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NEW GENISTEIN MONOGALACTOSIDE FROM THE AERIAL PART OF *Trifolium pratense*

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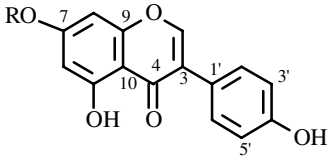
*Formononetin, prunetin, genistein, genistin, and a new genistein glycoside genistein-7-*O-β-*D-galactopyranoside were isolated from the aerial part of* Trifolium pratense *L. The structures of the isolated compounds were established based on chemical transformations and UV, ^1H, and ^{13}C NMR spectra.*

Key words: *Trifolium pratense* L., isoflavonoids, formononetin, prunetin, genistein, genistin, genistein-7-*O*-β-D-galactopyranoside, inermin-3-*O*-β-D-galactopyranoside.

Investigations of the pharmacological properties of clover isoflavonoids showed that these compounds are highly effective antitumor agents [1], possess antioxidant properties [2], and lower the risk of cardiovascular diseases [3]. Therefore, plants of the genus *Trifolium* (Fabaceae, bean), which contain large quantities of isoflavonoids [4], are of great interest for phytochemical research.

Herein we present results from research on the composition of isoflavonoids from the aerial part of field clover collected in Surgut region (Tyumen oblast, Khanty-Mansiisk Autonomous District) in July 2006.

The $CHCl_3$ fraction produced flavonoids **1** and **2**. Direct comparison with an authentic specimen of **1** identified it as formononetin (7-hydroxy-4′-methoxyisoflavone) [5].



2, 4

2: R = CH_3

4: R = β-D-Gal*p*

Flavonoid **2** had absorption maxima in the UV spectrum at 263, 292, and 335 nm that were characteristic of isoflavones [6, 7]. A bathochromic shift of the absorption maxima was not observed in the spectra recorded with added sodium acetate. This indicated that there was not a substituent in the C-7 position. A bathochromic shift by 10 nm was noted for the spectrum recorded with added $AlCl_3$. This was indicative of the presence of a phenol hydroxyl on C-5.

The PMR and ^{13}C NMR spectra (Table 1) showed that **2** was an isoflavone with substituted C-5, C-7, and C-4′ positions. The PMR spectrum had resonances for protons of two phenol hydroxyls (δ_H 9.7 and 12.9 ppm) that were characteristic of C-4′ and C-5 hydroxyls, respectively, and resonances for methoxyl protons at δ_H 3.8 ppm.

Compound **2** was identified as 5,4′-dihydroxy-7-methoxyisoflavone (prunetin).

Compounds **3**, **4**, and **5** were produced from the ethylacetate fraction.

Compound **3** had characteristic UV, PMR, and ^{13}C NMR spectra and was identified by comparison with an authentic sample as 5,7,4′-trihydroxyisoflavone (genistein) [6].

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TABLE 1. PMR and ^{13}C NMR Spectra of **2** and **4**

C atom	**2**		**4**	
	δ_C, ppm	δ_H (J/Hz)	δ_C, ppm	δ_H (J/Hz)
Aglycon				
2	153.6	8.34 s	153.6	8.34 s
3	121.4		121.4	
4	180.2		180.2	
5	157.6		157.6	
6	115.7	6.36 d (2.0)	115.7	6.36 d (2.0)
7	98.6		98.6	
8	93.7	6.59 d (2.0)	93.7	6.59 d (2.0)
9	157.6		157.6	
10	104.6		104.6	
1′	122.4		122.4	
2′/6′	130.0	7.35 d (8.1)	130.0	7.35 d (8.1)
3′/5′	115.2	6.38 d (8.4)	115.2	6.83 d (8.4)
4′	162.1		162.1	
OCH_3	56.3	3.83 s		
5-OH		12.92 s		12.92 s
4′-OH		9.72 s		9.72 s
Gal*p*				
1″			100.1	5.11 d (6.4)
2″			73.2	3.28 - 3.77
3″			76.5	3.28 - 2.77
4″			69.7	3.28 - 3.77
5″			77.2	3.28 - 3.77
6″			60.3	3.28 - 3.77

UV (EtOH, λ_{max}, nm: 264, 290, 332) and PMR and ^{13}C NMR spectra of **4** showed that it was an isoflavone glycoside [6]. The aglycon had a glycosylated hydroxyl on C-7 because the UV spectrum with added sodium acetate did not give a bathochromic shift of the absorption maxima. Acid hydrolysis of **4** produced genistein and D-galactose (TLC, GC).

The resonance of the anomeric proton of D-galactose appeared as a doublet with SSCC 6.7 Hz. The chemical shift of the resonance for C-1 in the sugar part with δ 100.8 ppm in the ^{13}C NMR spectrum (Table 1) was consistent with the α,β-configuration of galactose [6, 7].

Compound **4** had the structure genistein-7-*O*-β-D-galactopyranoside and is a new natural compound [8].

Spectral data of **5** were analogous to those of **4**. Hydrolysis produced genistein and D-glucose [6, 7]. Spectral data, chemical transformations, and comparison with an authentic sample showed that **5** was genistein-7-*O*-β-D-glucopyranoside (genistin).

EXPERIMENTAL

TLC used Sorbfil PTSKh-P-A-UV plates and the solvent systems $CHCl_3$:EtOAc (9:1, 1), $CHCl_3$:C_2H_5OH (9:1, 2), $CHCl_3$:EtOAc:C_2H_5OH (6:1:3, 3), and *n*-BuOH:EtOH:H_2O (5:3:2, 4).

