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FLAVONOID CELLOBIOSIDES FROM *SALVIA ULIGINOSA*

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Key Word Index—*Salvia uliginosa*; Lamiaceae; petals; copigment; apigenin 7-*O*-cellobioside; apigenin 7-*O*-cellobioside-4′-*O*-glucoside; apigenin 7,4′-*O*,*O*-diglucoside.

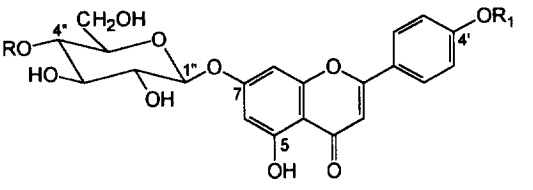
Abstract—Two new flavone glycosides with an unusual interglycosidic linkage have been isolated from the petals of *Salvia uliginosa* and identified by NMR spectroscopy as apigenin 7-*O*-β-D-glucopyranosyl-(1‴ → 4″)-β-D-glucopyranoside and apigenin 7-*O*-β-D-glucopyranosyl-(1‴ → 4″)-β-D-glucopyranoside-4′-*O*-β-D-glucopyranoside. The NMR and UV spectra of these compounds were compared with those of the known compound, apigenin 7,4′-*O*,*O*-di-β-D-glucopyranoside, which was isolated from the same source.

INTRODUCTION

Many species in the genus *Salvia* L. are noted for their brightly coloured flowers, which are typically pink to red or purple to blue. The pigments which give rise to blue flower colours are known to be complex, and may consist of several components including a coloured anthocyanin, a colourless flavonoid copigment, and one or more metal ions [1, 2]. A typical example is the pigment protodelphin isolated from the deep blue flowers of *S. patens*, which comprises the anthocyanin delphinidin 3-(6″-*p*-coumaroylglucoside)-5-(6″-malonylglucoside), the flavone apigenin 7,4′-*O*,*O*-diglucoside and magnesium [3]. *S. uliginosa* Benth., a perennial herb native to South America, was first cultivated at the Royal Botanic Gardens, Kew, as long ago as 1913 because of its beautiful sky-blue flowers [4]. In the present paper, the isolation and characterisation of potential flavonoid copigments from extracts of these flowers are described. Two of the compounds were found to be new apigenin derivatives with the disaccharide, *O*-β-D-glucosyl-(1 → 4)-glucose (cellobiose), attached at C-7.

RESULTS AND DISCUSSION

The methanolic extract of *S. uliginosa* petals was analysed for flavonoid glycosides by means of HPLC with photodiode array detection. One of the compounds present, **1**, gave apigenin and glucose on hydrolysis, and had a UV spectrum characteristic of apigenin 7-*O*-glycosides [5]. Its R_t was less than that of apigenin 7-*O*-glucoside, a standard used for comparison. Comparison of R_f values obtained by PC in three solvent systems (BAW, 15% HOAc and H_2O), indicated that those for **1** were less than those for apigenin 7-*O*-glucoside in every case. These data appeared to be contradictory, as although the lower R_f value in BAW and shorter R_t were consistent with the identification of **1** as an apigenin 7-*O*-diglucoside, a diglucoside would normally be expected to have a greater R_f value in 15% HOAc than the corresponding monoglucoside. Isolation and purification of **1** were achieved by means of prep PC and semi-prep HPLC, and the structure solved using NMR spectroscopy. The ^{1}H NMR spectrum, obtained with a carefully dried sample of **1** freshly dissolved in DMSO-d_6, contained two distinctive groups of resonances. Those at δ 12.95 (*s*, 5-OH), 7.95 (*d*, J = 8.9 Hz, H-2′,6′), 6.95



