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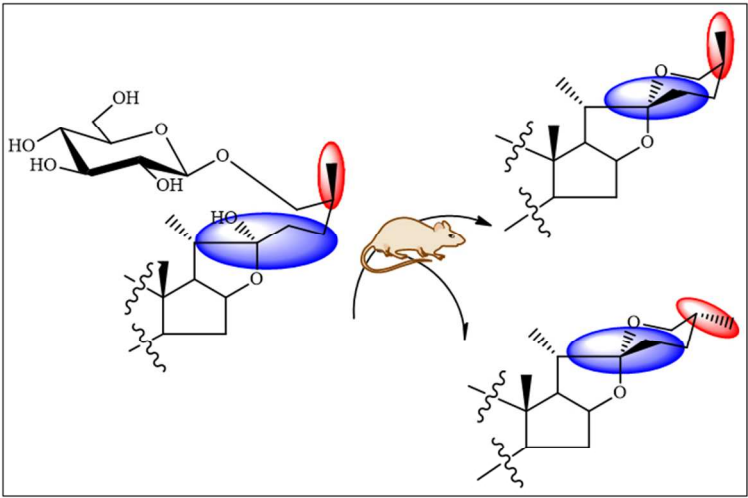


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Graph abstract. Stereoisomerism metabolites found in rats after oral administration of timosaponin B-II
254x190mm (96 x 96 DPI)

**Stereoisomerism metabolites found in rats after oral administration
of timosaponin B-II using HPLC-Q-TOF-MS and NMR method**

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19 Abstract

20 Timosaponin B-II (TB-II), a representative furostanol saponin from Rhizoma Anemarrhenae, has been
21 used to treat diabetes and senile dementia. To better understand the action mechanism of TB-II, it is
22 indispensable to study its metabolism profile *in vivo*. A HPLC-Q-TOF-MS and NMR method for the
23 analysis of TB-II and its metabolites in rat urine has been developed. Five derivatives of TB-II
24 including one novel compound (timosaponin BIII-d) were synthesized and used as the reference
25 compounds. In rat urine, a total of 13 metabolites were identified after oral administration of TB-II and
26 eight of them were confirmed by NMR. The metabolic pathways of TB-II were mainly
27 deglycosylation, F-ring isomerization, and even stereoisomerism at C₂₅. It is important for
28 understanding metabolism of TB-II in rats to clarify its metabolic pathways *in vivo*, which will
29 also provide useful information and reference for similar study in humans. Moreover, the
30 metabolites could contribute to obtain the better compounds than TB-II for the development of new
31 drug in future.

32

33 **Key words:** timosaponin B-II; metabolism; stereoisomerism; HPLC-Q-TOF-MS; NMR

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Introduction

As an important component of many well-known traditional Chinese prescriptions, Rhizoma Anemarrhenae has been clinically employed for the treatment of diabetes and senile dementia for hundreds of years. Timosaponin B-II (TB-II), a natural bioactive steroid glycoside (**Fig. 1**), is one of the major active ingredients in Rhizoma Anemarrhenae. According to the recent pharmacological researches, it has been reported that TB-II has a neuronal protective and anti-inflammatory effects possibly by suppressing the production of pro-inflammatory factors IL-1, IL-6 and TNF- α [1-3]. It could also improve the ability of learning and memory by promoting scavenging of superoxide radicals in the A β -induced dementia models [4]. Moreover, there is a possibility that it is regarded as a potential lead drug candidate used for senile dementia since TB-II could significantly ameliorate the learning and memorizing abilities in memory-deficit rat models [5].

Attention has been raised concerning the potential anti-dementia effects of TB-II. However, it was poorly absorbed via the gastrointestinal tract with the absolute oral bioavailability being only $1.1 \pm 0.3\%$ [6, 7]. Therefore, it is important to investigate the profile of metabolite of TB-II obtained after *in vivo* metabolism to understand its action mechanism. As of today, there are only limited studies performed, and most metabolites were only predicted by their mass spectra information [8], it is not convincing for the structure identification without NMR information.

