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Antibacterial and Antibiofilm Activity of Flavonoid and Saponin Derivatives from *Atriplex tatarica* against *Pseudomonas aeruginosa*

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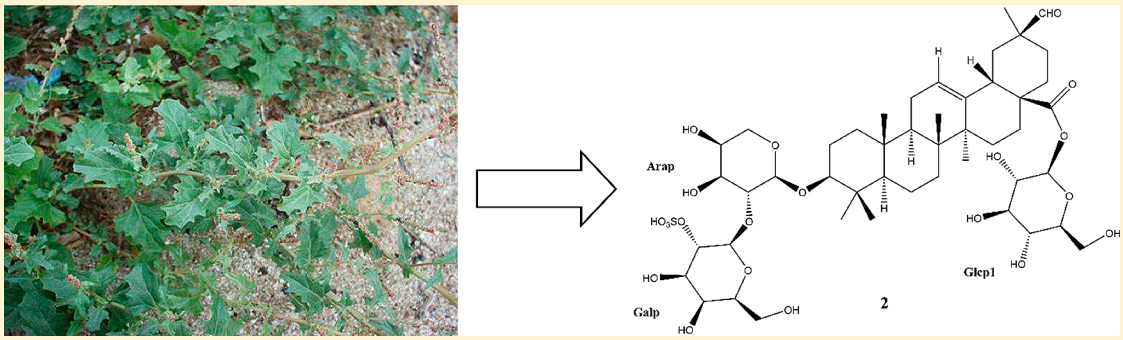
Supporting Information



ABSTRACT: A new flavonoid glucoside derivative, patuletin 3-*O*-(2-*O*-feruloyl)-*β*-D-glucuronopyranosyl-(1→2)-*β*-D-glucopyranoside, named atriplexin IV (**1**), and three new triterpenoid saponin derivatives, two sulfonylated, *β*-D-glucopyranosyl-3-*O*-(2-*O*-sulfo-*β*-D-galactopyranosyl)-(1→2)-*α*-L-arabinopyranoside-30-alolean-12-en-28-oate (**2**), named atriplexogenin I, *β*-D-glucopyranosyl-3-*O*-(2-*O*-sulfo-*β*-D-galactopyranosyl)-(1→2)-*α*-L-arabinopyranoside)-30-hydroxyolean-12-en-28-oate (**3**), named atriplexogenin II, and *β*-D-glucopyranosyl-3-*O*-(*β*-D-glucopyranosyl-(1→2)-*β*-D-galactopyranosyl-(1→2)-*α*-L-arabinopyranoside)-30-alolean-12-en-28-oate (**4**), named atriplexogenin III, were isolated by silica gel column and semipreparative HPLC chromatography from the *n*-butanol extract of the salt marsh plant *Atriplex tatarica*. In addition, two known secondary metabolites, patuletin3-*O*-*β*-D-apiofuranosyl-(1‴→2″)-*β*-D-glucopyranoside (**5**) and patuletin 3-*O*-5‴-*O*-feruloyl-*β*-D-apiofuranosyl-(1‴→2″)-*β*-D-glucopyranoside (**6**), were isolated for the first time from *A. tatarica*. The structures of the isolated compounds were elucidated by 1D and 2D NMR, HRESIMS, IR, and UV data. Antibacterial activity by the microdilution method and antibiofilm activity against *P. aeruginosa* were assessed. Compound **5** possesses significant antibacterial activity, while the most potent antibiofilm agent is compound **2**.

Atriplex tatarica L. is an annual plant of the Amaranthaceae (formerly Chenopodiaceae) family, section Sclerocalymma. The genus comprises nearly 270 species, mostly distributed in dry and semidry habitats of North America, central Asia, and southern Australia, while several species are found in continental saline habitats of the Northern Hemisphere.[1] According to the Euro+MedPlantbase, the species is a member of the *A. tatarica* aggregate, together with five other *Atriplex* species (*A. laciniata* L., *A. lasiantha* Boiss., *A. recurva* d'Urv., *A. tornabenei* Tineo, and *A. zahlensis* Mouterde).

The species is procumbent to erect, whitish, multibranched, and up to 150 cm in height. The leaves are triangular-rhombic to triangular-hastate; inflorescence is terminal, paniculate, or spicate and more or less leafless.[2] The species exhibits visible heterocarpy in terms of variations in size and color of its fruit, and the achene has a smooth and glossy testa.[3] *A. tatarica* is frequently found within southeastern Europe, through the Pannonian lowland to Central Europe, occupying saline habitats, such as solonchaks and solonetz sandy and alluvial trails, as well as village roadsides and clayish banks of rivers, swamps, and lakes.[4] In Serbia, *A. tatarica* is present on different salt-affected soils spread in the Pannonian part of the country, the Vojvodina Province, as well as within the nitrophilous and tramped communities.[5]

Earlier chemical investigation of *Atriplex* species revealed flavonoids, saponins, and alkaloids as the main secondary metabolites.[6] Among the flavonoids, characteristic glycosides of isorhamnetin from *A. farinosa*,[7] patuletin, quercetagetin, and spinacetin from *A. littoralis*,[8,9] and sulfonylated flavonoids of

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kaempferol and quercetin 3-*O*-sulfo-7-*O*-arabinopyranoside from *A. hortensis* were found.[10] Among the saponins, the main compounds were the triterpenoids longispinogenin and saikogenin F from *A. semibaccata*[11] and hederagenin and oleanolic acid types from *A. stylosa*.[12]

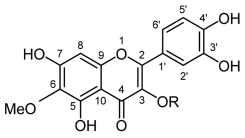
Previous investigations of patuletin and its derivatives showed anti-inflammatory and antiproliferative activities,[13,14] while bidesmosidic triterpenoids mostly possessed anti-inflammatory, cytotoxic, and antimicrobial activities.[15−19]

Pseudomonas aeruginosa is a Gram-negative bacterium that can cause serious infections in immunocompromised patients, and it is also a major cause of nosocomial infections, septic shock, bronchopneumonia, and wound infections. *P. aeruginosa* adopts a biofilm mode of growth in the lungs, kidneys, and urinary tract. When the bacterium has already formed a biofilm, it becomes extremely difficult to destroy the infection, and the bacteria are then more resistant to antibiotic therapy, as well as to the host immune response.[20] Some natural products can disrupt bacterial biofilms and thus make the bacteria more sensitive to different antibiotics,[21−24] since the phase of bacterial adherence is obstructed.[25] It has been found that inhibition of biofilm formation is easier to realize than inhibition of the already formed biofilm. This may decrease the chance of a later infection.[26]

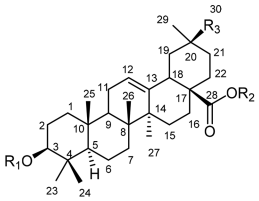
In this work, the isolation and structural elucidation of three new saponins and three flavonoid glycosides, one new, are reported, and their antibacterial and antibiofilm activities against *P. aeruginosa* are discussed.

