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FULL PAPER CHEMISTRY & BIODIVERSITY

Five New Furostanol Glycosides from the Fruits of *Tribulus terrestris* with NO Production Inhibitory Activity

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Eight furostanol glycosides including five undescribed compounds, named tribufurostanosides A-E (**1**-**5**), and three known ones (**6**-**8**) were isolated from the fruits of *Tribulus terrestris* L. Their chemical structures were determined by the IR, HR-ESI-MS, 1D-, and 2D-NMR spectra. Furostanols **1**-**8** significantly inhibited nitric oxide production in LPS activated RAW 264.7 cells with IC_{50} values ranging from 14.2 to 64.7 μM, compared to that of the positive control compound, dexamethazone (IC_{50} 13.6 μM).

Introduction

Tribulus terrestris is a valuable medicinal plant in Vietnam used in folk medicine to treat kidney tonic, eye pain, acne, back pain, red throat, and dysentery.[1] The leaves and fruits of *T. terrestris* have anti-inflammatory, antioxidant, antidiabetic, anticancer, testosterone-boosting, and liver protective effects.[2] In our previous paper, fifteen compounds including four undescribed spirostan glycosides have been reported from the branches and leaves of this plant.[3] This paper further reported eight furostanol glycosides including five undescribed compounds from MeOH extract of the fruits of *T. terrestris* and their NO production inhibition activity in LPS activated RAW 264.7 cells.

Results and Discussion

The *T. terrestris* fruits were extracted with methanol and further isolated using various chromatographic methods to get eight furostanol saponins (**1**-**8**). The known compounds were identified to be (25*R*)-26-*O*-(*β*-D-glucopyranosyl)-5*α*-furostan-12-one-3*β*,22*α*,26-triol 3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside (**6**),[4] (25*R*)-26-*O*-(*β*-D-glucopyranosyl)-5*α*-furostan-20(22)-ene-12-one-3*β*,26-diol 3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside (**7**),[5, 6] and (25*R*)-26-*O*-(*β*-D-glucopyranosyl)-5*α*-furostan-12-one-3*β*,22*α*,26-triol 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyra-noside (**8**),[7] by 1D- and 2D-NMR spectral data in comparison with those reported in the literature. Compounds **6** and **8** have been isolated from the fruits of *T. terrestris* and compound **7** has been reported from the leaves of this plant.

Compound **1** (Figure 1) was isolated as a white amorphous powder, which showed IR absorption bands at 3401, 1707, 1448, and 1068 cm^{-1}, suggesting for the presence of hydroxy, carbonyl, double bond, and C-O-C functionalities, respectively. The molecular formula of **1** was $C_{51}H_{82}O_{25}$, as determined from ion peaks at *m/z* 1093.5042 $[M-H]^-$ (calcd. for $[C_{51}H_{81}O_{25}]^-$: 1093.5072) and *m/z* 1129.4835 $[M+^{37}Cl]^-$ (calcd. for $[C_{51}H_{82}O_{25}{}^{35}Cl]^-$: 1129.4839) on the HR-ESI-MS, indicating eleven degrees of unsaturation. The ^1H NMR spectrum of **1** revealed three quaternary methyl [δ_H 0.96, 1.18, 1.40 (each 3H, s)], one secondary methyl (δ_H 0.97, d, J = 6.6 Hz), one olefinic proton at δ_H 4.35 (t, J = 7.8 Hz), two methine carbinol groups [δ_H 3.68 (m, H-3) and 4.75 (m, H-16)], and one oxygenated methylene group (δ_H 3.69 and 3.44, H_2-26), which were assigned for the aglycone of **1**.[8-10] In addition, four anomeric

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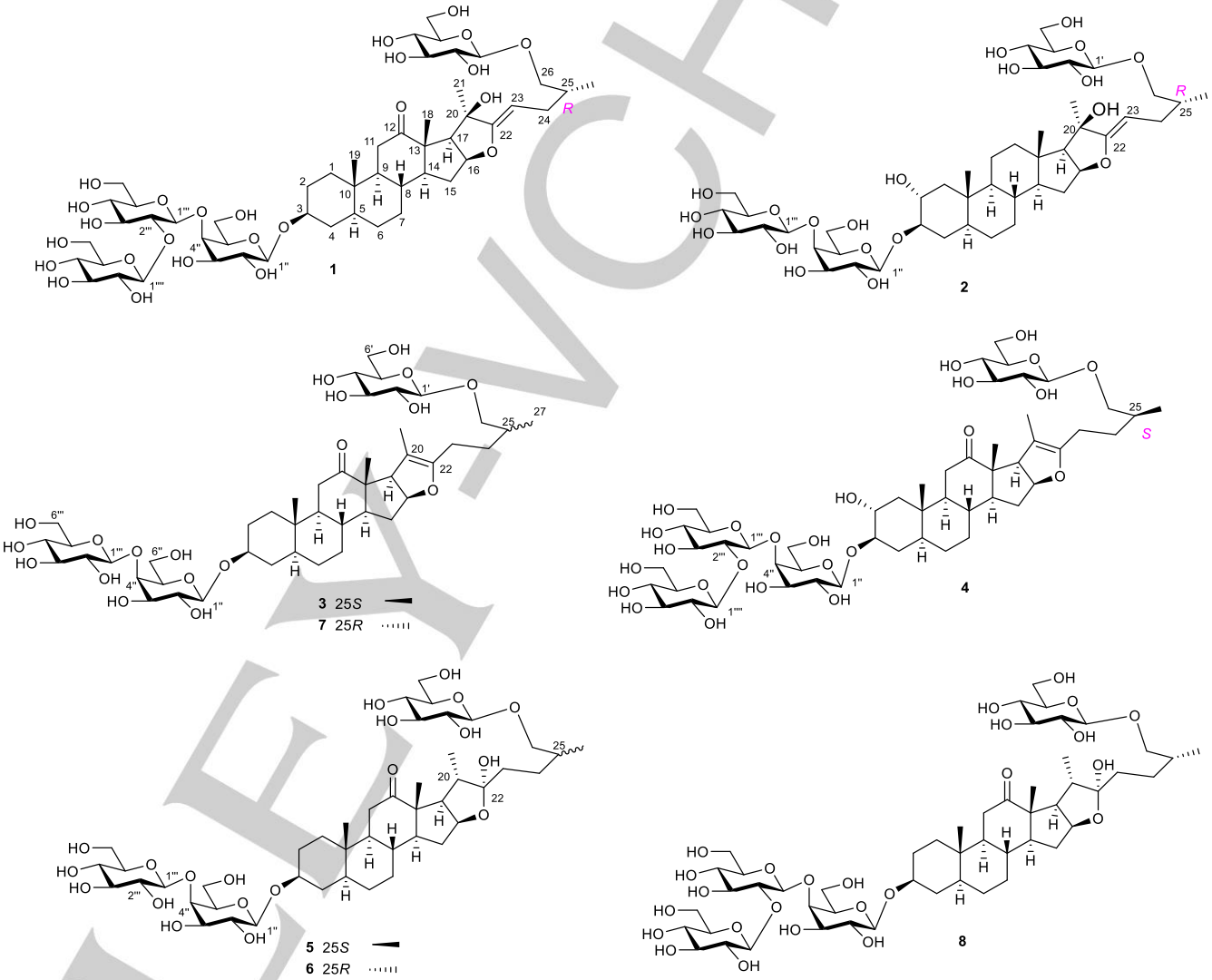

