Spinonin, a Novel Glycoside from *Ononis spinosa* subsp. *leiosperma*

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The roots of *Ononis spinosa* subsp. *leiosperma* (Leguminosae) afforded a new glycoside, spinonin (**1**), possessing a novel skeleton, in addition to the known isoflavonoid glycoside, ononin [7β-(glucosyloxy)formononetin] (**2**) and the known pterocarpan, 7-demethoxy-7-D-(glucosyloxy)-homopterocarpin (**3**). The structure of the new isolate was elucidated by spectral methods including ^{1}H and ^{13}C NMR, COSY, APT, HETCOR, HMBC, NOESY, CD, FABMS, HRMS, EIMS, CIMS, and some chemical reactions. Spinonin was inactive against a number of human cancer cell lines and HIV-1 reverse transcriptase. The compounds **1** and **3** showed weak activity against *Pseudomonas aeruginosa,* whereas **2** was active against β-hemolytic *Streptococcus.*

Ononis species are pharmacologically active and have been used for centuries as folk remedies in Turkey to aid the passage of kidney stones and as diuretic agents.[1] They are widespread in the Mediterranean region of Europe, and there are 18 *Ononis* species in Turkey, four of which are endemic.[2] Several plants of this genus have been shown to possess antibiotic, antifungal, antipyretic, antiinflammatory, and antiseptic properties[3−7] and have been used in the treatment of rheumatism, urinary tract infections, and skin diseases.[8] *Ononis spinosa* has been used to treat urethritis and as an aperient, a diuretic, and a detergent and has been shown to have antibacterial, analgesic, antiinflammatory, antiviral, and cytotoxic activities. It was first studied in 1955 by Barton and Overton, who reported the isolation of some triterpenoids.[9] In 1978, Haznagy *et al.* isolated sterol, triterpene, isoflavone, and pterocarpan derivatives from *O. spinosa.*[10] However, a literature survey showed that *O. spinosa* subsp. *leiosperma* has not hitherto been studied chemically and biologically. In this paper we report the isolation and characterization of a new glucoside, spinonin (**1**), with a novel skeleton. The structures of the known compounds, ononin [7β-(glucosyloxy)formononetin) (**2**)[10] and 7-demethoxy-7-D-(glucosyloxy)homopterocarpin (**3**)[10] were elucidated by comparison with reported spectral data. Spinonin was subjected to cytotoxic, HIV-1 reverse transcriptase, and antimicrobial bioassays.

An extract of the aerial parts of *O. spinosa* subsp. *leiosperma* afforded the known compounds, ononin [7β-(glucosyloxy)formononetin) (**2**) and 7-demethoxy-7-D-(glucosyloxy)homopterocarpin (**3**) and the new isolate, spinonin (**1**). The structure of spinonin (**1**) was elucidated by a combination of spectral methods, including 1D and 2D NMR techniques, and by some chemical transformations. The spectral data of **1** indicated the presence of an aromatic glucoside. The IR spectrum showed aromatic bands at 3050, 1605, 1580 sh, and 1505 cm^{-1}. The ^{1}H-NMR spectrum of **1** displayed aromatic signals at δ 7.62 (1H, d, J = 8.5 Hz), 7.48 (1H, br s), and 6.97 (1H, br d, J = 8.5 Hz). The multiplicity of the aromatic signals demonstrated a 1,2,4-trisubstituted aromatic ring. The (*o,m*) splitting patterns of the overlapped signals at δ 7.36 (2H, br d, J = 8.5 Hz) and 7.09 (2H, br d, J = 8.5 Hz), together with the methine signals at δ 131.6 and 116.1 in the APT spectrum indicated the presence of another *p*-substituted aromatic ring, one of the substituents being a hydroxyl group. The anomeric proton resonated at δ 5.70 (1H, br d, J = 6.5 Hz), showing that there was a monosaccharide unit in the molecule with a β orientation. Other signals of the sugar appeared between δ 4.00 and 4.50 as overlapped multiplets. HRFABMS indicated the molecular formula of $C_{23}H_{24}O_{10}$ for spinonin. The fragments at *m/z* 459 [M − H]$^-$ and *m/z* 297 [M − H − 162]$^-$ in the negative ion FABMS spectrum, together with the anomeric carbon signal at δ 102.1, the methine signals at δ 74.7, 78.8, 71.2, 75.2, and the methylene signal at δ 62.4 in the APT spectrum, indicated that the monosaccharide unit was glucose, which was also established by hydrolysis and cochromatographic TLC studies with standard sugars.[11] In addition, the singlet at δ 6.53 and the doublet at δ 6.46, together with the carbonyl signal at δ 173.7, the methine signals at δ 84.8 and 113.9, and the quaternary olefinic carbon signal at δ 163.6 in the APT spectrum, indicated the presence of a 2,3-dihydro-3-oxofuran ring (IR_{KBr} 1720 cm^{-1}) in the molecule. Furthermore, the two doublets of doublets at δ 3.38 (1H, J = 2.5, 14.5 Hz) and 2.85 (1H, J = 6.0, 14.5 Hz) in the ^{1}H-NMR spectrum implied the presence of a CH_2 group between the rings, which was coupled with the methine resonance at δ 6.46 (1H, dd, J = 2.5, 6.0 Hz) in the spin decoupling and COSY experiments, as an ABX system. The doublet at δ 6.97 (H-10) was collapsed to a singlet on irradiation of the signal at δ 7.62 (H-11), while irradiation of H-10 simplified the doublet at δ 7.62 to a singlet and sharpened the broadened singlet at δ 7.48 (H-8). Irradiation of the doublets at δ 7.36 (H-2′,6′) and at δ 7.09 (H-3′,5′) collapsed each other mutually to broadened singlets. The COSY spectrum of **1** also delineated the homonuclear couplings between H-1″ (δ 5.70) and the overlapped signals (δ 4.50−4.00). HET-