To take into account the advantages of NMR information for the structure confirmation, this work explored the use of a practical approach to synthesize some derivatives of TB-II to act as the reference compounds for LC-MS analysis. According to the characteristics of chemical structure of TB-II and the previous LC-MS analysis results [8], the major biotransformation pathway of TB-II *in vivo* was deglycosylation. Therefore, in this work, three timosaponin standards (TB-II, timosaponin B-III, and

timosaponin A-III) as well as five deglycosylation derivatives of TB-II including a novel one (timosaponin BIII-d) were obtained (**Fig. 1, 1-5**) and used as the reference compounds. Furthermore, a high performance liquid chromatography in combination with quadrupole-time-of-flight mass spectrometry (HPLC-Q-TOF-MS) method was established to rapidly investigate the metabolism in rat urine following oral administration of TB-II.

Materials & methods

Chemicals & Reagents

Hydrazine acetate was purchased from Sigma-Aldrich (Beijing, China). 1, 8-Diazabicyclo [5, 4, 0]-7-ene (DBU), Trichloroacetonitrile, and Trimethylsilyltrifluoromethanesulphonate (TMSOTf) were purchased from Ourchem Chemical Co., Ltd (Shanghai, China). Chloroform-*d*, pyridine-*d*₅ and DMSO-*d*₆ were obtained from Cambridge Isotope Laboratories, Inc. (MA, USA). Acetonitrile and formic acid of LC-MS grade were purchased from Dikma Technologies Inc. (CA, USA). All other analytical chemical reagents of analytical grade were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Deionized water was purified using a Milli-Q system (MA, USA). Silica gel (FCP, 200-300 Mesh) was used for flash chromatography.

Three reference standards timosaponin A-III, timosaponin B-III, TB-II were previously isolated from the Rhizoma Anemarrhenae in our laboratory and their chemical structures were unambiguously identified by comparison of their NMR and MS data with those in previous literature [9-11], the purities were all more than 98% by HPLC determination.

Sample Preparation

All animal procedures were conducted in accordance with the guidelines from the Review Committee of Animal Care and Use at the Shanghai Institute of Materia Medica (Shanghai, China). Six male

Sprague-Dawley rats (200-220 g; Shanghai SLAC Laboratory Animal Co., Shanghai, China) were kept in an environmentally controlled breeding room for 1 week and fasted 12 h (but with access to water) before starting the experiments.

Urine samples were obtained from six rats that have been on treatment with TB-II of 300 mg/kg using metabolic cages. A 600 μ L aliquot of mixed drug-containing urine samples was loaded onto a Solid Phase Extraction (SPE) cartridge, which was preconditioned with 2 mL methanol and 2 mL water. Then the cartridge was washed with 1 mL of water and the analyte eluted with 1 mL of methanol. The eluted solution was evaporated to dryness in a water bath at 37 $^{\circ}$ C under a gentle stream of nitrogen and the residue was reconstituted in 200 μ L methanol. The resulting solution was centrifuged at 12,000 rpm for 10 min at 4 $^{\circ}$ C and 1.0 μ L of supernatant was injected into the chromatographic system for LC-MS/MS analysis. Blank samples as controls were prepared with the same method as the drug-containing samples.

Instruments and analytical conditions

^1H , ^{13}C -NMR spectra and two dimension NMR spectra (HSQC and HMBC) were obtained on Bruker DRX-400 (Bruker, Germany) spectrometer at 26 $^{\circ}$ C, with $\text{DMSO}-d_6$, $\text{pyridine}-d_5$ or $\text{chloroform}-d$ as the solvents.

The chromatography analytical procedures were performed on an Agilent 1260 Series (Agilent, Santa Clara, CA, USA) LC system equipped with a binary pump, an online degasser, an auto plate-sampler, and a thermostatically controlled column compartment. The separation was carried out on an Agilent poroshell 120 EC- C_{18} column (100 mm \times 2.1 mm, 2.7 μ m; Agilent, CA, USA). The binary gradient elution system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) and separation was achieved using the following gradient: 8-15% B at 0-5

min, 15-24% B at 5-10 min; 24-28% B at 10-15 min; 28-50% B at 15-30 min; 50-90% B at 30-45 min. The composition was then held at 90% B for 5 min and returned to initial conditions and maintained 6 min for equilibration. The flow rate was $0.35 \text{ mL} \cdot \text{min}^{-1}$ and the injection volume was $1.0 \mu\text{L}$.