	R	R_1
1	β-D-glc	H
2	H	β-D-glc
3	β-D-glc	β-D-glc

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(*d*, J = 8.9 Hz, H-3′,5′), 6.85 (*s*, H-3), 6.83 (*d*, J = 2.0 Hz, H-8) and 6.47 (*d*, J = 2.0 Hz, H-6), represented aromatic protons, and were consistent with the identification of **1** as an apigenin 7-*O*-glycoside [6]. The second group, appearing between δ 3.00 and 5.50, comprised both non-exchangeable and exchangeable resonances of glycosidic origin. Two anomeric proton resonances were noted at δ 4.31 (*d*, J = 7.8 Hz) and 5.16 (*d*, J = 7.8 Hz), and used as the starting point for the complete assignment of the non-exchangeable glycosyl resonances. This was achieved by identifying two sets of sequential connectivities from DQF-COSY data, as illustrated in Fig. 1. The ^{1}H chemical shift and coupling constant data summarised in Table 1 were consistent with the identification of each glycosyl moiety as β-D-glucose [7]. Selective excitation of the δ 4.31 resonance using 1D XSROESY gave ROE connectivities to protons of both glucose moieties, but not to any aromatic protons [8]. This confirmed the identity of **1** as a diglucoside, and the assignment of the anomeric proton resonances at δ 4.31 and 5.16 to the terminal and primary glucose respectively. All of the exchangeable hydroxyl resonances were readily assigned from the DQF-COSY spectrum (Fig. 1). The terminal glucose had free 2-OH, 3-OH, 4-OH and 6-OH groups, whereas the primary glucose had free 2-OH, 3-OH and 6-OH groups but lacked a 4-OH group, indicating that this position was blocked. This data enabled the interglucosidic linkage to be defined as 1‴ → 4″. Additional confirmation was provided by analysis of corresponding ^{13}C resonance assignments obtained from $^1J(^1H, ^{13}C)$ correlations in an HSQC experiment. The C-4″ resonance of the primary glucose at δ 79.4 was found to be shifted downfield by +9.6 ppm compared with the C-4‴ resonance of the terminal glucose at δ 69.8 (Table 1). In addition, the C-3″ and C-5″ ^{13}C resonances of the primary glucose were also slightly upfield-shifted, a secondary consequence of the 1‴ → 4″ linkage. Compound **1** was therefore determined to be apigenin 7-*O*-β-D-glucopyranosyl-(1‴ → 4″)-β-D-glucopyranoside, a new flavonoid glycoside.

Two further flavone glycosides from the crude extract of *S. uliginosa* petals, **2** and **3**, also showed low mobility on paper chromatograms in the solvent BAW, with R_f values of 0.22 and 0.08, respectively, although they exhibited high mobility in 15% HOAc. Their UV spectra and HPLC R_ts were identical, and both yielded apigenin and glucose on hydrolysis. UV spectroscopy using standard shift reagents suggested that **2** and **3** were substituted at both the 7- and the 4′-positions [5]. Both compounds were purified by prep PC and semi-prep HPLC. The ^{1}H NMR spectrum of **2** comprised aromatic resonances typical of an apigenin derivative together with both exchangeable and non-exchangeable glycosyl resonances. Those corresponding to the B-ring aromatic protons of **2**, at δ 8.07 (*d*, J = 8.9 Hz, H-2′,6′) and 7.21 (*d*, J = 8.9 Hz, H-3′,5′) were downfield-shifted, consistent with substitution at the 4′ position [6]. Two anomeric pro-

