Two New Sulfated Furostanol Saponins from *Tribulus terrestris*

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The known furostanol saponins methylprotodioscin and protodioscin and two new sulfated saponins, sodium salt of 26-O-β-glucopyranosyl-22α-methoxy-(25R)-furost-5-ene-3β,26-diol-3-O-α-rhamnopyranosyl-(1→2)-β-4-O-sulfo-glucopyranoside (methylprototribestin) and sodium salt of 26-O-β-glucopyranosyl-22α-hydroxy-(25R)-furost-5-ene-3β,26-diol-3-O-α-rhamnopyranosyl-(1→2)-β-4-O-sulfo-glucopyranoside (prototribestin) have been isolated from the aerial parts of *Tribulus terrestris* L. growing in Bulgaria. The structures of the new compounds were elucidated on the basis of 1D and 2D (DQF-COSY, TOCSY, HSQC-TOCSY, HSQC, HMBC, ROESY) NMR data, ESI mass spectra and chemical transformation.

Introduction

Tribulus terrestris L. (Zygophyllaceae) is an annual herb of world wide distribution. The plant is used in the folk medicine in India, China, Bulgaria and other countries against various diseases, including impotence (Tomova *et al.,* 1981; Xu *et al.,* 2000; Wu *et al.,* 1999). Recently, antitumoral activity and effects on cardiovascular system have been also found (Xu *et al.,* 2000).

Tribulus terrestris growing in Bulgaria is a source for the industrial production of the original preparation "Tribestan" produced by Sopharma Joint Stock Co., Bulgaria. Tribestan consists of the n-BuOH extract of the aerial parts of the same plant and is successfully applied for treatment of sexual deficiency (Tomova *et al.,* 1981).

Furostanol and spirostanol saponins, flavonoid glycosides, alkaloids and some amides have been reported to occur in this plant (Tomova *et al.,* 1981; Xu *et al.,* 2000; Wu *et al.,* 1999; Saleh *et al.,* 2000).

In the present paper we report the isolation and structure elucidation of two new sulfated furostanol saponins **3** and **4**, found in the aerial parts of *T. terrestris* together with the known saponins methylprotodioscin (**1**) and protodioscin (**2**).

Materials and Methods

General

IR spectra were obtained on a Bruker IFS 113V in KBr disks. The NMR spectra were recorded in CD_3OD or pyridine-d_5 on a Bruker DRX 600 or Bruker DRX 250 spectrometer. ESI mass spectra: Bruker ESQUIRE-LC quadrupole ion trap instrument (Bruker-Franzen, Bremen, Germany) connected to an orthogonal electrospray ion source (Hewlett-Packard, Palo Alto, USA). TLC: aluminium sheets, silica gel 60 F254 (Merck), bands were detected under UV light and by spraying with Ehrlich reagent; liquid vacuum chromatography (LVC): silica gel 60 (Merck); medium-pressure liquid chromatography (MPLC): LiChroprep RP-8 (40–63 μm, 31 × 2.5 cm i.d., Merck).

Plant material

T. terrestris L. (aerial parts) was collected in July, 1998 in the region of the city of Ljubimetz, Bulgaria. The plant material was authenticated by Dr. R. Taskova and a voucher specimen (No SOM $/_{CO}$ 216) was deposited at the Herbarium of the Institute of Botany, Bulgarian Academy of Sciences, Bulgaria.

Extraction and isolation

Air dried and powdered plant material (5.5 kg) was extracted with 70% EtOH (3 × 24 h × 6 l) at room temperature. The combined EtOH solutions were concentrated to a small volume (2 l) and extracted in succession with chloroform (3 × 24 h × 1 l) and n-BuOH (3 × 24 h × 1 l). The n-BuOH layer was concentrated to dryness giving the crude saponin extract (CSE, 100.0 g). LVC of CSE (10.0 g) over silica gel (20.0 g) using $CHCl_3$-MeOH-H_2O (6:1:0.1 to 1:1:0.1) yielded fractions F1-F7. F4 (0.7 g) was subjected to MPLC with MeOH-H_2O (4:7 to 4:1,v/v) to give fractions S1-S10. S7 (0.1 g) on silica gel CC eluting with $CHCl_3$-MeOH-H_2O (6:1:0.1 to 1:1:0.1) afforded 20 fractions. The fractions eluted with $CHCl_3$-MeOH-H_2O (2:1:0.1) were further worked up by prep. TLC ($CHCl_3$-MeOH-H_2O, 65:35:10) to obtain methylprotodioscin (**1**, 16 mg) and protodioscin (**2**, 8 mg). F7 (0.7 g) was subjected to MPLC with MeOH-H_2O (3:7 to 4:1) to obtain fractions P1-P8. P5 (150 mg) on silica gel CC using $CHCl_3$-MeOH-H_2O (6:1:0.1 to 1:1:0.1) gave 14 fractions. The fractions (43 mg) eluted with $CHCl_3$-MeOH-H_2O (2:1:0.1) on prep.TLC ($CHCl_3$-MeOH-H_2O, 65:35:10) gave methylprototribestin (**3**, 12 mg) and a mixture (M, 20 mg) of methylprototribestin (**3**) and prototribestin (**4**). Prep.TLC of M using dioxane-CH_3CN-EtOAc-H_2O (5:5:10:2) led to pure **4** (6 mg).