Spots of flavonoids on plates were viewed under a UV light in a UFS-254/365 chromatographic irradiator and detected by spraying with a mixture of alcoholic vanillin (3%) and conc. HCl (4:1). Monosaccharides were detected by spraying chromatograms with H_2SO_4 with subsequent heating at 100-110°C. Column chromatography was performed on silica gel (KSKG, particle size 100/160 μm). PMR and ^{13}C NMR spectra were recorded on a Bruker AVACE AV300 instrument at

operating frequency 300 (PMR) and 75 MHz (^{13}C NMR). UV spectra were recorded on a SF-2000 spectrophotometer. Melting points were determined in capillaries in H_2SO_4.

Extraction and Isolation of Flavonoids. The air-dried ground aerial part (1.0 kg) was extracted five times with ethanol (85%) at room temperature. The resulting extract was condensed by evaporation in vacuo and treated successively with hexane, $CHCl_3$, EtOAc, and *n*-BuOH in a separatory funnel. The resulting fractions were evaporated to dryness in vacuo to produce $CHCl_3$ (2.2 g), EtOAc (29.5 g), and BuOH (35.0 g) fractions.

The $CHCl_3$ fraction (2.2 g) was chromatographed over a column (2.5 × 55 cm) of silica gel (66.0 g) using $CHCl_3$:hexane ($CHCl_3$ content increasing along a gradient from 70 to 100%) and then $CHCl_3$:EtOH (from 0 to 6%). Fractions of 50 mL were collected. Elution by $CHCl_3$:hexane (90:10) produced prunetin (70 mg); $CHCl_3$:EtOH (98:2), formononetin (50 mg).

The EtOAc fraction (10.00 g) was chromatographed over a column (3.5 × 90 cm) of silica gel (200 g). Compounds were eluted by $CHCl_3$:EtOH (EtOH content increasing along a gradient from 0 to 20%). Fractions of 100 mL were collected. Elution by $CHCl_3$:EtOH (8%) produced genistein-7-*O*-β-D-galactopyranoside (350 mg) and genistein-7-*O*-β-D-glucopyranoside (genistin) (25 mg).

Formononetin (7-hydroxy-4′-methoxyisoflavone, 1), $C_{16}H_{12}O_4$, $[M]^+$ 268, mp 261-263°C. UV spectrum (EtOH, λ_{max}, nm): 250, 263, 295.

PMR spectrum (300 MHz, C_5D_5N, δ, ppm, J/Hz): 3.57 (3H, s, OCH_3), 6.99 (2H, d, J = 9.0, H-3′,5′), 7.07 (1H, br.s, H-8), 7.14 (1H, dd, J = 9.0, 2.0, H-6), 7.72 (2H, d, J = 9.0, H-2′,6′), 8.10 (1H, s, H-2), 8.40 (1H, d, J = 9.0, H-5).

Prunetin (2), (5,4′-dihydroxy-7-methoxyisoflavone), $C_{16}H_{12}O_5$, $[M]^+$ 284, mp 233-234°C, UV spectrum (EtOH, λ_{max}, nm): 263, 292, 335. Table 1 lists the PMR and ^{13}C NMR spectra.

Genistein (5, 7, 4′-trihydroxyisoflavone, 3), $C_{15}H_{10}O_5$, $[M]^+$ 270, mp 299-302°C, UV spectrum (EtOH, λ_{max}, nm): 264, 290, 332.

PMR spectrum (300 MHz, C_5D_5N, δ, ppm, J/Hz): 6.46 (1H, d, J = 2.0, H-6), 6.55 (1H, d, J = 2.0, H-8), 7.06 (2H, d, J = 8.5, H-3′,5′), 7.54 (2H, d, J = 8.5, H-2′,6′), 7.92 (1H, s, H-2).

Genistein-7-*O*-β-D-galactopyranoside (4), $C_{21}H_{20}O_{10}$, $[M]^+$ 432, mp 258-259°C, UV spectrum (EtOH, λ_{max}, nm): 264, 290, 332. Table 1 lists the PMR and ^{13}C NMR spectra.

Acid Hydrolysis of 4. A solution of **4** (25 mg) in a mixture (20 mL) of HCl (5%) and ethanol (1:1) was boiled for 2 h. The resulting precipitate of the aglycon was filtered off and recrystallized from alcohol to afford genistein (7 mg), mp 261-263°C. TLC (system 4) and GC (trimethylsilyl ether) identified in the hydroysate D-galactose. The trimethylsilyl ether was prepared by dissolving the carbohydrate in pyridine, silylating with BSTFA for 20 min, evaporating to dryness, and dissolving the dry solid in hexane.

Genistein-7-*O*-β-D-glucopyranoside (genistin, 5), $C_{21}H_{20}O_{10}$, $[M]^+$ 432, mp 260-261°C, UV spectrum (EtOH, λ_{max}, nm): 264, 290, 332.

PMR spectrum (300 MHz, DMSO-d_6, δ, ppm, J/Hz): 3.28-3.77 (sugar protons), 5.11 (1H, d, J = 6.4, H-1″), 6.36 (1H, d, J = 2.0, H-6), 6.59 (1H, d, J = 2.0, H-8), 6.83 (2H, d, J = 8.4, H-3′,5′), 7.35 (2H, d, J = 8.1, H-2′,6′), 8.34 (1H, s, H-2), 9.72 (1H, s, 5-OH), 12.92 (1H, s, 4′-OH).

Acid Hydrolysis of 5. Glycoside **5** (25 mg) was hydrolyzed by the aforementioned method. The resulting solid aglycon was filtered off and recrystallized from alcohol to afford genistein (9 mg), mp 261-263°C. TLC and GC (trimethylsilyl ether) identified in the hydrolysate D-glucose.

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