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New transformation pathway and cytotoxic derivatives from the acid hydrolysis of timosaponin B III

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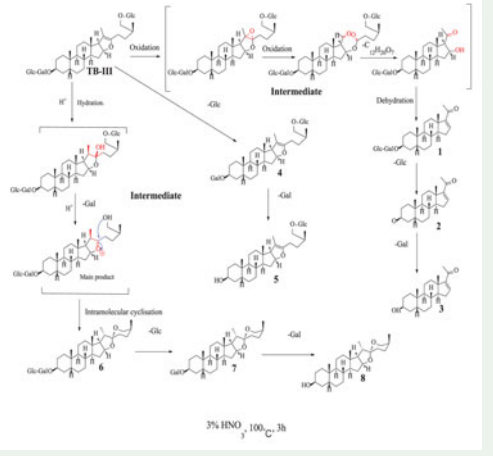
ABSTRACT

Timosaponin B III is a major bioactive steroidal saponin isolated from *Anemarrhena asphodeloides* Bge. To potentially discover derivatives with better biological activity, timosaponin B III was structurally modified via acid hydrolysis to yield one new (**2**, timopregnane A I) C_{21} steroidal glycoside and seven known compounds. Their structures were elucidated on the basis of NMR spectroscopy and mass spectrometry. All eight compounds were evaluated for cytotoxic activity against MCF7, SW480, HepG2, and SGC7901 cell lines *in vitro*. As a result, compounds **6** and **7** showed significant activity (IC_{50} 2.94–12.2 μM) against all tested cell lines. Structure–activity relationships of these compounds were investigated and the preliminary conclusions were provided. Moreover, a new transformation pathway was discovered in the acid hydrolysis of timosaponin B III for the first time.



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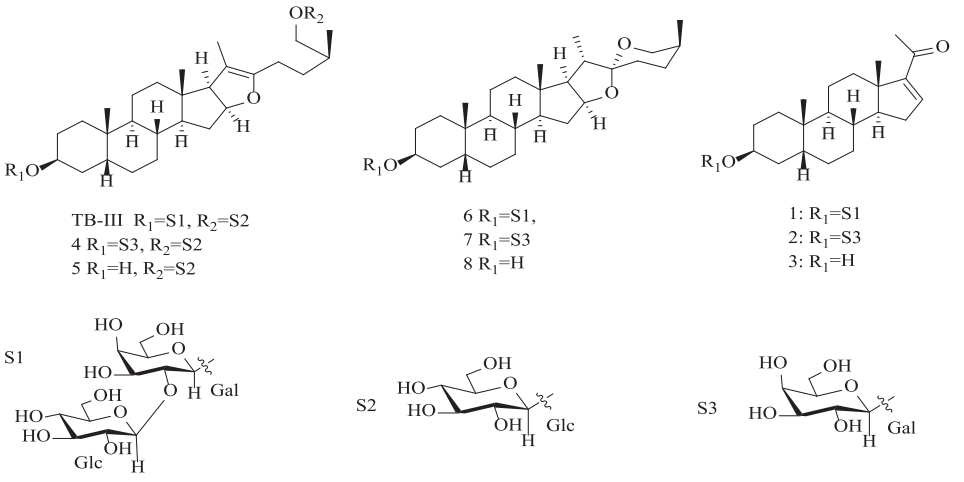

Figure 1. Structures of TB-III and compounds **1**–**8**.

1. Introduction

Timosaponin B III (TB-III) is a major steroidal saponin originally isolated from *Anemarrhena asphodeloides* Bge. In recent pharmacological studies, TB-III exhibited numerous bioactivities, including anti-inflammatory (Kim et al. 2009), anti-platelet aggregative (Zhang et al. 1999) and anti-depressive (Jiang et al. 2014) effects, but antitumor activity of TB-III has not been reported. A study on the metabolism of TB-III reported several biotransformation pathways, including deglycosylation, hydroxylation, oxidation, and ring cleavage (Jiang et al. 2014). Because the metabolites were produced in an *in vivo* acidic or enzymatic environment, *in vitro* acid hydrolysis of TB-III is a rational approach to study the mechanism of drug action as well as to search for potential active anticancer compounds.

In the present studies, TB-III was subjected to structural modification by acid hydrolysis to yield one new (timopregnane A I, **2**) C_{21} steroidal glycoside and seven known compounds (**1**, **4**–**9**). Two C_{21} steroidal glycosides and their aglycone (**1**–**3**) were obtained by acid hydrolysis of TB-III for the first time, and the transformation pathways to all hydrolytic derivatives are discussed herein. Furthermore, these compounds were evaluated for cytotoxic activity by the MTT method.