■ RESULTS AND DISCUSSION

Semipreparative reversed-phase HPLC separation of fractions obtained from column chromatography (CC) of the aerial parts of *A. tatarica* revealed one new flavonol glycoside (**1**) and three new triterpenoid saponin derivatives (**2**−**4**). The isolated compounds were identified according to 1D and 2D NMR, UV, IR, and MS data. The known compounds patuletin 3-*O*-*β*-D-apiofuranosyl-(1‴→2″)-*β*-D-glucopyranoside (**5**) (from *Spinacia oleracea*)[27] and patuletin 3-*O*-(5‴-*O*-feruloyl)-*β*-D-apiofuranosyl-(1‴→2″)-*β*-D-glucopyranoside (**6**) (from *A. littoralis*)[6] were found in *A. tatarica* for the first time.



1 R=Glcp(2-1)-GlucuronAp(2-*O*)-feruloyl



2 R_1=Arap(2-1)Galp(OSO$_3$H), R_2=Glcp1, R_3=CHO
3 R_1=Arap(2-1)Galp(OSO$_3$H), R_2=Glcp1, R_3=CH$_2$OH
4 R_1=Arap(2-1)Galp(2-1)Glcp2, R_2=Glcp1, R_3=CHO

Compound **1** was isolated as a yellow powder. Its UV maxima at λ_{max} at 254 (sh), 272, and 354 nm indicated the presence of a highly conjugated system. IR absorptions at 1651 and 3417 cm^{-1} indicated the presence of carbonyl and hydroxy groups, respectively. Compound **1** showed a deprotonated molecular ion [M − H]$^-$ in the negative HRESIMS at *m*/*z* 845.1795 and, together with the ^{13}C NMR data, indicated a molecular formula of $C_{38}H_{38}O_{22}$. The UV spectrum of compound **1** was characteristic for 3-OH-substituted flavonols. Free hydroxy groups at C-3′, C-4′, C-5, and C-7 were determined after the addition of shift reagents. The bathochromic shift (26 nm) of band I upon treatment with a methanolic NaOAc/H_3BO_3 solution confirmed the presence of a catechol moiety. Upon treatment with a methanolic $AlCl_3$/HCl solution, the bathochromic shifts of bands I (57 nm) and II (21 nm) were observed, indicating a free 5-OH group. The presence of a free hydroxy group at C-7 was proposed on the basis of the bathochromic shift of band II (20 nm) after addition of NaOAc.[28]

The ^{1}H and ^{13}C NMR data of compound **1** (Table 1, Figures S1 and S2, Supporting Information) were similar to those of known compound **6** (Table 1).[6] Patuletin as aglycone and the glucosyl and feruloyl moieties were identified by comparison of the NMR data with known compounds **5** and **6**. Different ^{1}H NMR resonances for the second sugar moiety indicated that the apiose moieties in **5** and **6** were replaced by a glucuronic acid. From the HMBC correlations H-1″/C-3, H-1‴/C-2″, and H-2‴/C-9⁗, the following linkage pattern was deduced: the glucosyl moiety was attached to C-3, the linkage between the glucosyl and glucuronic acid moieties was 1 → 2, and finally, the feruloyl moiety was connected to C-2‴ of the glucuronic acid unit (Figures S6 and S7, Supporting Information). Signals of the glucosyl moiety had similar chemical shifts to those in compounds **5** and **6** (Table 1). The HMBC correlations H-4‴, H-5‴/C-6‴ confirmed a 6‴-COOH group, and the NOE correlations H-1‴, H-5‴/H-3‴ confirmed the axial protons in the glucuronic acid moiety (Figures S4 and S6, Supporting Information). The two *trans* olefinic proton signals at δ_H 7.39 (d, *J* = 15.9 Hz, H-7) and δ_H 6.15 (d, *J* = 15.9 Hz, H-8), as well as signals at δ_H 6.74 (d, *J* = 1.5 Hz, H-2), δ_H 6.62 (dd, *J* = 8.3; 2.2 Hz, H-6), δ_H 6.54 (d, *J* = 8.1 Hz, H-5), and δ_H 3.71 (s, OMe), were characteristic of the feruloyl moiety (Table 1, Figure S1, Supporting Information). On the basis of all spectroscopic data, the structure of atriplexin IV (**1**) was established as a new patuletin derivative, patuletin 3-*O*-(2‴-*O*-feruloyl)-*β*-D-glucuronopyranosyl(1→2)-*β*-D-glucopyranoside.

Compound **2** was obtained as a white, amorphous powder. The negative HRESIMS data exhibited a deprotonated molecular ion [M − H]$^-$ at *m*/*z* 1005.4381 (calcd for $C_{47}H_{74}O_{21}S$−H 1005.4371). The ^{1}H NMR spectrum of **2** showed signals for six methyl groups at δ_H 0.78, 0.85, 0.95, 0.96, 1.06, and 1.19 (s, 3H), an oxymethyne proton δ_H 3.16 (dd, *J* = 4.3, 11.9 Hz), an olefinic proton at δ_H 5.33, and a formyl proton at δ_H 9.45 (Figure S8, Supporting Information). In the ^{13}C NMR spectrum, the olefinic carbons resonated at δ_C 124.7 and 144.5, and the ester and formyl carbonyl carbons at δ_C 177.5 and 208.0, respectively (Figure S9, Supporting Information). In comparison to the literature, compound **2** was recognized as an oleanane-type saponin.[15]

