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Steroidal saponins from the aerial parts of *Tribulus alatus* Del.

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Dedicated to the memory of Prof. Ivano Morelli.

Abstract

Six steroidal glycosides (**1**–**6**) were isolated from the aerial parts of *Tribulus alatus* Del. (Zygophyllaceae), together with one known cholestane, one spirostane, and six flavonol glycosides. Among them, **1** and **2** possess a furostane-type aglycone, **3** and **6** a cholestane structure, and **4** and **5** a spirostane skeleton. Their structural elucidation was accomplished by extensive spectroscopic methods including 1D (^{1}H, ^{13}C, ^{13}C DEPT, TOCSY, ROESY) and 2D NMR experiments (DQF-COSY, HSQC, HMBC) as well as ESI-MS analysis.

Keywords: *Tribulus alatus*; Zygophyllaceae; Furostane saponins; Cholestane saponins; Spirostane saponins

1. Introduction

The genus *Tribulus* (Zygophyllaceae family) comprises about 25 species that grow as shrubs or herbs in subtropical areas around the world (Hegnauer, 1973). Among them is *T. alatus* Del. (syn. *T. longipetalus* Viv.), an annual or biennial prostate herb, found in dry sandy soil along roads in warm-temperate zone of Egypt; the fruits of this plant are used in Pakistan for the treatment of urinary disorders and cough (Täckholm, 1974; Ghazanfar, 1994). An old phytochemical study on this plant evidenced the presence of steroidal saponins (Nag et al., 1979), while no biological report is present in the literature. A survey concerning the secondary metabolites showed that steroidal saponins (Achenbach et al., 1996; Cai et al., 2001; Bedir et al., 2002; De Combarieu et al., 2003), lignanamides (Li et al., 1998), alkaloids (Wu et al., 1999), and flavonoids (Saleh et al., 1982; Louveaux et al., 1998) are the typical constituents of the genus, and in particular of *T. terrestris*, a well known and largely distributed herbal drug, used as food supplements to improve performance in sports and for the treatment of impotency (De Combarieu et al., 2003).

The aim of our work was to carry out the phytochemical investigation of *T. alatus* aerial parts and herein we report the structural characterization of six new steroidal glycosides (**1**–**6**) from the MeOH extract of the title plant, on the basis of extensive spectroscopic and spectrometric analysis, including 2D NMR and ESI-MS spectra. One known cholestane, one spirostane, and six flavonol glycosides were also isolated and characterized.

2. Results and discussion

The phytochemical study of *T. alatus* aerial parts led to the isolation of six new steroidal saponins **1**–**6**, together with the known pentandroside A (Hamed et al., 2004),

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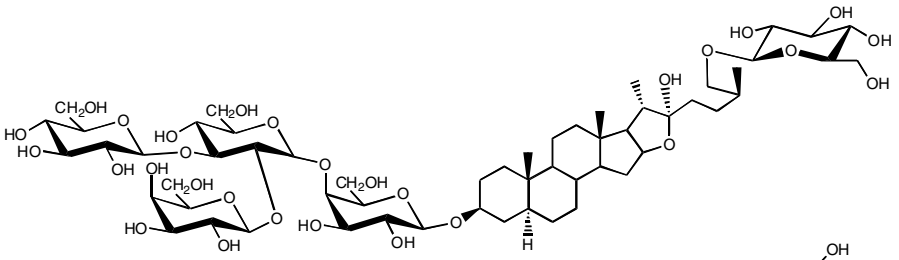
(25*S*)-5α-spirostan-3β-ol-3-*O*-β-D-glucopyranosyl-(1 → 2)-*O*-[β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (Achenbach et al., 1994), isorhamnetin 3-*O*-(6″-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside (Romussi et al., 1988), kaempferol 3-*O*-(3″,6″-di-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside (Liu et al., 1999), kaempferol 3-*O*-(3″-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside (Liu et al., 1999), tribuloside (Leitão et al., 2000), quercetin 3-*O*-β-D-glucopyranoside (Agrawal, 1989), and kaempferol 3-*O*-β-D-glucopyranoside (Agrawal, 1989).

Compound **1** had the molecular formula $C_{57}H_{96}O_{29}$, as determined by ^{13}C-, ^{13}C-DEPT, positive-ion ESI-MS, and



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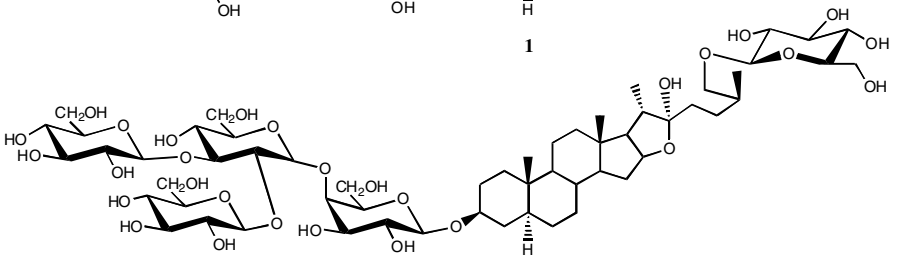

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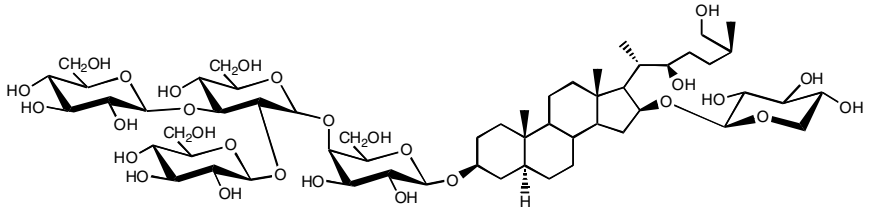