Mass spectrometry was performed using an Agilent 6530B Q-TOF mass spectrometer (Agilent, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) interface, and was operated in positive ion mode with parameters set as follows: capillary voltage, 4000 V; fragmentor, 150 V; pressure of nebulizer, 35 psi; drying gas temperature, 300°C ; sheath gas temperature, 350°C . Nitrogen was used as sheath and drying gas at a flow rate of $8.0 \text{ L} \cdot \text{min}^{-1}$ and $11.0 \text{ L} \cdot \text{min}^{-1}$, respectively. Data were collected in centroid mode and the mass range was set at m/z 50-1200 using the extended dynamic range and the collision energy was optimized at 25 V.

Synthesis of some derivatives

The details of synthetic procedures for compounds **1-5** and their NMR information are shown in Supplementary Materials

Results and Discussion

Chemistry

The structures of compounds **1-4** were confirmed by ^1H -NMR, ^{13}C -NMR, and HR-MS spectral data. The significant differences between **1** and **2**, as well as **3** and **4**, are the chemical shifts of H-27 and H-26 because of the configuration of C-25 [12, 13], that is, as for 25*S* configuration, the chemical shifts of H-27 were in the lower field than that of 25*R* (0.98 ppm, $J=6.4 \text{ Hz}$ for 25*S* while 0.77 ppm, $J=6.2 \text{ Hz}$ for 25*R*) and the difference between two protons of H-26 was more distinct ($\Delta_{26-\text{H}}=0.65 \text{ ppm}$ for 25*S* while $\Delta_{26-\text{H}}=0.10 \text{ ppm}$ for 25*R*) (**Fig. 2**).

One novel acid hydrolysis product of timosaponin BIII-d was also obtained. Compound **5** was

obtained as a white amorphous powder. Its molecular formula, $C_{39}H_{64}O_{13}$ (m/z 741.4422; calcd. for $C_{39}H_{65}O_{13}^+$, 741.4420) was deduced by positive HR-ESI/MS. Furthermore, the ESI-MS/MS (positive-ion mode) of **5** showed the characteristic ion peaks at m/z 741.4 $[M+H]^+$, 579.4 $[M+H-162]^+$, and 417.3 $[M+H-162-162]^+$, suggesting the presence of two hexose units. The 1H and ^{13}C NMR spectral data of **5** (Table 1) were almost identical to those standard compound of timosaponin B-III, possessing the characteristic signals of furostanol saponin such as four methyl signals at δ_H 0.71 (s, Me-18), 0.85 (s, Me-19), 1.64 (s, Me-21) and 1.04 (d, $J=6.8$ Hz, Me-27), two olefinic carbons at δ_C 152.9 (C-22) and 104.1 (C-20). However, two signals for anomeric protons at δ_H 4.89 (d, $J=7.6$ Hz), 4.84 (d, $J=7.7$ Hz) suggested the presence of only two sugar moieties. By analysis of the 1D and 2D NMR spectra, they were identified as β -galactose and β -glucose units, respectively. The HMBC spectrum showed correlations between H-1' (δ_H 4.89) of galactose unit and C-3 (δ_C 75.7), between H-1'' (δ_H 4.84) of glucose unit and C-26 (δ_C 75.8) of the aglycone moiety (Fig. 3). Thus, **2** was determined to be (25*S*)-26-*O*- β -D-glucopyranosyl-20(22)-ene-5 β -furost-3-*O*- β -D-galactopyranoside, which was simply named timosaponin BIII-d.

HPLC-MS/MS characterization of the synthetic products and commercial standards

In order to standardize the reference compounds, the retention time and MS spectral data of synthetic products and commercial standards were analyzed. Meanwhile, we have summarized the fragmentation patterns and chromatographic retention behaviors that will be valuable for the subsequent on-line elucidation of structurally related compounds.