Methylprototribestin (**3**): Amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3445, 1653, 1260, 1227, 1100, 1034, 913, 892, 832, 812 (intensity 913 < 832); ESI-MS (in MeOH): *m/z* 1041 $[M + Na]^+$; ^1H and ^{13}C-NMR: Table I.

Prototribestin (**4**): Amorphous powder. IR ν_{max}^{KBr} cm^{-1}: 3416, 1625, 1249, 1033, 914, 837 (intensity 914 < 837); ESI-MS (in MeOH): *m/z* 1027 $[M+Na]^+$; ^1H-NMR (Py-d_5), δ (ppm): 0.90 (s, 3H, 18-Me), 0.99 (d, *J* = 6.5 Hz, 3H, 27-Me), 1.04 (s, 3H, 19-Me), 1.36 (d, *J* = 6.9 Hz, 3H, 21-Me), 1.75 (d, *J* = 6.1 Hz, 3H, Rha-Me), 4.83 (d, *J* = 7.8 Hz, 1H, H-1‴), 4.89 (d, *J* = 7.7 Hz, 1H, H-1′), 6.25 (brs, 1H, H-1″).

Interconversion between **3** *and* **4**: a. A solution of **3** (20 mg) in 20 ml acetone:water (7:3) was heated at 70° for 1 h. The reaction mixture was concentrated under vacuum and subjected to multiple prep.TLC (dioxane-CH_3CN- EtOAc-water, 5:5:10:2) to obtain pure **4** (7 mg).

b. A methanolic solution of **4** (20 mg) was refluxed for 6 h and concentrated under reduced pressure. Multiple prep.TLC ($CHCl_3$-MeOH-H_2O, 65:35:10) gave pure **3** (15 mg).

Acid hydrolysis of **3**: Compound **3** (5 mg) was refluxed with 10% aqueous HCl (4 ml) for 4 h. After extraction with ethyl ether, the aqueous solution was treated with $BaCl_2$ to give a white precipitate of $BaSO_4$.

Solvolysis of **3**: A solution of saponin **3** (20 mg) in dioxane-pyridine, 1:1 (4 ml) was heated at 160 °C for 2 h. The reaction mixture was evaporated to dryness and purified by TLC ($CHCl_3$-MeOH-H_2O, 65:35:10) to obtain the desulfated saponin **5** (5 mg). ESI-MS (in MeOH): *m/z* 885 $[M+H]^+$; ^1H and ^{13}C NMR: Table I.

Results and Discussion

Investigation of the CSE of the aerial parts of *T. terrestris* growing in Bulgaria led to the isolation of four furostanol saponins. Two of them were the known methylprotodioscin (**1**) and protodioscin (**2**). The remaining two were proved to be new sulfated saponins named methylprototribestin (**3**) and prototribestin (**4**).

Compounds **1** and **2** were identified by comparison of their mass, ^1H and ^{13}C NMR spectral data with those reported in the literature for methylprotodioscin and protodioscin.

The structure elucidation of the new compounds was accomplished by detailed examination of their 1D and 2D NMR spectra, mass spectral studies and confirmed by chemical transformation. The assignments of all carbons and protons (Table I) was achieved through a combination of homonuclear proton-proton 2D (DQF-COSY, TOCSY, NOESY) and heteronuclear proton-carbon 2D (HSQC, HMBC and HSQS-TOCSY) experiments.