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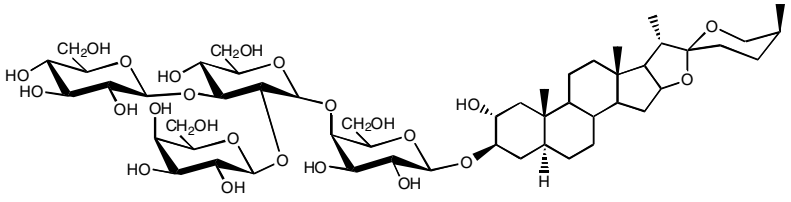

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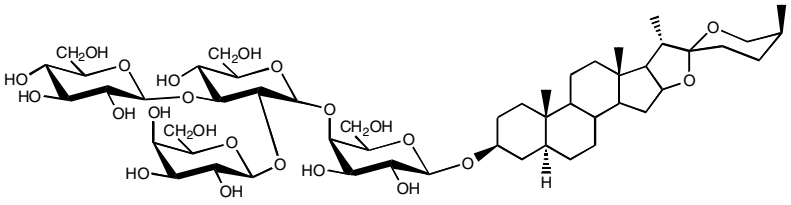

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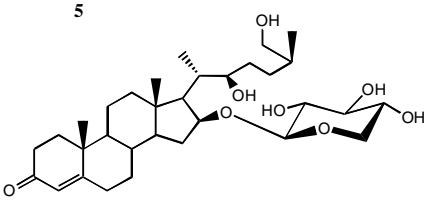

6

elemental analysis. The ESI-MS showed the $[M+Na]^+$ ion at *m/z* 1267 and prominent fragments at *m/z* 1105 $[M+Na\text{-}162]^+$, 943 $[M+Na\text{-}162\text{-}162]^+$, 781 $[M+Na\text{-}162\text{-}162\text{-}162]^+$, 619 $[M+Na\text{-}162\text{-}162\text{-}162\text{-}162]^+$, attributable to the sequential loss of four hexose residues. The ^1H NMR spectral data of **1** (see Section 3) contained signals of two three-proton singlets at δ 0.83 (δ_C 16.8) and 0.88 (δ_C 12.9) and two three proton doublets at δ 1.02 ($J = 6.0$ Hz, δ_C 16.0) and 0.97 ($J = 6.5$ Hz, δ_C 17.2), corresponding to the typical furostanol C-18, C-19, C-21, and C-27 methyl protons (Wang et al., 1997). Other characteristic features were the hemiketalic function at C-22 (δ_C 112.8), two secondary alcoholic functions (δ_C 79.4 and 83.5) and one primary alcoholic group at δ_C 76.0. The above ^1H and ^{13}C NMR spectral data, together with 2D NMR studies (DQF-COSY, HSQC, and HMBC experiments), were completely in agreement with (25*S*)-5α-furostan-3β,22α,26-triol as the aglycone of compound **1**. The α-configuration of C-22 hydroxyl group was defined by a ROE correlation between the H-20 proton (δ 2.20 m) with the H-23 protons (δ 1.87 *m*, 1.65 *m*). The 25*S* stereochemistry was deduced by comparison with 25*S* furostan glycosides isolated from *T. terrrestris* (Wang et al., 1997) and *T. cistoides* (Achenbach et al., 1994). The sugar portion of **1** contained, in the ^1H NMR spectrum (Table 1) five anomeric proton signals (δ 4.26, *d*, $J = 8.0$ Hz; 4.41, *d*, $J = 7.5$ Hz; 4.62, *d*, $J = 8.0$ Hz; 4.70, *d*, $J = 8.0$ Hz; 4.91, *d*, $J = 7.5$ Hz). The remaining 1D sugar spectral region of **1** was complex since most of the chemical shifts were overlapped. 1D-TOCSY experiments, together with the DQF-COSY spectrum, led us to establish the proton sequence within these sugar fragments that were identified as two β-galactopyranoside and three β-glucopyranoside. The HSQC experiment allowed the assignments of all the carbon resonances and therefore the identification of the sugars as two terminal β-glucopyranosyl units, one terminal β-galactopyranose, a 2,3-disubstituted β-glucopyranose, and a β-galactopyranose glycosilated at C-4. The position of the sugar units was unambiguously defined by the HMBC experiment in which long-range correlations were observed between $H\text{-}1_{galII}$–C-3, $H\text{-}1_{glcI}$–$C\text{-}4_{galI}$, $H\text{-}1_{galIII}$–$C\text{-}2_{glcI}$, $H\text{-}1_{glcII}$–$C\text{-}3_{glcI}$, $H\text{-}1_{glcIII}$–C-26. The configurations of the sugar units were assigned after hydrolysis of **1** with 1 N HCl. The hydrolysate was trimethylsilated, and GC retention times of each sugar were compared with those of authentic sugar samples prepared in the same manner. In this way the sugar units of **1** were determined to be D-galactose and D-glucose in the ratio 2:3. Thus, the structure of **1** was established as (25*S*)-26-*O*-β-D-glucopyranosyl-5α-furostan-3β,22α,26-triol-3-*O*- β-D-galactopyranosyl-(1 → 2)-*O*-[β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside.

