A New Triterpenoid Saponin from *Ononis spinosa* and Two New Flavonoid Glycosides from *Ononis vaginalis*

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The new triterpenoid saponin 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-(1→2)-β-D-glucuronopyranosyl]-3β,22α-dihydroxyolean-13-en-11-one has been isolated from *Ononis spinosa*. The new flavonoid glycoside 3-*O*-[2-*O*-(*E*)-p-coumaroyl-β-D-galactopyranosyl]-7-*O*-β-D-glucopyranosylkaempferol and the new pterocarpan glucoside 3,4-di-*O*-β-D-glucopyranosyl-4-hydroxymedicarpin have been obtained from *Ononis vaginalis*. The structures were determined primarily by NMR spectroscopy. The assignment of NMR signals was performed by means of ^{1}H-^{1}H COSY, ROESY, TOCSY, HMQC HMQC-COSY and HMBC experiments.

Key words: Ononis spinosa, *Ononis vaginalis*, Triterpenoid Saponin, Flavonoid Glycosides

Introduction

Ononis species (Fabaceae) are widespread in the Mediterranian region of Europe, in West Asia and North Africa. Several plants of this genus possess antibiotic, antifungal, antipyretic, antiinflammatory and antiseptic properties and have been used in the folk medicine for the treatment of rheumatism, urinary tract infections and skin diseases [1]. *Ononis spinosa* has been shown to have antibacterial, analgetic, antiinflammatory, antiviral and cytotoxic activities. Triterpenes, flavones, isoflavones and pterocarpans have been isolated from *Ononis spinosa*. The roots of *Ononis spinosa* are used as tea drug "Radix Ononides" [2].

Ononis vaginalis Vahl. possesses antibiotic and slightly antiviral properties. Some flavonoids like apigenin, trifolin and eupatilin have been isolated from the flowers of *Ononis vaginalis*. Eupatilin exhibits cytotoxic activity against human carcinoma of nasopharynx [3]. In a previous publication [4] we reported on the isolation of a new bishomoflavone and its glucoside from *Ononis vaginalis*. Now we desribe the isolation and structure elucidation of the new triterpenoid saponin **1** from Radix Ononides, the new flavonoid glycoside **2** and pterocarpan glucoside **3** from *Ononis vaginalis*.

Results and Discussion

The butanol extracts of Radix Ononides and the whole plant of *Ononis vaginalis* Vahl. were obtained as described in the experimental section. The crude saponin of Radix Ononides was purified by column chromatography on Sephadex LH-20 and RP-18 material and yielded the triterpenoid saponin **1** (Fig. 1).

The liquid secondary ion mass spectrum (LSI-MS) of **1** exhibited the $[M-1]^-$ ion at *m/z* 909. The fragment ions of *m/z* 763 $[M-1-146]^-$, 631 $[M-1-146-132]^-$ and 455 $[M-1-146-176]^-$ showed the sequential loss of a desoxyhexose, pentose and uronic acid moiety. The $[M+Na]^+$ ion at *m/z* 933.4847 of the high resolution electrospray ionization mass spectrum (HR-ESI-MS) yielded the molecular formula $C_{47}H_{74}O_{17}Na$.

The ^{1}H and ^{13}C NMR data indicated 3β,22α-dihydroxyolean-13-en-11-one as aglycone. The signals of the axial and equatorial oriented protons of the aglycone were assigned by ROESY experiments. The proton signal of H-22$_{ax}$ at δ3.32 showed a cross peak to the protons of the axial methyl group 30 (δ0.76) in the ROESY spectrum. The ^{13}C downfield shift of the C-9 signal ($\Delta\delta + 16.9$) in comparison with 3β,22α-dihydroxyolean-12-ene (sophoradiol, C-9, δ47.7, $CDCl_3$) is characteristic for oleans with an 11-keto function [5]. The ^2J HMBC cross peak be-