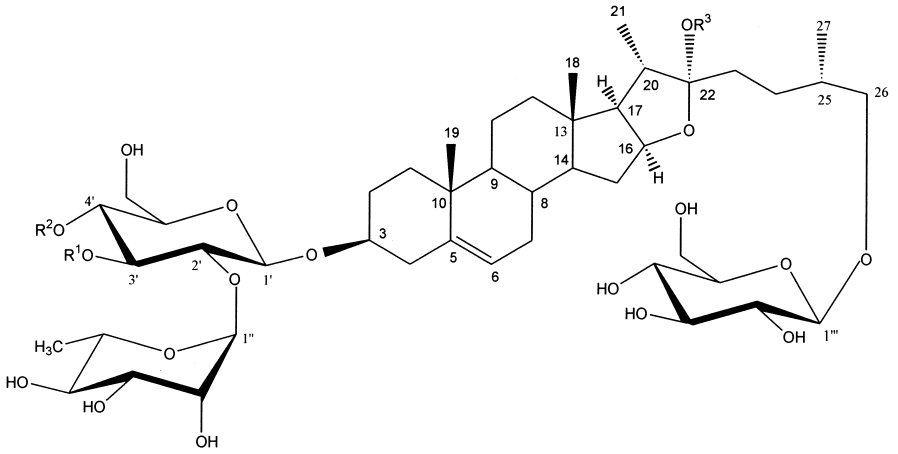
Methylprototribestin (**3**), $C_{46}H_{75}O_{21}SNa$, was suggested to be a furostanol derivative by the Ehrlich reaction. The IR spectrum exhibited a characteristic S-O absorption band at 1225 cm^{-1} and bands at 913, 832 and 812 cm^{-1} (intensity 913 < 832), indicative of a 25R-furostane steroidal structure (Silva *et al.,* 1999). Its glycosidic nature was shown by the IR absorptions at 3445 and 1034 cm^{-1}. The ESI mass spectrum (MeOH) showed the quasi molecular ion $[M+Na]^+$ at *m/z* 1041. Acid hydrolysis of **3**, followed by treatment with barium

Table I. ^{1}H and ^{13}C NMR data for compounds **3** and **5** in CD_3OD, δ in ppm, (*J* in Hz).

	3[a]		**5**[b]	
C	δ H[c]	δ C	δ H[c]	δ C
1	1.92–1.84 m, 1.23–1.04 m	38.7	1.94–1.84 m, 1.11–1.04 m	38.6
2	1.99–1.89 m, 1.66–1.55 m	30.9	1.96–1.87 m, 1.66–1.59 m	30.7
3	3.66–3.56 m	79.5	3.70–3.55 m	79.1
4	2.48–2.42 m, 2.34–2.25 m	39.7	2.40–2.50 m, 2.28–2.35 m	39.5
5	–	142.0	–	141.9
6	5.41–5.36 m	122.8	5.31–5.25 m	122.6
7	2.05–1.96 m, 1.60–1.52 m	33.3	2.07–1.98 m, 1.64–1.55 m	33.3
8	1.32–1.24[d] m, 1.70–1.61[d] m, 2.01–1.94[d] m	32.90[d] 32.93[d]	1.63–1.61 m	32.6
9	1.02–0.93 m	51.8	1.02–0.93 m	51.7
10	–	38.2	–	38.0
11	1.61–1.48 m	22.1	1.64–1.52 m	22.1
12	1.83–1.74 m, 1.25–1.15 m	41.0	1.86–1.80 m, 1.27–1.23 m	40.7
13	–	42.0	–	44.4
14	1.18–1.10 m	57.9	1.09–1.02 m	56.3
15	1.32–1.24[d] m, 1.70–1.61[d] m, 2.01–1.94[d] m	32.90[d] 32.93[d]	2.20–2.14 m, 1.45–1.39 m	35.2
16	4.40–4.35 m	82.6	4.80–4.68 m	85.6
17	1.76–1.71 m	65.2	2.55–2.45 m	65.5
18	0.84 s	17.0	0.71 s	14.6
19	1.05 s	20.0	1.05 s	19.9
20	2.18 q ($J \approx 7.0$)	41.3	–	103.6
21	1.01 d ($J = 7.0$)	16.3	1.60 s	11.9
22	–	114.1	–	152.9
23	1.87–1.79 m, 1.67–1.59 m	31.5	2.18–2.07 m, 1.38–1.28 m	24.1
24	1.64–1.55 m, 1.20–1.11 m	29.1	1.28–1.22 m	32.0
25	1.79–1.70 m	35.1	1.82–1.72 m	34.1
26	3.73 t ($J = 7.5$), 3.44–3.36 m	76.2	3.78–3.65 m, 3.45–3.33 m	75.8
27	0.95 d ($J = 6.8$)	17.4	0.95 d ($J = 6.4$)	17.3
OMe	3.14 s	47.8	–	–
1′	4.52 d ($J = 7.9$)	100.4	4.48 d ($J = 7.2$)	100.5
2′	3.45 dd ($J = 7.9, 9.0$)	79.2	3.43–3.31 m	79.0
3′	3.79 t ($J = 9.0$)	78.1	3.52–3.43 m	79.4
4′	4.14–4.10 m	77.9	3.36–3.22 m	71.8[e]
5′	3.44–3.36 m	76.1	3.36–3.17 m	77.7[f]
6′	3.87 dd ($J \approx 12.5, 2.0$) 3.73 dd ($J \approx 12.5, 5.2$)	62.6	3.93–3.79 m, 3.73–3.55 m	62.7
1″	5.22 d ($J \approx 1.3$)	102.2	5.19brs	102.2
2″	3.92–3.90 m ($J \approx 3.0, 1.3$)	72.3	3.96–3.88 m	72.2
3″	3.69–3.64 m	72.5	3.72–3.62 m	72.3
4″	3.39 t ($J = 9.7$)	74.1	3.47–3.34 m	73.9
5″	4.16–4.08 m	69.9	4.20–4.08 m	69.7
6″	1.24 d ($J = 6.4$)	18.1	1.21 d ($J = 6.0$)	18.0
1‴	4.24 d ($J = 7.9$)	104.7	4.22 d ($J = 7.9$)	104.5
2‴	3.19 dd ($J = 7.9, 9.0$)	75.3	3.24–3.14 m	75.1
3‴	3.35 t ($J = 9.0$)	78.3	3.36–3.17 m	78.1
4‴	3.28 t ($J = 9.0$)	71.9	3.36–3.22 m	71.6[e]
5‴	3.29–3.23 m	78.0	3.36–3.17 m	77.9[f]
6‴	3.86 dd ($J \approx 12.0, 2.2$) 3.66 dd ($J \approx 12.0, 5.2$)	63.0	3.93–3.79 m, 3.73–3.55 m	62.7