The negative ion ESI-MS of **2** (molecular formula $C_{57}H_{96}O_{29}$, as determined by ^{13}C-, ^{13}C-DEPT, and elemental analysis) showed a quasi-molecular ion peak at *m/z* 1243 $[M-H]^-$, together with ion at *m/z* 1081 $[M-H\text{-}162]^-$, 919 $[M-H\text{-}162\text{-}162]^-$, 757 $[M-H\text{-}162\text{-}162\text{-}162]^-$, 595 $[M-H\text{-}162\text{-}162\text{-}162\text{-}162]^-$, indicating that **2** was an isomer of **1**. The ^{13}C NMR spectrum showed 57 signals, of which 27 were assigned to a steroidal moiety and 30 to the saccharide portion. Analysis of NMR spectral data of **2** suggested that it had the same aglycone of **1**, while the sugar moiety was the point of difference. The structures

Table 1
^1H and ^{13}C NMR data of the sugar moieties of compounds **1** and **2** (CD_3OD, 600 MHz)[a]

Position	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
D-Gal I				
1	4.41 *d* (7.5)	102.7	4.41 *d* (7.5)	102.7
2	3.70 *dd* (8.5, 7.8)	72.6	3.70 *dd* (8.5, 7.8)	73.0
3	3.65 *dd* (8.5, 4.0)	73.1	3.60 *dd* (8.5, 4.0)	73.4
4	4.05 *dd* (4.0, 2.5)	80.1	4.05 *dd* (4.0, 2.5)	80.4
5	3.53 *m*	75.2	3.54 *m*	75.6
6a	3.94 *dd* (12.0, 2.0)	60.9	3.95 *dd* (12.0, 2.0)	61.4
6b	3.64 *dd* (12.0, 4.5)		3.65 *dd* (12.0, 4.5)	
D-Glc I				
1	4.62 *d* (8.0)	104.8	4.62 *d* (8.0)	104.7
2	3.78 *dd* (9.5, 8.0)	81.1	3.78 *dd* (9.5, 8.0)	81.3
3	3.78 *t* (9.5)	87.8	3.79 *t* (9.5)	88.0
4	3.37 *t* (9.5)	71.0	3.35 *t* (9.5)	70.8
5	3.59 *m*	77.6	3.60 *m*	77.2
6a	3.95 *dd* (12.0, 3.5)	63.2	3.95 *dd* (12.0, 3.5)	63.3
6b	3.62 *dd* (12.0, 5.0)		3.62 *dd* (12.0, 5.0)	
D-Gal II				
1	4.91 *d* (7.5)	105.1		
2	3.67 *dd* (8.0, 7.5)	72.0		
3	3.53 *dd* (8.0, 4.0)	75.2		
4	3.89 *dd* (4.0, 2.5)	70.5		
5	3.40 *m*	76.3		
6a	4.00 *dd* (12.0, 2.5)	62.0		
6b	3.70 *dd* (12.0, 4.5)			
D-Glc II				
1	4.70 *d* (8.0)	104.1	4.95 *d* (8.0)	104.3
2	3.29 *dd* (9.5, 8.0)	75.2	3.21 *dd* (9.0, 8.0)	75.3
3	3.38 *t* (9.5)	77.8	3.38 *t* (9.0)	77.5
4	3.39 *t* (9.5)	71.4	3.40 *t* (9.0)	71.0
5	3.40 *m*	77.9	3.38 *m*	78.0
6a	3.95 *dd* (12.0, 2.5)	62.5	3.93 *dd* (12.0, 3.5)	62.8
6b	3.65 *dd* (12.0, 5.5)		3.65 *dd* (12.0, 5.0)	
D-Glc III				
1	4.26 *d* (8.0)	104.8	4.70 *d* (8.0)	104.2
2	3.21 *dd* (9.0, 8.0)	75.3	3.30 *dd* (9.5, 8.0)	75.3
3	3.38 *t* (9.0)	77.8	3.38 *t* (9.5)	77.4
4	3.30 *t* (9.0)	71.4	3.38 *t* (9.5)	71.5
5	3.28 *m*	77.9	3.39 *m*	78.3
6a	3.92 *dd* (12.0, 3.0)	62.5	3.93 *dd* (12.0, 3.0)	62.5
6b	3.66 *dd* (12.0, 5.0)		3.62 *dd* (12.0, 5.5)	
D-Glc IV				
1			4.26 *d* (8.0)	104.5
2			3.21 *dd* (9.0, 8.0)	75.2
3			3.38 *t* (9.0)	77.4
4			3.32 *t* (9.0)	71.7
5			3.29 *m*	77.9
6a			3.84 *dd* (12.0, 3.0)	62.8
6b			3.70 *dd* (12.0, 5.0)	

[a] *J* values are in parentheses and reported in Hz; chemical shifts are given in ppm; assignments were confirmed by DQF-COSY, 1D-TOCSY, HSQC, and HMBC experiments.

of the oligosaccharide moieties of **2** were deduced using 1D-TOCSY and DQF-COSY experiments (Table 1). Thus, the chemical shifts of the sugar resonances were attributable to one β-galactopyranoyl (δ 4.41) and four β-glucopyranosyl (δ 4.26, 4.62, 4.70, 4.95) units. The absence of any glycosidation shift for three β-glucopyranose suggested that these sugars were the terminal units. Glycosidation shifts were observed for C-4_{gal} (80.4 ppm), C-2_{glcI} (81.3 ppm), and C-3_{glcI} (88.0 ppm). A cross-peak due to long-range correlation (HMBC) between H-1_{gal} and C-3 (79.4 ppm) of the aglycone indicated that galactose was the residue linked to C-3 of the aglycone; a cross-peak between H-1_{glcI} and C-4_{gal} (80.4 ppm) indicated that 2,3-disubstituted glucose I was the second unit of the tetrasaccharide chain at C-3 of the aglycone. Similarly, the cross-peak between H-1_{glcIV} and C-26 (76.0 ppm) confirmed that one glucose unit was the hexose unit linked to C-26 of the aglycone. Also in this case, the configuration of the sugar units was determined by acid hydrolysis of **2** followed by GC analysis. In this way the sugar units of **2** were determined to be D-galactose and D-glucose in the ratio 1:4. On the basis of these evidences, **2** was identified as (25*S*)-26-*O*-β-D-glucopyranosyl-5α-furostan-3β,22α,26-triol-3-*O*-β-D-glucopyranosyl-(1 → 2)-*O*-[β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside.

