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COUMARINS FROM THE FLOWERS OF *Trifolium repens*

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Trifolium repens L. (white clover), Fabaceae, is one of the most important and widely distributed forage legumes in the world [1, 2]. In addition to its high forage quality, the flowers and aerial parts are used medicinally due to their anti-inflammatory, anti-diarrheal, and mildly analgesic properties [3, 4]. The biological activity of this species is associated primarily with the flavonoids (mainly glycosides of flavonol derivatives) [5–7], bicoumarins [8], triterpene saponins [9], phenolic acids [10], and condensed tannins [11]. To date, there has been a study of the coumarins in white clover, but it was incomplete. Additionally, the majority of available literature data was published before 1970 [12–14]. Therefore, these papers, we describe the isolation from flowers of *T. repens* and structural elucidation of five coumarins. The compounds were identified using spectroscopic methods (UV, IR, MS, ^{1}H and ^{13}C NMR) and co-chromatography (TLC, PC) with standards. The identity of the sugar moiety was confirmed by the product of acid hydrolysis. All these data were in agreement with the literature data. Our chemical investigation of the flowers of *T. repens* led to the isolation of umbelliferone (**1**), scopoletin (**2**), repensin B (**3**), daphnoretin (**4**), and daphnorin (daphnoretin 7-*O*-β-D-glucoside) (**5**). Compounds **2** and **5** were isolated from *T. repens* for the first time.

Plant Material. The flowers of *Trifolium repens* L. were collected in June, 2002 from two year-old plants cultivated in Lodz, Poland. The seeds were authenticated and provided for the initiation of cultivation by Prof. Z. Staszewski, Plant Breeding and Acclimatization Institute of the Polish Academy of Sciences at Radzikow (Poland). Voucher specimens were deposited in the Herbarium of the Department of Pharmacognosy, Medical University of Lodz.

General Experimental Procedure. The melting points were determined on a Boethius apparatus and are uncorrected. The IR spectra were recorded in KBr pellets on a Mattson FTIR instrument. UV spectra were measured on a Perkin–Elmer Lambda 25 spectrophotometer in EtOH. ^{1}H (500 MHz) and ^{13}C (125 MHz) spectra were recorded in DMSO-d_6 on a Bruker DRX spectrometer, using TMS as internal standard. FAB-MS spectrum was scanned on a Finnigan MAT 95 (in glycerin, Cs^{+}, 13 keV). Preparative column chromatography (CC) was performed on silica gel 35–70 or 50–100 mesh (Macherey-Nagel), MN-Polyamid SC-6 (Macherey-Nagel) and Sephadex LH-20 (Sigma-Aldrich). Thin layer chromatography (TLC) was performed on silica gel plates (DC-Alufolien Kieselgel 60 G, Merck) using three solvent systems: S-1 benzene–EtOAc (1:1, v/v), S-2 benzene–acetone (10:3, v/v), and S-3 $CHCl_3$–MeOH (10:1, v/v). Paper chromatography (PC) was carried out on Whatman No.1 using two solvent systems: S-4 *n*-butanol–AcOH–H_2O (4:1:5, v/v/v, organic phase) and S-5 (15% AcOH, v/v).

Extraction and Isolation. The flowers of *T. repens* (360 g) were extracted with petrol and $CHCl_3$ (Soxhlet apparatus), and subsequently extracted with MeOH (7 × 2 L) and 70% aqueous MeOH (2 × 2 L) at room temperature, each for 8 h. The combined methanol extracts were suspended in 1 L of water and partitioned successively with Et_2O, EtOAc, and *n*-BuOH. The chloroform-soluble fraction (2.5 g) was first separated by CC on Sephadex (MeOH as eluent) to yield sterol and coumarin fractions. The coumarin fraction was then eluted on a silica gel column (eluent: C_6H_6–EtOAc (v/v) with EtOAc gradient). From the fraction eluted with C_6H_6–EtOAc 7:3, v/v and the fraction eluted with C_6H_6–EtOAc 3:2, v/v, compound **1** (18 mg) and compound **2** (12 mg) were obtained, respectively. The diethyl ether-soluble fraction (4.9 g) was subjected to polyamide column chromatography (eluent: C_6H_6–MeOH (v/v) with MeOH gradient) to afford three fractions (A–C). Fraction A (eluted with C_6H_6–MeOH 8.5:1.5, v/v) was re-chromatographed on a silica gel column with C_6H_6–EtOAc (4:1), v/v to afford compound **4** (10 mg). From fractions B (eluted with C_6H_6–MeOH 7:3, v/v) and C (eluted with C_6H_6–MeOH 6.5:3.5, v/v) compounds **3** (12 mg) and **5** (20 mg) were obtained, finally purified after crystallization from MeOH.