These data will provide a scientific basis for identification of the metabolites in biological samples. Positive ion mode was selected for HPLC-MS/MS analysis in this study, as it provides extensive structural information via collision-induced dissociation (CID). A typical MS total ion chromatogram

(TIC) of eight reference compounds studied in the experiment is shown in Supporting Information, **Fig. S13 (a)**. Positive ESI analysis of **S2** gave the $[M+H]^+$ ion at m/z 903.4948 ($C_{45}H_{75}O_{18}^+$, 0 ppm). The MS/MS CID fragmentation of the m/z 903 ion yielded three product ions at m/z 741.4417 ($C_{39}H_{65}O_{13}^+$, -0.4 ppm), 579.3887 ($C_{33}H_{55}O_8^+$, -0.7 ppm) and 417.3363 ($C_{27}H_{45}O_3^+$, 0 ppm) (**Fig. 4a**) through consecutive losses of three hexose units ($162\text{ Da} \times 3$), respectively. The ions at m/z 417 continued to yield the ion at m/z 399.3254 ($C_{27}H_{43}O_2^+$, -1.0 ppm) by loss of a H_2O (18 Da). In addition, the fragment ion at m/z 273.2208 ($C_{19}H_{29}O^+$, -1.8 ppm) was attributed to a skeleton residue by the cleavage of E-ring due to the presence of a 16,22-epoxy residue and a 22,26-epoxy residue, and then the ion continued to lose a molecule of H_2O to yield an ion at m/z 255.2109 ($C_{19}H_{27}^+$, 0.9 ppm). Based on the above data, compound **S2** was confirmed as timosaponin B-III. The proposed fragmentation pathway for compound **S2** is shown in **Fig. 4b**.

Compound **S5** yielded the $[M+H]^+$ ion at m/z 579.3892 ($C_{33}H_{55}O_8^+$, 0.2 ppm). And as the **S2** did, its MS/MS spectra produced four prominent fragment ions at m/z 417.3362, 399.3253 (low), 273.2215 and 255.2107 (**Fig. 4c**), originating from losses of hexose unit (162 Da), H_2O (18Da) and E-ring cleavage, respectively. However, there were two compounds (timosaponin A-I and its isomer) with the elementary composition of $C_{33}H_{54}O_8$ among the eight reference compounds, compound **S5** was ultimately identified as isotimosaponin A-I by a single injection analysis (Supporting Information, **Fig. S13-c**), and the compound **S6** was confirmed as timosaponin A-I. Similarly, the isomers (**S3** & **S4** and **S7** & **S8**) were successively differentiated as shown in **Fig. S13-b** and **Fig. S13-d**.

Taking the same identification methods described above, the eight reference compounds were unambiguously identified as TB-II, timosaponin B-III, timosaponin BIII-d, timosaponin A-III, isotimosaponin A-I, timosaponin A-I, isosarsasapogenin, and sarsasapogenin respectively. The

retention time, ESI-MS/MS data and fragmentations of eight standard compounds are summarized in Table 2.

The ESI-MS/MS data of these compounds shared some common features, such as the neutral losses of terminal hexose units (162 Da), H₂O (18 Da), and the partial cleavage of E-ring. It could be inferred that the ions at m/z 273 (C₁₉H₂₉O⁺) and 255 (C₁₉H₂₇⁺) were a pair of characteristic product ions of timosaponin and they could be used for structural elucidation of these derivatives with similar skeletons. Furthermore, we could tentatively conclude that the retention time of spirostanols were longer than furostanols on the reversed phase liquid chromatography (RPLC) column, and 25S was longer than that of 25R.

Identification of the metabolites in rat urine by HPLC-MS/MS

Excluding the influence of endogenous matrix in the biological sample, 13 peaks were tentatively predicted to be parent compound or metabolites of TB-II by comparing drug-containing samples with the corresponding control samples directly (Fig. 5). By comparing the accurate masses of peaks appearing in the chromatograms of drug-containing urine with those previously identified reference compounds, all the eight reference compounds (peaks 2, 3, 5, 9, 10, 11, 12 and 13) in urine were found, namely one parent compound and seven metabolites could be identified unambiguously. Besides, another five possible metabolites were also inferred according to the obtained exact molecular weight and its MS/MS information.