Compound **3** displayed molecular formula $C_{56}H_{96}O_{28}$ by means of MS, ^{13}C-, ^{13}C-DEPT NMR data, and elemental analysis. Its ESI-MS revealed a molecular ion at *m/z* 1215 $[M-H]^-$, 1053 $[M-H-162]^-$, 891 $[M-H-162-162]^-$, 729 $[M-H-162-162-162]^-$, 597 $[M-H-162-162-162-132]^-$ attributable to the sequential loss of three hexose and a pentose residue. The ^{13}C- and ^{13}C-DEPT NMR spectra showed 56 carbon signals, of which 27 carbon signals were assigned to a cholestane aglycone and 29 carbon signals to a sugar portion. The ^{1}H NMR spectrum of **1** showed for the aglycone moiety two tertiary methyl proton signals at δ 0.90 (δ_C 13.0) and 0.92 (δ_C 13.8), two methyl doublets at δ 0.93 ($J = 6.5$ Hz, δ_C 12.2) and 0.95 ($J = 6.5$ Hz, δ_C 17.3) and three methine protons indicative of secondary alcoholic functions at δ 3.70 (1H, *m*, H-3), 3.72 (1H, *br d*, $J = 8.0$ Hz, H-22), and 4.16 (1H, *ddd*, $J = 9.0$, 7.0, 4.5 Hz, H-16). Signals attributable to H_2-26 resonated at δ 3.35 (*dd*, $J = 11.0$, 6.0 Hz) and 3.48 (*dd*, $J = 11.0$, 6.5 Hz). The ^{13}C NMR spectrum of **3** also suggested for the aglycone moiety of **3** a cholestane skeleton with three secondary alcoholic functions (74.4, 79.4, and 83.1 ppm) and one primary alcoholic group (68.5 ppm). On the basis of HSQC and HMBC experiments the aglycone of **3** was characterized as (22*S*,25*S*)-5α-cholestan-3β,16β,22,26-tetraol (Jin et al., 2004). In addition to the aglycone signals, the ^{13}C NMR spectrum exhibited 29 signals attributable to the sugar portion made up of four hexopyranosyl and one pentopyranosyl units. The C-3 and C-16 carbon signals exhibited both glycosidation shifts (79.3 and 83.1 ppm, respectively). Analysis of the NMR data (Section 3) for the sugar portion of compound **3** and comparison with those of **2** revealed that these compounds had the same saccharide chain linked at C-3. From 1D-TOCSY, DQF-COSY, HSQC, and HMBC experiments it was possible to deduce the occurence of a β-xylopyranosyl unit at C-16 (cross-peak between δ 4.12 and 83.1 ppm). The configuration of the sugar units was determined as reported for compound **1**. Therefore, compound **3** was identified as (22*S*,25*S*)-16-*O*-β-D-xylopyranosyl-5α-cholestan-3β,16β,22,26-tetraol-3-*O*-β-D-glucopyranosyl-(1 → 2)-*O*-[β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside.

Compound **4** had the molecular formula $C_{51}H_{84}O_{24}$, as determined by ^{13}C-, ^{13}C-DEPT, positive-ion ESI-MS, and elemental analysis. The ^{13}C NMR showed 51 signals of which 27 were assigned to an aglycone moiety and 24 to the saccharide portion. The ^{1}H NMR spectra of **4** (see Section 3) showed two three-proton singlet signals at δ 0.82 and 0.92, two three-proton doublet signals at δ 0.83 ($J = 6.5$ Hz) and 0.98 ($J = 6.0$ Hz), and three methine proton signals indicative of secondary alcoholic function at δ 3.50 (1H, *m*, H-3), 3.87 (1H, *m*, H-2), and 4.40 (1H, *ddd*, $J = 9.5$, 7.0, 5.0 Hz, H-16). The remaining resonances in the ^{1}H and ^{13}C NMR spectra were completely in accordance with those of a 25*S*-5α-spyrostane type sapogenol glycosilated at C-3 (Agrawal et al., 1985). Thus, the genin structure of **4** was assigned as (25*S*)-5α-spirostan-2α,3β-diol (neogitogenin). Comparison of the NMR spectroscopic data of compound **4** (Section 3) with those of **1** showed these to be identical in the sugar portion linked at C-3. Therefore, the structure of (25*S*)-5α-spirostan-2α,3β-diol-3-*O*-β-D-galactopyranosyl-(1 → 2)-*O*-[β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside was assigned to compound **4**.

Compound **5** ($C_{51}H_{84}O_{23}$) was identified as a further spirostanol derivative possessing a saccharide chain at C-3. Comparison of the NMR spectroscopic data of compound **5** (Section 3) with those of **4** showed these to be identical in the sugar portion but different in the aglycone moiety. In particular, the spectroscopic data for the aglycone moiety of **5** were identical to those of (25*S*)-5α-spirostan-3β-ol (neotigogenin) (Agrawal et al., 1985). Thus, compound **5** was determined to be (25*S*)-5α-spirostan-3β-ol-3-*O*-β-D-galactopyranosyl-(1 → 2)-*O*-[β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside.

The ESI-MS of compound **6** ($C_{32}H_{52}O_8$, as determined by ^{13}C-, ^{13}C-DEPT, positive-ion ESI-MS, and elemental analysis) showed the $[M+Na]^+$ ion at *m/z* 587 and a fragmentation pattern due to the loss of one pentose unit (*m/z* 455 $[M+Na-132]^+$). Analysis of ^{1}H and ^{13}C NMR data (see Section 3) of the aglycon portion of **6** in comparison with those of **3** suggested that the β-hydroxy group at C-3 gave place to an α,β-unsaturated carbonyl function in **6**. Thus the aglycone of **6** was identified as (22*S*,25*S*)-16β,22,26-trihydroxycholest-4-en-3-one. Identification of the saccharide unit was performed by analysis of NMR data: the chemical shifts, the multiplicity of signals, and the values of the coupling constants were in agreement with the presence of a β-xylopyranoside portion linked at C-16 of the aglycon as revealed from the HMBC cross-peak between H-1_{xyl} (δ 4.16) and C-16 (83.1 ppm). After hydro-

lysis of **6** with 1 N HCl and its trimethylsilylation for GC analysis, the retention time of the sugar was comparable with that of an authentic sample of D-xylose. On the basis of these data, **6** was identified as (22*S*,25*S*)-16β,22,26-trihydroxycholest-4-en-3-one-16-*O*-β-D-xylopyranoside.

