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Saponins from *Allium minutiflorum* with antifungal activity

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Abstract

Three saponins, named minutoside A (**1**), minutoside B (**2**), minutoside C (**3**), and two known sapogenins, alliogenin and neoagigenin, were isolated from the bulbs of *Allium minutiflorum* Regel. Elucidation of their structure was carried out by comprehensive spectroscopic analyses, including 2D NMR spectroscopy and mass spectrometry. The structures of the new compounds were identified as (25R)-furost-2α,3β,6β,22α,26-pentaol 3-*O*-[β-D-xylopyranosyl-(1 → 3)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl] 26-*O*-β-D-glucopyranoside (**1**), (25S)-spirostan-2α,3β,6β-triol 3-*O*-β-D-xylopyranosyl-(1 → 3)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-galactopyranoside (**2**), and (25R)-furost-2α,3β,5α,6β,22α,26-esaol 3-*O*-[β-D-xylopyranosyl-(1 → 3)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl] 26-*O*-β-D-glucopyranoside (**3**). The isolated compounds were evaluated for their antimicrobial activity. All the novel saponins showed a significant antifungal activity depending on their concentration and with the following rank: minutoside B > minutoside C ≫ minutoside A. No appreciable antibacterial activity was recorded. The possible role of these saponins in plant–microbe interactions is discussed.

Keywords: *Allium*; Saponins; Furostanol-type; Spirostanol-type; Minutosides; Antifungal activity; Structure–activity relationships

1. Introduction

Saponins are a major family of secondary metabolites that occur in a wide range of plant species (Osbourn, 1996). These compounds, called phytoanticipins (Osbourn, 1999), are present constitutively in plants and seem to be involved in plant disease resistance because of their well-known antimicrobial activity (Papadopoulou et al., 1999; Bouarab et al., 2002; Wittstock and Gershenzon, 2002).

In our work on the discovery of bioactive saponins from *Allium* species (Lanzotti, 2005), we examined *Allium minutiflorum* Regel. This species is an Iranian bulbous perennial plant known as wild onion and used for food preparation. We isolated and determined the structure of three new furostanol and spirostanol saponins, named minutosides A–C (**1**–**3**), along with the known sapogenins, alliogenin and neoagigenin. The stereostructure of these compounds was elucidated by extensive NMR techniques and chemical methods. Moreover, we evaluated the possible involvement of these saponins in resistance of *A. minutiflorum* to pathogens.

2. Results and discussion

Bulbs of *A. minutiflorum* were air dried under controlled temperature (22–25 °C) and exhaustively extracted with hexane, $CHCl_3$, $CHCl_3$–MeOH (9:1) and MeOH. Both $CHCl_3$–MeOH (9:1) and MeOH extracts were separated by MPLC and HPLC techniques, affording the new compounds minutoside A (76.5 mg kg^{-1}), B (83.5 mg kg^{-1}), and C (9.3 mg kg^{-1}), together with the sapogenins neoagigenin (99.0 mg kg^{-1}) and alliogenin (2.1 mg kg^{-1}).

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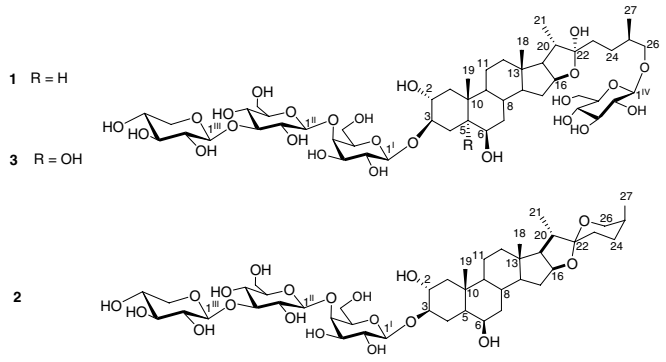

Chart 1. Chemical structures of minutoside A (**1**), minutoside B (**2**), and minutoside C (**3**).

Table 1
^{1}H NMR data of the aglycone portions of minutosides A (**1**), B (**2**), and C (**3**) (500 MHz, CD_3OD)

Position	**1**	**2**	**3**
	δ_H (int., mult., *J* in Hz)	δ_H (int., mult., *J* in Hz)	δ_H (int., mult., *J* in Hz)
1a	2.18[a]	2.04[a]	2.28 (1H, *dd*, 11.5, 7.5)
b	1.18[a]	0.93[a]	1.48 (1H, *dd*, 11.5, 4.0)
2	3.50[a]	3.53[a]	3.55
3	3.72[a]	3.37[a]	3.73[a]
4a	1.75[a]	1.65[a]	1.53[a]
b	1.43[a]	1.83[a]	1.37[a]
5	1.70[a]	1.22[a]	
6	4.05 (1H, *bs*)	4.03 (1H, *bs*)	4.05 (1H, *bs*)
7a	1.85[a]	1.19[a]	1.76[a]
b	1.82[a]	1.75[a]	1.62[a]
8	1.98 (1H, *m*)	2.00 (1H, *m*)	1.27 (1H, *m*)
9	0.81[a]	0.79[a]	1.45[a]
11a	1.61 (1H, *dd*, 10.5, 2.5)	1.55[a]	1.37 (1H, *dd*, 10.5, 2.5)
b	1.47 (1H, *m*)	1.43[a]	1.30 (1H, *m*)
12a	1.71[a]	1.78[a]	1.75[a]
b	1.19[a]	1.21[a]	1.18[a]
14	1.15 (1H, *m*)	1.19[a]	1.22 (1H, *m*)
15a	1.98[a]	2.00[a]	1.97[a]
b	1.26[a]	1.30[a]	1.22[a]
16	4.55 (1H, *q*, 5.5)	4.41(*td*, 6.3, 6.3, 7.8)	4.61 (1H, *q*, 5.5)
17	1.75[a]	1.75[a]	1.74[a]
18	0.87 (3H, *s*)	0.82 (3H, *s*)	0.80 (3H, *s*)
19	1.09 (3H, *s*)	1.08 (3H, *s*)	1.00 (3H, *s*)
20	1.90[a]	1.89[a]	2.08 (1H, *dq*, 6.5, 5.5)
21	1.02 (3H, *d*, 6.6)	1.00 (*d*, 7.0)	0.81 (3H, *d*, 6.6)
23a	1.70[a]	1.74[a]	1.70[a]
b	1.30[a]	1.59[a]	1.68[a]
24a	1.66[a]	1.63[a]	1.63[a]
b	1.28[a]	1.83v	1.22[a]
25	1.59 (1H, *m*)	1.62[a]	1.73 (1H, *m*)
26a	3.88 (1H, *dd*, 9.5, 3.9)	3.94 (*dd*, 11.3, 3.0)	3.86 (1H, *dd*, 9.5, 3.9)
b	3.30[a]	3.28[a]	3.20[a]
27	0.98 (3H, *d*, 6.6)	1.11 (*d*, 6.3)	0.96 (3H, *d*, 6.6)

