FLAVONOIDS FROM ELICITOR-TREATED CELL SUSPENSION CULTURES OF CEPHALOCEREUS SENILIS

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Abstract—Two new flavonoid glycosides, 6,7-dihydroxy-5-methoxyflavone 7-O-β-D-glucopyranoside and (2S)-6,7-dihydroxy-5-methoxyflavanone 7-O-β-D-glucopyranoside, together with two known compounds baicalein and baicalein 7-O-β-D-glucopyranoside, were isolated from chitin-elicited cell suspension cultures of Cephalocereus senilis. All structures, which were established by spectroscopic methods as well as chemical modifications, share the same unusual substitution pattern of the aurone previously described from the same culture. Each has an unsubstituted B-ring and 5,6,7-oxygenation in the A-ring, thus suggesting that a novel cinnamate:CoA ligase may be functional under these circumstances.

INTRODUCTION

Previous HPLC studies from our group demonstrated that five major phenolic compounds were synthesized de novo in chitin-treated cell suspension cultures of Cephalocereus senilis (old man cactus) [1]. However, to date only one of the five compounds has been fully characterized; that compound was a new aurone, 4,5-methylenedioxy-6-hydroxyaurone (cephalocerone, 1), a compound which showed antibacterial activity and may be a phytoalexin [2]. We report here the structures of four of the other chitin-induced flavonoids, two of which are new.

RESULTS AND DISCUSSION

Four flavonoids were isolated from chitin-treated cells of old man cactus suspension cultures, including two new compounds, 6,7-dihydroxy-5-methoxyflavone 7-O-β-D-glucopyranoside (3) and (2S)-6,7-dihydroxy-5-methoxyflavanone 7-O-β-D-glucopyranoside (4), as well as the known baicalein (2a) and baicalein 7-O-β-D-glucopyranoside (2). These compounds were not detected in unelicited cells in culture.

The UV spectra of 2 showed typical absorptions for flavones. The CI mass spectrum gave a molecular ion at m/z 432 and the EI mass spectrum gave an A-ring fragment at m/z 168 and a B-ring fragment at m/z 102, suggesting that there are three hydroxyl substituents in the A-ring and none in the B-ring. The 1H NMR data supported this substitution pattern: the spectrum exhibited two singlets for aromatic protons at 6.708 and 7.02.

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(one for an A-ring proton and another for H-3), as well as signals for five B-ring protons. The 13C NMR data were identical with those reported for a known flavone, namely baicalein 7-O-β-D-glucopyranoside [3]. Compound 2a, the aglycone of 2, was identified by direct UV spectral and co-TLC comparison with a standard sample of baicalein. The 13C NMR signals of 2a are assigned in Table 1.

Spectral data for 3 indicated it to be a flavone with a substitution pattern similar to 2 and 2a, namely three substituents in the A-ring and none in the B-ring. The three substituents in 3 were identified as OMe (δ3.80), OH (δ8.87) and OR (R = sugar). The sugar moiety was determined to be β-D-glucose by 13CNMR and by acid hydrolysis. To assign the positions of substitution within the A-ring, compound 3 was hydrolysed to give 3a, methylation of 3a afforded 3b. By 1H NMR 3b was identified as 5,6,7-trimethoxyflavone as the two singlets at δ6.80 and 6.66 exhibited by 3b were unambiguously assigned to H-8 and H-3, respectively by comparison with the chemical shifts of authentic standards (Table 2). Therefore, the 1H NMR signals at δ7.31 and δ6.79 of 3 are assigned to H-8 and H-3, respectively. The assignment of substituents to sites 5, 6 and 7 was carried out as follows. The hydroxyl group in 3 was clearly not sited at C-5 as no AlCl3/HCl shift was observed in the absorption spectrum. Its 1H NMR chemical shift (δ8.87) in DMSO-d6, however, equated closely with that of the OH-6 in 2 (δ8.63) and is quite distinct from that of OH-7 which failed to enhance the H-8 signal by irradiating the δ3.80 methoxyl signal. In a control experiment, irradiation of the H-3 signal at δ6.62 did produce an enhancement of the H-2’, H-6’ signals at δ7.89. The 13C NMR spectrum also supported the presence of a OMe-5 in that the C-4 resonance appeared at δ175.9 [11], and the methoxyl signal at δ6.10 is consistent with the presence of two ortho substituents [12]. Together these data define 3 as 6,7-dihydroxy-5-methoxyflavone 7-O-β-D-glucopyranoside.