The physicochemical and spectral data of the isolated compounds are reported below.

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Umbelliferone (1), $C_9H_6O_3$. White needles, mp 225–228°C (MeOH) (lit. [15] mp 226–227°C, [16] mp 226–228°C). TLC R_f 0.73 (S-1), 0.66 (S-2), 0.69 (S-3).

Scopoletin (7-Hydroxy-6-methoxycoumarin) (2). $C_{10}H_8O_4$. White needles, mp 200–204°C (MeOH) (lit. [17] mp 204–206°C). TLC R_f 0.55 (S-1), 0.56 (S-2), 0.71 (S-3). UV spectrum (EtOH, λ_{max}, nm): 229, 254, 298, 346. IR (KBr, ν, cm^{-1}): 3285 (OH), 2946, 1705 (C=O, α-pyrone), 1561, 1291, 1141. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 9.90 (1H, s, OH-7), 7.87 (1H, d, J = 9.5, H-4), 7.17 (1H, s, H-5), 6.73 (1H, s, H-8), 6.17 (1H, d, J = 9.5, H-3), 3.78 (3H, s, OCH_3) [17]. ^{13}C NMR (125 MHz, DMSO-d_6, δ): 162.1 (C-2), 151.3 (C-6), 149.7 (C-7), 145.8 (C-9), 145.3 (C-4), 111.8 (C-3), 111.1 (C-10), 109.7 (C-5), 103.0 (C-8), 56.4 (OCH_3) [18].

Repensin B (7,5′-Dihydroxy-3,6′-bicoumarin) (3). $C_{18}H_{10}O_6$. White needles, mp 324–327°C (MeOH), TLC R_f 0.30 (S-1), 0.23 (S-2), 0.45 (S-3). PC R_f 0.85 (S-4), 0.66 (S-5). FAB-MS, *m/z* ($I_{rel.}$, %): 322 (M^-, 44), 321 (65), 275 (20), 183 (100). UV spectrum (EtOH, λ_{max}, nm): 262 sh, 329. IR (KBr, ν, cm^{-1}): 3440–3265 (OH), 2924, 1712 (C=O, α-pyrone), 1683, 1615, 1590, 1112. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 10.64 (2H, s, OH-7, 5′), 7.97 (1H, d, J = 9.5, H-4′), 7.94 (1H, s, H-4), 7.56 (1H, d, J = 8.4, H-5), 7.55 (1H, d, J = 8.4, H-7′), 6.91 (1H, d, J = 8.5, H-8′), 6.81 (1H, dd, J = 8.4, 1.5, H-6), 6.77 (1H, br.s, H-8), 6.20 (1H, d, J = 9.5, H-3′). ^{13}C NMR (125 MHz, DMSO-d_6, δ): 161.5 (C-2), 160.3 (C-2′), 159.4 (C-5′), 159.3 (C-9), 155.3 (C-7), 153.3 (C-9′), 144.9 (C-4, 4′), 129.9 (C-5), 129.3 (C-7′), 115.0 (C-3), 113.5 (C-6), 112.9 (C-8′), 111.6 (C-10′), 111.3 (C-10, 3′), 110.3 (C-6′), 102.1 (C-8) [8].