Figure 1. Chemical structures of compounds **1** – **8**

protons were identified at δ_H 4.24 (H-1′), 4.39 (H-1″), 4.01 (H-1‴), and 4.68 (H-1⁗) (each d, J = 7.8 Hz), suggesting four sugar moieties.[6] The ^{13}C NMR and HSQC spectra of **1** indicated 51 carbons, including 24 of four sugar moieties and 27 of a furostanol aglycone (Tables 1 and 2).[10] The NMR data of aglycone of **1** were similar to the corresponding data of tuberoside I,[11] except for the methylene group at C-12 was replaced by a ketone group (δ_C 215.2). The NMR assignments were revealed by HSQC, ^{1}H-^{1}H COSY, and HMBC correlations (Figure 2, Tables 1 and 2). The ketone group at C-12, the hydroxy group at C-20, and the double bond at C-22/C-23 were confirmed by HMBC correlations from H_3-18 (δ_H 1.18) to C-12 (δ_C 215.2)/C-13 (δ_C 55.8)/C-14 (δ_C 58.2)/C17 (δ_C 59.6), from H_3-21 (δ_H 1.40) to C-17 (δ_C 59.6)/C-20 (δ_C 83.6)/C-22 (δ_C 157.8), and from H-23 (δ_H 4.35) to C-20/C-22. The C-26 carbon signal was shifted downfield (δ_C 76.0) suggesting that one glucose moiety (δ_C 104.6, 75.2, 78.2, 71.7, 77.9, 62.8) was linked to C-26 by an ether linkage. This was further indicated by HMBC correlations from H-1′ (δ_H 4.24) to C-26 (δ_C 76.0) and from H_2-26 (δ_H 3.69/3.44) to C-1′ (δ_C 104.6). The remaning sugar moieties were similar to that of (25*R*)-26-*O*-(*β*-D-glucopyranosyl)-5*α*-furostan-12-one-3*β*,22*α*,26-triol 3-*O*-*β*-D-gluco-pyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside **8**)[7] suggested by the ^{1}H, ^{13}C NMR, HSQC, ^{1}H-^{1}H COSY, and HMBC correlations in comparison with the reported data (Tables 1 and 2, Figure 2). All the sugar linkages must be in the *β*-form as suggested from the large coupling constants (J = 7.8 Hz) of the anomeric protons. Acid hydrolysis of **1** gave D-glucose and D-galactose, which were identified by comparison with authentic samples via TLC, and from the positive sign of the optical rotations.[12, 13] The small difference between the two H-26 proton signals (δ_H 3.69 and 3.44, Δ = 0.25 ppm) suggested (25*R*)-configuration.[10, 13, 14] In the NOESY spectrum, the cross peaks of H-3 (δ_H 3.68) with H-5 (δ_H 1.16), H-5 with H-9 (δ_H 1.12), H_3-21 (δ_H 1.18) with H-16*α* (δ_H 4.75) suggested *anpha/axial* orientation for H-3 and H-5, and *β*-OH group at C-20

(Figuure 3).[10] In addition, NOESY cross peak of H_3-21 and H_2-23 (δ_H 4.35) suggested *Z*-configuration of the $\Delta^{22,23}$ double bond.[11] From the above evidence, compound **1** was determined to be (25*R*,22*Z*)-26-*O*-(*β*-D-glucopyranosyl)-5*α*-furostan-22-ene-12-one-3*β*,20*β*,26-triol 3-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopy-ranoside and named tribufurostanoside A.

Table 1. ^{13}C NMR spectral data for compounds **1** - **5** in CD_3OD

No.	1	2	3	4	5	No.	1	2	3	4	5
1	37.8	45.8	37.8	45.4	37.7	**26-*O*-glc**					
2	30.3	71.5	30.3	71.2	30.3	1′	104.6	104.5	104.7	104.7	104.6
3	79.1	85.2	79.2	84.8	79.2	2′	75.2	75.2	75.2	75.2	75.2
4	35.2	34.1	35.3	34.0	35.3	3′	78.2	78.2	78.1	78.2	78.1
5	45.8	45.8	45.8	45.6	45.8	4′	71.7	71.7	71.7	71.7	71.7
6	29.6	29.0	29.6	28.8	29.6	5′	77.9	77.9	77.9	77.9	77.9
7	32.4	34.1	32.9	32.7	32.1	6′	62.8	62.8	62.8	62.8	62.8
8	35.2	35.2	35.5	34.8	35.5	**3-*O*-gal**					
9	57.5	55.5	57.2	56.9	57.1	1′′	102.7	103.2	102.9	102.9	102.9
10	37.4	37.9	37.4	38.4	37.3	2′′	73.2	73.0	73.2	72.9	73.2
11	38.5	21.8	39.0	39.1	38.8	3′′	75.6	75.8	75.5	75.6	75.5
12	215.2	40.4	216.3	215.8	216.0	4′′	80.5	79.4	79.2	80.4	79.2
13	55.8	41.7	58.7	57.1	56.8	5′′	75.2	75.1	75.3	75.5	75.2
14	58.2	57.8	55.7	55.5	57.1	6′′	60.9	61.5	61.3	61.2	61.3
15	33.6	33.1	34.5	34.5	32.7	**4′′-*O*-glc**					
16	83.5	84.9	84.1	84.1	80.7	1′′′	104.9	106.2	106.1	104.3	106.1
17	59.6	68.3	57.1	58.7	55.3	2′′′	85.0	75.8	75.7	84.7	75.6
18	14.2	14.2	14.6	14.6	16.6	3′′′	78.2	78.3	78.3	78.2	78.3
19	12.2	13.7	12.2	13.2	12.2	4′′′	70.8	72.0	72.0	70.9	72.0
20	83.6	78.1	104.3	104.7	41.4	5′′′	77.6	78.2	78.2	77.7	78.0
21	16.0	21.4	11.5	11.5	14.7	6′′′	62.0	63.0	63.2	62.2	63.1
22	157.8	162.9	153.9	154.0	111.8	**2′′′-*O*-glc**					
23	97.9	92.6	24.1	24.1	36.9	1′′′′	106.2			106.1	
24	30.0	30.2	32.0	32.0	28.6	2′′′′	76.3			76.2	
25	35.4	35.5	34.3	34.3	34.9	3′′′′	78.7			78.7	
26	76.0	76.1	76.0	76.0	76.0	4′′′′	71.8			71.7	
27	17.6	17.6	17.2	17.2	17.4	5′′′′	77.9			77.9	
						6′′′′	63.3			63.2	