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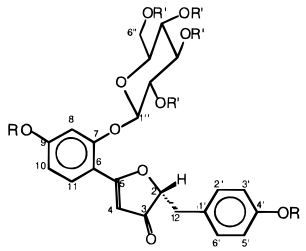
Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1**, **1a**, and **1b**

	1[a]		**4**[a]		**5**[a]	
position	^{1}H[b,f]	^{13}C (APT)[d,f]	^{1}H[b,f]		^{1}H[c]	^{13}C[e]
2	6.46, br d	84.8 (−)	6.26, br dd		6.47, ddd	84.7
3		173.7 (+)				173.4
4	6.53, br s	113.9 (−)	6.56, d		6.51, d	115.2
5		166.7 (+)				166.4
6		112.0 (+)				109.5
7		157.8 (+)				159.2
8	7.48, br s	103.9 (−)	7.35−7.10, m		7.46, d	102.7
9		163.6 (+)				164.2
10	6.97, br d	111.3 (−)	7.35−7.10, m		6.77, ddd	108.9
11	7.62, d	132.0 (−)	7.35−7.10, m		7.57, d	131.5
12	3.38, br dd	39.6 (+)	3.27, dd		3.34, dd	39.5
12′	2.85, br dd		2.86, dd		2.81, dd	
1′		127.7 (+)				129.4
2′,6′	7.36, br d	131.6 (−)	7.35−7.10, m		7.275, br d	131.8
4′		158.2 (+)				158.1
3′,5′	7.09, br d	116.1 (−)	7.35−7.10, m		6.83 br d	114.3
1″	5.70, br d	102.1 (−)	5.90, br d		5.72, d	102.3
2″	4.50−4.00, m	74.7 (−)	6.00−5.60, m		4.50−4.10, m	74.9
3″	4.50−4.00, m	78.8 (−)	6.00−5.60, m		4.50−4.10, m	79.8
4″	4.50−4.00, m	71.2 (−)	6.00−5.60, m		4.50−4.10, m	71.7
5″	4.50−4.00, m	79.0 (−)	6.00−5.60, m		4.50−4.10, m	79.8
6″	4.50−4.00, m	62.4 (+)	4.55, m		4.50−4.10, m	62.8
OAc			2.00, s	9-OMe	3.81, s	56.0
OAc			2.03, s	4′-OMe	3.52, s	55.3
OAc			2.04, s			
OAc			2.09, s			
OAc			2.17, s			
OAc			2.22, s			

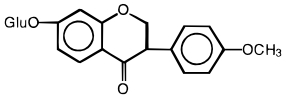
[a] Pyridine-d_5. [b] 200 MHz. [c] 300 MHz. [d] 50.32 MHz. [e] 75.43 MHz. [f] J (Hz), $J_{2,12} = 3.5$; $J_{2,12'} = 6.0$; $J_{12,12'} = 14.5$; $J_{2,4}$=1.5; $J_{10,11}$=8.5; $J_{2',3'} = J_{5',6'} = 8.5$; $J_{1'',2''} = 6.5$. (−), (+), negative and positive signals in the APT spectrum.