The presence of steroidal glycosides having furostane, cholestane, and spirostane skeleton in *T. alatus* is in accordance with literature data, reporting that these compounds are typical metabolites of *Tribulus* genus (Achenbach et al., 1994, 1996; Bedir et al., 2002). In particular, pentandroside A and (25*S*)-5α-spirostan-3β-ol-3-*O*-β-D-glucopyranosyl-(1 → 2)-*O*-[β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside were previously isolated also from *T. pentandrus* and *T. cistoides*, respectively (Hamed et al., 2004; Achenbach et al., 1996). The aglycon (22*S*,25*S*)-16β,22,26-trihydroxycholest-4-en-3-one occurred only in *T. pentandrus* and *T. cistoides*, while saponins with furostane and spirostane skeletons are present in all *Tribulus* species previously studied. All flavonoids isolated are also characteristic components of the genus. Tribuloside was identified before from *T. terrestris* (Bhutani et al., 1969); kaempferol 3-*O*-(3″-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside and kaempferol 3-*O*-β-D-glucopyranoside were obtained from *T. pentandrus*, while isorhamnetin 3-*O*-(6″-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside and quercetin 3-*O*-β-D-glucopyranoside were isolated from both the previous cited species (Saleh et al., 1982). An exception is kaempferol 3-*O*-(3″,6″-di-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside that to our knowledge is the first example of di-coumaroylated flavonol glycoside isolated from a *Tribulus* species.

3. Experimental

3.1. General procedures

Optical rotations were measured on a Perkin–Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. Elemental analysis was obtained from a Carlo Erba 1106 elemental analyzer. UV spectra were recorded on a Perkin–Elmer-Lambda 12 spectrophotometer. All the 2D NMR spectra were acquired in CD_3OD in the phase-sensitive mode with the transmitter set at the solvent resonance and TPPI (time proportional phase increment) used to achieve frequency discrimination in the ω_1 dimension. The standard pulse sequence and phase cycling were used for DQF-COSY, TOCSY, HSQC, HMBC, and ROESY experiments. The NMR data were processed on a Silicon Graphic Indigo2 Workstation using UXNMR software. Column chromatographies were performed over Sephadex LH-20 (Pharmacia, Uppsala, Sweden), silica gel (63–200 μm, Merck, Darmstadt, Germany), polyamide S6 (Riedel-de Haën AG, Seelze, Hannover), and cellulose pulver S (Riedel-de Haën AG, Seezle-Hannover, Germany); HPCPC (High Performance Centrifugal Partition Chromatography) was performed on an EverSeiko CPC240 chromatograph; HPLC separations were conducted on a Shimadzu LC-8A (Shimadzu Corporation, Kyoto, Japan) series pumping system equipped with a Waters R401 refractive index detector and with a Waters μ-Bondapak C_{18} column (Waters, Milford, MA). TLC was performed on precoated Kieselgel 60 F_{254} plates (Merck, Darmstadt, Germany); compounds were detected by $Ce(SO_4)_2/H_2SO_4$ (Sigma–Aldrich, Milano, Italy) solution. GC analyses were performed using a Dani GC 1000 instrument on a L-CP-Chirasil-Val column (0.32 mm × 25 m).

3.2. Plant material

The aerial parts of *T. alatus* Del. were collected in May 2002 in Nasr-City, Cairo, Egypt, and identified by Prof. Nabil El Hadidi of the Faculty of Science, Cairo University, Egypt. A voucher specimen (number 3978/1) is deposited at Herbarium Horti Botanici Pisani (Flora Aegyptiaca), Pisa, Italy.

3.3. Extraction and isolation

The dried aerial parts of *T. alatus* (2 kg) were finely powdered and exhaustively extracted with 70% MeOH, by maceration at room temperature. The crude methanolic extract was evaporated to dryness under reduced pressure (435 g), suspended in distilled water, and successively partitioned between chloroform, ethylacetate, and *n*-butanol. Each extract was collected and evaporated to dryness to give chloroform (15 g), ethyl acetate (8 g), and *n*-butanol (34 g) extracts, respectively. The *n*-butanol extract (34 g) was dissolved in the least amount of methanol and the saponin fraction was precipitated by addition of acetone. The residue obtained (9.8 g) was subjected to column chromatography using silica gel and eluting with chloroform followed by increasing concentrations of MeOH (between 1% and 50%) in $CHCl_3$. Fractions of 30 ml were collected, analyzed by TLC (silica gel plates, in $CHCl_3$ or mixtures $CHCl_3$–MeOH 99:1, 49:1, 97:3, 9:1, 4:1), and grouped into 16 fractions (A–P). Fractions *I* and *J* (1.2 g) were subjected to Sephadex LH-20 column using *n*-BuOH–*i*-PrOH–H_2O (4:1:5, upper phase) as mobile phase to give (25S)-5α-spirostan-3β-ol-3-*O*-β-D-glucopyranosyl-(1 → 2)-*O*-[β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (38.6 mg) together with one fraction (600 mg) that was further separated by RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (4:1) to give pure compounds **4** (3.5 mg, $t_R = 16$ min) and **5** (2.8 mg, $t_R = 30$ min). Fraction *K* (1.3 g) was purified by HPCPC with $CHCl_3$–MeOH–H_2O-*i*-PrOH (5:6:4:1) in which the stationary phase consisted of the upper phase (descending mode, flow rate 3 ml min^{-1}, 900 rpm rotor speed), with fractions of 3 ml collected. HPCPC fractions 70–77 (136 mg) and 136–170 (20 mg) were subjected separately to RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (3:2) to yield pure compounds **3** (2.0 mg, $t_R = 5$ min) and **2** (10.2 mg, $t_R = 8$ min) from fraction 70–77 and with