[*] Overlapped signals, [a] Recorded in 150 MHz, [b] Recorded in 600 MHz, glc: *β*-D-glucopyranose, gal: *β*-D-galactopyranose.

The IR spectrum of **2** suggested the presence of hydroxy (3399 cm^{-1}), double bond (1449 cm^{-1}), and ether (1075 cm^{-1}) functionalities. The HR-ESI-MS exhibited a quasi molecular ion peak $[M+Na]^+$ at *m/z* 957.4687 (calcd. for $[C_{45}H_{74}O_{20}Na]^+$: 957.4666, Δ=+2.2 ppm), determining the molecular formula of $C_{45}H_{74}O_{20}$ and nine degree of unsaturation. The NMR spectra of **2** were similar to those of **1** except the lost of the ketone and signals of one sugar, and the additional signals due to a hydroxy group at C-2, suggesting a furostanol glycoside (Tables 1 and 2).[8-10] Three sugar moieties were identifed from the anomeric signals at (δ_C/δ_H) 104.5/4.25 (1H, d, *J* = 7.8 Hz), 103.2/4.37 (1H, d, J = 7.8 Hz), and 106.2/4.53 (1H, d, *J* = 7.8 Hz), indicating the lost of one glucose unit compared to **1**. The sugar moieties were confirmed as 26-*O*-*β*-D-glucopyranosyl and 3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside by comparing the NMR data of **2** with those of **1**, and further evident by HSQC, COSY, and HMBC spectra. Protons H-1′ (δ_H 4.25), H-1′′ (δ_H 4.37), and H-1′′′ (δ_H 4.53) showed HMBC correlations with C-26 (δ_C 76.1), C-3 (δ_C 85.2), and with C-4′′ (δ_C 79.4), respectively, confirming one glucose sugar linked to C-26, the other glucose sugar linked to C-4′′ of the galactose unit, and the galactose unit attached to C-3. The additional hydroxy group at C-2 was confirmed by COSY cross peaks of H-1/H-2/H-3/H-4, and the down field shift of C-1, C-2, and C-3 compared to those of **1** (Table 1). Furthermore, the carbon chemical shifts of the ring A perfectly matched those of cistocardin,[15] suggesting 2*α*-OH group and both H-2 and H-3 were in *axial* orientation. This was further supported by NOESY cross peaks of H-3/H-5 and H_3-19/H-2 (Figure 3). The 20-OH group and $\Delta^{22,23}$ double bond were confirmed by HMBC correlations as shown in Figure 2. The NOESY cross peak between H*α*-16 and H_3-21, and between H_3-21 and H-23 suggested 20*β*-OH group and *Z*-