Three anomeric proton signals that were observed at δ_H 4.55 (d, *J* = 6.2 Hz), 4.79 (d, *J* = 7.6 Hz), and 5.35 (d, *J* = 8.0 Hz) displayed HSQC correlations with anomeric carbon signals at δ_C 105.4, 102.5, and 95.9, respectively (Table 2, Figures S8 and S13, Supporting Information), which were characteristic of the three monosaccharide units. Analysis of the acid hydrolysate (HPLC comparison with standards) as well as COSY and HSQC spectra of compound **2** revealed *β*-D-glucopyranosyl (Glcp1), *β*-D-galactopyranosyl (Galp), and *α*-L-arabinopyranosyl (Arap) moieties (Table 2, Figures S10, S13, Supporting Information). More precisely, a bidesmosidic structure was proposed (Figure 2).[16] The *β*-anomeric configurations for the D-galactopyranosyl and D-glucopyranosyl units were determined by their $^3J_{H1/H2}$ coupling constants of 7−8 Hz, and the *α*-anomeric configuration for the L-arabinopyranosyl was

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Table 1. ^{13}C and ^{1}H NMR Data of Compounds 1, 5, and 6 (125.80 MHz for ^{13}C and 500.26 MHz for ^{1}H, Methanol-d_4)

	1		5		6	
C/H	δ_C	δ_H (J in Hz)[a]	δ_C	δ_H (J in Hz)[a]	δ_C	δ_H (J in Hz)[a]
2	158.4		158.5		158.3	
3	134.6		134.8		134.4	
4	179.7		180.0		179.5	
5	153.8		153.8		154.0	
6	132.5		132.7		132.5	
7	153.5		154.0		153.5	
8	94.8	6.25, s	94.9	6.48, s	94.8	6.15, s
9	157.9		158.6		157.9	
10	106.4		106.5		106.8	
1′	123.4		123.5		123.4	
2′	117.4	7.53, d (2.0)	117.4	7.69, d (2.0)	117.5	7.69, d (2.0)
3′	146.0		146.1		146.0	
4′	149.8		149.9		149.3	
5′	116.2	6.85, d (8.5)	116.3	6.87, d (8.5)	116.0	6.84, d
6′	123.4	7.41, dd (2.2, 8.3)	123.4	7.64, dd (2.1, 8.5)	123.0	7.51, dd (2.0, 8.5)
OMe-6	61.1	3.88, s	61.1	3.87, s	61.0	3.84, s
Glcp[b]						
1″	98.8	5.90, d (7.9)	101.5	5.49, d (7.7)	101.0	5.72, d (7.8)
2″	79.9	3.97, dd (1.0, 10.0)	76.9	3.95, dd (8.0, 9.5)	75.1	4.04, dd (8.0, 9.5)
3″	73.0	3.85, m	75.5	3.69, m	75.6	3.72, m
4″	70.6	3.89, m	70.8	3.82, d (3.0)	70.8	3.85, m
5″	77.3	3.49, t (6.0)	77.3	3.44, t (6.0)	77.3	3.45, t (6.0)
6″	62.1	3.60, 3.54 m	62.2	3.60, m	62.1	3.63, m
				3.57, m		3.57, m
Apif[c]						
1‴			110.8	5.46, d (1.3)	109.5	5.47,d (1.3)
2‴			78.2	4.06, d (1.3)	78.3	3.87, m
3‴			81.0		79.5	
4‴			75.6	4.03, d (9.5)	75.3	4.29, m
				3.70, d (9.5)		3.66, d (9.5)
5‴			66.4	3.63, d (11.5)	70.1	4.49, m
				3.73, d (11.5)		4.32, d (11.2)
GlucuronAp[d]						
1‴	99.6	5.30, d (8.0)				
2‴	74.8	4.99, dd (9.1, 8.2)				
3‴	75.9	3.75, t (9.1)				
4‴	73.4	3.69, m				
5‴	76.4	4.01, d (9.5)				
6‴	173.3					
feruloyl						
1⁗	127.5				127.5	
2⁗	111.1	6.74, d (1.5)			111.6	6.92, d (2.0)
3⁗	149.1				149.4	
4⁗	150.4				149.8	
5⁗	116.3	6.54, d (8.1)			116.4	6.76, d (8.1)
6⁗	123.6	6.62, dd (2.2, 8.3)			124.2	6.83, dd (1.5, 8.0)
7⁗	146.7	7.39, d (15.9)			146.6	7.22, d (15.8)
8⁗	115.6	6.15, d (15.9)			115.0	6.06, d (15.8)
9⁗	168.7				169.0	
OMe-3	56.2	3.71, s			56.4	3.86, s

[a] J values are given in parentheses. [b] Glcp = glucopyranosyl. [c] Apif = apiofuranosyl. [d] GlucuronAp = glucuronopyranosyl.

determined according to the $^{3}J_{H1/H2}$ coupling constant of 6.2 Hz (Table 2). The HMBC cross-peak between anomeric signals at δ_H 4.55 (α-L-Arap) and C-3 at δ_C 91.2 was unambiguous proof that the L-arabinopyranosyl unit was attached to the aglycone at C-3 via C-1_{Arap}, and the HMBC correlation H-1_{Galp}/C-2_{Arap} confirmed the disaccharide β-D-galactopyranosyl-(1→2)-L-arabinopyranoside moiety at C-3 (Figure S16, Supporting Information). The chemical shifts of an anomeric proton at δ_H 5.35 and anomeric carbon at δ_C 95.9 suggested that the D-glucopyranosyl unit was attached by an ester linkage to C-28, which was confirmed by the HMBC cross-peak between H-1_{Glcp1}/C-28. The linkages of saccharide units to the triterpenoid skeleton were additionally confirmed by the NOESY correlations H-3/H-1_{Arap}, H-2_{Arap}/H-1_{Galp}, and

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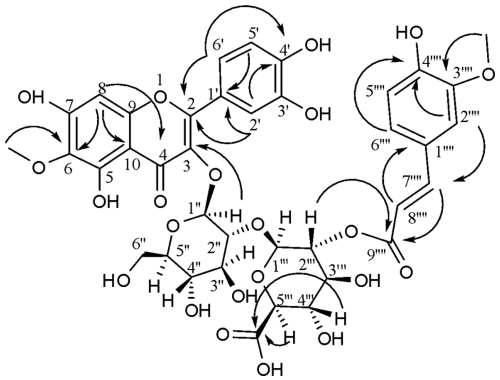

Figure 1. Characteristic HMBC correlations for flavonoid **1**.