2. Results and discussion

The eight derivatives were obtained by acid hydrolysis (3% nitric acid) of TB-III at 100 °C for 3 h. One new C21 steroidal glycoside timopregnane A I (2), together with seven known compounds, timopregnane A (1) (Zhu et al. 2012), 3β-hydroxy-5β-pregn-16(17)-ene-20-one (3) (Blunt and Stothers 1977), timosaponin B III-d (4) (Fu et al. 2015), timosaponin B III-b (5) (Liu et al. 2013), timosaponin A III (6) (Lu et al. 2016), timosaponin A I (7) (Zhao et al. 2015) and sarsapogenin (8) (Blunt and Stothers 1977) were obtained (Figure 1).

Compound **2** was obtained as a white amorphous powder. The molecular formula was determined as $C_{27}H_{42}O_7$ from a quasi-molecular ion peak at *m/z* 501.2821 $[M+Na]^+$ (calcd. for 501.2823) in the HR-ESI-MS spectrum. The ^{1}H NMR spectrum of **2** (Table S1) showed three tertiary methyls at δ_H 0.93 (s, CH_3-18), 0.86 (s, CH_3-19) and

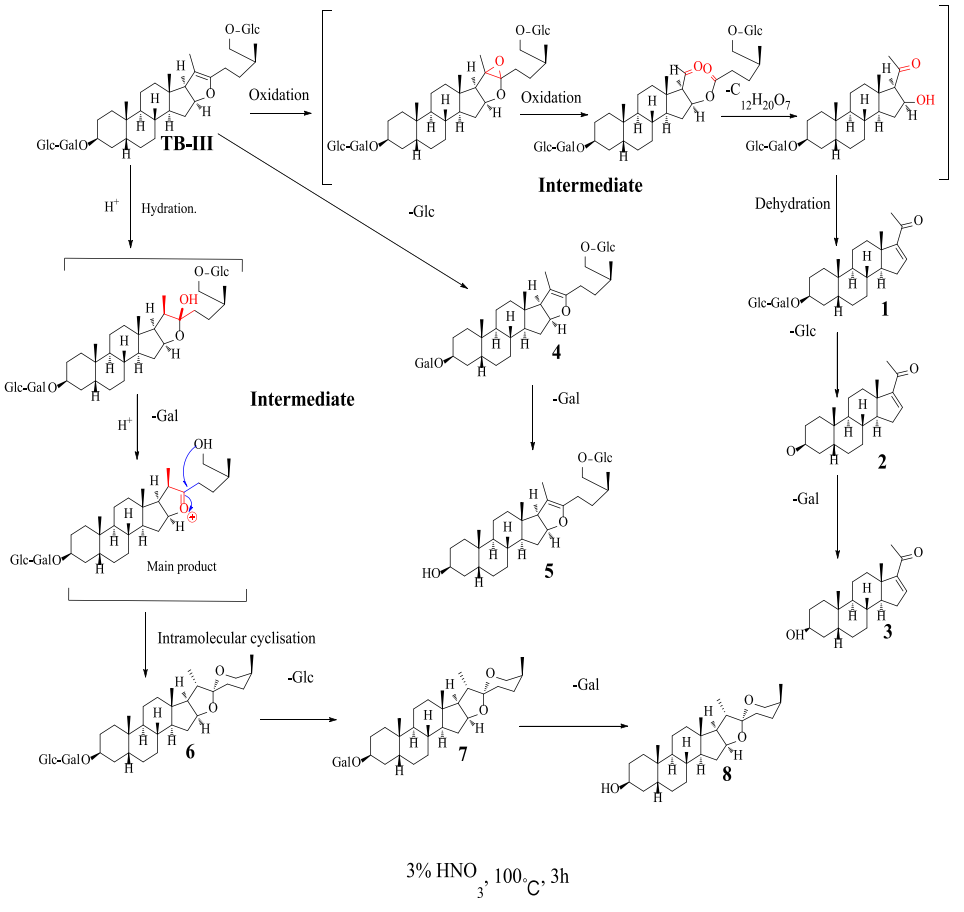

Figure 2. The proposed pathways to the hydrolysis products (**1**–**8**) from TB-III.