Table 2. ^{1}H NMR spectral data for compounds **1** - **5** in CD_3OD

No.	1	2	3	4	5
1	1.02*/1.62-1.63 (m)	0.94*/2.00 (dd, 12.6; 4.8)	1.03*1.63-1.64 (m)	0.98*/1.88 (dd, 12.6 4.8)	1.02/1.62-1.63 (m)
2	1.53*/1.90-2.00 (m)	3.64-3.65 (m)	1.53*/1.90-2.00 (m)	3.67-3.68 (m)	1.53*/1.90-2.00 (m)
3	3.67-3.69 (m)	3.48-3.49 (m)	3.67-3.69 (m)	3.49-3.50 (m)	3.68-3.69 (m)
4	1.34 (dd, 11.4, 11.4)	1.39-1.40 (m)	1.35 (dd, 11.4, 11.4)	1.42-1.43 (m)	1.35 (dd, 11.4, 11.4)
	1.75-1.77 (m)	1.43-1.44 (m)	1.76-1.77 (m)	1.81-1.82 (m)	1.77 (m)
5	1.15-1.17 (m)	1.18-1.19 (m)	1.17-1.19 (m)	1.24-1.25 (m)	1.16-1.17 (m)
6	1.40 - 1.42 (m)	1.30*/1.39-1.42 (m)	1.40-1.42 (m)	1.39-1.45 (m)	1.40-1.42 (m)
7	1.02*/1.81-1.83 (m)	1.74*/2.15-2.16 (m)	1.02*/1.81-1.83 (m)	1.02*/1.81-1.83 (m)	1.02*/1.81-1.83 (m)
8	1.99-2.01 (m)	1.53-1.54 (m)	1.98-2.00 (m)	1.97-1.99 (m)	1.99-2.01 (m)
9	1.12-1.14 (m)	0.75-0.76 (m)	1.13-1.14 (m)	1.21-1.22 (m)	1.14-1.15 (m)
11	2.17 (dd, 13.8, 6.6)	1.40 (m)	2.21 (dd, 13.8 4.8)	2.22 (dd, 13.8, 5.4)	2.20 (dd, 14.4, 4.8)
	2.58 (dd, 13.8, 13.8)	1.57 (m)	2.54 (dd, 13.8 13.8)	2.58 (t, 13.8)	2.52 (dd, 14.4, 13.8)
12	-	1.28*/1.94-1.95 (m)	-	-	-
14	1.44-1.45 (m)	1.12-1.14 (m)	1.40-1.42 (m)	1.40-1.42 (m)	1.47-1.48 (m)
15	1.63-1.81 (m)	0.97-0.98 (m)/1.72*	1.64*/2.31-2.32 (m)	1.64*/2.31-2.32 (m)	1.48*2.10-2.11 (m)
16	4.75-4.76 (m)	4.85-4.87 (m)	4.68-4.69 (m)	4.69 (m)	4.51-4.53 (m)
17	2.70 (d, 6.6)	1.93-1.94 (m)	3.22*	3.23*	2.50-2.52 (m)
18	1.18 (s)	0.84 (s)	0.98 (s)	0.98 (s)	1.11 (s)
19	0.96 (s)	0.89 (s)	0.97 (s)	1.00 (s)	0.97 s
20	-	-	-	-	2.00-2.01 (m)
21	1.40 (s)	1.50 (s)	1.59 (s)	1.59 (s)	1.09 (d, 7.2)
23	4.35 (t, 7.8)	4.35 (t, 7.8)	2.12-2.17 (m)	2.12-2.16 (m)	1.65-1.76 (m)
24	1.99-2.20 (m)	1.97 (m)/ 2.10 (m)	1.28*/1.68-1.69 (m)	1.28*/1.62-1.64 (m)	1.31*/1.62-1.63 (m)
25	1.87-1.89 (m)	1.83-1.84 (m)	1.78-1.79 (m)	1.78-1.79 (m)	1.75-1.76 (m)
26	3.44 (dd, 9.0, 5.4)	3.44 (dd, 9.0, 5.4)	3.33*	3.33*	3.36*
	3.69*	3.68*	3.79 (dd, 9.0, 5.4)	3.79 (dd, 9.0, 5.4)	3.79 (dd, 9.6, 6.0)
27	0.97 (d, 6.6)	0.94 (d, 7.2)	0.97 (d, 7.0)	0.97 (d, 7.0)	0.96 (d, 6.0)
26-*O*-glc					
1′	4.24 (d, 7.8)	4.25 (d, 7.8)	4.25 (d, 7.8)	4.25 (d, 7.8)	4.26 (d, 7.8)
2′	3.20 (dd, 9.0, 7.8)	3.20 (dd, 9.0, 7.8)	3.20 (dd, 9.0, 7.8)	3.20 (dd, 9.0, 7.8)	3.20 (dd, 9.0, 7.8)
3′	3.35*	3.35*	3.36*	3.35*	3.36*
4′	3.25*	3.29*	3.29*	3.27*	3.29*
5′	3.35*	3.25*	3.35*	3.25*	3.35*
6′	3.68*	3.69*	3.68 (dd, 12.0, 5.4)	3.68*	3.68 (dd, 12.0, 2.4)
	3.87 (dd, 12.0, 2.4)	3.87*	3.87*	3.87 (dd, 12.0, 2.4)	3.88 (dd, 12.0, 5.4)
3-*O*-gal					
1′′	4.39 (d, 7.8)	4.37 (d, 7.8)	4.37 (d, 7.8)	4.39 (d, 7.8)	4.37 (d, 7.8)
2′′	3.60*	3.58*	3.50 (dd, 9.0, 7.8)	3.69*	3.50*
3′′	3.53*	3.58*	3.53 *	3.53*	3.54*
4′′	4.01 (d, 3.0)	4.07 (d, 3.0)	4.06 (d, 3.0)	4.03 (d, 3.0)	4.06 (d, 3.0)
5′′	3.52*	3.58*	3.57*	3.55*	3.58*
6′′	3.61*/3.93*	3.68*/3.86*	3.62*/3.88*	3.67*/3.99*	3.63*/ 3.88*
4′′-*O*-glc					
1′′′	4.55 (d, 7.8)	4.53 (d, 7.8)	4.53 (d, 7.8)	4.58 (d, 7.8)	4.53 (d, 7.8)
2′′′	3.54*	3.28*	3.29*	3.53*	3.30*
3′′′	3.60*	3.35*	3.32*	3.60*	3.33*
4′′′	3.40*	3.23*	3.23*	3.38*	3.23*
5′′′	3.40*	3.26*	3.30*	3.40*	3.30*
6′′′	3.81*	3.62*	3.60*	3.81 (dd, 12.0, 5.4)	3.61*
	3.97 (dd,12.0, 1.8)	3.91*	3.92 (dd,12.0, 2.4)	3.98 (dd, 12.0, 1.8)	3.92 (dd, 12.0, 2.4)
2′′′-*O*-glc					
1′′′′	4.68 (d, 7.8)			4.68 (d, 7.8)	
2′′′′	3.28*			3.28*	
3′′′′	3.35*			3.35*	
4′′′′	3.30*			3.31*	
5′′′′	3.26*			3.26*	
6′′′′	3.81 (dd, 12.0, 4.8)			3.60*	
	3.97 (dd, 12.0, 1.8)			3.92 (dd, 11.4, 1.8)	

[*] Overlapped signals, [a] Recorded in 150 MHz, [b] Recorded in 600 MHz, glc: β-D-glucopyranose, gal: β-D-galactopyranose.

geometry of $\Delta^{22,23}$ double bond. The large *J* values (7.8 Hz) of the anomeric protons at δ_H 4.25, 4.37, and 4.53 suggested *β*-form for all the glycosidic linkages. The small difference between the two H-26 proton signals (δ_H 3.68 and 3.44, Δ = 0.24 ppm) suggested (25*R*)-configuration.[10, 13, 14] Acid hydrolysis of **2** gave D-glucose and D-galactose, identified by comparison with authentic samples via TLC, and from the positive sign of the optical rotations.[11, 12] Thus, compound **2** was determined to be (25*R*,22*Z*)-26-*O*-(*β*-D-glucopyranosyl)-5*α*-furostan-22-ene-2*α*,3*β*,20*β*, -26-tetraol 3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside and named tribufurostanoside B.