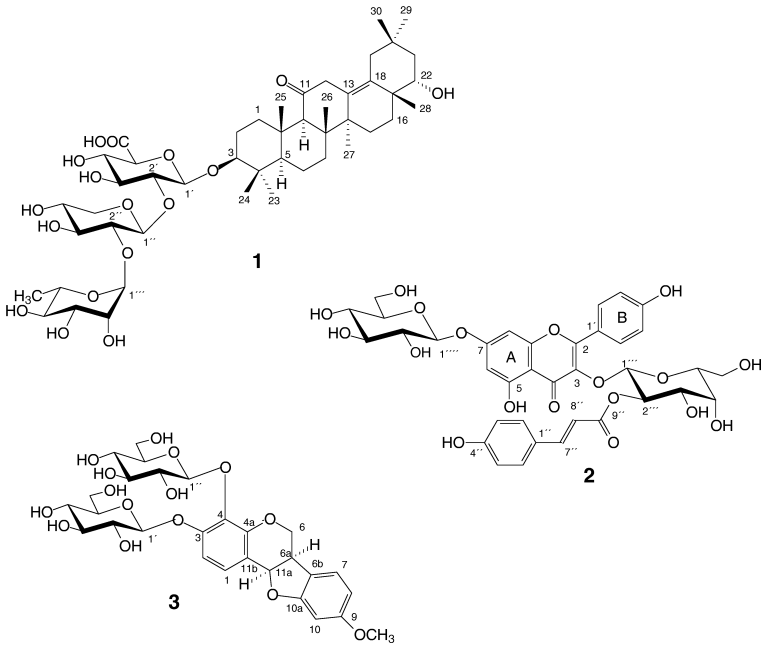

Fig. 1. 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-(1→2)-β-D-glucuronopyranosyl]-3β,22α-dihydroxy-olean-13-en-11-one (**1**) from *Ononis spinosa* and 3-*O*-[2-*O*-(*E*)-p-coumaroyl-β-D-galactopyranosyl]-7-*O*-β-D-glucopyranosylkaempferol (**2**), 3,4-di-*O*-β-D-glucopyranosyl-4-hydroxymedicarpin (**3**) from *Ononis vaginalis*.

tween H-9 (δ2.45) and C-11 (δ211.9) proves the 11-keto function. The position of the double bond (C-13 → C-18) was determined by the HMBC-cross peaks 3H-28 (δ0.98)/C-18 (δ135.1) and 2H-12 (δ1.38)/C-13 (δ134.1). Taking account of the ^{13}C glycosidation shifts of the C-3, C-2 and C-4 signals, the other ^{13}C NMR data of the aglycone are in good agreement with abrisapogenol J [5]. In this way the configuration in position 22 of abrisapogenol J should be 22α-hydroxy and not 22β.

Three anomeric proton signals at δ4.37 (d, J = 7.2 Hz), 4.83 (d, J = 6.9 Hz) and 5.18 (d, J = 1.4 Hz) indicated the presence of three monosaccharide units bounded as glycosides. By use of ^{1}H,^{1}H-COSY-45 and TOCSY spectra and the determiantion of the D-form for glucuronic acid and xylose and the L-form for rhamnose (as described in the experimental section), the individual monosaccharides were identified as D-glucuronopyranose, D-xylopyranose and L-rhamnopyranose. The coupling constants of the anomeric proton signals of D-glucuronopyranose (J = 7.2 Hz) and D-xylopyranose (J = 6.9 Hz) are in agreement with a $\beta-$configuration. The linkage of the saccharide unit to the aglycone of **1** was determined by means of HMBC spectra. The cross peaks of the ^3J long range couplings between H-1' glucuronic acid (δ4.37)/C-3 aglycone (δ91.1) indicated the point of linkage to the sapogenin. The ROESY cross peak between H-1" xylose (δ4.83)/H-2' glucuronic acid (δ3.56) and the downfield shift of the glucuronic acid C-2 signal ($\Delta\delta$ + 3.6) in comparison with unsubstituted glucuronic acid (C-2, δ75.5, CD_3OD) proved the linkage of xylose at position 2 of glucuronic acid [6]. The ROESY cross peak H-1"' rhamnose (δ5.18)/H-2" xylose (δ3.38) and the downfield shift of the C-2 signal of xylose ($\Delta\delta$ + 3.9) compared with unsubstituted xylose (C-2, δ75.0, CD_3OD) indicated that the terminal rhamnose is bounded at position 2 of xylose [7].