The UV spectral data for 3 and 3a were readily interpretable on the basis of their proposed structures and especially when the data were compared to the spectra of 2 and 2a. The long wavelength band for 2a is at 326 nm for the A-ring chromophore and, as expected, shifts hypsochromically to 314 nm when the 7 position is glycosylated. Similarly, the less intense A-ring band at 314 nm for 3a shifts hypsochromically to 306 nm when the 7 position is glycosylated as in 3. The AlCl3 and AlCl3/HCl spectra of 2, 2a and 3, 3a are not comparable because 2 and 2a contain the usual OH-5 and C=κetone complex which remains stable in the presence of acid while 3a forms a 6,7-diOAH--AlCl3 complex exhibiting a band at 393 nm. This band is replaced by another at 372 nm on the addition of HCl. Such residual effects, probably due to the HCl itself, have been noted previously with flavones lacking a free OH-5 [10].

Compound 4 exhibited typical 1H and 13C NMR signals for a flavone: an 1H NMR ABX pattern was observed for Hα-3, Hα-3 and H-2, and 13C NMR signals at δ78.4 and 44.9 were representative of C-2 and C-3, respectively. As with 2, 2a and 3, 4 was also found to have an unsubstituted B-ring and three A-ring substituents: OMe (δ3.73), OH (δ8.27) and O-glucosyl; the CI mass spectrum gave a [M + 1] at m/z 499 which was in accord with a flavane containing these substituents. Compound 4 has the same substitution pattern as 3, i.e. OMe at C-5, OH at C-6, and O-glucosyl at C-7 on the basis of the following evidence: (i) 1H NMR of 4: OH-6 at δ8.27; (ii) NOE of aglycone 4a: no enhancement of the signal for H-8 by irradiating the OMe signal (δ3.73) and O-glucosyl; the CI mass spectrum gave a [M + 1] at m/z 499 which was in accord with a flavane containing these substituents. Compound 4 has the same substitution pattern as 3, i.e. OMe at C-5, OH at C-6, and O-glucosyl at C-7 on the basis of the following evidence: (i) 1H NMR of 4: OH-6 at δ8.27; (ii) NOE of aglycone 4a: no enhancement of the signal for H-8 by irradiating the OMe signal (δ3.73) and O-glucosyl; the CI mass spectrum gave a [M + 1] at m/z 499 which was in accord with a flavane containing these substituents. Compound 4 has the same substitution pattern as 3, i.e. OMe at C-5, OH at C-6, and O-glucosyl at C-7 on the basis of the following evidence: (i) 1H NMR of 4: OH-6 at δ8.27; (ii) NOE of aglycone 4a: no enhancement of the signal for H-8 by irradiating the OMe signal (δ3.73) and O-glucosyl; the CI mass spectrum gave a [M + 1] at m/z 499 which was in accord with a flavane containing these substituents. Compound 4 has the same substitution pattern as 3, i.e. OMe at C-5, OH at C-6, and O-glucosyl at C-7 on the basis of the following evidence: (i) 1H NMR of 4: OH-6 at δ8.27; (ii) NOE of aglycone 4a: no enhancement of the signal for H-8 by irradiating the OMe signal (δ3.73) and O-glucosyl; the CI mass spectrum gave a [M + 1] at m/z 499 which was in accord with a flavane containing these substituents. Compound 4 has the same substitution pattern as 3, i.e. OMe at C-5, OH at C-6, and O-glucosyl at C-7 on the basis of the following evidence: (i) 1H NMR of 4: OH-6 at δ8.27; (ii) NOE of aglycone 4a: no enhancement of the signal for H-8 by irradiating the OMe signal (δ3.73) and O-glucosyl; the CI mass spectrum gave a [M + 1] at m/z 499 which was in accord with a flavane containing these substituents. Compound 4 has the same substitution pattern as 3, i.e. OMe at C-5, OH at C-6, and O-glucosyl at C-7 on the basis of the following evidence: (i) 1H NMR of 4: OH-6 at δ8.27; (ii) NOE of aglycone 4a: no enhancement of the signal for H-8 by irradiating the OMe signal (δ3.73) and O-glucosyl; the CI mass spectrum gave a [M + 1] at m/z 499 which was in accord with a flavane containing these substituents.
methoxyflavanone 7-O-β-D-glucopyranoside is assigned to 4.

A common feature of the four flavonoids reported here, as well as the previously reported aurone (1), is that they all have 5,6,7-oxygenation in the A-ring and an unsubstituted B-ring. This suggests that they may share the same biogenetic precursor as was proposed for the formation of the aurone, namely 2',4',6'-trihydroxychalcone [1]. Chitin elicitation of the culture appears to activate a biosynthetic pathway that bypasses p-coumaric acid in the formation of the chalcone. Paré et al. [1] suggested that two chalcone synthases are probably involved in flavonoid biosynthesis in elicited old man cactus tissue, one that uses p-coumaroyl CoA as substrate and another that uses cinnamoyl CoA, with the latter pathway resulting in flavonoids with unsubstituted B-rings. In most plants studied to date, the p-coumarate: CoA ligase which activates p-coumaric acid exhibits little or no activity with cinnamic acid [15,16]; thus a novel cinnamoyl: CoA ligase appears to be present in the elicited cultures of old man cactus. Enzyme purification studies are underway to establish the details of this potentially novel biosynthetic pathway to flavonoids.