[a] Overlapped with other signals.

Minutoside A (**1**, Chart 1), isolated as an amorphous solid in relatively high yield, showed a molecular formula of $C_{50}H_{84}O_{25}$, deduced by high-resolution FAB MS measurements, and confirmed by ^{13}C NMR data (Tables 2 and 3). Preliminary ^{1}H NMR analysis of **1** (CD_3OD, Tables 1 and 3) indicated a glycoterpene nature of the compound. In fact, the ^{1}H NMR spectrum showed two tertiary methyls (δ0.87 and 1.09), two secondary methyls (δ0.98 and 1.02), and four anomeric (δ4.25, 4.40, 4.63 and 4.95) protons. The ^{13}C NMR spectrum (Tables 2 and 3) showed, in addition, a diagnostic signal at δ 113.9, indicating the presence of a hemiacetal carbon and suggesting a furostane skeleton for the aglycone of **1**.

Combined analysis of 2D COSY and HOHAHA spectra of **1** allowed to detect six spin systems, two belonging to aglycone moiety, and the remaining four to four sugar residues. Consequently, each proton has been related to the directly bonded carbon through a HSQC spectrum.

Concerning the aglycone, the first spin system started from ring A and extended up to ring E protons, while the second spin system included the side chain protons (C-23–C-27). The HMBC cross peaks, reported in Fig. 1, allowed to connect the two spin systems through the quaternary C-22 and to build up the structure of the aglycone moiety as furostan-2,3,6,22,26-pentaol. The diaxial coupling between H-3 and H-5, detected in a ROESY spectrum (Fig. 1), indicated a *cis* orientation among them and a β-configuration of the hydroxyl at C-3. The α-orientation

Table 2
^{13}C NMR data of the aglycone portion of **1**, **2**, and **3** (125 MHz, CD_3OD)

Position	**1**	**2**	**3**
	δ_C (mult.)	δ_C (mult.)	δ_C (mult.)
1	47.1 (CH_2)	48.0 (CH_2)	45.9 (CH_2)
2	75.2 (CH)	73.0 (CH)	71.5 (CH)
3	84.8 (CH_2)	85.0 (CH_2)	83.0 (CH_2)
4	32.8 (CH_2)	32.7 (CH_2)	35.8 (CH_2)
5	48.0 (CH_2)	48.2 (CH_2)	74.9 (CH_2)
6	71.5 (CH_2)	71.6 (CH_2)	71.6 (CH_2)
7	40.7 (CH_2)	40.7 (CH_2)	38.4 (CH_2)
8	30.7 (CH)	30.7 (CH)	32.6 (CH)
9	55.5 (CH)	55.6 (CH)	45.9 (CH)
10	37.7 (C)	37.8 (C)	38.0 (C)
11	22.1 (CH_2)	22.1 (CH_2)	22.3 (CH_2)
12	41.0 (CH_2)	41.0 (CH_2)	41.1 (CH_2)
13	41.4 (C)	41.8 (C)	41.8 (C)
14	57.1 (CH)	57.1 (CH)	57.1 (CH)
15	31.4 (CH_2)	32.5 (CH_2)	31.4 (CH_2)
16	82.4 (CH)	82.3 (CH)	82.2 (CH)
17	63.0 (CH)	63.6 (CH)	63.8 (CH)
18	17.0 (CH_3)	17.1 (CH_3)	17.0 (CH_3)
19	17.3 (CH_3)	17.5 (CH_3)	17.1 (CH_3)
20	42.2 (CH)	43.5 (CH)	42.9 (CH)
21	16.3 (CH_3)	14.9 (CH_3)	16.3 (CH_3)
22	113.9 (C)	111.1 (C)	110.5 (C)
23	34.8 (CH_2)	26.9 (CH_2)	34.6 (CH_2)
24	29.0 (CH_2)	26.8 (CH_2)	29.8 (CH_2)
25	37.8 (CH)	31.5 (CH)	37.4 (CH)
26	75.5 (CH_2)	66.1 (CH_2)	75.3 (CH_2)
27	17.5 (CH_3)	17.0 (CH_3)	17.5 (CH_3)

Table 3
^{1}H and ^{13}C NMR data of the Sugar Portion of **1** and **2** (data extracted from **1**), and **3** (500 MHz and 125 MHz, CD_3OD)