H-26/H-1_{Glcp1}. Additional NOESY correlations of axial sugar protons H-1_{Arap}/Me-23, H-1_{Arap}/H-3, H-1_{Arap}/H-5_{Arap}, H-1_{Glcp1}/H-3_{Glcp1}, H-1_{Glcp1}/H-5_{Glcp1}, H-1_{Galp}/H-3_{Galp}, and H-1_{Galp}/H-5_{Galp} confirmed the conformation provided in Figure 2 (Figure S12, Supporting Information). The relative configuration of compound **2** was determined based on NOESY correlations. An axial orientation of H-3 was determined according to the coupling constants J = 11.9 and 4.3 Hz. Correlations in the NOESY spectrum of H-3/H-5, H-9 indicated that H-3, H-5, and H-9 were axial, i.e., α oriented; correlations Me-24/Me-25 and Me-25/Me-26 revealed β orientations of the methyl groups, and the correlation Me-27/Me-29 indicated an α orientation of Me-27 and *trans* connection of the D-ring (Figure 2). The presence of a sulfate group in compound **2** was determined by the HRESIMS data. The 80 Da [M(OSO_3H) − M(OH) = 80] higher mass as well as intensity of the [M − H + 2] ion, similar to the calculated value for sulfonylated compounds (28.0 exptl, 22.7% calcd), indicated the presence of an OSO_3H group. The position of a sulfate group was determined at C-2_{Galp} according to significant downfield shifting of H-2_{Galp} (∼0.5−0.7 ppm) and C-2_{Galp} (8−10 ppm), similar to saponins isolated from *Fagonia indica*.[15] Thus, atriplexogenin I (**2**) is β-D-glucopyranosyl-3-*O*-(2-*O*-sulfo-β-D-galactopyranosyl)-(1→2)-α-L-arabinopyranoside-30-alolean-12-en-28-oate, a new bidesmosidic derivative.

Compound **3** was obtained as a white, amorphous powder. The negative HRESIMS exhibited a deprotonated molecular ion [M − H]$^-$ at m/z 1007.4552 (calcd for $C_{47}H_{76}O_{21}S$−H 1007.4527). The ^{1}H NMR spectrum of compound **3** showed signals for six methyl groups at δ_H 0.80, 0.85, 0.89, 0.95, 1.05, and 1.17 (s, 3H), an oxymethyne proton at δ_H 3.17 (dd, J = 4.3, 11.9 Hz), two oxymethylene protons at δ_H 3.44 and 3.50, and an olefinic proton at δ_H 5.30 (Table 2, Figure S17, Supporting Information). In the ^{13}C NMR spectrum, oxygenated aliphatic carbons were observed at δ_C 66.4 and 91.5, the olefinic carbons at δ_C 124.3 and 144.6, and the ester carbonyl carbon at δ_C 178.1 (Table 2). According to the literature, compound **3** was recognized as an oleanane-type saponin.[15]

In comparison to compound **2**, compound **3** exhibited similar ^{1}H and ^{13}C NMR spectra. The only difference in the structure was the substituent at C-20, where a hydroxymethyl group was found instead of the formyl group present in compound **2**. This was confirmed by the HMBC cross-peaks of H-30/C-29, C-20 and Me-29/C-20, as well as ROESY correlations of H-18, H-19, H-21, H-22, and H-29/H-30 (Figures S20 and S22, Supporting Information). ^{1}H and ^{13}C NMR chemical shifts of the signals of sugar moieties were comparable to those of compound **2**, indicating the same sugar units in compound **3** (Table 2). The same HMBC and ROESY correlations confirmed the same positions of sugar moieties as in compound **2**. Additionally, identical evidence was found for the configurations of all sugar units, all methyl groups, and the sulfate group, as described for compound **2**. Thus, the structure of atriplexogenin II (**3**) was defined as β-D-glucopyranosyl-3-*O*-(2-sulfo-β-D-galactopyranosyl)-(1 → 2)-α-L-arabinopyranoside-30-hydroxyolean-12-en-28-oate, a new bidesmosidic derivative.

The presence of the formyl signal in the ^{1}H NMR spectrum of the starting *n*-butanol extract confirmed that compound **2** was not an artifact that emerged during the isolation process, since it is an oxidation product of compound **3**.

Compound **4** was obtained as a white, amorphous powder. The negative ion HRESIMS data exhibited a deprotonated molecular ion [M − H]$^-$ at m/z 1087.5321 (calcd for $C_{53}H_{84}O_{23}$−H 1087.5331). The ^{1}H NMR spectrum of compound **4** showed signals of six methyl groups at δ_H 0.80, 0.85, 0.95, 0.96, 1.05, and 1.20 (s, 3H), one oxymethyne proton at δ_H 3.17 (dd, J = 4.3, 11.9 Hz), an olefinic proton at δ_H 5.33, and a formyl proton at δ_H 9.45 (Table 2, Figure S23, Supporting Information). In the ^{13}C NMR spectrum, an oxygenated carbon at δ_C 91.4, the olefinic carbons at δ_C 124.7 and 144.4, and ester and formyl carbonyl carbons at δ_C 177.5 and 208.0, respectively, were present (Table 2, Figure S24, Supporting Information). Similar to compounds **2** and **3**, compound **4** was recognized as an oleanane-type saponin.[15]

The ^{1}H and ^{13}C NMR data of compound **4** revealed the presence of four anomeric signals at δ_H/δ_C 4.54/105.3, 4.74/103.3, 5.34/95.9, and 4.59/106.0, which indicated four monosaccharide units (Table 2, Figure S29, Supporting Information). In comparison to compound **2**, instead of the sulfo group at C-2_{Galp}, an additional D-glucopyranosyl unit (Glcp2) was attached (Figure 2), deduced from the HRESIMS data and molecular formula. The HMBC correlation of H-1_{Glcp2}/C-2_{Galp} and chemical shift of H-2_{Galp} at δ_H 3.41 (in comparison to **2**) confirmed its position, i.e., a D-glucopyranosyl-(1→2)-D-galactopyranosyl linkage. Additionally, the HMBC correlation of H-1_{Galp}/C-2_{Arap} confirmed the D-galactopyranosyl-(1→2)-L-arabinopyranoside linkage. Similar to compound **2**, where a disaccharide unit was attached to C-3, a trisaccharide moiety was found at C-3 in compound **4**, according to the HMBC correlations of H-3/C-1_{Arap} and H-1_{Arap}/C-3 (Figure S31, Supporting Information). The ^{1}H and ^{13}C NMR chemical shifts of the remaining H and C atom signals from sugar moieties, respectively, were comparable to those of compounds **2** and **3**, indicating the same sugar units in compound **4** (except those of the H-2_{Galp} and C-2_{Galp}, since a sulfo group was present at C-2_{Galp} in compounds **2** and **3**). The same ROESY and HMBC correlations confirmed the same positions of sugar moieties as in compound **2** (Figure 2, Figures S27 and S30, Supporting Information). Thus, the structure of atriplexogenin III (**4**) was defined as β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-α-L-arabinopyranoside-30-alolean-12-en-28-oate, a new bidesmosidic derivative.