COR experiments enabled the assignments of the proton bearing carbons to be made. Observation of the polarization transfers from H-2′,H-6′ to C-4′ and C-12 and from H_2-12 to C-2, C-1′ and C-2′,6′ in the HMBC spectrum indicated that the 12-CH_2 group was located between the *p*-substituted aromatic ring and the 2,3-dihydrofuran ring. The resonance of H-2 was coupled with C-5, and H-4 with C-2, C-5, and C-6 showing the (C-5−C-6) linkage of the furan ring and the 1,2,4-trisubstituted aromatic ring. H-11 was coupled with C-5, C-7, and C-9, and H-8 with C-7, C-6, C-9, and C-10. In addition, correlations between H-1″ and C-4″ and C-7 indicated that the sugar moiety was linked to the aglycon at C-7. In the NOESY experiments, observation of NOEs between H-8 and H-1″, H-11 and H-4, and H-2′,H-6′ and H-3′,H-5′ supported the proposed structure. Acetylation of **1** afforded **4**, which showed six acetoxy methyl signals at δ 2.00, 2.03, 2.04, 2.09, 2.17, and 2.22 (Table 1). The HRMS spectrum of the acetyl derivative supported this structure, giving a molecular ion peak at m/z 713.209 corresonding to $C_{35}H_{37}O_{16}$.

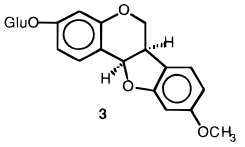
The location of the sugar unit was unambiguously established through the preparation of the dimethyl ether derivative **5**. ^{1}H- and ^{13}C-NMR spectra of **5** exhibited two additional singlet resonances at δ 3.81 (3H) and 3.52 (3H), and 56.0 and 55.3, respectively, indicating the presence of two methoxy groups (Table 1). Spectral analysis of **5** showed that the two methoxy groups were located at C-4′ and C-9. In the NOESY spectrum of **5**, NOEs were observed between the C-9−OCH_3 and H-8 and H-10, and between the C-4′−OCH_3 and H-3′,H-5′, between H-8 and H-1″, between H-4 and H-11, and between H-10 and H-11. The CIMS spectrum of **5** gave a molecular peak at m/z 489 $[M + 1]^+$ corresponding to $C_{25}H_{28}O_{10}$. In the CD studies (Figure 1), the ΔE values (Table 3), proved the β-orientation of H-2, and hence permitted the determination of the absolute configuration at C-2 as *R* shown in **1**.[12] This is the first isolation of a natural product with this skeleton.



1 R=H
4 R=R'=Ac
5 R=Me, R'=H



2



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Spinonin was tested against a number of human cancer cell lines, including BC1, Lu1, KB-V(+VLB), and SKNSH in which it was inactive. The isolate was also inactive in an HIV-1 reverse transcriptase inhibition assay.

Spinonin, as well as compounds **2** and **3** were tested against the Gram-positive bacteria *Bacillus subtilis* ATTC 6633, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATTC 12228, *Enterococcus faecalis* ATCC 29212, and beta-hemolytic *Streptococcus* 48 (clinical isolate); the Gram-negative bacteria *Proteus mirabilis* ATTC 14153, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, and *Klebsiella*

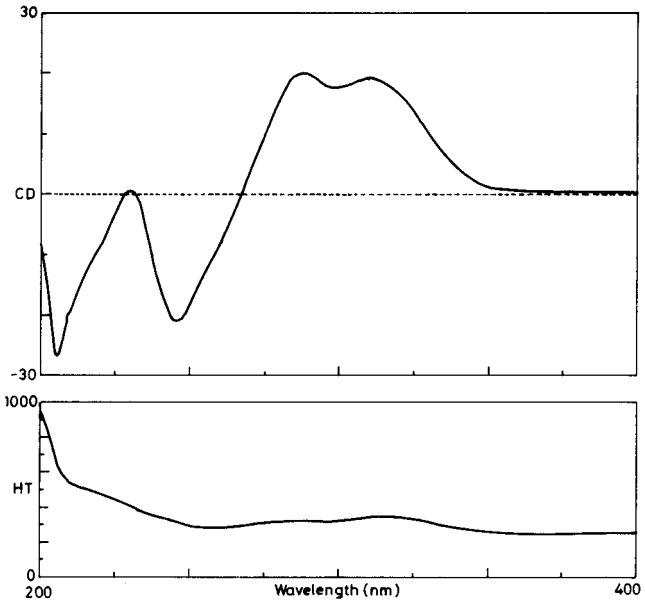

Figure 1. CD curve of **1**.