Position	**1**		**3**	
	δ_H (int., mult., *J* in Hz)	δ_C (mult.)	δ_H (int., mult., *J* in Hz)	δ_C (mult.)
GAL I				
1^{I}	4.40 (1H, *d*, 7.4)	102.8 (CH)	4.39 (1H, *d*, 7.4)	102.8 (CH)
2^{I}	3.68 (1H, *dd*, 7.4, 8.1)	75.8 (CH)	3.69 (1H, *dd*, 7.4, 8.1)	75.8 (CH)
3^{I}	3.53[a]	80.0 (CH)	3.53[a]	78.3 (CH)
4^{I}	4.04 (1H, *bd*, 2.2)	81.1 (CH)	4.05 (1H, *bd*, 2.2)	79.9 (CH)
5^{I}	3.90[a]	67.2 (CH)	3.90[a]	67.2 (CH)
6^{I}a	3.59[a]	62.9 (CH_2)	3.59[a]	62.9 (CH_2)
6^{I} b	3.67[a]		3.67[a]	
GLC II				
1^{II}	4.63 (1H, *d*, 7.3)	104.4 (CH)	4.62 (1H, *d*, 7.3)	104.5 (CH)
2^{II}	3.78 (1H, *dd*, 7.3, 8.1)	78.1 (CH)	3.78 (1H, *dd*, 7.3, 8.1)	78.1 (CH)
3^{II}	3.75 (1H, *dd*, 7.3, 8.1)	87.8 (CH)	3.72 (1H, *dd*, 7.3, 8.1)	87.8 (CH)
4^{II}	3.32[a]	75.3 (CH)	3.32[a]	75.3 (CH)
5^{II}	3.68[a]	70.3 (CH)	3.67[a]	70.4 (CH)
6^{II}a	3.80[a]	63.0 (CH_2)	3.80[a]	63.1 (CH_2)
6^{II} b	3.55[a]		3.55[a]	
XYL III				
1^{III}	4.95 (1H, *d*, 7.4)	104.3 (CH)	4.94 (1H, *d*, 7.4)	104.3 (CH)
2^{III}	3.28[a]	75.7 (CH)	3.28[a]	75.7 (CH)
3^{III}	3.55[a]	78.3 (CH)	3.55[a]	78.3 (CH)
4^{III}	3.61 (1H, *ddd*, 2.1, 7.8, 8.8)	71.3 (CH)	3.61 (1H, *ddd*, 2.1, 7.8, 8.8)	71.8 (CH)
5^{III}a	3.30[a]	71.0 (CH_2)	3.30[a]	71.0 (CH_2)
5^{III}b	3.92[a]		3.92[a]	
GLC IV				
1^{IV}	4.25 (1H, *d*, 7.5)	104.9 (CH)		
2^{IV}	3.40 (1H, *t*, 7.5)	78.1 (CH)		
3^{IV}	3.30[a]	77.5 (CH)		
4^{IV}	3.61 (1H, *dd*, 6.8, 6.5)	77.9 (CH)		
5^{IV}	3.40 (1H, *m*)	71.7 (CH)		
6^{IV}a	3.68 (1H, *bd*, 11.5)	62.8 (CH_2)		
6^{IV}b	3.84 (1H, *bd*, 11.5)			

[a] Overlapped with other signals.

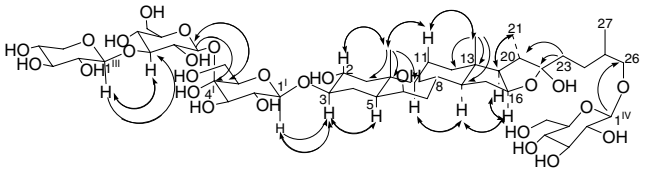

Fig. 1. Selected HMBC (H → C) and ROESY (H ↔ H) correlations exhibited by compound **1**.

of 2-OH has been determined by the ROESY cross-peak of H-2 with H_3-19, while a β-orientation of 6-OH has been defined by the small coupling constants observed for H-6 (δ4.05, *bs*) indicative of its equatorial nature. The 25R stereochemistry of the side chain was deduced by the resonances of protons and carbons at C-25, C-26 and C-27, and by the vicinal couplings between H-25, and the two H-26, in comparison with the literature data (Dong et al., 2001). The following ROESY correlations (Fig. 1), H-11/H_3-19, H-11/H_3-18, H-9/H-14, H-14/H-16, H-16/H-17, and H-17/H_3-21 completed the relative stereochemistry of **1** with the usual B/C *trans*, C/D *trans*, D/E *cis*, and C-20α stereochemistry (Dong et al., 2001; Fattorusso et al., 2002). On the basis of those data, the stereostructure of the aglycone has been determined as depicted in formula. Finally, the stereochemistry at C-22 has been assigned as α by accurate analysis of NMR data, and comparison with other compounds previously described (Fattorusso et al., 2002; Corea et al., 2003). However, as observed for other furostanol saponins (Fattorusso et al., 2002; Corea et al., 2003), compound **1**, left in aqueous solution overnight at room temperature, gave rise to the equilibrated mixture of the two hemiacetals at C-22 (22α-OH and 22β-OH, 40% and 60%, respectively).