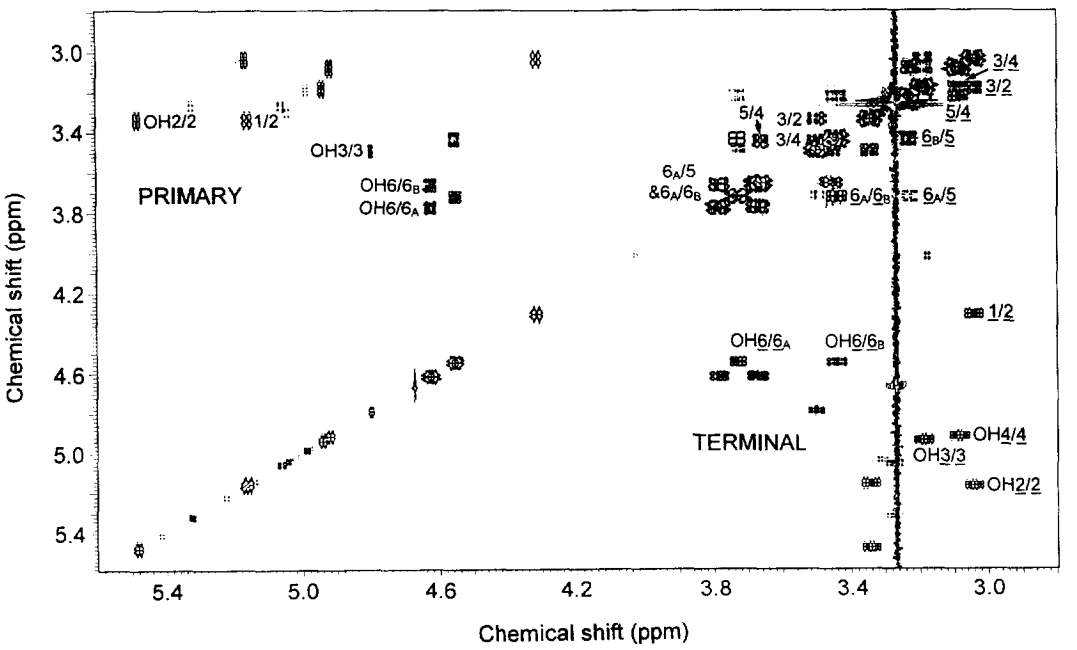

Fig. 1. 500 MHz DQF-COSY spectrum of **1** (apigenin 7-*O*-β-D-glucopyranosyl-(1‴ → 4″)-β-D-glucopyranoside), showing sequential ^{1}H–^{1}H connectivities associated with resonances of glycosidic origin. Cross-peaks corresponding to the primary and terminal glucose units are labelled above and below the diagonal respectively, for reference purposes. Assignments for the terminal glucose unit are shown underlined. The absence of an OH4/4 cross-peak for the primary glucose unit should be noted. Unlabelled cross-peaks of low intensity correspond to a contaminant present in trace amounts.

Table 1. ^{1}H and ^{13}C NMR chemical shift assignment and coupling constant data for compound **1** (δ in DMSO-d_6, 500 MHz, 37°)

H	δ(^{1}H)	δ(^{1}H) Exchangeable	δ(^{13}C)
3	6.85 *s*		102.8
6	6.47 *d* (2.0)		99.3
8	6.83 *d* (2.0)		94.7
2′,6′	7.95 *d* (8.9)		128.5
3′,5′	6.95 *d* (8.9)		115.8
1″	5.16 *d* (7.8)		99.4
2″	3.34 *m*	5.48 *d* (5.4)	72.5
3″	3.49 *m*	4.80 *d* (2.3)	74.6
4″	3.45 *m*		79.4
5″	3.66 *m*		74.8
6″	3.67 *m*	4.63 *t* (5.7)	59.8
	3.78 *dd*		
1‴	4.31 *d* (7.8)		102.9
2‴	3.04 *m*	5.17 *d* (4.9)	73.0
3‴	3.19 *m*	4.94 *d* (4.9)	76.4
4‴	3.08 *m*	4.92 *d* (5.5)	69.8
5‴	3.21 *m*		76.7
6‴	3.44 *m*	4.55 *t* (5.3)	60.8
	3.73 *ddd*		