Additionally, the antibacterial and antibiofilm activities of the isolated compounds were examined since similar triterpenoids exhibited significant antimicrobial activities.[17−19]

The antibacterial activities of compounds isolated from *A. tatarica* are shown in Table 3. Minimal inhibitory concen-

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Table 2. ^{13}C and ^{1}H NMR Data of Compounds 2−4 (125.80 MHz for ^{13}C and 500.26 MHz for ^{1}H, Methanol-d_4)

	2		3		4	
C/H	δ_C	δ_H (J in Hz)[a]	δ_C	δ_H (J in Hz)[a]	δ_C	δ_H(J in Hz)[a]
1	40.0	1.00, m[b]	39.8	1.00, m[b]	40.0	1.00, m[b]
		1.63, m[b]		1.63, m[b]		1.63, m[b]
2	27.1	1.73, m[b]	28.4	1.73, m[b]	27.1	1.74, m[b]
		1.83, m[b]		1.84, m[b]		1.85, m[b]
3	91.2	3.16, dd(4.3, 11.9)	91.5	3.17, dd(4.5, 11.5)	91.4	3.17, dd(4.5, 11.5)
4	40.5		40.2		40.5	
5	57.1	0.78, m[b]	57.0	0.77, m[b]	57.1	0.80, m[b]
6	19.5	1.39, m[b]	18.5	1.40, m[b]	19.5	1.40, m[b]
		1.54, m[b]		1.53, m[b]		1.52, m[b]
7	34.0	1.32, m[b]	33.9	1.33, m[b]	34.0	1.32, m[b]
		1.49, m[b]		1.49, m[b]		1.49, m[b]
8	40.9		40.8		40.9	
9	49.2	1.59, m[b]	49.0	1.57, m[b]	49.1	1.58, m[b]
10	38.0		38.1		38.1	
11	24.7	1.91, m[b]	24.5	1.88, m[b]	24.7	1.91, m[b]
12	124.7	5.33, m	124.3	5.30, t (3.0)	124.7	5.33, m
13	144.5		144.6		144.4	
14	43.0		42.9		43.0	
15	29.0	1.10, m[b]	28.7	1.08, m[b]	29.1	1.09, m[b]
		1.75, m[b]		1.74, m[b]		1.79, m[b]
16	24.0	1.79, m[b]	24.1	2.08, m[b]	24.0	2.06, m[b]
		2.06, m[b]				
17	47.7		47.8		47.8	
18	43.7	2.64, dd (13.3, 4.9)	42.2	2.82, dd (13.5, 4.5)	43.7	2.64, dd (13.5, 4.5)
19	40.9	1.80, m[b]	42.8	1.38, m[b]	40.9	1.79, m[b]
		1.85, m[b]		1.62, m[b]		1.85, m[b]
20	47.8		36.1		47.7	
21	28.6	1.49, m[b]	29.9	1.31, m[b]	28.7	1.47, m[b]
		1.90, m[b]		1.49, m[b]		1.91, m[b]
22	33.8	1.66, m[b]	32.7	1.59, m[b]	33.8	1.30, m[b]
		1.73, m[b]		1.69, m[b]		1.69, m[b]
23	28.7	1.06, s	28.4	1.05, s	28.7	1.05, s
24	17.1	0.85, s	16.7	0.85, s	17.1	0.85, s
25	16.2	0.95, s	15.8	0.95, s	16.1	0.95, s
26	17.8	0.78, s	17.7	0.80, s	17.8	0.80, s
27	26.6	1.19, s	26.3	1.17, s	26.6	1.20, s
28	177.5		178.1		177.5	
29	24.4	0.96, s	28.0	0.89, s	24.4	0.96, s
30	208.0	9.45, s	66.4	3.44, m	207.9	9.45, s
				3.50, m		
Arap[c]						
1	105.4	4.55, d (6.2)	105.0	4.55, d (6.2)	105.3	4.54, d (6.2)
2	79.4	3.85, m[b]	78.6	3.85, m[b]	79.2	3.89, m[b]
3	73.5	3.83, m[b]	73.3	3.83, m[b]	73.4	3.88, m[b]
4	69.3	3.87, m[b]	68.8	3.87, m[b]	68.8	3.90, m[bb]
5	65.3	3.51, dd (2.0, 12.0)	65.1	3.51, dd (2.0, 12.0)	65.1	3.51, dd (2.0, 12.0)
		3.83, m[b]		3.82, m[b]		3.83, m[b]
Galp[d]						
1	102.5	4.79, d (7.6)	102.3	4.79, d (7.6)	103.3	4.74, d (7.6)
2	82.9	4.04, dd (7.9, 8.8)	82.4	4.04, dd (7.9, 8.8)	84.8	3.41, m
3	77.3	3.63, t (8.8)	77.2	3.63, t (8.8)	77.7	3.56, t (8.8)
4	71.7	3.26, m[b]	71.7	3.26, m[b]	71.8	3.25, m[b]
5	78.0	3.28, m[b]	77.8	3.29, m[b]	78.2	3.26, m[b]
6	62.9	3.61, dd (6.2,11.8)[g]	63.0	3.60, dd (6.2,11.8)[g]	63.1	3.62, dd (6.2,11.8)[g]
		3.82, dd (6.2,11.8)[g]		3.83, dd (6.2, 11.8)[g]		3.82, dd (6.2, 11.8)[g]
Glcp[e]						
1	95.9	5.35, d (8.0)	95.6	5.38, d (8.0)	95.9	5.34, d (8.0)
2	74.0	3.30, t	73.8	3.32, m[b]	74.1	3.30, m[b]
3	78.4	3.39, t (9.0)	78.2	3.40, t (9.0)	78.4	3.39, t (9.0)