The IR spectrum of **3** was similar to those of **1** suggesting the presence of hydroxy, ketone, double bond, and ether functionalities. The molecular formula of **3** was ditermined to be $C_{45}H_{72}O_{19}$ by the HR-ESI-MS [found *m/z* 917.4727 $[M+H]^+$, calcd. for $[C_{45}H_{73}O_{19}]^+$: 917.4740 (Δ=-1.4 ppm)], indicating ten degree of unsaturation. The NMR data of **3** were similar to the corresponding data of **1** except for the lost of signals of one sugar unit and the different signals of C-20, C-21, C-22, and C-23 (Table 1). The sugar moieties of **3** were closely resembling those of **2** suggesting they have the same sugar moieties. Which were further evident by ^{1}H-^{1}H COSY, HSQC, and HMBC analyses (Figure 2). The lost of one olefinic proton in comparison with compound **1** and the up field shift of C-21 (δ_C 11.5) suggested $\Delta^{20,22}$ double bond. [5, 6] Which was further confirmed by HMBC correlations from H_3-21 (δ_H 1.59) to C-17 (δ_C 57.1)/C-20 (δ_C 104.3)/C-22 (δ_C 153.9). The NMR data of **3** were very similar to those of **7**, except for the difference of protons H_2-26. The large difference between the two H-26 proton signals (δ_H 3.33 and 3.79, Δ = 0.46 ppm) suggested (25*S*)-configuration.[10, 13, 14] The sugar linkages should be *β*-form as suggested from large *J* coupling constants (7.8 Hz) of the anomeric protons (Table 2). Proton H-3 was in *α*-configuration determined by the similarity of NMR data of **3** with those of **1**, and by the NOESY correlation from H-3 and H-5 (Figure 3). Acid hydrolysis of **3** gave D-glucose and D-galactose, identified by comparison with authentic samples via TLC, and from the positive sign of the optical rotations.[12, 13] Thus, compound **3** was determined to be (25*S*)-26-*O*-(*β*-D-glucopyranosyl)-5*α*-furostan-20(22)-ene-12-one-3*β*,26-diol 3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside and named tribufurostanoside C.

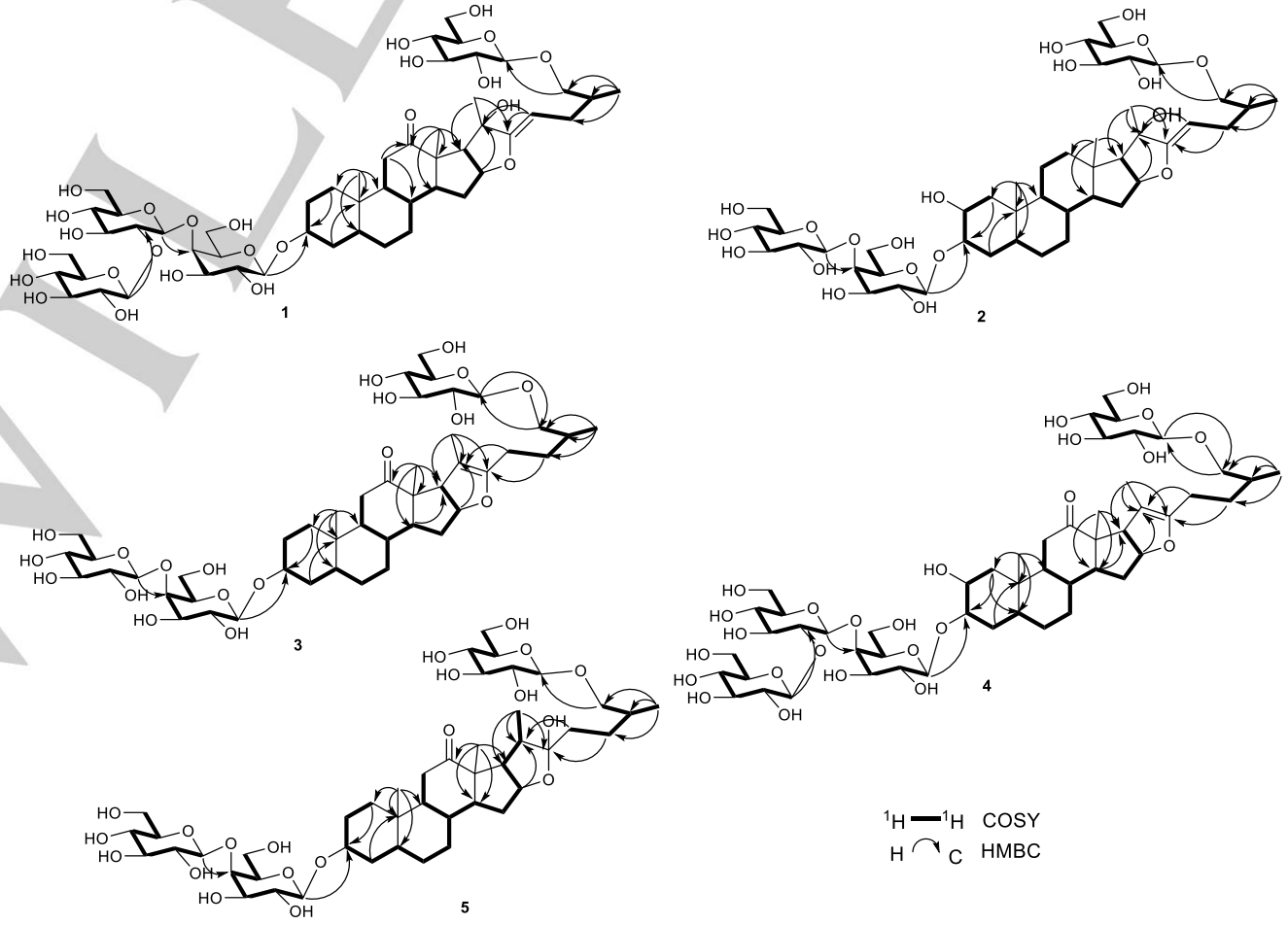

Figure 2. The key HMBC and ^{1}H-^{1}H COSY correlations of compounds **1** - **5**