[a]600MHz; [b]250MHz; [c]The ^{1}H-chemical shifts were extracted from the 2D HSQC-experiment; [d]The ^{1}H and ^{13}C chemical shifts of 8, 15 can not be assigned unambiguously; [e,f]Signals may be interchangeable within each column.

chloride gave a white precipitate of $BaSO_4$, confirming the existance of a sulfate group (Sanchez-Contreras *et al.,* 2000).



1 R^1 = H, R^2 = Rha, R^3 = Me
2 R^1 = R^3 = H, R^2 = Rha
3 R^1 = H, R^2 = SO_3Na, R^3 = Me
4 R^1 = R^3 = H, R^2 = SO_3Na
5 R^1 = R^2 = H, $\Delta^{20,22}$
6 R^1 = Glc, R^2 = H, R^3 = Me
7 R^1 = Glc, R^2 = R^3 = H

Fig. 1. Structure of compounds **1–7**.

The ^{1}H NMR spectrum of **3** revealed two three-proton singlets at δ 0.84 (δ_c 17.0) and 1.05 (δ_c 20.0) and two three-proton doublets at δ 1.01 (J = 7.0 Hz, δ_c 16.3) and 0.95 (J = 6.8 Hz, δ_c 17.4), corresponding to the typical furostanol C-18, 19, 21, 27 methyl protons (Silva *et al.,* 1999). The methyl signals at δ 1.24 d (J = 6.4 Hz, δ_c 18.1) and 3.14 s (δ_c 47.8) were attributed to the methyl group of rhamnose (Me-6″) and the 22-OMe in accordance with the HMBC correlations between Me-6″ and C-4″, 5″ and between 22-OMe and C-22, respectively. The signal of the quarternary carbon at δ 114.1 supported a furostane skeleton possessing an OMe group at C-22 (Debella *et al.,* 1999).

The DQF- COSY, TOCSY, HSQC-TOCSY experiments allowed the sequental assignment of the resonances of the sugar units, starting from the easily distinguished anomeric protons at δ 4.52 (d, J = 7.9 Hz, H-1′), 5.22 (d, J = 1.3 Hz, H-1″) and 4.24 (d, J = 7.9 Hz, H-1‴). Multiplet patterns, ^{1}H-^{1}H coupling constants and ^{13}C chemical shifts confirmed the presence of one α-rhamnopyranosyl and two β-glucopyranosyl units (Kalinovski *et al.,* 1984).