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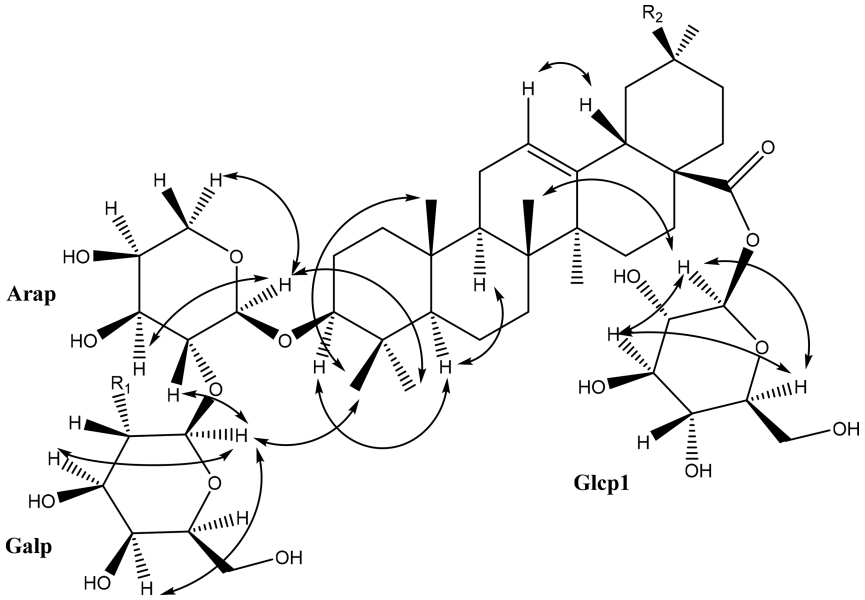
Table 2. continued

C/H	2		3		4	
	δ_C	δ_H (J in Hz)[a]	δ_C	δ_H (J in Hz)[a]	δ_C	δ_H(J in Hz)[a]
4	71.2	3.33, m[b]	71.0	3.34, m[b]	71.2	3.33, m[b]
5	78.8	3.33, m[b]	78.5	3.34, m[b]	78.9	3.33, m[b]
6	62.5	3.67, dd (5.0,12.0)[g]	62.3	3.67, dd (5.0,12.0)[g]	62.5	3.67, dd (5.0,12.0)[g]
		3.79,m[b]		3.81, m[b]		3.80,m[b]
Glcp2[f]						
1					106.0	4.59, d (7.6)
2					76.5	3.26, m[b]
3					77.8	3.37, t (9.0)
4					71.4	3.31, m[b]
5					78.9	3.31, m[b]
6					62.8	3.69, m[b]
						3.90, dd (2.0, 12.0)[g]

[a]J values are given in parentheses. [b]Signals overlapped; values obtained from the HSQC spectra. [c]Arap = arabinopyranosyl. [d]Galp = galactopyranosyl. [e]Glcp1 = glucopyranosyl 1. [f]Glcp2 = glucopyranosyl 2. [g]Coupling constants obtained from 2DJ spectra.



2 R_1=OSO_3H, R_2=CHO; **3** R_1=OSO_3H, R_2=CH_2OH; **4** R_1=(2-1)Glcp2, R_2=CHO

Figure 2. Characteristic NOESY correlations for new saponin derivatives **2**−**4**.

Table 3. Minimum Inhibitory (MIC) and Bactericidal Concentrations (MBC) of Compounds (μM)

bacteria		1	5	6	2	3	4	streptomycin	ampicillin
Micrococcus flavus	MIC	295.5	399.4	311.7	596.4	−[a]	919.1	344.2	716.3
	MBC	472.8	639.0	623.4	1192.8		1378.7	516.3	1146.1
Listeria monocytogenes	MIC	886.5	639.0	935.2	1988.1	−[a]	1148.9	344.2	1146.1
	MBC	1182.0	1198.1	1246.9	2485.1		1838.2	516.3	1432.7
Pseudomonas aeruginosa	MIC	295.5	399.4	311.7	994.0	496.0	689.3	344.2	2149.0
	MBC	472.8	639.0	623.4	1192.8	744.0	1378.7	516.3	3581.7
Escherichia coli	MIC	591.0	399.4	498.7	497.0	−[a]	919.1	344.2	716.3
	MBC	1182.0	639.0	623.4	596.4		1378.7	516.3	1432.7

[a]Not tested because of insufficient amount of compound **3**.

tration (MIC) and minimal bactericidal concentration (MBC) were determined using the microdilution method. Antibacterial activities of the compound and standard antibiotics (streptomycin and ampicillin) were tested against a panel of selected Gram-positive and Gram-negative bacteria. All compounds possessed antibacterial activities in the MIC range of 295.5−1988.1 μM, while the MBC values were 472.8−2485.1 μM. The standard antibacterial agents streptomycin and ampicillin, used as positive controls, possessed inhibitory activities of 344.2 μM and 716.3−2149.0 μM and bactericidal activities of

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516.3 μM and 1146.1−3581.7 μM, respectively. The strongest antibacterial activities against *Micrococcus flavus* and *Pseudomonas aeruginosa* were exhibited by compound **1**, better than both antibacterial controls, ampicillin and streptomycin. The strongest activities against *Listeria monocytogenes* and *Escherichia coli* were obtained for compound **5**, better than ampicillin and lower than streptomycin (Table 3). The most sensitive strains were *M. flavus* and *P. aeruginosa*, while *L. monocytogenes* was the most resistant bacterium.

The tested compounds exhibited antibiofilm activity against *P. aeruginosa* at sublethal concentrations at 0.5, 0.25, and 0.125 of MIC values (Table 4.). All the compounds reduced biofilm formation in the range of 20.07−58.06% at the concentration of 0.5 MIC, while some of them showed activity at the lowest tested concentration (0.125 MIC). The most promising antibiofilm agent among tested compounds was compound **2** (56.63−7.17%) at all tested concentrations, while compounds **5** (58.06 and 12.19%) and **4** (53.14 and 39.43%) possessed potential at 0.5 and 0.25 of MIC values. Streptomycin and ampicillin reduced biofilm by values in the range of 52.46−89.31% and 58.94−85.24%, at all tested sub-MIC, respectively. The results showed that tested compounds exhibited remarkable *P. aeruginosa* biofilm inhibition activity. The reduced antibiofilm activity toward a preformed biofilm is evidence that cells in a biofilm are more resistant to antimicrobial agents compared to free-floating cells.