ton resonances were observed at δ 5.03 (*d*, J = 7.3 Hz) and 5.07 (*d*, J = 7.8 Hz). Selective excitation of the H-3′,5′ resonance at δ 7.21 using 1D XSROESY gave a strong ROE to the anomeric proton resonance at δ 5.03, thus assigning it to a 4′-*O*-glucosyl moiety. Use of the same technique to selectively excite the H-8 resonance at δ 6.86 resulted in the observation of a strong ROE to the anomeric proton resonance at δ 5.07, thus confirming its assignment to a 7-*O*-glucosyl moiety. Analysis of ^1J(^{1}H, ^{13}C) correlations in an HSQC experiment allowed remaining ^{1}H and ^{13}C resonance assignments of both glycosyl moieties to be completed. Compound **2** was therefore identified as apigenin 7,4′-*O*,*O*-di-β-D-glucopyranoside, a known apigenin glycoside. The ^{1}H NMR spectrum of **3** was more complex than the corresponding spectra of **1** and **2**, but contained features common to them both. Aromatic resonances at δ 12.87 (*s*, 5-OH), 8.06 (*d*, J = 8.9 Hz, H-2′,6′), 7.21 (*d*, J = 8.9 Hz, H-3′,5′), 6.96 (*s*, H-3), 6.88 (*d*, J = 2.0 Hz, H-8) and 6.48 (*d*, J = 2.0 Hz, H-6), were consistent with an apigenin derivative substituted at the 4′ position [6]. Three anomeric proton resonances were found at δ 5.16 (*d*, J = 7.8 Hz), 5.03 (*d*, J = 7.3 Hz) and 4.32 (*d*, J = 7.8 Hz) and the site of attachment of the glycosyl moieties determined using 1D XSROESY experiments. Selective excitation at δ 5.16 resulted in ROE connectivities to the H-6 and H-8 protons of the A-ring, indicating attachment of the corresponding glycosyl moiety at C-7. In a similar experiment, selective excitation at δ 5.03 resulted in ROE connectivities to the H-3′,5′ protons of the B-ring indicating attachment of a second glycosyl moiety at C-4′. A complete set of glycosyl ^{1}H and ^{13}C resonance assignments for **3**, obtained from ^1J(^{1}H, ^{13}C) correlations in an HSQC experiment, and comparison with corresponding data for **1** and **2**, confirmed the presence in **3** of both 7-*O*-β-D-glucopyranosyl-(1‴ → 4″)-β-D-glucopyranoside and 4′-*O*-β-D-glucopyranoside units. Compound **3** was therefore determined to be apigenin 7-*O*-β-D-glucopyranosyl-(1‴ → 4″)-β-D-glucopyranoside-4′-*O*-β-D-glucopyranoside, a second new apigenin glycoside. The disaccharide unit comprising two glucose molecules linked 1 → 4 is commonly found in plants as a constituent of cellulose, and has been assigned the trivial name cellobiose. Compounds **1** and **3** may thus be more conveniently referred to as apigenin 7-*O*-cellobioside and apigenin 7-*O*-cellobioside-4′-*O*-glucoside respectively. These examples appear to represent the first definitive report of the occurrence of flavonoid cellobiosides, although in an earlier investigation concerning the Chinese medicinal herb 'Huai-Jiao', one constituent was identified as the 7-*O*-cellobioside of the isoflavonoid, genistein [9]. However, this proposal, and the precise identity of the plant material used in the study, require further confirmation. The number of cellobiosides cited in the literature representing other classes of natural product is very limited [10], and the potential for misidentification due to partial characterisation must be borne in mind. Cellobiose was first recorded as a glycosidic component in haemocorin, a substituted phenalenone derivative isolated from the roots of *Haemodorum corymbosum* Vahl (Haemodoraceae) [11, 12]. In this example, the disaccharide unit released from haemocorin by mild acid hydrolysis was identified as cellobiose on the basis of melting point, optical rotation, PC and chemical derivatisation [11].

Apigenin 7,4′-*O*,*O*-di-β-D-glucopyranoside (**2**) has been reported previously from a number of sources, including the petals of *S. patens*, where it was identified as a copigment in the blue pigment protodelphin [3]. It is conceivable that the same compound may function as the copigment of the anthocyanin component in *S. uliginosa* petals. This does not exclude the possibility however, that the apigenin cellobiosides **1** and **3** participate in this process. Resolution of this important question will be afforded by the identification of the anthocyanin components of *S. uliginosa* petals, work which is currently in progress.

EXPERIMENTAL

Plant material

Salvia uliginosa was grown outside at the Royal Botanic Gardens (RBG), Kew, during the summer of 1996 (accession number: 1987-524). Plants were harvested in October, and the petals carefully removed from the inflorescences to avoid contamination with

sepals and other organs of the same plant. The petals were freeze-dried immediately after selection. A voucher specimen of the plant is deposited in the Herbarium, RBG Kew.

General

^1H and ^{13}C NMR spectra were recorded at 500 and 125 MHz, respectively. Samples were dissolved in DMSO-d_6 with TMS as primary reference. A temp. of 37° was used for all NMR experiments.