Table 2. Heteronuclear Couplings and Diagnostic NOEs of Compound **1**

$^2J_{CH}$ and $^3J_{CH}$[a]	NOE correlations[b]
H-1″ and C-3″, C-7	H-1″ and H-8
H-8 and C-6, C-7, C-9, C-10	H-4 and H-11
H-11 and C-9, C-5, C-7	H-2′,H-6′ and H-3′,H-5′
H-4 and C-5, C-6, C-2	
H-2 and C-5	
H-12 and C-2, C-1′, C-2′, C-6′	
H-2′,6′ and C-12	

[a] From HMBC experiments. [b] From NOESY experiments.

Table 3. CD Spectral Data of Compound **1**[a]

$\Delta\epsilon$	λ	θ (mdeg)
0	363	0
6.64	310	19.074
6.11	298	17.5368
6.96	287	19.9649
0	267	0
−7.26	246	−20.8401
0	231	0
0.14	229	0.3894
0	228	0

[a] c 8.7 × 10^{-5} M, MeOH.

pneumoniae ATCC 4352, and the yeast *Candida albicans* ATCC 10231. Compounds **1** and **3** showed weak activity (MIC 200 μg/mL) against *P. aeruginosa*. The best effect among the three compounds was obtained with the compound **2** against β-hemolytic *Streptococcus* (MIC = 25 μg/mL).

Experimental Section

General Experimental Procedures. IR spectra were recorded on a Perkin-Elmer Model 983 in KBr; ^{1}H and ^{13}C NMR , APT, HETCOR were recorded at 200 MHz and 50.32 MHz on a Bruker AC-200L instrument at TUBITAK (Gebze); COSY, HETCOR, NOESY were performed at 300 MHz and 75.43 MHz on a Varian XL instrument, and HMBC was performed at 500/125 MHz on a GE Omega 500 instrument using standard GE programs at the University of Illinois at Chicago with TMS as internal standard. HRMS and EIMS were recorded on VG ZabSpec at TUBITAK (Gebze); CI, on Finnigan MAT 90 instrument in Chicago. FABMS was obtained using both VG ZabSpec and Finnigan MAT 90 instruments in a glycerol matrix. CD was performed in MeOH on a JASCO J-40A automatic recording instrument. The optical rotation was measured with a Perkin-Elmer model 241 polarimeter.

Plant Material. The roots of *O. spinosa* L. subsp. *leiosperma* (Boiss) were collected from the western part of Turkey (Çiçekliköy-Manisa) and identified by Dr. S. Onuk. A voucher specimen is deposited in the Herbarium of Faculty of Science, Ege University (no. 7433).

Extraction and Isolation. Dried and powdered plant material (900 g) was extracted with MeOH at room temperature, and the extract was evaporated *in vacuo*. After washing the crude extract with hexane and Me_2CO successively, it was applied on a Si gel column (5.5 × 100 cm) and eluted with the following solvent systems: A, $CHCl_3$−MeOH (65:30); B, $CHCl_3$−MeOH−H_2O (65:20:5); C, $CHCl_3$−MeOH−H_2O (65:30:5); D, $CHCl_3$−MeOH−H_2O (65:35:10); E, $CHCl_3$−MeOH−H_2O (65:35:10 + 10%MeOH), successively. The compound **2** was obtained during elution with $CHCl_3$−MeOH−H_2O (65:35:10) and purified by preparative TLC. Then, the fourth fraction was extracted with *n*-BuOH. This BuOH extract, which contained compounds **1**−**3,** was separated by Si gel column using the same solvent gradient. The isolates were further purified on a Sephadex LH 20 column (2.0 × 40 cm) eluting with CH_2Cl_2−MeOH (80:20). In this way, **1** (50 mg)**, 2** (12 mg), **3** (19 mg) were obtained.