The butanolic extract of the whole plant of *Ononis vaginalis* was subjected to successive column chromatography on silica gel. Further purification was achieved by MPLC (RP-18), Sephadex LH-20 column chromatography and HPLC (RP-18) to give pure flavonoid glycoside **2** and pterocarpan glucoside **3** (Fig. 1).

The HR-ESI-MS of **2** showed the $[M+Na]^+$ ion at *m/z* 779.1834 which led to the molecular formula $C_{36}H_{36}O_{18}Na$.

In the ^{1}H NMR spectrum of **2** the doublets of H-6 and H-8 were observed at δ6.40 and 6.65 with a meta coupling constant $^4J_{6,8}$ = 1.8 Hz. Both signals exhibited HMBC cross peaks to C-7 (δ164.6) which should be substituted by an oxygen due to its downfield shift. The ^{13}C signal of C-5 was found at δ162.8. The HMBC signal H-2' (δ7.98)/C-2 (δ158.9) proved the linkage of the B ring to the pyran ring. The four

signals at δ7.98, 6.83 and 7.37, 6.76 characterize the two AA'BB' spin systems of the flavonoid B ring H-2',H-6'/H-3',H-5' and the p-coumaric acid ester. The strong downfield shifts of the ^{13}C signals of C-4' B ring (δ161.8) and C-4" (δ161.3) indicated the presence of hydroxy groups. The coupling constant $^3J_{7'',8''} = 15.7$ Hz proved the (*E*)-configuration of the p-coumaric acid ester.

The hexose in position 3 of kaempferol is β-D-galactopyranose. The coupling constants $^3J_{1'''\mathrm{ax},2'''\mathrm{ax}} =$ 8.4 Hz (H-1"', δ5.55), $^3J_{2'''\mathrm{ax},3'''\mathrm{ax}} = 10.0$ Hz (H-3"', δ3.71), $^3J_{3'''\mathrm{ax},4'''\mathrm{eq}} = 3.0$ Hz (H-3"', δ3.71) are characteristic for β-D-galactopyranose. The HMBC signal H-1"' galactose (δ5.55)/C-3 kaempferol (δ135.3) indicated the linkage of β-D-galactopyranose at C-3 of kaempferol. The downfield shift of the H-2"' galactose signal (δ5.29) and the HMBC cross peak H-2"' galactose (δ5.29)/C-9" (*E*)-p-coumaroyl (δ168.7) confirmed the esterification in position 2 of galactose. The ^{1}H and ^{13}C NMR spectra of the second hexose are in agreement with β-D-glucopyranose which is bounded at position 7 of kaempferol. This is established by the HMBC cross peak H-1"" glucose (δ4.96)/C-7 aglycone (δ164.6).

The LSI mass spectrum of **3** exhibited the $[M-1]^-$ ion at m/z 609. The fragment ions at m/z 447 [M-1-162]$^-$ and 255 [M-1-2×162-30]$^-$ indicated the loss of hexose and 2×hexose plus HCHO. The HR-ESI-MS of **3** exhibited the $[M+Na]^+$ ion at m/z 633.1765 which is in agreement with the molecular formula $C_{28}H_{34}O_{15}Na$.

The ^{1}H and ^{13}C NMR spectra of **3** showed besides two β-D-glucopyranoses the pterocarpan aglucone 4-hydroxymedicarpin. In the ^{1}H NMR spectrum of **3** five aromatic signals were observed which belong to two different spin systems. The H-1 and H-2 signals were doublets at δ7.25 and 7.04 with an ortho coupling constant $^3J_{1,2} = 8.8$ Hz. The signal of H-7 (δ7.19) showed a coupling constant $^3J_{7,8} = 8.2$ Hz which confirmed the ortho coupling to H-8. The H-8 signal exhibited besides the ortho also a meta coupling $^4J_{8,10} = 2.2$ Hz to H-10 (δ6.37). The HMBC cross peaks H-1 (δ7.25)/C-3 (δ148.8), H-1/C4a (δ147.4), H-1/C-11a (δ79.7), H-2 (δ7.04)/C-4 (δ132.7) and H-2/C11b (δ114.8) were in agreement with the structure of the aglucone. The 9 position of the CH_3O group was confirmed by a HMBC signal CH_3 (δ3.73)/C-9 (δ161.9). The two free hydroxy groups in position 3 and 4 of 4-hydroxymedicarpin are glucosylated in compound **3**. The HMBC cross peak H-1' (δ4.87)/C-3 (δ148.8) indicated the linkage of glucose' at C-3 of the aglucone.