MeOH–H_2O (7.5:2.5) to give pure compound **1** (2.5 mg, $t_R = 11$ min) from fraction 136–170. The ethyl acetate extract (8 g) was purified by cellulose column to obtain 8 major fractions. Fraction 3 (550 mg) of the cellulose column was subjected to Sephadex LH-20 chromatography using *n*-BuOH–*i*-PrOH–H_2O (4:1:5, upper phase) as mobile phase to give 11 groups. Group 2 (260 mg) was purified by silica gel column with $CHCl_3$–MeOH 9:1 to give pure pentandroside A (14.5 mg) and compound **6** (7.6 mg). Group 7 (30 mg) was further separated by RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (1:1) to yield quercetin 3-*O*-β-D-glucopyranoside (5.0 mg, $t_R = 11$ min). Group 6 (230 mg) of the Sephadex column was subjected to silica gel column to yield tribuloside (20 mg) together with three fractions. Fractions 1 (27 mg) and 3 (25 mg) were chromatographed over RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (3:2) to give pure isorhamnetin 3-*O*-(6″-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside (7.1 mg, $t_R = 13$ min) and kaempferol 3-*O*-(3″-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside (1.8 mg, $t_R = 17$ min) from fraction 1 and with MeOH–H_2O (1:1) to give pure kaempferol 3-*O*-β-D-glucopyranoside (5.2 mg, $t_R = 10$ min) from fraction 3. Fraction 6 (170 mg) of the cellulose column was purified by HPCPC with $CHCl_3$–MeOH–H_2O (7:10:8) in which the stationary phase consisted of the upper phase (descending mode, flow rate 3 ml min^{-1}, 900 rpm rotor speed), with fractions of 3 ml collected. HPCPC fractions 60–61 (25 mg) was further separated by RP-HPLC on a C_{18} μ-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 ml min^{-1}) with MeOH–H_2O (6.5:3.5) to yield pure kaempferol 3-*O*-(3″,6″-di-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside (10.0 mg, $t_R = 12$ min).

3.3.1. (25S)-26-O-β-D-Glucopyranosyl-5α-furostan-3β,22α,26-triol-3-O-β-D-galactopyranosyl-(1 → 2)-O-[β-D-glucopyranosyl-(1 → 3)]-O-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (1)

White amorphous powder, $[\alpha]_D^{25}$: −61° (*c* 0.1, MeOH); ESI-MS in positive ion mode (*m*/*z*): 1267 $[M+Na]^+$, 1249 $[M+Na-18]^+$, 1105 $[M+Na-162]^+$, 943 $[M+Na-162-162]^+$, 781 $[M+Na-162-162-162]^+$, 619 $[M+Na-162-162-162-162]^+$; elemental analysis: C 54.93%, H 7.79% calcd. for $C_{57}H_{96}O_{29}$, C 54.97%, H 7.77%; ^1H NMR of the aglycone moiety (CD_3OD, 600 MHz): δ 0.83 (3H, *s*, Me-18), 0.88 (3H, *s*, Me-19), 0.97 (3H, *d*, *J* = 6.5 Hz, Me-27), 1.02 (3H, *d*, *J* = 6.0 Hz, Me-21), 2.20 (1H, *m*, H-20), 3.45 (1H, *dd*, *J* = 11.0, 6.0 Hz, H-26a), 3.70 (1H, *m*, H-3), 3.76 (1H, *dd*, *J* = 11.0, 6.5 Hz, H-26b), 4.38 (1H, *ddd*, *J* = 9.0, 7.0, 4.5 Hz, H-16); ^{13}C NMR of the aglycone portion (CD_3OD, 600 MHz): δ 12.9 (C-19), 16.0 (C-21), 16.8 (C-18), 17.2 (C-27), 22.3 (C-11), 28.7 (C-6), 29.0 (C-24), 30.5 (C-2), 31.4 (C-23), 32.7 (C-15), 33.4 (C-7), 34.8 (C-25), 35.2 (C-4), 36.5 (C-8), 38.2 (C-1), 38.3 (C-10), 41.1 (C-12), 41.2 (C-20), 41.4 (C-13), 46.2 (C-5), 55.9 (C-9), 57.5 (C-14), 65.3 (C-17), 76.0 (C-26), 79.4 (C-3), 83.5 (C-16), 112.8 (C-22). ^1H and ^{13}C NMR of the sugar moiety: see Table 1.

3.3.2. (25S)-26-O-β-D-Glucopyranosyl-5α-furostan-3β,22α,26-triol-3-O-β-D-glucopyranosyl-(1 → 2)-O-[β-D-glucopyranosyl-(1 → 3)]-O-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (2)

White amorphous powder, $[\alpha]_D^{25}$: −94° (*c* 0.1, MeOH); ESI-MS in negative ion mode (*m*/*z*): 1243 $[M-H]^-$, 1081 $[M-H-162]^-$, 919 $[M-H-162-162]^-$, 757 $[M-H-162-162-162]^-$, 595 $[M-H-162-162-162-162]^-$; elemental analysis: C 55.00%, H 7.75% calcd. for $C_{57}H_{96}O_{29}$, C 54.97%, H 7.77%; ^1H and ^{13}C NMR of the aglycone moiety were superimposable with those reported for compound **1**; ^1H and ^{13}C NMR of the sugar moiety: see Table 1.