EXPERIMENTAL

General. Mps: uncorr; TLC: (i) polygram polyamide-6 (Macherey-Nagel), the spots were visualized under UV light (365 nm) and by spraying with 1% AlCl₃ in EtOH; (ii) cellulose (EM Science); CC: polyamide-6-powder (Serva) and polyclar AT (GAF Co.); ¹H NMR: 500, 360 and 250 MHz; ¹³C NMR: 125, 90 and 62.5 MHz. EIMS: 70 eV.

Extraction and isolation. The cell suspension culture of \textit{Cephalocereus senilis} employed for this study was prepared as previously reported [2]. After 48 hr post-subculture, 80 flasks of culture (475 ml each) were elicited with chitin suspension. To each flask, 25 ml of sterile chitin suspension (5 mg ml⁻¹) were added. The cells were collected by filtration 24 hr after elicitation and sonicated in MeOH at 50° for 30 min (× 3). The MeOH soln was cooled under vacuum at 60° and partitioned between H₂O and CH₂Cl₂. EtOAc and n-BuOH, respectively. The concentrates of the EtOAc and n-BuOH fractions contained similar flavonoids (TLC) and were combined (8 g). The combined material was chromatographed on a polyclar AT (200 g) column eluted with H₂O and then H₂O–MeOH with increasing amounts of MeOH; 208 fractions of 15 ml were collected. Fractions 109–138 yielded a yellow ppt, which was purified on a polyamide-6-powder column eluted with the same H₂O–MeOH solvent system to give 2 (182 mg). Fractions 88–95 gave 3 (200 mg) as yellow cubes; the material was recrystallized from MeOH. Fractions 50–74 afforded 4 (190 mg) as fine needles. Compound 2a (80 mg) was purified from the CH₂Cl₂ fraction by a polyclar AT column eluted with the same H₂O–MeOH system and then a Sephadex LH-20 column eluted with MeOH.

\textbf{Acid hydrolysis.} Samples were dissolved in a small amount of MeOH and hydrolysed in 2 M HCl at 95°. The concentrates from the EtOAc extracts of the hydrolysates were purified by passing them through a polyamide column and the eluents analysed for aglycones. The water layers were used for sugar analysis (co-TLC with glucose) on cellulose TLC in pyridine–EtOAc–HOAc–H₂O (36:36:7:21).

\textbf{Methylation.} Samples were dissolved in MeOH and methylated with diazomethane which was generated from N-methyl-N-nitroso-p-toluene sulphonamide (Diazald, Aldrich). Excessive amounts of diazomethane in Et₂O were added to chilled samples which were kept on ice for 1 hr and then at room temp. overnight.

\textbf{Baizalein 7-O-β-D-glucopyranoside (2).} Yellow amorphous powder (MeOH), C₁₃H₂₁O₁₀, mp 212–214° (lit. [17] dec 208°, lit. [18] 222–223°). TLC (polyamide): MeOH (2:1), Rₚ 0.62. UV \(\lambda_{max} \text{nm} \): 245sh, 275, 315; + NaOMe: 241, 272, 315 (dec); + AlCl₃: 249, 283, 292sh, 342; + AlCl₃ + HCl: 249, 283, 292sh, 339, 402sh; + NaOAc: 276, 308sh, 400sh (dec); + NaOAc + H₂BO₃: 278, 320sh. EIMS \(m/z\) (rel. int.): 270 [M–Glcé]⁺ (86), 168 (30), 102 (24), 77 (11). CIMS \(m/z\) (rel. int.): 432 [M⁺] (17), 270 [M–Glcé]⁺ (76). ¹H NMR (250 MHz, DMSO-d₆, TMS, δ ppm): [aglycone moiety] 12.59 (1H, s, OH-5), 8.63 (1H, s, OH-6), 8.09 (2H, dd, J = 7.7, 1.6 Hz, H-2 and H-6), 7.60 (3H, m, H-3', H-4' and H-5'), 7.08 (1H, s, H-8 or H-3) and 7.02 (1H, s, H-3 or H-8); [glucosyl moiety] 5.48 (1H, br s, OH-2'), 5.35 (1H, d, J = 4.3 Hz, OH-3'), 5.16 (1H, dd, J = 5.2 Hz, OH-4'), 5.05 (1H, d, J = 7.1 Hz, H-1'), 4.73 (1H, t, J = 5.2 Hz, OH-6'), 3.78 (1H, m, H-6'), 3.52 (2H, m, H-6' and H-3'), 3.38 (2H, m, H-2' and H-5') and 3.25 (1H, m, H-4'). ¹³C NMR (62.5 MHz, DMSO-d₆, TMS): Table 1.