All pterocarpans are cis annulated. The coupling constant $^3J_{6a,11a} = 6.8$ Hz is in good agreement with $^3J_{6a,11a} = 7.0$ Hz of the cis annulated (-)-maackiain. The absolute configuration of maackiain was determined by X ray analysis and is 6a *R*, 11a *R*. The CD spectra of (-)-maackiain and other (-)-(6a *R*, 11a *R*)-pterocarpans are chracterized by a positive Cotton effect of the ^{1}Lb band at $\approx$ 280 nm and a negative Cotton effect of the ^{1}La band at $\approx$ 230 nm. [8, 9]. The CD spectrum of **3** showed the same positive (^{1}Lb band at 284 nm, Θ [mdegree] +18.8) negative (^{1}La band at 233 nm, Θ [mdegree] -39.8) pattern. Due to these data the pterocarpan glucoside **3** possesses (6a *R*, 11a *R*)-configuration. The aglucone of **3**, 4-hydroxymedicarpin, has been isolated before from the roots of the medicinal plant *Taverniera abyssinica* (Fabaceae) [10].

Experimental Section

General

Negative ion MS: MAT 8500 (Finnigan), matrix glycerol. ESI-MS: Micromass LCT, solvent MeOH. NMR: 500.13 MHz (^{1}H) and 125.76 MHz (^{13}C), reverse probehead, δ in ppm, solvent CD_3OD, CD_3OD signals were used as int. standard (^{1}H: 3.30, ^{13}C: 49.0), temp. 290 K, HMQC: phase-sensitive using TPPI (Time Proportional Phase Increment), BIRD (Bilinear Rotation Decoupling) sequence, GARP decoupled, HMBC: using TPPI, delay to achieve long range couplings: 71 msec ($J_{C,H} = 14$ Hz). CD: Jasco J-600, solvent MeOH.

CC: silica gel (0.063 – 0.2 mm); TLC: silica gel (0.25 and 1 mm precoated plates 60 F_{254}, Merck, 0.25 mm precoated plastic sheets SIL G/UV_{254} Macherey-Nagel, Düren, Germany), the spots were sprayed with 'triterpene reagent' (1% vanillin in 50% H_3PO_4), 'sugar reagent' (4% ethanolic aniline-4% ethanolic diphenylamine-H_3PO_4, 5:5:1 v/v/v) and phosphomolybdic acid reagent (Aldrich). MPLC (Büchi): RP-18 (0.015 mm), HPLC (Knauer): RP-18 (0.007 mm).

Isolation

Ononis spinosa

The tea drug Radix Ononides (Caesar & Loretz GmbH, Hilden, Germany) (250 g) was exhaustively extracted with EtOH (2 l). The extract was evaporated to dryness and partitioned between H_2O (500 ml) and cyclohexane (1 l). The aqueous phase was successively extracted with EtOAc (1 l) and *n*-BuOH (1 l). The butanolic extract was evaporated

Table 1. ^{1}H and ^{13}C NMR spectral data saponin **1** in CD_3OD.