Peak 1 eluted at 14.285 min, giving the [M+H]⁺ ion at m/z 593.3688 (C₃₃H₅₃O₉⁺, 0.7 ppm), which was 176 Da higher than that of sarsasapogenin/isosarsasapogenin. After losing a neutral fragment 176 Da (a glucuronide unit), the fragment ions at m/z 417.3366, 273.2211 and 255.2108 were the same as those of sarsasapogenin/isosarsasapogenin, suggesting that peak 1 may be the glucuronide conjugate of

sarsasapogenin/isosarsasapogenin. As for peaks 4 and 6, the molecular ions of peak 4 (m/z 759.4521) and peak 6 (m/z 597.4001) were 162 and 324 Da less than that of TB-II, respectively. Furthermore, the representative fragment ions also showed no difference with TB-II. Therefore, they were tentatively identified as deglycosylated TB-II and di-deglycosylated TB-II, respectively. Similarly, peak 7 could be tentatively identified as deglycosylated timosaponin BIII-d.

Peak 8 exhibited the same $[M+H]^+$ ion with peaks 5 and 9 at m/z 741.4422 ($C_{39}H_{65}O_{13}^+$, 0.3ppm), revealing that these three compounds were isomers. Moreover, they all showed the same fragments ions in the MS/MS spectra. On the basis of analysis of peaks 10 and 11, as well as peaks 12 and 13, we could infer that there may be an isomer of timosaponin A-III, which were isomerized only at C-25 in the process of metabolism *in vivo*. Therefore, peak 8 was tentatively identified as isotimosaponin A-III.

Conclusions

In this study, 13 metabolites in urine were detected by HPLC-Q-TOF-MS after an oral administration of TB-II to rats, and eight of them were identified unambiguously by the reference compounds, the proposed metabolic pathway of TB-II in rats is presented in **Fig. 6**. The metabolic pathways of TB-II were mainly deglycosylation and F-ring isomerization. Meanwhile, it is so interesting that the furostanol timosaponin mainly transform to spirostanol and even the isospirostanol timosaponin (configuration of C-25 will convert to *R*) *in vivo*. To the best of our knowledge, it is the first report of the isomerization behaviors. As described in the previous literature [14-16], the compounds of smilagenin (isosarsasapogenin) and 25-*R* spirostanol timosaponin have wide range of pharmacological activities, such as stimulating the gene expression of BDNF, improving memory, and antitumor activities. This means that the biological activity of TB-II could be attributed to the presence of the metabolites with stereoisomerism at C25. The results in this work are important

for the understanding of TB-II metabolism in rats and provide useful information and reference for similar study in humans.

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245

246 **Figures captions:**247 **Fig. 1.** Structures of the TB-II and its main available deglycosylated metabolites.

248 **Fig. 2.** ^1H -NMR (400 MHz) spectra (pyridine- d_5) (δ 0.6-1.2 ppm and δ 3.1-4.2 ppm) of
249 compounds with 25*S* (a) and 25*R* (b): as for 25*S* configuration, the chemical shifts of H-27 were in
250 the lower field than that of 25*R* (0.98 ppm, $J=6.4$ Hz for 25*S* while 0.77 ppm, $J=6.2$ Hz for 25*R*) and
251 the difference between two protons of H-26 was more distinct ($\Delta_{26\text{-H}}=0.65$ ppm for 25*S* while
252 $\Delta_{26\text{-H}}=0.10$ ppm for 25*R*).

253 **Fig. 3.** Key HMBC ($^1\text{H} \rightarrow ^{13}\text{C}$) correlations of compound **5**

254 **Fig. 4.** Representative MS/MS spectrum of timosaponin BIII (a) and its proposed fragmentation
255 pathway (b); the representative MS/MS spectrum of isotimosaponin A-I

256 **Fig. 5.** Total ion chromatograms (TICs) for rat blank urine (a) and urine after oral administration
257 of TB-II (b) in positive mode.

258 **Fig. 6.** Proposed metabolic pathways of TB-II in rat.