\textbf{Baizalein (2a).} Yellow amorphous powder, C₁₃H₁₉O₅, mp 263–264° (lit. [19] 264–265°). UV \(\lambda_{max} \text{nm} \): 252sh, 270, 326, 370sh; + NaOMe: 256, 363, 425sh; + AlCl₃: 250sh, 271, 282sh, 367; + AlCl₃ + HCl: 256sh, 282, 295sh, 348; + NaOAc: 256, 358, 428sh; + NaOAc + H₂BO₃: 264, 279sh, 357, 420sh. EIMS \(m/z\) (rel. int.): 270 [M⁺] (70), 269 [M–1⁺] (100), 242 (13), 168 (73), 140 (47), 135 (25), 112 (28), 105 (22), 102 (39), 77 (42), 69 (55). ¹H NMR (360 MHz, DMSO-d₆, TMS, δ ppm): 12.68 (1H, s, OH-5), 8.07 (2H, m, H-2' and H-6'), 7.59 (3H, m, H-3', H-4' and H-5'), 6.94 (1H, s, H-8 or H-3) and 6.65 (1H, s, H-3 or H-8). ¹³C NMR (90 MHz, DMSO-d₆, TMS): Table 1.

6,7-Dihydroxy-5-methoxyflavone 7-O-β-D-glucopyranoside (3). Light yellow cubes (MeOH), C₁₂H₁₀O₅, mp 156–158°. TLC (polyamide): MeOH–H₂O–H₂O (2:1), Rₚ 0.81. UV \(\lambda_{max} \text{nm} \): 240, 402, 270 (4:3:2), 306 (4:1:7); + NaOMe: 268, 291, 396; + AlCl₃: 240, 270, 306; + AlCl₃ + HCl: 240, 270, 306; + NaOAc: 269, 293, 394; + NaOAc + H₂BO₃: 270, 307. FABMS \(m/z\) (rel. int.): 447 [M⁺ + 1⁺] (19), 285 [M + 1⁺] (30), 270 [285–Me⁺] (6). ¹H NMR (500 MHz, DMSO-d₆, TMS, δ ppm): [aglycone moiety] 8.87 (1H, s, OH-6), 8.04 (2H, dd, J = 8.1, 1.6 Hz, H-2' and H-6'), 7.58 (3H, m, H-3', H-4' and H-5'), 7.31 (1H, s, H-8), 6.79 (1H, s, H-3), 3.80 (3H, s, OMe-5); [glucosyl moiety, assigned by C–H correlation spectrum] 5.45 (1H, d, J = 4.1 Hz, OH-2'), 5.19 (1H, d, J = 4.7 Hz, OH-3'), 5.14 (1H, d, J = 5.3 Hz, OH-4'), 5.10 (1H, d, J = 7.3 Hz, H-1'), 4.71 (1H, t, J = 5.6 Hz, OH-6'), 3.78 (1H,
6,7-Dihydroxy-5-methoxyflavone (3a). Acid hydrolysis of 3a gave 4a light yellow powder, C_{16}H_{12}O_{5}, mp 166–167°C. TLC (polyamide): MeOH–H$_2$O (2:1), R$_f$ 0.51. UV max nm: 245, 268, 314; +NaOMe: 246, 267, 363; +AlCl$_3$: 241, 277, 393; +AlCl$_3$ + HCl: 247, 272, 312, 327, +NaAc: 267, 364; +NaAc + H$_2$BO$_3$: 262, 301, 312, 327. $^1$H NMR (500 MHz, Me$_2$SO-d$_6$, δ ppm): 7.89 (2H, m, H-2' and H-6'), 7.47 (3H, m, H-3', H-4' and H-5'), 6.81 (1H, s, H-8), 6.62 (1H, s, H-3), 3.80 (3H, OMe). Glucose was identified by TLC in the aq. layer.

6,7-Trimethoxyflavone (4b). Methylation of 4a gave 4b. Powder, C_{18}H_{14}O$_6$. $^1$H NMR (250 MHz, CDCl$_3$, δ ppm): 7.40 (3H, m, H-2', H-3', H-4' and H-6'), 6.34 (1H, s, H-8), 5.39 (1H, dd, J = 3.0, 13.2 Hz, H-2), 3.93 (3H, OMe), 3.86 (3H, s, OMe), 3.81 (3H, s, OMe), 3.00 (1H, dd, J = 13.2, 16.7 Hz, H$_{ex}$-3) and 2.77 (1H, dd, J = 16.7, 3.0 Hz, H$_{ex}$-3). $^{13}$C NMR (62.5 MHz, CDCl$_3$): Table 1.

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