The long range correlation between H-1′ and the carbon signal at δ 79.5 fixed the attachment of the inner glucose to C-3 of the aglycone, while the correlation between H-1″ and the carbon signal at δ 79.2 showed that α-rhamnose is linked to C-2′ of this glucose. The crosspeaks between H-2′ and C-1′, 3′, 1″ provided additional evidence for the interglycosidic linkage at C-2′. The anomeric proton H-1‴ was long range coupled to C-26 and indicated that saponin **3** had a 26-O-β-glucopyranosyl group. One of the glucosidic protons (H-4′) in **3** was strongly deshielded and appeared at δ 4.12 (δ_c 77.9), suggesting that its geminal hydroxyl group is substituted and most probably bears the sulfate group.

A solvolysis (Palagiano *et al.,* 1996) of the saponin **3** (dioxane-pyridine, 160 °C, 2 h) afforded the less polar desulfated compound **5** having a C-20/C-22 double bond. Its furostanol nature was suggested by the positive Ehrlich reaction. Its ESI spectrum showed a quasi molecular ion at *m/z* 885 $[M+H]^+$. The molecular formula $C_{45}H_{72}O$, the pseudo furostanol structure **5** and the proton and carbon chemical shifts were deduced from its 1D and 2D (HSQC, HMBC) NMR and mass spectral data.

A comparison of the spectral data of **3** and **5** confirmed the position of the sulfate group at C-4′. As expected, compared to compound **5**, the C-4′ carbon signal in **3** appeared at lower field (+ 6.1 ppm), while higher field shifts were ob-

served for C-3′ (−1.3 ppm) and C-5′ (−1.1 ppm) (Watanabe *et al.,* 1983). In addition, the C-20/C-22 double bond in **5** caused changes in the chemical shifts of some carbons and protons.

In this way the structure **3** was unambiguously assigned to methylprototribestin.

Prototribestin **4**, $C_{45}H_{73}O_{21}SNa$, was found to be positive to the Ehrlich reaction. The presence of S-O group was indicated by the absorption band at 1249 cm^{-1} in its IR spectrum. The ^1H NMR spectrum exhibited the four methyl signals typical of a furostanol structure, and three anomeric protons suggesting the existance of three sugar units. However, no signal for OMe group was visible in the ^1H NMR spectrum.

Compound **4** was easily converted to methylprototribestin **3** on refluxing with MeOH, while **3** was transformed back to **4** on boiling with aqueous acetone. Analogous interconversion has been reported between 22-hydroxy and 22-methoxyfurostanol glycosides (Palagiano *et al.,* 1996). Hence, **4** was proved to be a 22-hydroxy analogue of **3.** The ESI mass spectrum (MeOH) showed a quasi molecular ion $[M+Na]^+$ at *m/z* 1027 supporting the proposed structure.

Based on these data the structure **4** was established to prototribestin.

Because the above mentioned interconversion between the 22-hydroxy and 22-methoxy furostanol glycosides is known to occur, the 22-OMe derivatives are usually considered to be artefacts formed from the 22-hydroxy compounds during the isolation procedure. For this reason the aerial parts of *T. terrestris* were extracted with pyridine-dioxane at room temperature (Debella *et al.,* 1999). TLC examination revealed the presence of **1–4** in the pyridine-dioxane extract (PDE) and proved the natural occurrence of **1** and **3**.

Tomova *et al.* assigned the structures of protodioscin (**2**) and protogracilin (**7**) to the main components of Tribestan only on the basis of some chemical transformations (Tomova *et al.,* 1981; Watanabe *et al.,* 1983). Our investigations showed that **1–4** are present in Tribestan in the same quantity and ratio as in the PDE and the CSE. Our TLC comparison of CSE, Tribestan and PDE confirmed that **1** and **3** predominate over **2** and **4** and could be considered as their main components. Furthermore, Tribestan was worked-up and the compounds corresponding to **1–4** isolated following the experimental procedure (see Materials and Methods) applied to CSE. An examination of the spectral data of the isolated compounds confirmed them to be identical to **1–4**. As expected, the NMR and MS spectra of **3** and **4** appeared to be quite different from those reported (Aquino *et al.,* 1986) for methylprotogracilin (**6**) and protogracilin (**7**). We have not found **6** and **7** in CSE and Tribestan so far.

Therefore, the structures of the main components of Tribestan and the CSE of *T. terrestris* growing in Bulgaria were established as methylprotodioscin (**1**) and methylprototribestin (**3**).

To the best of our knowledge, compounds **3** and **4** are the first sulfated furostanol saponins found in *T. terrestris*. It is interesting to note, that the spiro analogue of **3** and **4** had been already isolated from *T. terrestris* of Bulgarian origin (Matshenko *et al.,* 1990).

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