Concerning the saccharide portion, the analysis started with the association of the four anomeric protons (δ 4.25, 4.40, 4.63 and 4.95) with the relevant anomeric carbon signals (δ 104.9, 102.8, 104.5 and 104.3, respectively), through the HSQC experiment. The nature of the single monosaccharides and their sequence has been determined by combined analysis of 2D COSY, HOHAHA, HSQC, and HMBC experiments. Starting from the anomeric proton of each sugar unit, all the proton signals within each spin

system were recognized by COSY and HOHAHA spectra, and then connected to the relevant carbon by HSQC spectrum. Then, HMBC and ROESY spectra gave key information on the glycosidic linkages. The data obtained indicated that all sugars were in the pyranose form, three being hexoses and one pentose. Combined analysis of the coupling constants of each spin system, taken by ^{1}H NMR spectrum or by 1H subspectrum of 2D HOHAHA, together with informations on the spatial proximity of protons obtained by 2D ROESY (e.g. diaxial couplings between H-1^{I}–H-3^{I} and H-3^{I}–H-5^{I}) allowed the identification of two glucoses, one galactose, and one xylose. All sugars were determined as β-anomers on the basis of the large $J_{H\text{-}1\text{–}H\text{-}2}$ coupling constants. Furthermore, ROESY (H-4^{I}/H-1^{II}, H-3^{II}/H-1^{III}) and HMBC (C-4^{I}/H-1^{II}, C-3^{II}/H-1^{III}) correlations permitted the sequence deduction of a trisaccharide chain, that has been placed at C-3 of the aglycone on the basis of ROESY (H-3/H-1^{I}) and HMBC (C-3/H-1^{I}) cross peaks. Finally, the remaining β-glucose has been located at C-26 by considering the HMBC C-26/H-1^{IV} correlation.

To confirm the nature of the sugar units and to determine their absolute configuration, **1** has been subjected to acid hydrolysis (1 N HCl), followed by trimethylsilylation and GC analysis on a chiral column in comparison with both series of glucose, galactose, and xylose. By this procedure, the sugars were identified to belong to the commonly found D-series. This procedure has been applied to all new isolated compounds. All these data indicated the structure of **1** as (25R)-furost-2α,3β,6β,22α,26-pentaol 3-*O*-[β-D-xylopyranosyl-(1 → 3)-O-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl] 26-*O*-β-D-glucopyranoside.

Minutoside B (**2**, Chart 1), isolated as an amorphous solid, constituted the major compound of the saponin fraction. It showed a molecular formula of $C_{44}H_{72}O_{19}$, deduced by high-resolution FAB MS measurements, and confirmed by ^{13}C NMR data (Tables 2 and 3) which differed from **1** in the absence of a hexose moiety. Analysis of 2D NMR spectra of **2** indicated the same trisaccharide chain as **1**, composed by β-Gal, β-Glc, and β-Xyl. Differences between **1** and **2** were found in the aglycone portion, that in **2** has been easily determined as neoagigenin by NMR analysis and comparison with literature data (Kel'ginbaev et al., 1974).

Finally, interglycosidic linkages have been determined by key correlations in the HMBC spectrum which allowed the elucidation of the chemical structure of compound **2** as (25S)-spirostan-2α,3β,6β-triol 3-*O*-β-D-xylopyranosyl-(1 → 3)-O-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-galactopyranoside.

Minutoside C (**3**), $C_{50}H_{84}O_{26}$ by high-resolution FAB MS, was isolated in low yield. It was disclosed to be the 5-hydroxy analogue **1**. This has been first suggested by MS data, being the *m*/*z* value of minutoside A 16 amu less when compared to **3**. Indeed, inspection of 1D and 2D NMR data revealed that these two molecules differed uniquely for the chemical shifts of C/H atoms around C-5. In particular, this last carbon revealed to be an oxygenated quaternary atom (δ74.9) and, in addition, the α-orientation of OH-5 has been defined by the downfield shift observed for H1-α as compared to corresponding resonance of **1** (δ 2.28 in **3** vs δ 2.18 in **1**). These evidences defined the structure of **3** as (25R)-furost-2α,3β,5α,6β,22α, 26-esaol 3-*O*-[β-D-xylopyranosyl-(1 → 3)-O-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl]-26-*O*-β-D-glucopyranoside.

Minutoside C possesses as structural feature an OH-5α that is rare among furostanol saponins. Its aglycone moiety has been recently found in two saponins isolated from *A. elburzense*, elburzensosides A and B (Barile et al., 2004), differing from minutoside C in the saccharide chain linked at C-3.

Along with these compounds we isolated two sapogenins, alliogenin (Khristulas et al., 1970) and the rare neoagigenin (Kel'ginbaev et al., 1974).

The five isolated compounds were tested for their antimicrobial activity against a number of fungal and bacterial microorganisms. All saponins showed a significant antifungal activity depending on their concentration and, generally, the following rank was observed: minutoside B > minutoside C > neoagigenin > alliogenin ≫ minutoside A (Table 4). These results indicate that antifungal activity of minutosides B and C in some cases (i.e. *Trichoderma* sp.) may be comparable to that of common natural antibiotics and synthetic fungicides (Lorito et al., 1996; Schoonbeek et al., 2001). Several permanent alterations in the tested fungi, such as hyphal swelling and changes in the rate of sporulation were evident (data not shown). Minutoside B, that is one of the most abundant in the plant tissue (83.5 mg kg^{-1}), displayed the highest antifungal activity towards all fungi.

On the basis of our results, some structure–activity relationships within this class of antifungal agents were established. In particular, comparison of the chemical structure of the spirostanol saponin minutoside B with the corresponding furostanol saponin minutoside A, indicated the importance of a spirostanol-type aglycone for the antifungal activity. This is confirmed by the observation that neoagigenin, the sapogenin of minutoside B, also showed a considerable activity. A positive effect of a hydroxyl group at C-5 was evident by comparing the activities of minutosides A and C, which differ from each other by the presence of the 5-OH group on C. This structural feature is, thus, responsible of the higher activity showed by the furostanol saponin minutoside C. This observation is in contrast with a previous study reporting the absence of any antifungal activity for the furostanol saponins from *Tribulus terrestris* (Zhang et al., 2006).