Spinonin (1): $[\alpha]^{20}_D$ + 3.2° (*c* 1.46, MeOH); HR-FABMS *m/z* 461.143 37 $[M + H]^+$, calcd for $C_{23}H_{24}O_{10}$ 461.144 77; IR ν_{max} (KBr) 3420, 3050, 2950, 1720, 1605, 1580 sh, 1505, 1450, 1420, 1300, 1250, 1230, 1190, 1080, 840, 820 cm^{-1}; UV λ_{max} (MeOH) 313 (ϵ 1.60 × 10^6), 284 (ϵ 1.39 × 10^6), 216 (ϵ 2.07 × 10^6) nm; ^{1}H NMR (200 MHz and 300 MHz) and ^{13}C NMR (50.32 MHz and 75.43 MHz) in pyridine-d_5, see Table 1; HMBC (500 MHz and 125 MHz) and NOESY (300 MHz and 75.43 MHz) in pyridine-d_5, see Table 2; FABMS (positive ion) *m/z* 461 $[M + H]^+$; FABMS (negative ion) *m/z* 459 $[M - H]^-$, 297 $[M - H - Glu]^-$, 165 $[M - H - Glu - O{=}C_6H_4{=}C{=}C{=}O]^-$; CD [$\lambda$ (mdeg)] (MeOH), 363 (0), 310 (19.1), 298 (17.5), 287 (20.0), 267 (0), 246 (-20.8), 231 (0), 229 (0.39), 228 (0).

Acetylation of 1. Spinonin (**1**, 10 mg) in pyridine (1 mL) was treated with Ac_2O (1 mL) at room temperature for 24 h to afford **4** (5 mg): IR ν_{max} ($CHCl_3$) 3010, 2970, 2930, 1750, 1720, 1605, 1580 sh, 1505, 1450, 1375, 1230, 1170, 1070, 1040, 910, 760, 600 cm^{-1}; ^{1}H NMR (200 and 300 MHz) and ^{13}C NMR (50.32 MHz and 75.43 MHz) in pyridine-d_5, see Table 1; HREIMS *m/z* 713.2099, calcd for $C_{35}H_{36}O_{16}$ 713.2082.

Methylation of 1. Spinonin (**1**, 10 mg) was treated overnight with an excess of CH_2N_2 in Et_2O. The CH_2N_2 was obtained in the standard manner. The reaction mixture was concentrated *in vacuo* and **5** was recovered (5 mg) by preparative TLC on Si gel; ^{1}H NMR (300 MHz) and ^{13}C NMR (75.43 MHz) in pyridine-d_5, see Table 1; CIMS *m/z* 489 $[M + 1]^+$ (23), 327 [M + 1 − 162] (93), 121 $[C_8H_9O]$ (100).

Acidic Hydrolysis of 1. Spinonin (**1**) and the reference sugars were applied to a Si gel TLC plate. The plate was subjected to concd HCl vapors in a chromatography tank at 100 °C for 15 min. Subsequently, the plate was eluted with $CHCl_3$−MeOH−H_2O (65:30:10,

v/v), and the hydrolysis products were visualized by spraying with anisaldehyde reagent.

Evaluation of Antimicrobial Activity. Spinonin and the known compounds were tested against a panel of bacteria including the Gram-positive bacteria *B. subtilis* ATTC 6633, *S. aureus* ATCC 6538P, *S. epidermidis* ATTC 12228, *E. faecalis* ATCC 29212, and beta-hemolytic *Streptococcus* 48 (clinical isolate); the Gram-negative bacteria *P. mirabilis* ATTC 14153, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, and *K. pneumoniae* ATCC 4352, and the yeast *C. albicans* ATCC 10231.

The paper-disk diffusion method was used to select compounds active against these test microorganisms.[11] Overnight cultures of microorganisms were adjusted to approximately 10^6 cfu/mL according to MacFarland turbidity standards and spread over the appropriate media (Mueller-Hinton agar for *Staphylococci*, *B. subtilis*, and Gram-negatives; blood agar for *Streptococcus* and *Enterococcus*; Sabouraud agar for the yeast) in Petri dishes. Filter paper disks (0.5 cm) impregnated with MeOH solutions of the test compounds were placed on the seeded and air-dried surface of the media. After overnight incubation at 37 °C, the zones of inhibition around the disks were measured. The antimicrobial effects of compounds that produced zones of inhibition ≥ 5 mm were quantitated by using the broth dilution method, and MIC (minimal inhibitory concentration) values (μg/mL) were determined.[12] In both tests MeOH was used as a control.

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