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New Saponins from Timosaponin BIII by Acid Hydrolysis

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Timosaponin BIII (TBIII), a saponin isolated from the rhizome of *Anemarrhena asphodeloides*, exhibited potent antidepressive and anxiolytic activities in depressed mice. After administration of TBIII in rats, two main metabolites had been detected in the brain tissue, which had similar aglycone with TBIII and formed by elimination of two hexose moieties. In this study, we had hydrolyzed TBIII by some means and obtained 4 artificial steroidal saponins (three novel steroidal saponins: timosaponin BIII-a, timosaponin BIII-b, timosaponin BIII-c; and a known steroidal saponin: timosaponin BII-b) by the dilute-acid hydrolysis. Their structures were verified by means of mass spectrometry and nuclear magnetic resonance (NMR) spectral analyses.

Key Words: *Rhizoma anemarrhenae,* Steroidal saponin, Timosaponin BIII, Acid hydrolysis, NMR.

INTRODUCTION

Timosaponin BIII (also called anemarrhenasaponin IV or pseudoprototimosaponin AIII or timosaponin B) is a main activated compound of *Rhizoma anemarrhenae*[1,2]. TBIII had desirable hypoglycemic activity in streptozotocin-induced diabetic mice but showed no effects on glucose uptake and insulin release, which suggested that the hypoglycemic mechanism may be due to inhibition of hepatic gluconeogenesis and glycogenolysis[2]. In a activity study of timosaponin BIII, we found it had dramatically antidepressive and anxiolytic activities in depressed mice. After the intraperitoneal injection in rats, two main metabolites had been detected in the brain tissue, which formed by elimination of two hexose moieties and ring-opening reaction from the molecule of timosaponin BIII.

In the present study, we had performed dilute acid hydrolysis, Lewis acid catalyst and enzymatic hydrolysis of timosaponin BIII to find out the relationship between the structure and the antidepressive and anxiolytic activities. We had got 4 hydrolyzates from the sulphuric acid hydrolysis. The antidepressive and anxiolytic activities of these artificial products will be examined in future investigations.

EXPERIMENTAL

Timosaponin BIII was isolated from *Rhizoma anemarrhenae*, which was purchased from medicinal materials market of Shanghai (Shanghai City, China). HPLC grade acetonitrile was purchased from Dikma Company (Dikma, USA). Other reagents used are of analytical grade (Sinopharm Chemical Reagent Co. Ltd., China).

Optical rotations were measured with a Perkin-Elmer 343 polarimeter. NMR spectra were measured on Varian Mercury-400 (Varian, U.S.A), using Deuterium pyridine as solvents and TMS as an internal standard. HR-ESI-MS was carried out on a Micromass TOF Ultima (Micromass, U.K), the ionization mode was negative electrospray (ESI-). Semipreparative HPLC was performed on an Unimicro Technologies System (quaternary pump, China) with a Grace Apollo C_{18} (10 mm i.d. × 250 mm, ODS, 5 μm) semipreparative HPLC column, including an EasyGuard Kit C_{18} (4 mm × 2 mm) guard column; detector was UV.

A solution of timosaponin BIII (200 mg, 0.22 mmol) and sulfuric acid (450 μL, 8.28 mmol) in CH_3OH-H_2O (150 mL, 2:13). The mixture was stirred at 100 ºC for 7 h. The reaction solution was partitioned with *n*-butanol and the *n*-butanol solution was concentrated under reduced pressure to dryness. The residue was separated by semipreparative RP-HPLC using aqueous acetonitrile system (Fig. 1).

Chromatographic spectrometric conditions: The semipreparative HPLC was equipped with a reversed-phase column, including an C_{18} guard column. The column was maintained at 25 ºC. The elution gradient for the semi-preparative HPLC experiment was conducted using acetonitrile (A) and water (B). The UV detection wavelength was set at 205 nm. The initial elution condition was 50 % A (v/v), linearly changed to 100 % A (v/v) at 25 min.

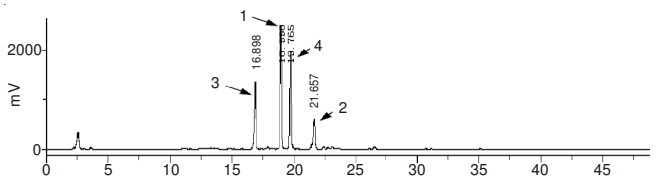

Fig. 1. HPLC profile of the reaction mixture of the hydrolysis of timosaponin BIII

RESULTS AND DISCUSSION

It gave 4 reaction products (Figs. 1 and 2), **1** (t_R 18.99 min, 4.6 mg), **2** (t_R 21.66 min, 4.5 mg), 3 (t_R 16.90 min), 4.4 mg and 4 (t_R 19.77 min, 16.6 mg). The quantitative yields of those compounds were 3.5, 3.5, 3.5 and 12.6 %, respectively. We report herein upon the structural elucidation of three new compounds (**1, 2, 3**) and of a known compound (**4**). The known steroidal saponin, timosaponin BII-b, was identified by a comparison of the observed $[\alpha]^{20}_D$, ^{1}H NMR, ^{13}C NMR, DEPT and HR-ESI-TOF-MS data with the literature values[3].