Daphnoretin (6-Methoxy-7-hydroxy-3,7′-bicoumarin) (4). $C_{19}H_{12}O_7$. White needles, mp 244–246°C (MeOH) (lit. [16] mp 244–245°C). TLC R_f 0.53 (S-1), 0.50 (S-2), 0.74 (S-3). PC R_f 0.87 (S-4), 0.62 (S-5). UV spectrum (EtOH, λ_{max}, nm): 267 sh, 345. IR (KBr, ν, cm^{-1}): 3432 (OH), 2922, 1721 (C=O, α-pyrone), 1615, 1286, 1129. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 10.32 (1H, s, OH-7), 8.04 (1H, d, J = 9.5, H-4′), 7.88 (1H, s, H-4), 7.70 (1H, d, J = 8.6, H-5′), 7.21 (1H, s, H-5), 7.19 (1H, d, J = 2.3, H-8′), 7.11 (1H, dd, J = 8.6, 2.3, H-6′), 6.86 (1H, s, H-8), 6.38 (1H, d, J = 9.5, H-3′), 3.80 (3H, s, OCH_3). ^{13}C NMR (125 MHz, DMSO-d_6, δ): 160.1 (C-2′), 159.7 (C-7′), 157.0 (C-2), 155.0 (C-9′), 150.3 (C-7), 147.4 (C-9), 145.7 (C-6), 144.1 (C-4′), 135.7 (C-3), 131.0 (C-4), 130.0 (C-5′), 114.4 (C-10′), 113.9 (C-3′), 113.5 (C-6′), 110.2 (C-10), 109.3 (C-5), 104.0 (C-8′), 102.8 (C-8), 56.0 (OCH_3) [19–21].

Daphnorin (Daphnoretin 7-*O*-β-D-Glucopyranoside) (5). $C_{25}H_{22}O_{12}$. White needles, mp 200–204°C (MeOH) (lit. [20] mp 200°C). PC R_f 0.72 (S-4), 0.79 (S-5). Acid hydrolysis of **5** produced daphnoretin and D-glucose. FAB-MS, *m/z* ($I_{rel.}$, %): 514 (M^-, 15), 353 (10), 339 (48), 325 (90), 311 (100). UV spectrum (EtOH, λ_{max}, nm): 265, 337. IR (KBr, ν, cm^{-1}): 3447 (OH), 2924, 1718 (C=O, α-pyrone), 1698, 1620, 1284. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 8.02 (1H, d, J = 9.5, H-4′), 7.84 (1H, s, H-4), 7.69 (1H, d, J = 8.6, H-5′), 7.25 (1H, s, H-5), 7.21 (1H, s, H-8), 7.21 (1H, d, J = 2.3, H-8′), 7.11 (1H, dd, J = 8.6, 2.3, H-6′), 6.36 (1H, d, J = 9.5, H-3′), 3.78 (3H, s, OCH_3), 5.06 (1H, d, J = 7.3, H-1″ of Glc), 3.66 (1H, dd, J = 9.4, 5.5, H-6_a″), 3.41 (1H, dd, J = 13.4, 5.4, H-6_b″), 3.26–3.28 (1H, m, H-3″), 3.13–3.14 (3H, m, H-2″, 4″, 5″) [22]. ^{13}C NMR (125 MHz, DMSO-d_6, δ): 159.9 (C-2′), 159.4 (C-7′), 156.9 (C-2), 155.0 (C-9′), 149 (C-7), 146.7 (C-9), 146.3 (C-6), 144.0 (C-4′), 137.0 (C-3), 129.9 (C-4, 5′), 114.5 (C-10′), 114.0 (C-3′), 113.7 (C-6′), 112.1 (C-10), 109.4 (C-5), 104.3 (C-8′), 103.0 (C-8), 99.6 (C-1″), 77.1 (C-5″), 76.7 (C-3″), 73.0 (C-2″), 69.5 (C-4″), 60.6 (C-6″), 56.0 (OCH_3).

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