3.3.3. (22S,25S)-16-O-β-D-Xylopyranosyl-5α-cholestan-3β,16β,22,26-tetraol-3-O-β-D-glucopyranosyl-(1 → 2)-O-[β-D-glucopyranosyl-(1 → 3)]-O-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (3)

White amorphous powder, $[\alpha]_D^{25}$: −50° (*c* 0.1, MeOH); ESI-MS in negative ion mode (*m*/*z*): 1215 $[M-H]^-$, 1053 $[M-H-162]^-$, 891 $[M-H-162-162]^-$, 729 $[M-H-162-162-162]^-$, 597 $[M-H-162-162-162-132]^-$; elemental analysis: C 55.27%, H 7.94% calcd. for $C_{56}H_{96}O_{28}$, C 55.25%, H 7.95%; ^1H NMR of the aglycone moiety (CD_3OD, 600 MHz): δ 0.90 (3H, *s*, Me-19), 0.92 (3H, *s*, Me-18), 0.93 (3H, *d*, *J* = 6.5 Hz, Me-21), 0.95 (3H, *d*, *J* = 6.5 Hz, Me-27), 3.35 (1H, *dd*, *J* = 11.0, 6.0 Hz, H-26a), 3.48 (1H, *dd*, *J* = 11.0, 6.5 Hz, H-26b), 3.70 (1H, *m*, H-3), 3.72 (1H, *br d*, *J* = 8.0 Hz, H-22), 4.16 (1H, *ddd*, *J* = 9.0, 7.0, 4.5 Hz, H-16); ^{13}C NMR of the aglycone portion (CD_3OD, 600 MHz): δ 12.2 (C-21), 13.0 (C-19), 13.8 (C-18), 17.3 (C-27), 21.8 (C-11), 29.6 (C-6), 30.7 (C-2), 31.0 (C-24), 33.2 (C-23), 33.3 (C-7), 35.3 (C-4), 36.3 (C-20), 36.6 (C-8), 37.1 (C-25), 37.2 (C-1), 37.6 (C-15), 39.0 (C-10), 41.4 (C-12), 43.0 (C-13), 46.0 (C-5), 56.0 (C-9), 56.1 (C-14), 59.1 (C-17), 68.5 (C-26), 74.4 (C-22), 79.4 (C-3), 83.5 (C-16); ^1H and ^{13}C NMR of the sugar moiety at C-3 were superimposable with those of compound **2**; ^1H NMR of the xylose moiety at C-16 (CD_3OD, 600 MHz): δ 3.16 (1H, *dd*, *J* = 9.5, 7.5 Hz, H-2), 3.18 (1H, *dd*, *J* = 12.0, 5.0, H-5b), 3.31 (1H, *t*, *J* = 9.5 Hz, H-3), 3.48 (1H, *m*, H-4), 3.86 (1H, *t*, *J* = 11.0 Hz, H-5a), 4.12 (1H, *d*, *J* = 7.6 Hz, H-1); ^{13}C NMR of the xylose moiety at C-16 (CD_3OD, 600 MHz): δ 66.9 (C-5), 71.3 (C-4), 75.4 (C-2), 78.0 (C-3), 107.3 (C-1).

3.3.4. (25S)-5α-Spirostan-2α,3β-diol-3-O-β-D-galactopyranosyl-(1 → 2)-O-[β-D-glucopyranosyl-(1 → 3)]-O-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (4)

White amorphous powder, $[\alpha]_D^{25}$: −45° (*c* 0.1, MeOH); ESI-MS in positive ion mode (*m*/*z*): 1103 $[M+Na]^+$, 941 $[M+Na-162]^+$, 779 $[M+Na-162-162]^+$, 617 $[M+Na-162-162-162]^+$; elemental analysis: C 56.61%, H 7.86% calcd. for $C_{51}H_{84}O_{24}$, C 56.65%, H 7.83%; ^1H NMR of the aglycone moiety (CD_3OD, 600 MHz): δ 0.82 (3H, *s*, Me-18), 0.83 (3H, *d*, *J* = 6.5 Hz, Me-27), 0.92 (3H, *s*, Me-19), 0.98 (3H, *d*, *J* = 6.0 Hz, Me-21), 3.33 (1H, *dd*, *J* = 11.0,

5.5 Hz, H-26a), 3.45 (1H, *dd*, $J = 11.0$, 6.5 Hz, H-26b), 3.50 (1H, *m*, H-3), 3.87 (1H, *m*, H-2), 4.40 (1H, *ddd*, $J = 9.5$, 7.0, 5.0 Hz, H-16); ^{13}C NMR of the aglycone portion (CD_3OD, 600 MHz): δ 13.5 (C-19), 14.6 (C-21), 17.0 (C-18), 17.2 (C-27), 22.1 (C-11), 26.2 (C-24), 26.8 (C-23), 27.0 (C-25), 29.4 (C-6), 32.3 (C-15), 32.8 (C-4), 33.6 (C-7), 35.3 (C-8), 37.4 (C-10), 40.5 (C-12), 40.9 (C-13), 42.5 (C-20), 45.5 (C-5), 45.7 (C-1), 55.6 (C-9), 57.3 (C-14), 63.4 (C-17), 67.4 (C-26), 70.0 (C-2), 81.9 (C-16), 84.4 (C-3), 110.3 (C-22); ^{1}H and ^{13}C NMR of the sugar moiety at C-3 were superimposable with those of compound **1**.