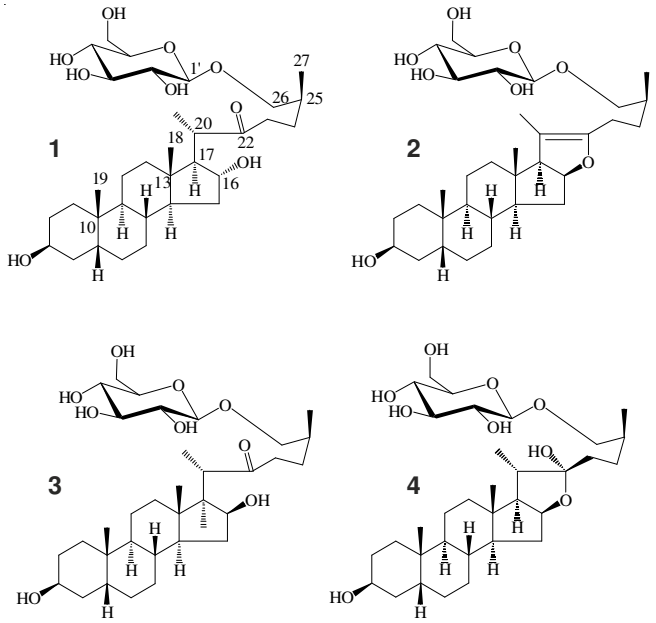

Fig. 2. Compounds **1, 2, 3** and **4** from hydrolysis of timosaponin BIII

Compound **1** was obtained as white amorphous powder, $[\alpha]^{20}_D$ -46.0 (*c* 0.05, pyridine). The molecular formula was determined as $C_{33}H_{56}O_9$ by HR-ESI-MS ($[M+Na]^+$ *m/z* 619.3816; calcd. 619.3817). The ^{13}C NMR spectrum (Table-1) of **1** displayed the presence of glycopyranosyl unit in addition to 26 carbon signals for the aglycone. The coupling constant of the anomeric proton at δ_H 4.85 (J = 7.7 Hz) showed a β-glycopyranoside. The 26 carbon atoms due to the aglycone were classified as 4 methyls, 12 methylenes, 14 methines and 3 quaternary carbon atoms using distortionless enhancement by polarization transfer (DEPT) spectrum analysis and ^{13}C NMR data. The ^{1}H and ^{13}C NMR spectra of the aglycone of **1** were closely resemble that of timosaponin BIII[4], except for the appearance of the carbonyl carbon at δ_C 214.86 and the absence of one double bond. The connectivities of the molecular fragments were established by a heteronuclear multiple bond correlation experiment (HMBC), where the long-range correlations were observed between the protons of H-20 (δ_H 2.80), H-21 (δ_H 1.23) and H-23 (δ_H 2.93, 2.75) and the carbonyl carbon (δ_C 214.86), whereas the protons of H-17 (δ_H 1.90) and the oxygenated carbon (δ_C 75.93), indicating that the carbonyl was located at C-22 and hydroxyl was placed at C-16. This experiment also clarified the site of glycosidation showing a long-range correlation between the anomeric proton of glucose at δ_H 4.85 (H-1') and the oxygenated carbon atom at δ_C 75.19 (C-26). The stereochemistry of **1** was determined from the coupling pattern in the ^{1}H NMR spectrum and detailed analysis of rotation frame overhauser effect spectroscopy (ROESY) data. The ROE cross-peaks of significant intensity between the H-18 at δ 0.71 and the H-16 at δ 4.29 indicated that 16-OH was a configuration. The *cis*-configuration between ring A and B was confirmed from the ROESY correlation of H-5 with H-19. The other significant ROESY correlations were shown on Fig. 3. Therefore, the structure of **1** was elucidated as shown and named timosaponin BIII-a, which had not previously been reported form natural sources or synthesis.