The two strains of the antagonistic fungus *Trichoderma harzianum* were much more sensitive than the fungal pathogens to minutosides B and C, and neoagigenin (Table 4). This result is consistent with previous findings which report a high sensitivity of *Trichoderma* spp. to saponins from *Medicago sativa* and *Panax quinquefolius* (Zimmer et al.,

Table 4
Antifungal activity of saponins isolated from *Allium minutiflorum* at three concentrations (1000, 100 and 10 ppm)

Fungal species	Minutoside A			Minutoside B			Minutoside C			Alliogenin			Neoagigenin		
	1000	100	10	1000	100	10	1000	100	10	1000	100	10	1000	100	10
Alternaria alternata	52 ± 3.9a	98 ± 7.8b	97 ± 8.2b	0a	62 ± 10.2b	89 ± 13.4b	0a	74 ± 5.2b	115 ± 6.9c	90 ± 6.5a	86 ± 7.7a	90 ± 7.7a	90 ± 9.5a	100 ± 68a	95 ± 7.8a
Alternaria porri	95 ± 6.3a	90 ± 8.9b	94 ± 6.3a	0a	77 ± 5.0b	85 ± 5.1b	0a	63 ± 5.5b	98 ± 4.8c	77 ± 6.5a	83 ± 6.5a	89 ± 6.4a	90 ± 9.0a	93 ± 5.5a	93 ± 5.2a
Botrytis cinerea	31 ± 5.5a	40 ± 6.8a	55 ± 9.3a	0c	21 ± 2.8b	42 ± 10.6c	27 ± 4.0a	98 ± 4.8c	73 ± 8.7c	30 ± 3.6a	52 ± 7.0a	59 ± 15.8a	31 ± 7.3a	31 ± 9.0a	66 ± 12.1a
Fusarium oxysporum	65 ± 7.6a	78 ± 10.2a	82 ± 6.9a	0a	71 ± 6.4 b	68 ± 5.3b	42 ± 4.7a	79 ± 8.6b	87 ± 9.2b	87 ± 8.5a	90 ± 7.3a	87 ± 8.6 a	0a	56 ± 6.5b	73 ± 7.6c
F. oxysporum f. sp. *lycopersici*	86 ± 8.5a	82 ± 10.9a	98 ± 22.3a	0a	73 ± 7.0 b	76 ± 6.2c	0a	62 ± 6.1b	84 ± 9.0c	62 ± 8.0a	80 ± 10.0a	85 ± 11.3 a	10 ± 2.8a	81 ± 6.4b	97 ± 9.3c
F. solani	68 ± 5.0a	74 ± 9.7a	78 ± 6.8a	0a	12 ± 2.8b	51 ± 4.9c	18 ± 2.4a	44 ± 5.6b	48 ± 5.8b	19 ± 3.2a	64 ± 9.9b	65 ± 6.3 b	0a	28 ± 6.6a	26 ± 4.8a
Pythium ultimum	72 ± 0.2a	81 ± 0.2b	100 ± 0.0c	34 ± 0.1a	89 ± 0.3b	98 ± 0.1b	43 ± 0.5a	100 ± 0.0b	100 ± 0.0b	19 ± 3.2a	100 ± 0.0a	100 ± 0.0a	90 ± 0.3a	100 ± 0.0a	100 ± 0.0a
Rhizoctonia solani	73 ± 0.0a	75 ± 0.0a	77 ± 0.0a	25 ± 0.0a	49 ± 0.0b	70 ± 0.0c	27 ± 0.0a	54 ± 0.0b	106 ± 0.1c	0a	60 ± 0.0b	70 ± 0.0c	0a	43 ± 0.1b	54 ± 0.1b
Trichoderma harzianum P1	58 ± 3.6a	104 ± 5.9b	104 ± 7.3b	0a	0a	0a	0a	0a	0a	0a	58 ± 5.4b	103 ± 8.3c	0a	0a	24 ± 1.2b
T. harzianum T39	83 ± 1.1a	96 ± 1.8a	94 ± 1.1a	0a	0a	55 ± 0.0b	0a	77 ± 1.4b	100 ± 2.7c	92 ± 2.0a	94 ± 2.0a	96 ± 1.8a	0a	0a	0a

Data indicate fungal growth in percentage compared to control (=100). Values are average of five replicates ± standard error. Different letters indicate significant differences (Duncan test; $P < 0.05$).

1967; Nicol et al., 2002). The Oomycete *Pythium ultimum*, instead, was the less sensitive to all saponins (Table 4). One of the mechanism by which saponins display an antimicrobial activity is based on their ability to form complexes with sterols present in the membrane of microorganisms. This causes damages in the membrane and the consequent collapse of cells (Morrissey and Osbourn, 1999). Tolerance of *P. ultimum* could be related with the lack of sterols in the membrane of oomycetes. In addition, all five saponins did not show any appreciable antimicrobial activity towards the selected bacteria (data not shown). Sensitivity of bacteria to saponins has often been reported (Cioaca et al., 1978; Mandala et al., 2005; Avato et al., 2006), although comparative studies between fungi and bacteria showed that bacteria are less sensitive in general (Wang et al., 2000; Mandala et al., 2005; Avato et al., 2006). The mechanisms underlying saponins antibacterial activities are still unclear.