The IR spectrum of compound **4** indicated the presence of OH, C=O, C=C, and C-*O*-C functional groups. The molecular formular of **4** was $C_{51}H_{82}O_{25}$ as determined by the HR-ESI-MS (found *m/z* 1093.5071 $[M-H]^-$, calcd. for $[C_{51}H_{81}O_{25}]^-$: 1093.5072), indicating eleven degrees of unsaturation. The NMR data of the aglycone of **4** were similar to the corresponding data of **3** except for the additional signals due to one glucose moiety (δ_C/δ_H: 106.1/4.68, 76.2/3.28, 78.7/3.35, 71.7/3.31, 77.9/3.26, 63.2/3.60 and 3.92) and hydroxy group at C-2 (δ_C/δ_H: 71.2/3.67). The NMR data of sugar moieties of **4** matched those of **1**. In addition, the HMBC correlations from H-1'''' (δ_H 4.68) to C-2''' (δ_C 84.7), from H-1''' (δ_H 4.58) to C-4'' (δ_C 80.4), from H-1'' (δ_H 4.39) to C-3 (δ_C 84.8), and from H-1' (δ_H 4.25) to C-26 (δ_C 76.0) further indicated 26-*O*-glucopyranosyl and 3-*O*-glucopyranosyl-(1→2)-glucopyranosyl-(1→4)-galactopyra-noside moieties. In addition, the NMR data of ring A of **4** matched those of **2**, and the NMR data of C, D, E rings of **4** matched those of **3** (Table 1), suggesting carbons C-2 and C-3 were oxygenated, ketone group at C-12, and the double bond at C-20/22. Which was further confirmed by HSQC, COSY, and HMBC correlations (Figure 2). The NOESY cross peaks of H-19/H-2, H-3/H-5, H-5/H-9 indicated β and α-orientation of H-2 and H-3, respectively (Figure 3). The large difference between the two H-26 proton signals (δ_H 3.33 and 3.79, Δ = 0.46 ppm) suggested (25*S*)-configuration.[10, 13, 14] The sugar linkages should be β-form as suggested from large *J* coupling constants of the anomeric protons (Table 2). Acid hydrolysis of **4** gave D-glucose and D-galactose, identified by comparison with authentic samples via TLC, and from the positive sign of the optical rotations.[11, 12] Thus, compound **4** was determined to be (25*S*)-26-*O*-(β-D-glucopyranosyl)-5α-furostan-20(22)-ene-12-one-2α,3β,26-triol 3-*O*-β-D-glucopyra-nosyl-(1→2)-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside and named tribufurostanoside D. The Scifinder database presents only 1 reference related to this compound, suggested by LC-MS and its structure has not been validated.[16] Therefore, compound **4** was considered as previously undescribed.