Extraction procedures

14 g of freeze-dried petals of *S. uliginosa* were extracted for 24 h in 900 ml 80% MeOH, and re-extracted for another 24 h period with 500 ml 80% MeOH. The extracts were combined after filtration and a sample analysed by HPLC for the presence of flavonoid glycosides. The combined extract was then concd. to ca 2 ml prior to prep. PC.

Isolation of flavonoid glycosides

Compounds **1**, **2** and **3** were isolated by means of two prep. PC steps using the solvents BAW (*n*-BuOH–H_2O–HOAc, 4:1:5, v/v, upper layer) and 15% aq. HOAc, followed by semiprep. HPLC. Sample purity was monitored throughout by analytical HPLC.

Analytical and semiprep. HPLC

A Waters HPLC system consisting of a LC 600 pump and 996 photodiode array detector was used in gradient elution mode. LiChrospher 100RP-18 columns of dimension 4.0 mm (i.d.) × 250 mm and 10.0 mm (i.d.) × 250 mm were used for analytical and semiprep. applications respectively. The solvent systems employed were 2% aq. HOAc (A) and MeOH, HOAc, H_2O (18:1:1) (B) (system I), or H_2O (A) and MeOH (B) (system II). Initial conditions for both solvent systems were $A = 75\%$ with a linear gradient reaching $A = 0\%$ at $t = 20$ min. Isocratic elution continued to $t = 23$ min, after which time the programme returned to the initial solvent composition. A flow rate of 1.0 ml min^{-1} and column temp. of 25° were maintained throughout for analytical HPLC, whereas a flow rate of 4.5 ml min^{-1} and column temp. of 30° were used for semiprep. HPLC.

Acid hydrolysis

A small amount of each compound was dissolved in 0.5 ml 50% MeOH in a test tube. 3 ml of 2 M HCl was added to each sample and the mixture heated in a boiling water bath for 30 min. The soln was cooled, 2 ml of EtOAc were added and mixed thoroughly with the hydrolysate. The upper EtOAc layer was removed with a pipette, evaporated to dryness, dissolved in 0.8 ml of 80% aq. MeOH and analysed for flavonoid aglycones by HPLC. The aq. bottom layer was evaporated to dryness and analysed for sugars by PC [5].

Apigenin 7-O-β-D-*glucopyranosyl*-(1‴ → 4″)-β-D-*glucopyranoside* (*Apigenin* 7-O-*cellobioside*) (**1**)

UV λ_{max} MeOH nm: 266, 337; + NaOH 272, 300 sh, 348 sh, 376; + $AlCl_3$ 274, 298 sh, 337, 380 sh; + $AlCl_3$ and HCl 274, 298 sh, 337, 378 sh; + NaOAc 256 sh, 266, 391; + NaOAc and H_3BO_3 266, 336. PC: R_f in BAW 0.23; R_f in 15% HOAc 0.15. HPLC: R_t in solvent I 15.7 min; R_t in solvent II 15.6 min. Acid hydrolysis for 30 min: apigenin and glucose. ^1H and ^{13}C NMR: see Table 1.

Apigenin 7,4′-O,O-*di*-β-D-*glucopyranoside* (*Apigenin* 7,4′-O,O-*diglucoside*) (**2**)

UV λ_{max} MeOH nm: 269, 317; + NaOH 288, 368 sh (decreased intensity); + $AlCl_3$ 279, 297, 332, 378 sh; + $AlCl_3$ and HCl 278, 296, 330, 374 sh; + NaOAc 269, 317; + NaOAc and H_3BO_3 269, 317. PC: R_f in BAW 0.22; R_f in 15% HOAc 0.50. HPLC: R_t in solvent I 11.2 min; R_t in solvent II 11.7 min. Acid hydrolysis for 15 min: apigenin, apigenin 7-*O*-glucoside, apigenin 4′-*O*-glucoside and glucose; for 30 min: apigenin and glucose. ^1H NMR (DMSO-d_6): δ 12.88 (1H, *s*, 5-OH), 8.07 (2H, *d*, $J = 8.9$ Hz, H-2′,6′), 7.21 (2H, *d*, $J = 8.9$ Hz, H-3′,5′), 6.97 (1H, *s*, H-3), 6.86 (1H, *d*, $J = 2.0$ Hz, H-8), 6.47 (1H, *d*, $J = 2.0$ Hz, H-6), 5.07 (1H, *d*, $J = 7.8$ Hz, H-1″), 5.03 (1H, *d*, $J = 7.3$ Hz, H-1‴), 3.72, 3.49 (2 × 2H, 2 × m, 2 × H-6″ and 2 × H-6‴), 3.47 (1H, *m*, H-5″), 3.41 (1H, *m*, H-5‴), 3.31 (2H, *m*, H-3″ and H-3‴), 3.30 (2H, *m*, H-2″ and H-2‴), 3.19 (2H, *m*, H-4″ and H-4‴); ^{13}C NMR (DMSO-d_6) (assignments of non-quaternary C atoms from HSQC): δ 128.3 (C-2′,6′), 116.7 (C-3′,5′), 104.1 (C-3), 100.0 (C-1″ and C-1‴), 99.7 (C-6), 95.1 (C-8), 77.0 (C-5″ and C-5‴), 76.4 (C-3″ and C-3‴), 73.1 (C-2″ and C-2‴), 69.8 (C-4″ and C-4‴), 60.6 (C-6″ and C-6‴).