The potent antifungal activity displayed by *A. minutiflorum* saponins, especially minutoside B, suggests that these preformed compounds, alone or in combination, may act as chemical barriers to fungal attacks. This hypothesis is also supported by the high content in the bulbs of both minutoside B and neoagigenin (Table 4). However, many fungi may attack plants by producing enzymes that degrade saponins into non-toxic molecules (Sandrock and Van Etten, 1998; Morrissey and Osbourn, 1999). For example, the fungus *Gaeumannomyces graminis* var. *avenae* is able to infect oat plants because it secretes avenacinase, an extracellular enzyme that detoxifies avenacins, the oat saponins (Bowyer et al., 1995). A resistance mechanism has also been found in the fungus *Armillaria mellea*, a plant pathogen that is able to degrade the antifungal isoflavone genistein into non-toxic metabolites (Curir et al., 2006). From this point of view, the high sensitivity of the two *T. harzianum* strains to *A. minutiflorum* saponins could depend on the low or null ability of this fungus to detoxify these compounds. It is known that *Trichoderma*, though it infects roots, is not pathogenic to plants because it limits infection to the superficial cell layers (Harman et al., 2004). Saponins and/or other plant antimicrobial compounds could be involved in limiting *Trichoderma* from further invading the plant tissue. More studies are needed to prove this hypothesis.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a Perkin–Elmer 192 polarimeter equipped with a sodium lamp (589 nm) and 10-cm microcell. High-resolution ESIMS experiments were performed on an Applied Biosystem API 2000 triple–quadrupole mass spectrometer. The spectra were recorded by infusion into the ESI source using MeOH as solvent. GC–MS analysis was performed on a Carlo Erba instru-

ment. FTIR spectra were run on a Perkin–Elmer 1600 spectrometer in KBr. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Unity Inova spectrometer at 500.13 and 125.77 MHz, respectively. Chemical shifts were referred to the residual solvent signal (CD_3OD: δ_H 3.31, δ_C 49.0). The multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. ^{1}H connectivities were determined by using COSY and HOHAHA experiments; the 2D HOHAHA experiments were performed in the phase-sensitive mode (TPPI) using the MLEV-17 (mixing time 125 ms) sequence for mixing. One-bond heteronuclear ^{1}H–^{13}C connectivities were determined with 2D HSQC pulse sequence with an interpulse delay set for $^{1}J_{CH}$ of 130 Hz. Two and three bond heteronuclear ^{1}H–^{13}C connectivities were determined with 2D HMBC experiments, optimised for $^{2-3}J_{CH}$ of 8 Hz. Nuclear Overhauser effect (NOE) measurements were performed by 2D ROESY experiments. Medium pressure liquid chromatography (MPLC) was performed on a Büchi 861 apparatus using LiChroprep RP-18 (40–63μm) columns. Prep. TLC on SiO_2 with BuOH:H_2O:CH_3COOH 60:25:15 (BAW) for development was used. Spots were visualized with cerium sulphate in 2 N H_2SO_4. HPLC in isocratic mode was performed on a Varian apparatus equipped with a RI-3 refractive index detector [semipreparative μ-Bondapack C_{18} column (7.8 mm × 300 mm, i.d.)].

3.2. Plant material

Wild samples of *A. minutiflorum* were collected in the Ardestan (2200 m), Isfhan provinces, Iran, in June 2003 and identified by Prof. S. Zarre, Department of Biology, University of Tehran. A voucher specimen (No. 1146) has been deposited at the Department of Pharmacognosy, Isfahan University of Medical Sciences.

3.3. Extraction and isolation

The bulbs were air-dried immediately after collection, under controlled temperature (22 °C) and without exposure of light, giving a dry weight of 1.8 kg. They were finely hand-cut and then exhaustively extracted, at room temperature, with the following solvents in this order: *n*-hexane, $CHCl_3$, $CHCl_3$–MeOH (9:1), and MeOH. Each solvent extraction stage was conducted for 1 day and was repeated four times using 3 l of solvent, under stirring.

The $CHCl_3$–MeOH (9:1) extract of bulbs was concentrated under vacuum to afford a crude organic extract (15.0 g), which was chromatographed by MPLC on a RP-18 column using a linear gradient solvent system from H_2O to MeOH. Preliminary NMR analyses of the eluates let us to select two interesting fractions eluted with H_2O–MeOH (1:9) and MeOH 100%. The first one was chromatographed by HPLC on a semipreparative C_{18} column with the mobile phase H_2O–MeOH (3:7) to give the new compounds **2** (150.4 mg, $t_R = 19.0$ min) and **3** (16.7 mg, $t_R = 14.0$ min) along with the known sapogenin alliogenin (Khristulas et al., 1970) in a pure form (3.7 mg). The second one, instead, was purified by HPLC using H_2O–MeOH (2:8) as eluent, affording the known sapogenin neoagigenin (Kel'ginbaev et al., 1974) in a pure form (178.2 mg).

The bulb MeOH extract (50.0 g) was partitioned between *n*-BuOH and H_2O in order to remove sugar compounds. The organic layer was filtered and then concentrated under vacuum giving a crude extract (35.6 g), which was chromatographed by MPLC on a RP-18 column using a linear gradient solvent system from H_2O to MeOH. Preliminary NMR studies of the eluates revealed that only one fraction contained saponin compounds. This fraction, eluted with H_2O–MeOH (35:65) was further purified by HPLC on a semipreparative C_{18} column with the mobile phase H_2O–MeOH (45:55), giving the pure new compound **1** (137.7 mg, $t_R = 30.0$ min).