2.25 (s, CH_3-21) and an olefinic proton at δ_H 6.67 (m). One anomeric proton at δ_H 4.85 (d, $J = 7.5$ Hz) confirmed the presence of one sugar moiety. The ^{13}C NMR spectrum (Table S1) showed 27 signals, including those for one anomeric carbon (δ_C 104.3), two olefinic carbons (δ_C 156.1, 145.1) and a carbonyl carbon (δ_C 196.7). These spectroscopic features suggested that, like the known identified compounds, **2** is a C_{21} steroidal glycoside containing with a carbonyl group. The spectroscopic data of **2** and **1** were quite similar, particularly for the aglycone. The two compounds differed only in the number of sugars attached at C-3 (one in **2**, two in **1**). In the HMBC spectrum of **2** (Figure S1), long-range corrections were observed between CH_3-21 (δ_H 2.25) and C-20 (δ_c 196.7); CH_3-18 (δ_H 0.93) and C-13 (δ_c 47.0), C-14 (δ_c 56.9), C-17 (δ_c 156.1); H-16 (δ_H 6.67) and C-14 (δ_c 56.9), C-15 (δ_c 32.7), C-17 (δ_c 156.1), C-20 (δ_c 196.7); CH_3-19 (δ_H 0.86) and C-1 (δ_c 31.2), C-5 (δ_c 37.5), C-10 (δ_c 35.9). In NOSEY spectrum, H-5 showed a correlation with the β-oriented methyl protons of CH_3-19, which confirmed the 5β configuration. Based on the above information, the aglycone structure of compound **2** was identified as 3β-hydroxy-5β-pregn-16(17)-ene-20-one.

The monosaccharide of **2** was confirmed as D-galactose following the acid hydrolysis of **2** and identification by HPLC analysis. The β-anomeric configuration of the sugar

was determined from its coupling constant. The sugar moiety was assigned at C-3 based on the observed HMBC correlation between Gal H-1' (δ_H 4.85) and C-3 (δ_C 74.8). Thus, compound **2** (timopregnane AI) was fully identified as 3-*O*-β-D-galactopyranosyl-(3β,5β)-pregn-16(17) -ene-20-one (Figure 1).

In this acid hydrolysis study, we found three obvious transformation pathways, **I**, **II**, and **III**. Metabolite **1** was likely produced from TB-III by opening of the E-ring followed by conversion to a C_{21} steroidal glycoside. In this transformation pathway (**I**), the decisive factor is the ready oxidation of the 20, 22-double bond. Subsequently, oxidative cleavage of this bond and loss of the ester on the C-17 OH, followed by dehydration would create the 16, 17-double bond in metabolites **1**–**3**. Compounds **4** and **5** are products of successive hydrolysis of the C-3 sugar chain (**II**). In the final pathway (**III**), the C-26 sugar chain is removed and the side chain at C-22 is cyclized to give spirostanol-type steroidal glycosides (**6**–**7**) and an aglycone (8). Figure 2 illustrates the proposed mechanisms for transformation of TB-III to **1**–**8**.

TB-III and its eight hydrolytic derivatives were evaluated for cytotoxic effects against MCF7, SW480, HepG2 and SGC7901 cell lines *in vitro* (Table S2), with **6** used as the positive control (Kang et al. 2011). Compounds **1**, **2**, **4**, and **5** as well as TB-III showed no toxicity toward the four cell lines ($IC_{50} > 100\ \mu M$). Compounds **6** and **7** exhibited significant anticancer effects against the four cell lines (IC_{50} 2.94–12.2 μM). Compounds **3** and **8** showed moderate cytotoxicity against three (MCF7, SW480, HepG2) and four cell lines, respectively. The results demonstrated that the acid hydrolysis products **3** and **6**–**8** from TB-III exhibited greater cytotoxic activity than the parent compound, which suggests that the inactive natural compound may incur certain bioactive effects through metabolic transformation. Moreover, acid hydrolysis of timosaponins from *A. asphodeloides* is a feasible method to obtain better biological activity.

The results of this study also provided preliminary information about the structure-activity relationships of these compounds. These steroidal saponins have three different aglycone skeletons, spirostanol, furostanol, and epipregnenolone saponins. In our study, the six-ring derivatives (**6**–**8**) exhibited much greater activity than the five-ring (TB-III, **4**, **5**) or four-ring (**1**–**3**) derivatives; thus, the steroidal saponins with an F-ring showed obviously increased cytotoxic activity. To investigate the role of the number of sugar moieties, we compared the activities of **1**–**3** and **6**–**8**. Compounds **1** and **2** showed no activity against the four cell lines, but their aglycone structure **3** exhibited moderate cytotoxicity against MCF7, SW480, and HepG2. However, the aglycone **8** was less potent than the di- and mono-glycosides, **6** and **7**, respectively. This result suggested that the number of sugar moieties might affect the activity, but the aglycone structure was more important in our study.