Apigenin 7-O-β-D-*glucopyranosyl*-(1‴ → 4″)-β-D-*glucopyranoside*-4′-O-β-D-*glucopyranoside* (*Apigenin* 7-O-*cellobioside*-4′-O-*glucoside*) (**3**)

UV λ_{max} MeOH nm: 269, 317; + NaOH 286, 368 sh (decreased intensity); + $AlCl_3$ 278, 299 sh, 332, 380 sh; + $AlCl_3$ and HCl 277, 298 sh, 330, 378 sh; + NaOAc 268, 316; + NaOAc and H_3BO_3 269, 317. PC: R_f in BAW 0.08; R_f in 15% HOAc 0.48. HPLC: R_t in solvent I 11.2 min; R_t in solvent II 11.7 min. ^1H NMR (DMSO-d_6): δ 12.87 (1H, *s*, 5-OH), 8.06 (2H, *d*, $J = 8.9$ Hz, H-2′,6′), 7.21 (2H, *d*, $J = 8.9$ Hz, H-3′,5′), 6.96 (1H, *s*, H-3), 6.88 (1H, *d*, $J = 2.0$ Hz, H-8), 6.48 (1H, *d*, $J = 2.0$ Hz, H-6), 5.16 (1H, *d*, $J = 7.8$ Hz, H-1″), 5.03 (1H, *d*, $J = 7.3$ Hz, H-1⁗), 4.32 (1H, *d*, $J = 7.8$ Hz, H-1‴), 3.78 (1H, *m*, H-6″), 3.72 (2 × 1H, 2 × *m*, H-6‴ and H-6⁗), 3.68 (1H, *m*, H-6″), 3.67 (1H, *m*, H-5″), 3.49 (1H, *m*, H-3″), 3.48 (1H, *m*, H-6⁗), 3.46 (1H, *m*, H-4″), 3.44 (1H, *m*, H-6‴), 3.41 (1H, *m*, H-

5''''), 3.33 (1H, *m*, H-2''), 3.31 (1H, *m*, H-3''''), 3.29 (1H, *m*, H-2''''), 3.21 (1H, *m*, H-5'''), 3.19 (1H, *m*, H-3'''), 3.18 (1H, *m*, H-4''''), 3.08 (1H, *m*, H-4'''), 3.04 (1H, *m*, H-2'''); ^{13}C NMR (DMSO-d_6) (assignments of non-quaternary C atoms from HSQC): δ 128.2 (C-2',6'), 116.6 (C-3',5'), 104.2 (C-3), 103.1 (C-1'''), 100.0 (C-1'''') 99.6 (C-6), 99.4 (C-1''), 94.9 (C-8), 79.6 (C-4''), 77.1 (C-5''''), 76.5 (C-5'''), 76.4 (C-3''''), 76.3 (C-3'''), 74.9 (C-5''), 74.6 (C-3''), 73.1 (C-2'''), 72.9 (C-2''''), 72.7 (C-2''), 69.9 (C-4'''), 69.6 (*C*-4''''), 60.8 (C-6'''), 60.6 (C-6''''), 59.8 (C-6'').

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