3.4. Minutoside A

(25R)-Furost-2α,3β,6β,22α,26-pentaol 3-*O*-[β-D-xylopyranosyl-(1 → 3)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl] 26-*O*-β-D-glucopyranoside (**1**); Yield: 137.7 mg; colorless amorphous solid; $[\alpha]_D^{25}$ − 30.7° ($c = 0.1$ MeOH); IR (KBr) ν_{max} 3410, 2930, 1150, 1045 cm^{-1}; ^{1}H NMR data, see Tables 1 and 3; ^{13}C NMR data, see Table 2. HRFABMS (negative ion): found *m/z* 1084.5301 $[M-H]^-$; calculated for $C_{50}H_{84}O_{25}$ *m/z* 1084.5325.

3.5. Minutoside B

(25S)-Spirostan-2α,3β,6β-triol 3-*O*-β-D-xylopyranosyl-(1 → 3)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-galactopyranoside (**2**); Yield: 150.4 mg; colorless amorphous solid; $[\alpha]_D^{25}$ − 35.2° ($c = 0.1$ MeOH); IR (KBr) ν_{max} 3413, 2932, 1150, 1043 cm^{-1}; ^{1}H NMR data, see Tables 1 and 3; ^{13}C NMR data, see Table 2. HRFABMS (negative ion): found *m/z* 904.4728 $[M-H]^-$; calculated for $C_{44}H_{72}O_{19}$ *m/z* 904.4747.

3.6. Minutoside C

(25R)-Furost-2α,3β,5α,6β,22α,26-esaol 3-*O*-[β-D-xylopyranosyl-(1 → 3)-*O*-β-D-glucopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl] 26-*O*-β-D-glucopyranoside (**3**); Yield: 16.7 mg, colorless amorphous solid; $[\alpha]_D^{25}$ − 41.7° ($c = 0.1$ MeOH); IR (KBr) ν_{max} 3400, 2934, 1159, 1048 cm^{-1}; ^{1}H NMR data, see Tables 1 and 3; ^{13}C NMR data, see Table 2. HRFABMS (negative ion): found *m/z* 1100.5313 $[M-H]^-$; calculated for $C_{50}H_{84}O_{26}$ *m/z* 1100.5332.

3.7. Determination of sugar absolute configurations

A solution of each isolated compound (1 mg) in 1 N HCl (0.25 ml) was stirred at 80 °C for 4 h. While cooling, the solution was concentrated under a stream of N_2. The residue was dissolved in 1-(trimethylsilyl)imidazole

(Trisil-Z) and pyridine (0.1 ml) and the solution was stirred at 60 °C for 5 min. The solution was dried with a stream of N_2, and the residue was separated by water and CH_2Cl_2 (1 mL, 1:1). The CH_2Cl_2 layer was analyzed by GC (Alltech L-Chirasil-Val column, 0.32 mm × 25 m; temperatures for injector and detector, 200 °C; temperature gradient system for the oven, 100 °C for 1 min and then raised to 180 °C; rate 5 °C/min). Peaks of the hydrolysate of **1**, **2**, and **3** were detected at 10.98, 13.98, and 14.66 min in the ratio of 1:1:2 for **1** and **3** and in the ratio of 1:1:1 for **2**. Retention times for authentic samples after being treated simultaneously with Trisil-Z were 10.98 (D-xylose) and 11.05 (L-xylose), 13.98 (D-galactose) and 13.75 (L-galactose), 14.66 (D-glucose) and 14.73 min (L-glucose). Co-injection of each hydrolysate with standard D-xylose, D-galactose, and D-glucose gave single peaks.

3.8. Biological assays

Antifungal activity of the saponins was tested on soil-borne pathogens (*Fusarium oxysporum*, *F. oxysporum* f. sp. *lycopersici*, *F. solani*, *P. ultimum* and *Rhizoctonia solani*), air-borne pathogens (*Botrytis cinerea*, *Alternaria alternata* and *A. porri*) and the biocontrol fungus *T. harzianum* (strains P1 and T39). Antibacterial activity was assessed against soil-borne pathogens (*Xanhomonas campestris* pv. *campestris*, *Agrobacterium tumefaciens* and *Streptomyces turgidiscabies*), foliar pathogens (*Pseudomonas syringae* pv. *syringae*, *Clavibacter michiganensis* pv. *michiganensis*) and biocontrol agents (*Bacillus mycoides* and *P. fluorescens*). Among these species, only *B. mycoides*, *C. michiganensis* and *S. turgidiscabies* were gram-positive. Microbes were obtained from the Department of Arboriculture, Botany and Plant Pathology, University of Naples "Federico II", Italy.

Antifungal activity was assessed by the in vitro spore germination test derived from Lorito et al. (1996). Briefly, a suspension of 10^3 spores was prepared in 50 μl of PDB (Potato Dextrose Broth) 0.1 strength with 15 μl of 5 mM potassium phosphate buffer (pH 6.7). Saponins were added to obtain three final concentrations (1000, 100 and 10 ppm). One hundred microlitres of each solution were placed in an ELISA (FALCON) 96-well plate and incubated at 25 °C. The number of germinated spores and the hyphal length were measured after 25 h. For *Pythium* and *Rhizoctonia*, the antifungal activity was evaluated by using plates of 9 cm of PDA (Potato Dextrose Agar) 0.1 strength added with the saponins at the three concentrations as above described, inoculated with a 5 mm plug containing the fungi grown on PDA for four days. Plates were incubated at 25 °C and the fungi radial growth was measured after 72 h. Antibacterial activity was assessed by the disc diffusion method. Plates of 9 cm were filled with 20 ml of M9 containing bacteria. A 5 mm disc of agar was removed from the centre of the plates and 50 μl of saponins solution at the three different concentrations were added to the wells. Plates were incubated at 25 °C and after 48 h the inhibition was measured.