3.3.5. (25S)-5α-Spirostan-3β-ol-3-O-β-D-galactopyranosyl-(1 → 2)-O-[β-D-glucopyranosyl-(1 → 3)]-O-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (5)

White amorphous powder, $[\alpha]_D^{25}$: −23° (*c* 0.1, MeOH); ESI-MS in positive ion mode (*m/z*): 1087 $[M+Na]^+$, 925 $[M+Na-162]^+$, 763 $[M+Na-162-162]^+$, 601 $[M+Na-162-162-162]^+$; elemental analysis: C 57.49%, H 7.96% calcd. for $C_{51}H_{84}O_{23}$, C 57.51%, H 7.95%; ^{1}H NMR of the aglycone moiety (CD_3OD, 600 MHz): δ 0.82 (3H, *s*, Me-18), 0.88 (3H, *s*, Me-19), 0.83 (3H, *d*, $J = 6.5$ Hz, Me-27), 1.00 (3H, *d*, $J = 6.5$ Hz, Me-21), 3.34 (1H, *dd*, $J = 11.0$, 5.5 Hz, H-26a), 3.48 (1H, *dd*, $J = 11.0$, 6.5 Hz, H-26b), 3.70 (1H, *m*, H-3), 4.41 (1H, *ddd*, $J = 9.0$, 7.0, 4.5 Hz, H-16); ^{13}C NMR of the aglycone portion (CD_3OD, 600 MHz): δ 12.6 (C-19), 15.0 (C-21), 16.8 (C-18 and C-27), 22.3 (C-11), 26.0 (C-24), 26.7 (C-23), 26.9 (C-25), 29.5 (C-6), 30.3 (C-2), 32.4 (C-15), 33.2 (C-7), 35.2 (C-4), 36.6 (C-8), 37.0 (C-10), 37.9 (C-1), 40.6 (C-12), 41.4 (C-13), 42.6 (C-20), 45.9 (C-5), 55.7 (C-9), 57.3 (C-14), 63.6 (C-17), 67.6 (C-26), 79.2 (C-3), 82.0 (C-16), 110.5 (C-22); ^{1}H and ^{13}C NMR of the sugar moiety at C-3 were superimposable with those of compound **1**.

3.3.6. (22S,25S)-16β,22,26-Trihydroxycholest-4-en-3-one-16-O-β-D-xylopyranoside (6)

White amorphous powder, $[\alpha]_D^{25}$: −12° (*c* 0.1, MeOH); UV_{max} (MeOH): 242 (log e 3.96); ESI-MS in positive ion mode (*m/z*): 587 $[M+Na]^+$, 527 $[M+Na-60]^+$, 455 $[M+Na-132]^+$; elemental analysis: C 68.04%, H 9.30% calcd. for $C_{32}H_{52}O_8$, C 68.06%, H 9.28%; ^{1}H NMR (CD_3OD, 600 MHz): δ 0.91 (3H, *d*, $J = 6.5$ Hz, Me-27), 0.93 (3H, *d*, $J = 6.5$ Hz, Me-21), 0.94 (3H, *s*, Me-18), 1.23 (3H, *s*, Me-19), 3.18 (1H, *dd*, $J = 9.0$, 7.5 Hz, H-2_{xyl}), 3.23 (1H, *dd*, $J = 11.0$, 5.0 Hz, H-$5b_{xyl}$), 3.31 (1H, *t*, $J = 9.0$ Hz, H-3_{xyl}), 3.37 (1H, *dd*, $J = 11.0$, 5.5 Hz, H-26a), 3.48 (1H, *m*, H-4_{xyl}), 3.49 (1H, *dd*, $J = 11.0$, 6.5 Hz, H-26b), 3.75 (1H, *br d*, $J = 8.0$ Hz, H-22), 3.82 (1H, *t*, $J = 11.0$ Hz, H-$5a_{xyl}$), 4.16 (1H, *d*, $J = 7.5$ Hz, H-1_{xyl}), 4.20 (1H, *ddd*, $J = 9.0$, 7.5, 4.5 Hz, H-16), 5.72 (1H, br s, H-4); ^{13}C NMR (CD_3OD, 600 MHz): δ 12.0 (C-21), 13.5 (C-18), 17.2 (C-27), 17.6 (C-19), 21.8 (C-11), 31.0 (C-24), 33.2 (C-23), 33.3 (C-7), 33.9 (C-2), 34.6 (C-6), 36.1 (C-20), 36.5 (C-1), 36.6 (C-8), 37.1 (C-25), 37.3 (C-15), 39.9 (C-10), 40.8 (C-12), 43.2 (C-13), 55.3 (C-9 and C-14), 58.6 (C-17), 66.7 (C-5_{xyl}), 68.2 (C-26), 71.2 (C-4_{xyl}), 74.1 (C-22), 75.2 (C-2_{xyl}), 78.0 (C-3_{xyl}), 83.1 (C-16), 107.2 (C-1_{xyl}), 123.9 (C-4), 176.0 (C-5), 203.0 (C-3).

The NMR and ESI-MS data of all other compounds match literature reports: pentandroside A (Hamed et al., 2004), (25*S*)-5α-spirostan-3β-ol-3-*O*-β-D-glucopyranosyl-(1 → 2)-*O*-[β-D-glucopyranosyl-(1 → 3)]-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (Achenbach et al., 1994), isorhamnetin 3-*O*-(6″-*E*-*p*-coumaroyl)-β-D-glucopyranoside (Romussi et al., 1988), kaempferol 3-*O*-(3″,6″-di-*O*-*E*-*p*-coumaroyl)-β-D-glucopyranoside, kaempferol 3-*O*-(3″-*E*-*p*-coumaroyl)-β-D-glucopyranoside (Liu et al., 1999), tribuloside (Leitão et al., 2000), quercetin 3-*O*-β-D-glucopyranoside, and kaempferol 3-*O*-β-D-glucopyranoside (Agrawal, 1989).

3.4. Acid hydrolysis of compounds 1–6 and GC analysis to determine absolute configuration of sugars

A solution (2.0 mg each) of compounds **1**–**6** in 1 N HCl (1 ml) was stirred at 80 °C in a stoppered reaction vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. Each residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 ml), and the solution was stirred at 60 °C for 5 min. After drying the solution, the residue was partitioned between water and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using an L-CP-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. Peaks of the hydrolysate were detected by comparison with retention times of authentic samples of D-glucose, D-xylose, and D-galactose (Sigma–Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

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