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260

261 **Table 1.** The ¹H (400 MHz) and ¹³C (100 MHz) NMR spectroscopic data for compound **5**
262 (pyridine-*d*₅)

Position	δ_C (ppm)	δ_H (ppm)	Position	δ_C (ppm)	δ_H (ppm)
1	31.5	1.50 (m), 1.47 (o)	22	152.9	-
2	27.3	2.02 (m), 1.10 (m)	23	31.1	2.07 (m), 1.56 (m)
3	75.7	4.25 (o)	24	24.1	2.26 (m), 1.04 (o)
4	34.9	2.12 (m), 1.46 (o)	25	34.2	1.97 (m)
5	37.5	2.03 (o)	26	75.8	4.05 (o), 3.50 (m)
6	27.5	1.29 (m), 1.13 (o)	27	17.7	1.04 (d, <i>J</i> =6.8 Hz)
7	27.6	1.57 (m), 1.09 (o)	Gal		
8	35.7	1.46 (o)	1'	104.4	4.89 (d, <i>J</i> =7.6 Hz)
9	40.7	1.31 (m)	2'	73.2	4.49 (m)
10	35.7	-	3'	77.4	4.11 (o)
11	21.8	1.36 (m), 1.21 (o)	4'	70.8	4.63 (m)
12	40.6	1.76 (m), 1.20 (o)	5'	76.0	4.20 (o)
13	44.4	-	6'	62.9	4.48 (o), 4.11 (o)
14	55.2	0.88 (o)	Glu		
15	31.9	1.87 (m), 1.49 (m)	1''	105.7	4.84 (d, <i>J</i> =7.7 Hz)
16	85.1	4.85 (o)	2''	75.0	4.05 (o)
17	65.2	2.51 (m)	3''	79.1	4.25 (o)
18	14.8	0.71 (s)	4''	72.2	4.25 (o)
19	24.4	0.85 (s)	5''	79.0	3.97 (m)
20	104.1	-	6''	63.3	4.58 (m), 4.42 (m)
21	12.3	1.64 (s)			

263 Note: (o) Overlapped with other signals

264 **Table 2.** Identification of the metabolites in rat urine by HPLC-Q/TOF-MS/MS

Peak NO.	t _R (min)	Formula	Experimental Mass (<i>m/z</i>) ^a	Calculated Mass (<i>m/z</i>)	Error (ppm) ^b	Error (mDa) ^c	MS/MS Fragments (<i>m/z</i>)	Proposed compound
1	14.285	C ₃₃ H ₅₂ O ₉	593.3688	593.3684	0.7	0.4	417.3366, 273.2211, 255.2108	Sarsasagenin/Isosarsasapogenin glucuronide
2	16.165	C ₄₅ H ₇₆ O ₁₉	921.5055	921.5054	0.1	0.1	903.4952, 741.4415, 579.3883, 417.3363, 399.3262, 273.2205, 255.2113	TB-II *
3	22.009	C ₄₅ H ₇₄ O ₁₈	903.4948	903.4948	0	0	741.4417, 579.3887, 417.3363, 399.3261, 273.2208, 255.2109	Timosaponin BIII *
4	23.280	C ₃₉ H ₆₆ O ₁₄	759.4521	759.4525	-0.5	-0.4	741.4420, 579.3889, 417.3362, 399.3253, 273.2211, 255.2115	Deglycosylated TB-II
5	25.068	C ₃₉ H ₆₄ O ₁₃	741.4422	741.4420	0.3	0.2	579.3890, 417.3373, 273.2207, 255.2103	Timosaponin BIII-d *
6	27.142	C ₃₃ H ₅₆ O ₉	597.4001	597.3997	0.7	0.4	579.3899, 417.3371, 273.2214, 255.2101	Di-deglycosylated TB-II
7	27.955	C ₃₃ H ₅₄ O ₈	579.3899	579.3891	1.4	0.8	417.3370, 399.3266, 273.2216, 255.2112	Deglycosylated timosaponin BIII-d
8	30.902	C ₃₉ H ₆₄ O ₁₃	741.4422	741.4420	0.3	0.2	579.3894, 417.3373, 399.3269, 273.2218, 255.2110	Isotimosaponin AIII
9	32.935	C ₃₉ H ₆₄ O ₁₃	741.4411	741.4420	-1.2	-0.9	579.3884, 417.3352, 399.3254, 273.2209, 255.2101	Timosaponin AIII *
10	38.068	C ₃₃ H ₅₄ O ₈	579.3892	579.3891	0.2	0.1	417.3362, 273.2215, 255.2107	Isotimosaponin AI *
11	38.423	C ₃₃ H ₅₄ O ₈	579.3895	579.3891	0.7	0.4	417.3360, 273.2211, 255.2109	Timosaponin AI *
12	44.979	C ₂₇ H ₄₄ O ₃	417.3367	417.3363	1.0	0.4	273.2213, 255.2103	Isosarsasapogenin *
13	47.011	C ₂₇ H ₄₄ O ₃	417.3372	417.3363	2.2	0.9	273.2217, 255.2112	Sarsasapogenin *