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References

Avato, P., Bucci, R., Tava, A., Vitali, C., Rosato, A., Bialy, Z., Jurzysta, M., 2006. Antimicrobial activity of saponins from *Medicago* sp.: structure–activity relationship. Phytother. Res. 20, 454–457.

Barile, E., Zolfaghari, B., Sajjadi, S.E., Lanzotti, V., 2004. Saponins of *Allium elburzense*. J. Nat. Prod. 67, 2037–2042.

Bouarab, K., Melton, R., Peart, J., Baulcombe, D., Osbourn, A.E., 2002. A saponins-detoxifying enzyme mediates suppression of plant defense. Nature 418, 889–892.

Bowyer, P., Clarke, B.R., Lunness, P., Daniels, M.J., Osbourn, A.E., 1995. Host range of a plant pathogenic fungus determined by a saponin detoxifying enzyme. Science 267, 371–374.

Cioaca, C., Margineanu, C., Cucu, V., 1978. The saponins of *Hedera helix* with antibacterial activity. Pharmazie 33, 609–610.

Corea, G., Fattorusso, E., Lanzotti, V., 2003. Saponins and flavonoids of *Allium triquetrum*. J. Nat. Prod. 66, 1405–1411.

Curir, P., Dolci, M., Corea, G., Galeotti, F., Lanzotti, V., 2006. The plant antifungal isoflavone genistein is metabolized by *Armillaria mellea* Vahl to give non-fungitoxic products. Plant Biosyst. 140, 156–162.

Dong, M., Feng, X., Wang, B., Wu, L., Ikejima, T., 2001. Two novel furostanol saponins from the rhizomes of *Dioscorea panthaica* Prain et Burkill and their cytotoxic activity. Tetrahedron 57, 501–506.

Fattorusso, E., Iorizzi, M., Lanzotti, V., Taglialatela-Scafati, O., 2002. Chemical composition of shallot (*Allium ascalonicum* Hort.). J. Agric. Food Chem. 50, 5686–5690.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M., 2004. *Trichoderma* species-opportunistic, avirulent plants symbionts. Nature Rev. 2, 43–56.

Kel'ginbaev, A.N., Gorovits, M.B., Abubakirov, N.K., 1974. Steroid saponins and sapogenins of *Allium*. VII. Structure of neoagigenin and agigenin. Khim. Prir. Soedin. 6, 801–802.

Khristulas, F.S., Gorovits, M.B., Luchanskaya, V.N., Abubakirov, N.K., 1970. New steroidal sapogenin from *Allium giganteum*. Khim. Prir. Soedin. 6, 489–490.

Lanzotti, V., 2005. Bioactive saponins from *Allium* and *Aster* plants. Phytochem. Rev. 4, 95–110.

Lorito, M., Woo, S.L., D'Ambrosio, M., Harman, G.E., Hayes, C.K., Kubicek, C.P., Scala, F., 1996. Synergistic action between cell wall degrading enzymes and membrane affecting compounds. Molec. Plant–Microbe Interact. 9, 206–213.

Mandala, P., Sinha Babub, S.P., Mandal, N.C., 2005. Antimicrobial activity of saponins from *Acacia auriculiformis*. Fitoterapia 76, 462–465.

Morrissey, J.P., Osbourn, A.E., 1999. Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbiol. Mol. Biol. Rev. 63, 708–724.

Nicol, R.W., Traquair, J.A., Bernards, M.A., 2002. Ginensosides as host resistance factors in American ginseng (*Panax quinquefolius*). Can. J. Bot. 80, 557–562.

Osbourn, A.E., 1996. Saponins and plant defence – a soap story. Trends Plant Sci. 1, 4–9.

Osbourn, A.E., 1999. Antimicrobial phytoprotectants and fungal pathogens: a commentary. Fung. Gen. Biol. 26, 163–168.

Papadopoulou, K., Melton, R.E., Leggett, M., Daniels, M.J., Osbourn, A.E., 1999. Compromised disease resistance in saponins-deficient plants. Proc. Natl. Acad. Sci. USA 96, 12923–12928.

Sandrock, R.W., Van Etten, H.D., 1998. Fungal sensitivity to and enzymatic degradation of the phytoanticipin α-tomatine. Phytopathology 88, 137–143.

Schoonbeek, H., Del Sorbo, G., De Waard, M.A., 2001. The ABC transporter BcatrB affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. Molec. Plant–Microbe Interact. 14, 562–571.

Wang, Y., McAllister, T.A., Yanke, L.J., Cheeke, P.R., 2000. Effect of steroidal saponin from *Yucca schidigera* extract on ruminal microbes. J. Appl. Microbiol. 88, 887–896.

Wittstock, U., Gershenzon, J., 2002. Costitutive plant toxins and their role in defense against herbivores and pathogens. Curr. Opin. Plant Biol. 5, 1–8.

Zhang, J.D., Zheng, X., Yong-Bing, C., Hai-Sheng, C., Lan, Y., Mao-Mao, A., Ping-Hui, G., Yan, W., Xin-Ming, J., Yuan-Ying, J., 2006. Antifungal activities and action mechanisms of compounds from *Tribulus terrestris* L.. J. Ethnopharmacol. 103, 76–84.

Zimmer, D.E., Pedersen, M.W., McGuire, D.F., 1967. A bioassay for alfalfa saponins using the fungus *Trichoderma viride*. Pers. ex. Fr. Crop. Sci. 7, 223–224.