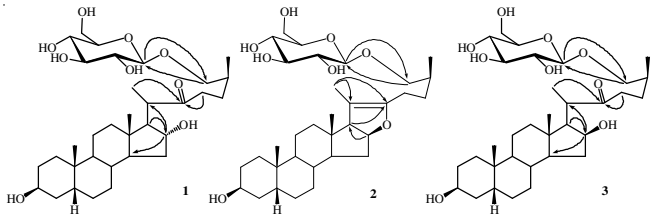

Fig. 3. Key HMBC of 1, 2 and 3

Compound **2** was isolated as white amorphous powder, $[\alpha]^{20}_D$-66.0 (*c* 0.05, pyridine), had a molecular formula of $C_{33}H_{55}O_8$ by the positive ion HR-ESI-TOF-MS (Found *m/z*: 601.3713 $[M+Na]^+$; calcd. for $C_{33}H_{55}O_8Na$: 601.3711). The ^{13}C NMR spectrum and DEPT experiment showed the presence of 4 methyls, 12 methylenes, 13 methines and 4 quarternary carbons. The presence of a b-glycopyranosyl moiety was determined by the anomeric proton at δ_H 4.87 (J = 7.7 Hz) and the carbon signal at δ_C 105.19. Its NMR spectral data were very similar to those of timosaponin BIII[4] indicating the same basic skeleton for **2**. In the HMBC spectra, long-range correlations were observed from δ_H 4.87 (H-1') to δ_C 75.22 (C-26) and *vice veisa*. Accordingly, the structure of **2** was elucidated as shown and named timosaponin BIII-b, which also had not previously been reported form natural sources or synthesis.

Compound **3** was a white amorphous powder, $[\alpha]^{20}_D$ -36.0 (*c* 0.05, pyridine). It showed a molecular formula of $C_{33}H_{56}O_9$, as established by HR-ESI-TOF-MS (Found *m/z*: 619.3815 $[M+Na]^+$; calcd. for $C_{33}H_{56}O_9Na$: 619.3817). Its NMR spectral data were very similar to those of **1** indicating the same basic skeleton for **3**. Its ROESY spectrum were closely resemble that of **1**, except for the absence of the correlation between the H-18 at δ 0.76 and the H-16 at δ 4.09. It indicated that **1** and **3** were epimers and 16-OH of **3** was β configuration. The other significant ROESY correlations were shown on Fig. 4. Therefore, the structure of **3** was elucidated as shown and named timosaponin BIII-c, which had not previously been reported form natural sources or synthesis.

TABLE-1
NMR SPECTRAL DATA FOR **1, 2, 3** AND **4** IN PYRIDINE-d_5 (δppm) (^{1}H: 400 MHz ; ^{13}C: 100 MHz)

