =$ OMe $R_3 = R_4 = H$
(4) $R_1 = R_2 = R_3 = R_4 = OMe$
(5) $R_1 = R_2 = R_3 = OMe$; $R_4 = OH$
(6) $R_1 = OMe$; $R_3 = OH$; $R_2 = R_4 = H$

the observed and calculated spectra; Fig. 2 lists the calculated chemical shifts and coupling constants for the $ABCDX_3$ system.

Flavone B (**2**), $C_{15}H_6O_2(OCH_3)_2(OH)_2$, mp 254–255°, was according to its physical properties and particularly its mass spectrum, the ring B phenol corresponding to flavone A. This was confirmed by the smooth conversion of flavone B into flavone A with diazomethane.

Flavone C (**3**), $C_{15}H_7O_2(OCH_3)_2(OH)$, mp 179–180°, according to its mass spectrum lacked a ring B substituent, as present in both flavones A and B, and this was borne out in the NMR spectrum. This showed complex three- and two-proton multiplets centred respectively at δ 7.57 and 7.96, characteristics of a monosubstituted phenyl residue, in addition to singlets at δ 6.45 (C-3) and 6.68 (C-6). The placement of one ring A methoxyl at C-7 rather than C-6 cannot be derived from spectroscopic evidence and is based on analogy with flavone (**4**) from *A. paniculata* [14] and of wightin (**5**) and echinoidinin (**6**) from *A. wightiana* and *A. echinoides* [15, 16].

EXPERIMENTAL

Extraction of flavones (**1**), (**2**) *and* (**3**). Tissue cultures of *Andrographis paniculata* (200 g wet wt), showing root formation after growth on modified White's medium containing NAA (0.2 mg/ml) [9], were extracted × 5 with MeOH–H_2O (4:1). The combined extracts (800 ml) were evapd to remove MeOH, the residue diluted with H_2O (100 ml) and extracted with EtOAc (5 × 50 ml). The EtOAc extract was dried and evapd *in vacuo*, affording an oily residue (162 mg). This was separated by PLC on Merck Si Gel 60 F_{254} plates (0.25 nm), using $CHCl_3$–MeOH (9:1), EtOAc–C_6H_{14} (4:1), and EtOAc–CCl_4 (4:1). In this way the 3 flavones were obtained: (A) 12.1 mg; (B) 4.5 mg; (C) 1.2 mg.

Flavone A (**1**), yellow needles from EtOAc, had mp 190–191°. MS: *m/e* 328.0934 (67), M^+, $C_{18}H_{16}O_6$; 313.0638 (100), $C_{17}H_{13}O_6$; 285.0768 (10), $C_{16}H_{13}O_5$; 181.0144 (19), $C_8H_5O_5$; 153.0189 (23), $C_7H_5O_4$; 135.0460 (2), $C_8H_7O_2$. NMR ($CDCl_3$): δ 3.92, 3.95 (3H, 6H singlets, 3 × $OC\underline{H}_3$'s); 6.43 (1H, *s*, C-3); 7.05 (1H, *s*, C-6); for δ and *J* values for C-3′, C-4′, C-5′ and C-6′, see Fig. 2. UV: λ_{max} (MeOH) 273 (ε 27000), 330 (ε 11100); λ_{max} (MeOH + $AlCl_3$ or $AlCl_3$ + HCl) 283, 296 (sh), 342, 410 nm. IR cm^{-1}: ν_{max} (CCl_4) 1660 (C=O), 1615 (methoxylated aromatic ring). (Found: C, 66.6; H, 5.0. $C_{18}H_{16}O_6$ requires: C, 65.85; H, 4.9%).

Flavone B (**2**), yellow needles from EtOAc–C_6H_{14}, had mp 254–255°. MS: *m/e* 314 (41) M^+; 299 (100) $M^+ - CH_3$; 271 ($M^+ - CH_3$, CO); 181 (34) $A^{+\cdot} - CH_3$; 153 (88) $A^{+\cdot} - CH_3$, −CO; 121 (14) $B_2^{+\cdot}$; 118 (20) $B_1^{+\cdot}$ UV nm: λ_{max} (MeOH) 273 (ε 27000), 340 (ε 11600 nm); λ_{max} (MeOH + $AlCl_3$ or $AlCl_3$ + HCl) 282, 293, 347, 402. Positive Gibbs test, λ_{max} 688 nm. IR cm^{-1}: ν_{max} (CCl_4) 1650, 1610.

Methylation of flavone B (1 mg) in MeOH (2 ml) with excess ethereal CH_2N_2 and purification by PLC, afforded flavone A whose UV and MS were essentially identical with those of flavone A of natural provenance.

Flavone C (**3**), yellow needles from $CHCl_3$–C_6H_{14}, had mp 179–180°. MS: *m/e* 298 (40) M^+; 283 (100) $M^+ - CH_3$; 255 (20) $M^+ - CH_3$, −CO; 181 (20) $A^{+\cdot} - CH_3$; 153 (88) $A^{+\cdot} - CH_3$, −CO. NMR ($CDCl_3$): 3.95, 3.96 (3H, singlets, $OC\underline{H}_3$'s), 6.45 (1H, *s*, C-3); 6.68 (1H, *s*, C-6); 7.58 (3H, *m*, C-3′, C-4′, C-5′); 7.98 (2H, *m*, C-2′, C-6′), UV nm: λ_{max} (MeOH) 276 (ε 31300), 340 (ε 6750); λ_{max} (MeOH + $AlCl_3$ or $AlCl_3$ + HCl) 285 sh, 295, 330 sh, 410. Gibbs test negative.

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