3. Experimental

3.1. General experimental procedures

ESI-MS and HR-ESI-MS experiments were performed on an Agilent 1100 Series LC/MSD Trap SL mass spectrometer and Agilent 6520B Q-TOF spectrometer, respectively. NMR spectra were recorded on Bruker ACF-300 and -500 spectrometers using pyridine-d_5 as solvent with tetramethylsilane (TMS) as the internal reference. The chemical shifts are

given in δ (ppm) and coupling constants in Hz. Analytical HPLC was performed on a Shimadzu LC-20AT with SPD-20A UV/VIS detector, using a YMC-Pack ODS-A column (250 mm $\times$4.6 mm, 5 μm). Semi-preparative HPLC was performed on a Shimadzu LC-6AD with SPD-20A UV/VIS detector, using an YMC-Pack ODS-A column (250 mm $\times$20 mm, 5 μm). Column chromatography (CC) was carried out on silica gel (200–300 mesh, Qingdao, China), Silica gel 60 (230–400 mesh, Merck) and all chemical reagents used in the studies were produced by Nanjing Chemical Reagent Co. Ltd. (Nanjing, China).

3.2. Preparation of derivatives

The TB-III (1 g) was dissolved in 500 mL nitric acid aqueous solution (3% nitric acid). The mixture was stirred at 100 °C for 3 h. The reaction solution was neutralized with bicarbonate, partitioned with ethyl acetate, and concentrated under reduced pressure to dryness. The residue was separated by silica gel eluted with $CHCl_3$:MeOH (40:1 $\sim$ 2:1, v/v) to give four fractions (Fr.1–4). Fr.1 was further separated by silica gel CC to yield **3** (30 mg) and **8** (42 mg). Fr.2 was further separated by silica gel CC and semi-preparative HPLC to give **2** (10 mg), **7** (15 mg), and **5** (25 mg). Fr.3 was further separated by ODS CC and semi-preparative HPLC to yield **1** (20 mg), **4** (18 mg), and **6** (13 mg).

3.2.1. Timopregnane A I

White amorphous powder, $[\alpha]_D^{25}$ -40.8 ($c = 0.12$, MeOH), ^{1}H-NMR (500 MHz, C_5D_5N) δ_H and ^{13}C-NMR (125 MHz, C_5D_5N) δ_C see Table S1. HR-ESI-MS *m/z* 501.2821 ($[M + Na]^+$, calcd for $C_{27}H_{42}O_7$, 501.2823).

3.3. Acid hydrolysis of 2 and sugar analysis

Compound **2** (2 mg) was hydrolyzed with 2 M HCl-MeOH (5 ml) under reflux for 3 h. The reaction mixture was diluted with H_2O and extracted with $CHCl_3$. The water layer was neutralized with Na_2CO_3, concentrated, and subjected to TLC analysis together with an authentic sample of D-galactose, and developed with CH_2Cl_2-MeOH-H_2O (15: 6: 1). Detection was carried out with 5% vanillin-sulfuric acid spray.

3.4. Bioassay of cytotoxic activity

Human breast adenocarcinoma MCF7 cells, human colon adenocarcinoma SW480 cells, human hepatocellular carcinoma HepG2 cells, and human gastric cancer SGC7901 cells were obtained from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. MCF7, SW480, and SGC7901 cells were cultured in RPMI-1640 medium (Gibco, California) supplemented with 10% fetal bovine serum (Gibco, Invitrogen Corporation, NY), 100 U/ml benzyl penicillin, and 100 U/ml streptomycin in a humidified environment with 5% CO_2 at 37 °C. HepG2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen Corporation, NY)

supplemented with 10% fetal bovine serum (Gibco, Invitrogen Corporation, NY), 100 U/ml benzyl penicillin, and 100 U/ml streptomycin in a humidified environment with 5% CO_2 at 37 °C.

Cell toxicity was measured by a colorimetric assay using MTT as described previously (Liu et al. 2016). Experiments were carried out in triplicate in a parallel manner. Control cells were treated with culture media containing 0.1% DMSO. After incubation for 24 h, absorbance (A) was measured at 570 nm.

Cell viability (%) was calculated using the following equation:

Cell viability (%) = (Atreatment/Acontrol) × 100%.

IC_{50} (the concentration that caused 50% inhibition of cell proliferations) was calculated by the Logit method.

4. Conclusion

In summary, this study reports the isolation, structural elucidation, and cytotoxic activity of hydrolytic derivatives of TB-III. One new C_{21} steroidal glycoside was isolated together with seven known compounds. Notably, a transformation pathway from TB-III to epipregnenolone derivatives was demonstrated for the first time. This discovery provides new thoughts for studying the metabolism of timosaponins *in vitro* and *in vivo*. The cytotoxic activities of the nine compounds were evaluated against MCF7, SW480, HepG2 and SGC7901 cell lines. Compounds **6** and **7** exhibited significant cytotoxic activity and may merit further research as potential anticancer leads.

Supplementary material

Supplementary material relating to this article is available online, alongside Tables S1–S2 and Figures S1–S28.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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