Position	^{1}H	^{13}C	Position	^{1}H	^{13}C
1ax/eq	0.98/2.33	39.5	26	0.79	19.0
2ax/eq	1.73/1.94	27.2	27	1.34	20.8
3ax	3.14	91.1	28	0.98	17.3
4		40.6	29	0.98	32.7
5ax	0.70	56.2	30	0.76	25.4
6ax/eq	1.43/1.58	18.7	GlcA		
7ax/eq	1.58/1.46	34.5	1’	4.37 d, 7.2	105.6
8		44.3	2’	3.56	79.1
9ax	2.45	64.6	3’	3.56	79.0
10		37.8	4’	3.41	74.0
11		211.9	5’	3.49	76.6
12ax/eq	1.38	45.1	6’		176.9[a)]
13		134.1	Xyl		
14		44.3	1”	4.83 d, 6.9	103.0
15ax/eq	1.73/1.26	26.8	2”	3.38	78.9
16ax/eq	1.48/1.83	32.7	3”	3.41	78.9
17		41.6	4”	3.41	71.7
18		135.1	5”	3.10/3.81	66.5
19ax/eq	1.70/2.16	38.8	Rha		
20		33.1	1”’	5.18 d, 1.4	102.0
21ax/eq	1.44/1.38	44.4	2”’	3.91	72.1
22ax	3.32	78.2	3”’	3.73	72.3
23	1.08	28.6	4”’	3.38	74.2
24	0.84	16.7	5”’	4.08	69.7
25	1.16	17.0	6”’	1.24	18.2

GlcA = β-D-glucuronopyranose, Xyl = β-D-xylopyranose, Rha = α-L-rhamnopyranose, [a)] carboxylate form.

Table 2. ^{1}H and ^{13}C NMR spectral data of kaempferol glycoside **2** in CD_3OD.

Pos.	^{1}H	^{13}C	Pos.	^{1}H	^{13}C
2		158.9	5”	6.76 d, 8.4	116.9
3		135.3	6”	7.37 d, 8.4	131.3
4		179.5	7”	7.57 d, 15.7	146.8
5		162.8	8”	6.27 d, 15.7	115.2
6	6.40 d, 1.8	100.7	9”		168.7
7		164.6	Gal		
8	6.65 d, 1.8	95.7	1”’	5.55 d, 8.4	100.9
9		157.9	2”’	5.29	74.3
10		107.6	3”’	3.71 dd, 10.0, 3.0	73.3
1’		122.4	4”’	3.85	70.4
2’	7.98 d, 8.6	132.4	5”’	3.49	77.8
3’	6.83 d, 8.6	116.4	6”’	3.65	62.1
4’		161.8	Glc		
5’	6.83 d, 8.6	116.4	1””	4.96 d, 6.9	101.1
6’	7.98 d, 8.6	132.4	2””	3.45	74.7
1”		127.2	3””	3.52	77.5
2”	7.37 d, 8.4	131.3	4””	3.42	71.2
3”	6.76 d, 8.4	116.9	5””	3.47	78.3
4”		161.3	6””	3.64/3.85	62.4

Gal = β-D-galactopyranose, Glc = β-D-glucopyranose.

under reduced pressure at 42 °C (54 g) and purified by CC on Sephadex LH-20 (MeOH-H_2O 75:15) to give three frs. I (22 g), II (10 g) and III (1.5 g). A part of fr. III (250 mg) was subjected to column chromatography on RP-18 (MeOH-H_2O 6:4) and yielded the triterpene saponin **1** (12 mg).

Table 3. ^{1}H and ^{13}C NMR spectral data of pterocarpan glucoside **3** in CD_3OD.

Pos.	^{1}H	^{13}C	Pos.	^{1}H	^{13}C
1	7.25 d, 8.8	127.9	OCH_3	3.73	55.9
2	7.04 d, 8.8	112.2	Glc		
3		148.8	1’	4.87 d, 7.2	103.7
4		132.7	2’	3.52	75.0
4a		147.4	3’	3.47	77.5
6	3.62/4.34	68.0	4’	3.39	71.3
6a	3.61	40.9	5’	3.42	78.4
6b		117.5	6’	3.72/3.91	62.5
7	7.19 d, 8.2	126.0	Glc		
8	6.46 dd, 8.2, 2.2	107.4	1”	4.95 d, 7.7	105.2
9		161.9	2”	3.49	75.8
10	6.37 d, 2.2	97.6	3”	3.47	77.9
10a		161.8	4”	3.41	71.2
11a	5.55 d, 6.8	79.7	5”	3.19	78.4
11b		114.8	6”	3.66/3.76	62.3

Glc = β-D-glucopyranose.