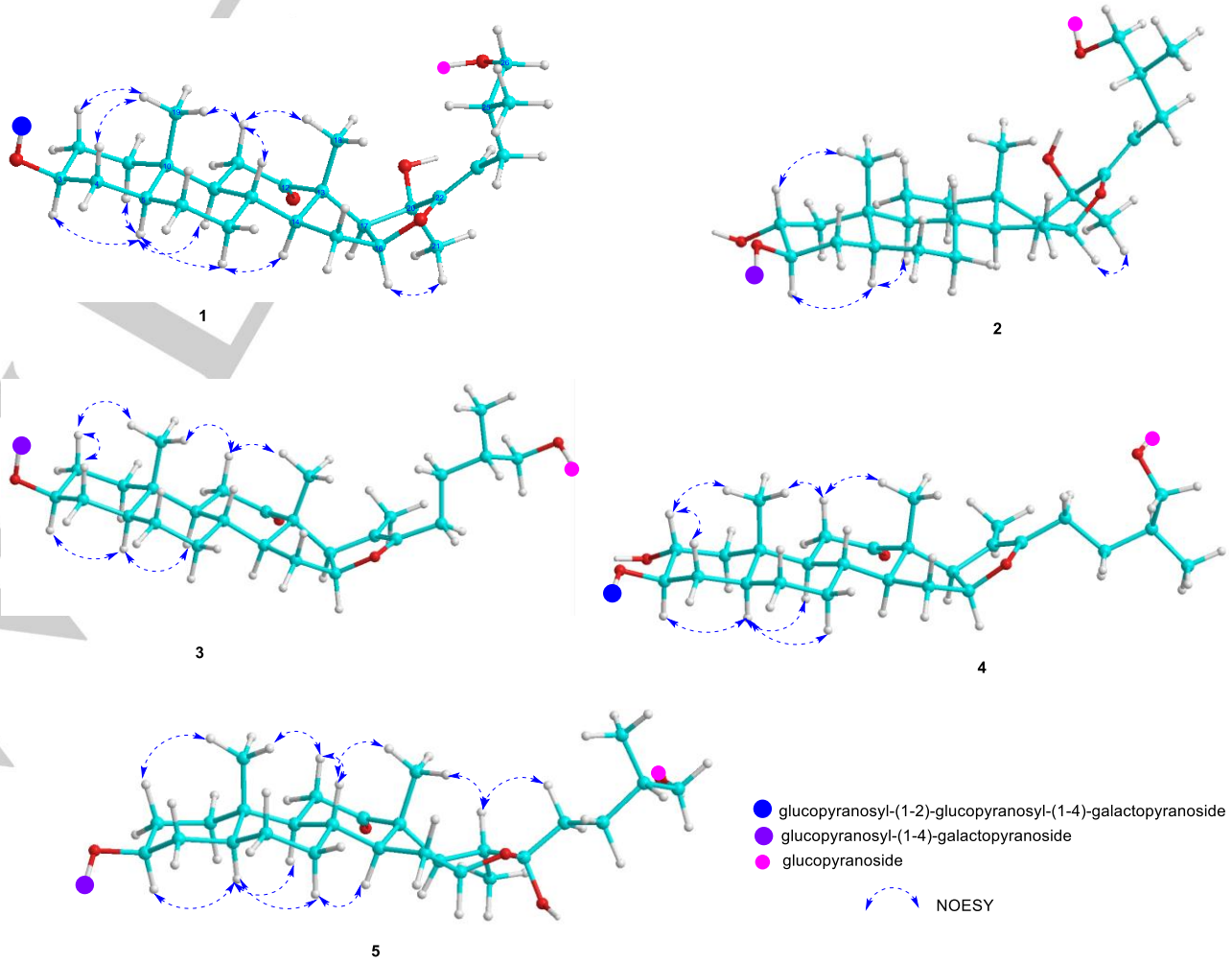

Figure 3. The key NOESY correlations of aglycones of compounds **1** - **5**

The IR spectrum of **5** showed absorption bands at 3405, 1705, 1450, and 1071 cm^{-1}, corresponding to the hydroxy, ketone, double bond, and ether functionalities, respectively. Its molecular formula was determined as $C_{45}H_{74}O_{20}$ by the HR-ESI-MS. (found *m/z* 933.4706 $[M-H]^-$, calcd. for $[C_{45}H_{73}O_{20}]^-$: 933.4701, Δ=+0.5 ppm), indicating nine degree of unsaturation. The NMR spectra of **5** were closely resembling those of **6** suggesting a furostanol glycoside bearing three sugar moieties and one ketone group at C-12.[4] All the NMR assignments of **5** (Tables 1 and 2) were supported by 1D- and 2D NMR spectra in comparison with those of (25*R*)-26-*O*-(*β*-D-glucopyranosyl)-5*α*-furostan-12-one-3*β*,22*α*,26-triol 3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside (**6**).[4] Two quaternary methyl groups [δ_C/δ_H: 16.6/1.11 (s) and 12.2/0.97 (s)], two secondary methyl groups [δ_C/δ_H: 14.7/1.09 (d, *J* = 7.2 Hz) and 17.4/0.96 (*J* = 7.0 Hz), one ketone (δ_C 216.0 C-12), and a hydroxy group at C-22 (δ_C 111.8) were identified (Tables 1 and 2). The location of C=O and 22-OH groups were detemined by HMBC correlations from H_3-18 (δ_H 1.11) to C-12/C-13/C-14/C-17 and from H_3-21 (δ_H 1.09) to C-17/C-20/C-22 (Figure 2). One glucose attached to C-26 by an ether linkage as evident by HMBC correlations from H-1′ (δ_H 4.26) to C-26 (δ_C 76.0) and from H_2-26 (δ_H 3.36 and 3.79) to C-1′ (δ_C 104.6). In addition, the HMBC correlations from H-1‴ to C-4″ and from H-1″ to C-3 further confirmed 3-*O*-glucopyranosyl-(1→4)-galactopyranoside moiety. The NOESY cross peaks between H-3 and H-5, between H-21 and H-16, and between H-20 and H-23 (Figure 3) suggested H-3, H-5, and 22-OH were in *α*-orientation, and H-20 was in *β*-orientation. All the glycosidic linkages were in *β*-from as determined by the large coupling constant (7.8 Hz) of the anomeric protons (Table 2). The large difference between the two H-26 proton signals (δ_H 3.36 and 3.79, Δ = 0.43 ppm) suggested (25*S*)-configuration.[10, 13, 14] Acid hydrolysis of **5** gave D-glucose and D-galactose, identified by comparison with authentic samples via TLC, and from the positive sign of the optical rotations.[12, 13] Thus, compound **5** was determined as (25*S*)-26-*O*-(*β*-D-glucopyranosyl)-5*α*-furostan-12-one-3*β*,22*α*,26-triol 3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-galactopyranoside and named tribufurostanoside E.

Compounds **1**-**8** were evaluated for their NO production inhibitory activity in LPS stimulated RAW 264.7 cells. These compounds did not show significant cytotoxic activity (Table S1), and were further screened for their NO production effects in LPS stimulated RAW 264.7 cells. As shown in Table 3, compounds **1**-**8** showed significantly effects with IC_{50} value of 17.2, 38.7, 16.6, 14.2, 52.4, 53.7, 25.8, and 64.7 *μ*M, respectively, compared to that of the positive control compound, dexamethasone, which showed IC_{50} value of 13.6 *μ*M. Regarding the relationship between structure and activity, the results suggested that the $\Delta^{20,22}$ and $\Delta^{22,23}$ double bonds may play important role in the NO production inhibitory activity of the furostanol saponins. These results are consistent with previous papers that furostanol saponins shows NO inhibitoty and inflammatory activities.[17-19]

Table 3. NO inhibitory effects in LPS-activated RAW 264.7 cells of the isolated compounds

Compounds	NO inhibition (IC_{50}, *μ*M)
1	17.2 ± 1.0
2	38.7 ± 1.5
3	16.6 ± 1.1
4	14.2 ± 1.3
5	52.4 ± 1.2
6	53.7 ± 0.7
7	25.8 ± 1.0
8	64.7 ± 1.5
Dexamethasone*	13.6 ± 1.1

[*]positive control compound

Conclusions

Five undescribed (**1**-**5**) and three known furostanol glycosides (**6**-**8**) were isolated from the methanol extract of fruits of *T. terrestris*. Their chemical structures were elucidated by IR, HR-ESI-MS, 1D- and 2D NMR spectra in comparison with the reported data. These results are completely consistent with previous reports that furostanol glycosides are the main component of *T. terrestris*.[20-27] In addition, all the isolates showed significantly NO production inhibitory activity in LPS stimulated RAW 264.7 cells with IC_{50} values ranging from 14.2 to 64.7 *μ*M. Regarding the relationship between structure and biological activity, the above results suggested that the furostanol glycosides with $\Delta^{20(22)}$ double bond (**3**, **4**, and **7**) or Δ^{22} (**1** and **2**) double bond shows stronger activity compared to that of componds without these double bonds (**5**, **6**, and **8**).

Experimental Section

General

The optical rotations were measured on a Jasco P2000 polarimeter. The infrared spectra (IR) were recorded on a Spectrum Two FT-IR spectrometer. The high-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was acquired on an Agilent 6530 Accurate Mass Q-TOF LC/MS. The NMR spectra were recorded on a Bruker 600 MHz spectrometer. Semi-preparative high-performance liquid chromatography (HPLC) were run on an Agilent 1260 system including binary pump, autosampler, DAD detector, and semi-preparative HPLC column YMC J'sphere ODS-H80 (4 *μ*m, 20 × 250 mm). Isocratic mobile phase with the flow rate of 2.5 mL/min was used in Semi-prep-HPLC.

The compound was monitored at wavelengths of 205, 230, 254, and 280 nm. Flash column chromatography was performed using silica gel, reversed phase C-18, and diaion HP-20 resins as stationary phase. Thin layer chromatography was carried out on pre-coated silica gel 60 F_{254} and RP-18 F_{254S} plates. The spots were detected by spraying with aqueous solution of H_2SO_4 5% followed by heating with a heat gun.

Plant material

The fruits of *Tribulus terrestris* L., were collected in Nha Trang, Khanh Hoa, Vietnam, in September 2022 and identified by Dr Nguyen The Cuong, Institute of Institute of Ecology and Biological Resources. A voucher specimen (NCCT-P107) was deposited at the Institute of Marine Biochemistry, VAST.