No.	1		2		3		4	
	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1	1.58 (m), 1.93 (m)	30.47	1.89 (m)	30.64	1.52 (m), 1.88 (m)	30.52	1.73 (m), 1.61 (m)	28.61
2	1.91 (m), 1.33 (m)	27.12	1.94 (m), 1.33 (m)	27.14	1.92 (m), 1.19 (m)	27.19	1.88 (m), 1.14 (m)	27.16
3	4.42 (m)	66.06	4.42 (m)	66.07	4.41 (m)	66.07	4.38 (m)	66.01
4	1.84 (m), 1.67 (m)	36.95	1.63 (m), 1.55 (m)	34.41	1.92 (m), 1.75 (m)	37.54	1.85 (m), 1.54 (m)	30.62
5	1.71 (m)	37.00	2.17 (m)	37.04	2.17 (m)	37.08	1.96 (m)	37.03
6	1.32 (m), 1.18 (m)	26.58	1.34 (m), 1.18 (m)	26.97	1.39 (m), 1.19 (m)	26.65	1.54 (m)	
7	1.75 (m), 1.63 (m)	28.59	1.76 (m), 1.59 (m)	28.65	1.78 (m), 1.62 (m)	28.61	1.33 (m)	26.87
8	1.36 (m)	35.37	1.52 (m)	35.27	1.38 (m)	35.45	1.04 (m)	35.59
9	1.84 (m)	40.00	1.80 (m)	40.12	1.39 (m)	40.08	1.35 (m)	40.10
10	-	35.42	-	35.57	-	35.49	-	35.59
11	1.39 (m), 1.23 (m)	21.00	1.79 (m), 1.40 (m)	21.38	1.35 (m), 1.15 (m)	20.97	1.42 (m), 1.29 (m)	21.22
12	1.38 (m),1.30 (m)	40.39	1.39 (m), 1.27 (m)	40.15	1.58 (m), 1.30 (m)	39.59	1.79 (m), 1.18 (m)	40.47
13	-	44.49	-	43.88	-	44.01	-	41.26
14	1.55 (m)	53.71	1.49 (m)	54.82	1.62 (m)	53.36	1.14 (m)	56.48
15	2.01 (m), 1.56 (m)	34.32	1.89 (m), 1.51 (m)	31.39	2.0 (m), 1.60 (m)	34.37	2.01 (m), 1.41 (m)	32.43
16	4.29 (m)	75.93	4.88 (m)	84.59	4.09 (m)	75.22	4.58 (m)	81.22
17	1.90 (d, 3.2)	63.39	2.55 (d, 10.1)	64.68	2.23 (dd, 6.4, 10.4)	62.17	2.01 (m)	64.02
18	0.71 (s)	13.65	0.75 (s)	14.45	0.76 (s)	14.93	0.94 (s)	16.76
19	1.03 (s)	24.19	1.05 (s)	24.26	1.0 1(s)	24.24	1.04 (s)	24.26
20	2.80 (m)	49.34	-	103.59	2.84 (dq, 6.6, 10.4)	47.09	2.25 (m)	40.66
21	1.23 (d, 6.7)	16.78	1.67 (s)	11.83	1.49 (d, 6.95)	17.28	1.37 (m)	16.48
22	-	214.86	-	152.38	-	214.63	-	110.65
23	2.93 (m), 2.75 (m)	38.89	1.56 (m)	34.41	2.76 (m)	38.91	2.09 (m)	37.15
24	2.06 (m), 1.91 (m)	27.92	2.23 (m), 1.04 (m)	23.62	2.06 (m), 1.92 (m)	27.92	2.07 (m), 1.69 (m)	28.33
25	2.01 (m)	33.56	1.63 (m)	33.68	2.01 (m)	33.56	1.92 (m)	34.42
26	4.07 (m, 6.5), 3.52 (dd, 9.4)	75.19	4.11 (dd, 5.7, 9.2), 3.52 (dd, 6.9, 9.2)	75.22	4.33 (m), 3.56 (dd, 6.3, 9.5)	75.01	4.08 (m), 3.48 (m)	75.38
27	1.02 (d, 7.4)	17.44	1.08 (m)	17.16	1.04 (d, 6.6)	17.41	1.07 (d)	17.44
1′	4.85 (d, 7.7)	105.02	4.87 (m, 7.7)	105.19	4.88 (d, 7.75)	105.10	4.84 (d)	105.10
2′	4.06 (m)	75.21	4.07 (m)	75.21	4.09(m)	74.95	4.01 (m)	75.21
3′	4.28 (m)	78.50	4.28 (m)	78.53	4.29(m)	78.58	4.23 (m)	78.59
4′	4.27 (m)	71.64	4.28 (m)	71.68	4.28(m)	71.70	4.23 (m)	71.65
5′	3.98 (m)	78.58	4.00 (m)	78.60	4.01(m)	78.63	3.94 (m)	78.49
6′	4.59 (m), 4.42 (m)	62.75	4.59 (m), 4.43 (m)	62.82	4.61 (brd, 11.6), 4.44 (m)	62.86	4.55 (m), 4.39 (m)	62.77

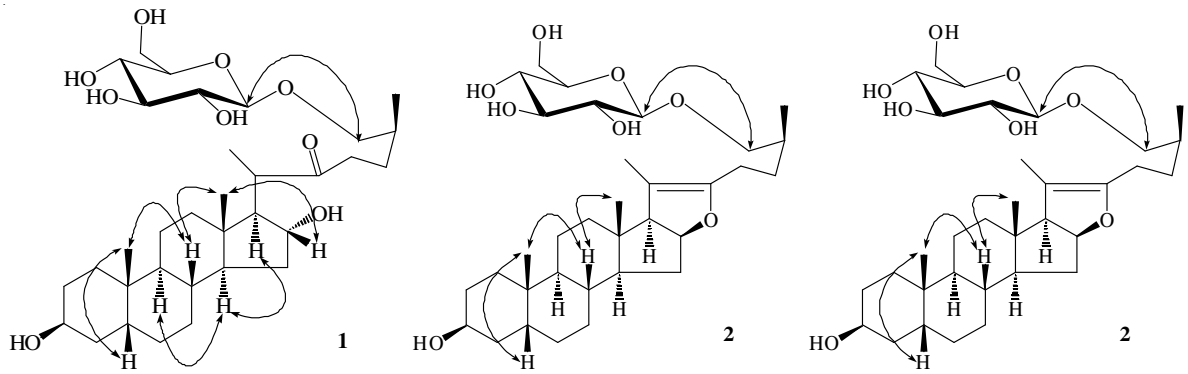

Fig. 4. Key ROESY correlations of **1, 2** and **3**

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