Ononis vaginalis

Ononis vaginalis Vahl. was collected in 1999 nearby Burg El-Arab Egypt and identified by Dr. M. Elgebaly from the National Research Centre (NRC) Cairo. A voucher specimen of the plant is deposited at the Herbarium of the NRC, Department of Chemotaxonomy. Dried powder of the whole plant (2 kg) was exhaustively extracted with 80% MeOH (10 l) to give after evaporation the crude extract (50 g), which was successively partitioned between H_2O and *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH. The butanolic fr. was evaporated under reduced pressure at 42 °C (7 g). The butanolic extract was chromatographed on silica gel eluting with $CHCl_3$-MeOH-H_2O with increasing polarity to afford flavonoid glycoside fr. IV (200 mg) which was subjected to MPLC using RP-material and MeOH-H_2O 40:60 as eluent. Further chromatography on Sephadex LH-20 eluting with MeOH yielded pure flavonoid glycoside **2** (10 mg) and pterocarpan fr. V (6 mg) which was purified by RP-18 HPLC (MeOH-H_2O 50:50) to give the pure pterocarpan glucoside **3** (1.5 mg).

(*R*)-2-Butylglycosides

A sample (*ca.* 250 μg) of the saponin **1** was hydrolysed with 0.5 ml 5% HCl for at least 3 h at 80 °C. After evaporation of the acid under red. pres., 0.5 ml (*R*)-2-BuOH was added, dried HCl gas was bubbled through the soln. for 30 s and the reaction mixture was heated for 3 h at 80 °C under N_2 in a sealed vessel. Trimethylsilylation was performed with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide overnight. (*R*)-2-butyl-L-GlcA: R_t 82.55, R_i 2095; (*R*)-2-butyl-D-GlcA: R_t 81.97, R_i 2085; (*R*)-2-butyl-L-Xyl: R_t 54.45, R_i 1982; (*R*)-2-

butyl-D-Xyl: R_t 55.24, R_i 1987; (*R*)-2-butyl-L-Rha: R_t 52.23, R_i 1854. Identification of the sugars were done by comparison of the R_i values and co-injection with the appropriate standard. R_i according to [11]. Consequently it was shown for the saponin **1** that glucuronic acid and xylose belong to the D- and rhamnose to the L-series.

Spectroscopic data

3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-xylopyranosyl-(1→2)-β-D-glucuronopyranosyl]-3β,22α-dihydroxyolean-13-en-11-one (**1**): $C_{47}H_{74}O_{17}$ (910). For ^{1}H and ^{13}C NMR: see Table 1. – LSI-MS (negative ion mode): *m/z* (%) = 909 [M-H]$^-$ (80), 763 [M-H-Rha]$^-$ (15), 631 [M-H-Rha-Xyl]$^-$ (15) and 455 [M-H-Rha-Xyl-GlcA]$^-$ (12). HR-ESI-MS: m/z = 933.4847 [M+Na]$^+$, calcd. for $C_{47}H_{74}O_{17}Na$ 933.4823 (Δ + 2.6 ppm).

3-*O*-[2-*O*-(*E*)-p-coumaroyl-β-D-galactopyranosyl]-7-*O*-β-D-glucopyranosylkaempferol (**2**): $C_{36}H_{36}O_{18}$ (756). For ^{1}H and ^{13}C NMR: see Table 2. – HR-ESI-MS: m/z = 779.1834 [M+Na]$^+$, calcd. for $C_{36}H_{36}O_{18}Na$ 779.1799 (Δ + 4.5 ppm).

3,4-Di-*O*-β-D-glucopyranosyl-4-hydroxymedicarpin (**3**): $C_{28}H_{34}O_{15}$ (610). CD: Θ (mdegree) = 284 nm (+18.8), 233 nm (−39.8). – For ^{1}H and ^{13}C NMR: see Table 3. – LSI-MS (negative ion mode): *m/z* (%): 609 [M-H]$^-$ (19), 447 [M-H-Glc]$^-$ (48), 255 [M-H-2xGlc-HCHO]$^-$ (100). HR-ESI-MS: m/z = 633.1765, calcd. for $C_{28}H_{34}O_{15}Na$ 633.1795 (Δ − 4.7 ppm).

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