Table 4. Effects of Isolated Compounds on Inhibition of the Biofilm Formation of *P. aeruginosa* (PAO1)

	inhibition of biofilm formation[a]		
compound	0.5 MIC (% ± SE)	0.25 MIC (% ± SE)	0.125 MIC (% ± SE)
1	43.0 ± 0.6	26.2 ± 0.4	1.8 ± 0.1
5	58.1 ± 0.4	12.2 ± 0.7	NE[b]
6	20.1 ± 0.3	4.3 ± 0.1	NE[b]
2	56.6 ± 1.7	45.9 ± 0.7	7.2 ± 0.1
3	26.5 ± 0.4	21.9 ± 0.4	12.5 ± 0.1
4	53.4 ± 0.6	39.4 ± 0.7	NE[b]
ampicillin	65.3 ± 0.7	58.9 ± 0.5	85.2 ± 0.4
streptomycin	52.5 ± 0.5	74.6 ± 0.4	89.3 ± 0.4

[a]Inhibition of the biofilm formation values were calculated as (mean A_{620} control well − mean A_{620} treated well)/(mean A_{620} control well) × 100. Values are expressed as the means ± SE. [b]NE = no effect.

■ EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Rudolph Research Analytical AUTOPOL IV automatic polarimeter. UV spectra were recorded using a GBC Cintra 40 UV/vis spectrometer. IR spectra were recorded on a Thermo Scientific Nicolet 6700 FT-IR spectrometer using the ATR technique. All NMR spectra were recorded on a Bruker Avance III 500 spectrometer at 500.26 for ^{1}H and 125.80 MHz for ^{13}C, with methanol-d_4 as solvent and tetramethylsilane as reference. HRESIMS data were obtained on an Agilent Technologies 6210 time-of-flight LC/MS system equipped with an ESI interface (ESITOFMS). The solvent was MeOH, and the mobile phase was 0.2% HCOOH−CH_3CN (1:1), at a 0.2 mL/min flow rate. The ESI was operated in the negative mode, and nitrogen was used as the drying gas (12 L/min) and nebulizing gas at 350 °C (45 psi). The OCT RF voltage was set to 250 V, and the capillary voltage was set to 4.0 kV. The voltages applied to the fragmentor and skimmer were 140 and 60 V, respectively. Scanning was performed from 100 to 1500 *m/z* (mass-to-charge ratio). Analytical TLC was carried out on silica gel 60 GF_{254} 20 × 20 cm plates with a layer thickness of 0.25 mm (Merck). For CC, Merck silica gel (Si gel) (particle size 0.063−0.200 mm), $CHCl_3$, MeOH, and water were used. Semipreparative HPLC was performed on an Agilent 1100 series instrument equipped with a diode array detector (DAD). The column used was a Zorbax Eclipse XDB-C18 column (i.d. 9.4 mm × 250 mm, particle size 5 μm). Monosaccharides in hydrolysates were determined on a Dionex ICS 3000 DP liquid chromatography system equipped with a quaternary gradient pump with an electrochemical detector (gold as working and Ag/AgCl as reference electrode). The anion-exchange column was a CarboPac PA100 pellicular column (i.d. 4 mm × 250 mm). Solvents for HPLC analysis were of chromatographic grade. Standards of D-glucose, D-galactose, D-glucuronic acid, and L-arabinose were purchased from Tokyo Chemical Industry, TCI (Belgium). All aqueous solutions were prepared using Ultrapure TKA deionized water.

Plant Material. The plant material was collected in July 2016 (during the full flowering phase of the plant) from a salt marsh situated in northern Serbia, near the town of Novi Bečej (45°38′02″ N, 20°12′38″ E). The plant voucher was authenticated by one of the authors (Z.D.-S.) and deposited at the Herbarium of the Department of Agricultural Botany, University of Belgrade, Republic of Serbia.

Extraction and Isolation. The air-dried aerial parts were powdered (105.0 g) and extracted with MeOH (3 × 500 mL, 1 h) at room temperature with the use of an ultrasonic bath. The extracts were combined, filtered, and concentrated under reduced pressure to give 18.9 g of residue. A portion (17.9 g) was suspended in H_2O (100 mL) and washed with CH_2Cl_2 (3 × 100 mL). The water layer was extracted with *n*-BuOH (3 × 100 mL). The butanol extract residue (1.94 g), obtained after evaporation, was dissolved in MeOH and fractionated by a combination of isocratic and gradient CC on silica gel with $CHCl_3$−MeOH−H_2O eluent. Thirty-seven fractions were obtained after TLC analysis. Fraction 35 (89.7 mg) was used for the isolation of compounds **1** and **5**, fraction 28 (66.3 mg) for compound **6**, fraction 23 (51.6 mg) for compounds **2** and **3**, and fraction 20 (93.5 mg) for compound **4**. These fractions were further subjected to semipreparative HPLC-DAD separation on an Eclipse XDB-C18 (250 × 9.4 mm, 5 μm) column to purify compounds. The gradient program 1 was used for flavonoids (**1**, **5**, and **6**): 0−20 min 5−16% B, 20−28 min 16−40% B, 28−30 min 40−99% B, 30−35 min 99% B, 35−36 99−5% B, and 36−41 min 5% B. Solvent A was 0.2% HCOOH in H_2O (v/v), while solvent B was MeCN. The flow rate was 4 mL/min, and detection wavelengths were 260 and 280 nm. Compound **1** was further purified using the following HPLC program 2: 0−25 min 10−20% B, 25−26 min 20−10% B, and 26−31 min 10%. The detection wavelength was 280 nm. For saponins (**2**, **3**, **4**), gradient program 3 was used: 0−15 min 5−50% B, 15−25 min 50−100% B, 25−27 min 100−5% B, and 27−32 min 5% B. The detection wavelength was 210 nm. Finally, 1.9 mg (**1**), 6.6 mg (**2**), 1.6 mg (**3**), and 2.9 mg (**4**) were obtained. The known compounds spatuletin 3-*O*-*β*-D-apiofuranosyl(1‴→2″)-*β*-D-glucopyranoside (**5**) and patuletin 3-*O*-5‴-*O*-feruloyl-*β*-D-apiofuranosyl(1‴→2″)-*β*-D-glucopyranoside (**6**) were obtained in quantities of 4.9 and 2.6 mg, respectively.