Extraction and isolation

The dried fruits (0.3 kg) of *T. terrestris* were minced and ultrasonic extracted with MeOH to obtain the MeOH extract (TF1, 86 g). This was suspended in water and then partitioned with EtOAc to get EtOAc extract (TF2, 4.7 g) and water layer (TF3). The water layer was isolated on a Diaion HP20 eluting with MeOH/H_2O (25%, 50%, 75%, and 100% MeOH) to get four fractions, TF3A-TF3D. Fraction TF3C and TF3D were combined (TF3E, 36 g) and isolated on a silica gel column eluting CH_2Cl_2/MeOH (7/1) to get three fractions, TF3E1-TF3E3. Fraction TF3E2 (15.7 g) was isolated on a silica gel column eluting with CH_2Cl_2/MeOH/H_2O (3/1/0.1) to get three fractions, TF3E2A-TF3E2C. Fraction TF3E2A (2.5 g) was chromatographed on an YMC R18 column eluting with acetone/water (1/1.5) to give five fractions, TF3E2A1-TF3E2A5. Fraction TF3E2A4 (210 mg) was isolated on the HPLC eluting with 27% ACN to give compounds **7** (15.2 mg, t_R 50.9 min) and **3** (13.9 mg, t_R 53.4 min). Fraction TF3E2B (1.7 g) was isolated on a YMC R18 column eluting with acetone/water (1/2) to get two fractions, TF3E2B1 and TF3E2B2. Fraction TF3E2B1 (174 mg) was isolated on the HPLC eluting with 20% ACN to give compounds **5** (26.5 mg, t_R 55.8 min) and **6** (13.9 mg, t_R 60.9 min). Fraction TF3E2B2 (72 mg) was purified on the HPLC eluting with 20% ACN to give compound **2** (15.1 mg, t_R 58.8 min). Fraction TF3E2C (6.2 g) was isolated on an YMC R18 column eluting with acetone/water (1/2) to get three fractions, TF3E2C1-TF3E2C3. Fraction TF3E2C1 (408 mg) was purified on the HPLC eluting with 18% ACN to give compound **8** (59.0 mg, t_R 62.5 min). Fraction TF3E2C3 (490 mg) was isolated on the HPLC eluting with 60% MeOH in water to give compounds **1** (13.3 mg, t_R 55.8 min) and **4** (13.5 mg, t_R 60.9 min).

Tribufurostanoside A (**1**)

A white amorphous powder; $[\alpha]_D^{25}$: +7.4 (*c* 0.1, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3401, 2926, 1707, 1448, 1367, 1165, 1065. HR-ESI-MS *m/z* 1093.5042 $[M-H]^-$, calcd. for $[C_{51}H_{81}O_{25}]^-$: 1093.5072 (Δ=-2.7 ppm); *m/z* 1129.4835 $[M+^{37}Cl]^-$, calcd. for $[C_{51}H_{82}O_{25}{}^{35}Cl]^-$: 1129.4839 (Δ=-0.3 ppm); *m/z* 1131.4844 $[M+^{37}Cl]^-$, calcd. for $[C_{51}H_{82}O_{25}{}^{37}Cl]^-$: 1131.4809 (Δ=+3.1 ppm); ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data are shown in Tables 1 and 2 (Figures S1-S12).

Tribufurostanoside B (**2**)

A white amorphous powder; $[\alpha]_D^{25}$: -51.9 +4.9 (*c* 0.1, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3399, 2929, 1653, 1449, 1163, 1075. HR-ESI-MS *m/z* 957.4687 $[M+Na]^+$, calcd. for $[C_{45}H_{74}O_{20}Na]^+$: 957.4666 (Δ=+2.2 ppm); ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data are shown in Tables 1 and 2 (Figures S13-S23).

Tribufurostanoside C (**3**)

A white amorphous powder; $[\alpha]_D^{25}$: +4.8 (*c* 0.1, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3408, 2926, 1704, 1449, 1130, 1057; HR-ESI-MS *m/z* 917.4727 $[M+H]^+$, calcd. for $[C_{45}H_{73}O_{19}]^+$: 917.4740 (Δ=-1.4 ppm); ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data are shown in Tables 1 and 2 (Figures S24-S34).

Tribufurostanoside D (**4**)

A white amorphous powder; $[\alpha]_D^{25}$: +5.3 (*c* 0.1, MeOH); IR (KBr) ν_{max} (cm^{-1}):3402, 2927, 1704, 1168, 1071; HR-ESI-MS *m/z* 1093.5071 $[M-H]^-$, calcd. for $[C_{51}H_{81}O_{25}]^-$: 1093.5072, (Δ=-0.1 ppm); *m/z* 1129.4818 $[M+^{35}Cl]^-$, calcd. for $[C_{51}H_{82}O_{25}{}^{35}Cl]^-$: 1129.4839 (Δ=-1.9 ppm), *m/z* 1131.4842 $[M+^{37}Cl]^-$, calcd. for $[C_{51}H_{82}O_{25}{}^{37}Cl]^-$: 1131.4809, (Δ=+2.9 ppm), *m/z* 1095.5260 $[M+H]^+$, calcd. for $[C_{51}H_{83}O_{25}]^+$: 1095.5218 (Δ=+3.8 ppm); ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data are shown in Tables 1 and 2 (Figures S24-S34).

Tribufurostanoside E (**5**)

A white amorphous powder; $[\alpha]_D^{25}$: +8.2 (*c* 0.1, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3405, 2932, 1705, 1450, 1373, 1164, 1071; HR-ESI-MS *m/z* 933.4706 $[M-H]^-$, calcd. for $[C_{45}H_{73}O_{20}]^-$: 933.4701 (Δ=+0.5 ppm); ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz) data are shown in Tables 1 and 2 (Figures S35-S46).

*Acid hydrolysis of compounds **1**-**5***

Acid hydrolysis of compounds **1**-**5** were the same as described in previous work[12, 13] referred to Supplementary information.

Nitric oxide assay

The NO assay protocol is the same as described in previous papers [28-30] referred to Supplementary information.

Supplementary Material

Supporting information for this article is available on the WWW under http://dx.doi.org/....

Additional references cited within the Supporting Information.[12, 13, 28-30]

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Author Contribution Statement

Kiem PV, Tai BH, Yen PH, Giang LD designed experiments, elucidated chemical structures and wrote the paper. Quoc NV, Hoang NH, Cuc NT, Huong PTT, Dung DT, Trang DT extracted and isolated compounds and prepared sample for bioassay.

Keywords

Tribulus terrestris, Zygophyllaceae, furostanol glycoside, tribufurostanosides A-E, NO inhibitory activity

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Entry for the Graphical Illustration

Twitter Text

- Isolation and determination of five undescribed and three known furostan glycosides from the fruits of *Tribulus terrestris* with their NO production inhibitory activity in LPS-stimulated RAW264.7 macrophages.

- Keywords: *Tribulus terrestris*, Zygophyllaceae, furostanol glycoside, tribufurostanosides A-E, NO inhibitory activity

- Corresponding author's name: Phan Van Kiem, and Twitter account: PVKiem_IMBC.