Acid Hydrolysis of Compounds. Compounds **1**−**6** (1.0 mg each) were hydrolyzed with 2 M HCl (1.0 mL) for 30 min at 100 °C. After cooling, each mixture was neutralized to pH 7 with solid $NaHCO_3$, filtered through an HPLC 0.45 μm filter, and analyzed on a Dionex ICS 3000 DP LC system with electrochemical detection. Standard solutions of D-glucose, D-galactose, D-glucuronic acid, and L-arabinose were prepared in ultrapure H_2O at concentrations of 20 μg/mL with the addition of 70 mg of NaCl (the same quantity of NaCl remained in the samples after neutralization). The specialized program used for analysis of monosaccharides was as follows: 0−5.0 min, isocratic elution with 15% A, 85% C; 5.0−5.1 min, gradient to 15% A, 2% B, 83% C; 5.1−12.0 min, isocratic elution with 15% A, 2% B, 83% C; 12.0−12.1 min, gradient to 15% A, 4% B, 81% C; 12.1−20.0 min, isocratic elution with 15% A, 4% B, 81% C; 20.0−20.1 min, gradient to 20% A, 20% B, 60% C; 20.1−30.0 min, isocratic elution with 20% A, 20% B, 60% C, where A is 600 mM NaOH, B is 600 mM NaOAc, and C is ultrapure H_2O. The flow rate was 0.7 mL/min.

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Before analysis, the system was preconditioned with 15% A, 85% C for 30 min.

Acid Hydrolysis of Crude Saponin and Flavonoid Mixture. The crude saponin and flavonoid mixture (300 mg), rich in compounds **1**, **2**, **3**, and **4**, was hydrolyzed with 1 M HCl (30 mL) for 3 h at 100 °C. After cooling, the mixture was extracted with EtOAc (3 × 30 mL). The aqueous layer was then neutralized with solid $NaHCO_3$ and freeze-dried. The identity of the monosaccharides was confirmed by comparison of TLC retention times to those of standard compounds, using MeCOEt−*i*-PrOH−Me_2CO−H_2O (20:10:7:6) as a mobile phase. Thereafter, preparative TLC using the same solvent system was performed, and optical rotations of the isolated sugars were measured.

The absolute configurations were determined after comparison of their optical rotations to those of commercial standard compounds: D-glucose {$[\alpha]^{21}_D$ +54 (*c* 0.2, H_2O); standard: +59 (*c* 0.2, H_2O)}, D-galactose{$[\alpha]^{20}_D$ +60 (c 0.2, H_2O); standard: +63 (c 0.2, H_2O)}, D-glucuronic acid {$[\alpha]^{20}_D$ +11 (c 0.2, H_2O); standard: +13 (c 0.2, H_2O)}, and L-arabinose {$[\alpha]^{20}_D$ +107 (*c* 0.2, H_2O); standard: +114 (*c* 0.2, H_2O)}.

Antibacterial Activity. For the evaluation of antibacterial activity, Gram-negative bacteria *Escherichia coli* (ATCC 35210) and *Pseudomonas aeruginosa* (ATCC 27853) and Gram-positive bacteria *Listeria monocytogenes* (NCTC 7973) and *Micrococcus flavus* (ATCC 10240) were used. These were obtained from the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stankovic", Belgrade, Serbia. The antibacterial assay was carried out by a microdilution method[29,30] using a previously described procedure.[31]

Bacterial Strains, Growth Media, and Culture Conditions. The *P. aeruginosa* PA01 used in this study is from the collection of the Mycoteca, Institute for Biological Research "Sinisa Stankovic", Belgrade, Serbia. Bacteria were routinely grown in Luria−Bertani (LB) medium (1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract) with shaking (220 rpm) and cultured at 37 °C.

Biofilm Formation. The effects of different concentrations of investigated compounds (0.5, 0.25, and 0.125 of MIC) on biofilm-forming ability were tested on polystyrene flat-bottomed microtiter 96-well plates as already described,[32,33] with some modifications. A volume of 100 μL of overnight culture of *P. aeruginosa* (inoculum size 1×10^8 CFU/mL) was added to each well of the plates in the presence of 100 μL of subinhibitory concentrations (sub-MIC) of compounds or 100 mL of medium (control). After incubation for 24 h at 37 °C, each well was washed twice with sterile phosphate-buffered saline (PBS) (pH 7.4), dried, and treated for 10 min with 0.1% crystal violet in order to determine the biofilm mass. The content of the wells was homogenized and the absorbance at 625 nm was read on a Sunrise Tecan ELISA reader. The experiment was performed in triplicate and repeated twice, and values are presented as the mean values with standard error.

Atriplexin IV (1): yellow powder; $[\alpha]^{22}_D$ −75 (*c* 2.0, MeOH); UV, (MeOH) λ_{max} (log ε) 254sh (4.23), 272 (4.16), 354 (4.28); (NaOMe), 272 (4.27), 388 (4.43); ($AlCl_3$), 277 (4.44), 328 (4.37), 400 (4.13); ($AlCl_3$ + HCl), 275 (4.45), 301sh (4.46), 331 (4.40), 372sh (4.32), 395sh (4.28); 411sh (4.13), (NaOAc), 274 (4.27), 329 (4.29), 382sh (4.04); (NaOAc + H_3BO_3), 267 (4.43), 333 (4.32), 380 (4.22) nm; IR ν_{max} 3417, 2958, 1700, 1651, 1601, 1516, 1471, 1372, 1297, 1271, 1200, 1182, 1068, 816, cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 845.1795 $[M - H]^-$ (calcd for $C_{38}H_{38}O_{22}$−H 845.1782).

Atriplexogenin I (2): amorphous powder; $[\alpha]^{22}_D$ 12 (*c* 1.2, MeOH); IR ν_{max} 3414, 2930, 1731, 1648, 1456, 1387, 1371, 1258, 1226, 1074, 1031, 996, 817 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 1005.4381 $[M - H]^-$ (calcd for $C_{47}H_{74}O_{21}S$−H 1005.4371).

Atriplexogenin II (3): amorphous powder; $[\alpha]^{22}_D$ 6 (*c* 1.0, MeOH); IR ν_{max} 3360, 2926, 1723, 1629, 1598, 1515, 1454, 1373, 1256, 1172, 1071, 1030, 996, 817 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 1007.4552 $[M - H]^-$ (calcd for $C_{47}H_{76}O_{21}S$−H 1007.4527).

Atriplexogenin III (4): amorphous powder; $[\alpha]^{22}_D$ 19 (*c* 1.8, MeOH): IR ν_{max} 3337, 2924, 1732, 1588, 1455, 1360, 1258, 1172, 1075, 1028, 613 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 1087.5321 $[M - H]^-$ (calcd for $C_{53}H_{84}O_{23}$−H 1087.5331).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.8b00970.

NMR spectra of the isolated compounds (PDF)

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Notes

The authors declare no competing financial interest.

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