265 ^a Gave [M+H]⁺ as quasi-molecular ions.266 ^b Differences between the measured and calculated values.267 ^c Milli-Dalton, differences between the measured and calculated values.

268 * Identified with the reference compounds.

269

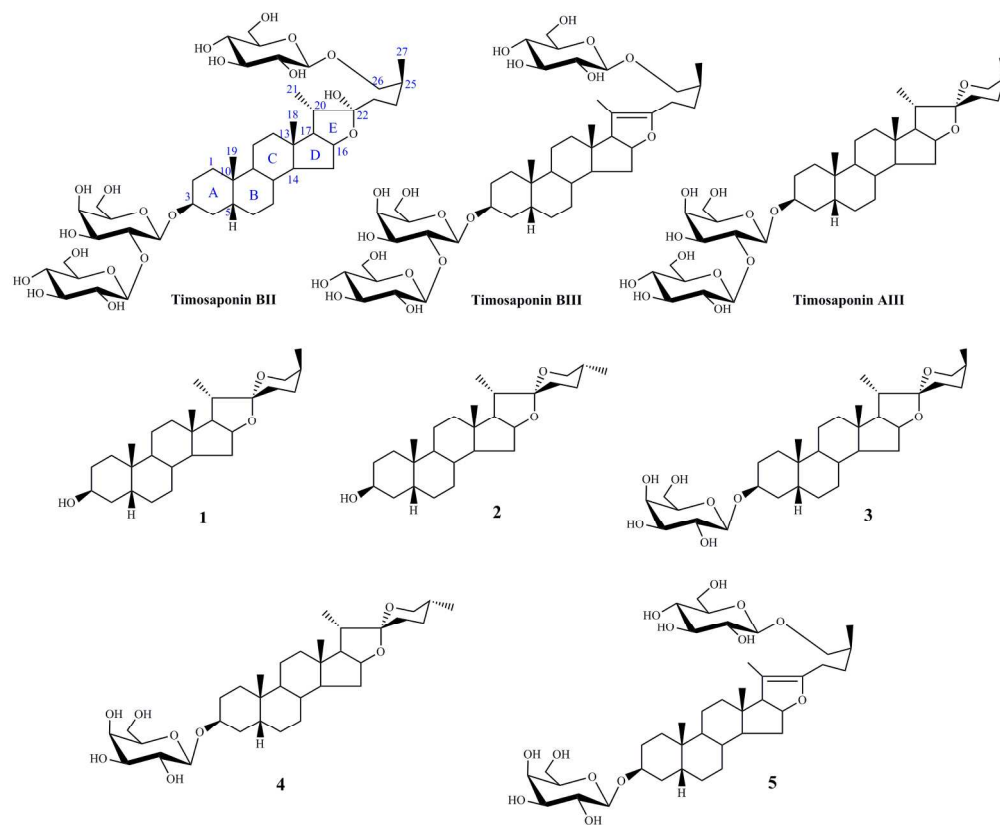


Fig. 1. Structures of the TB-II and its main available deglycosylated metabolites.
181x148mm (300 x 300 DPI)

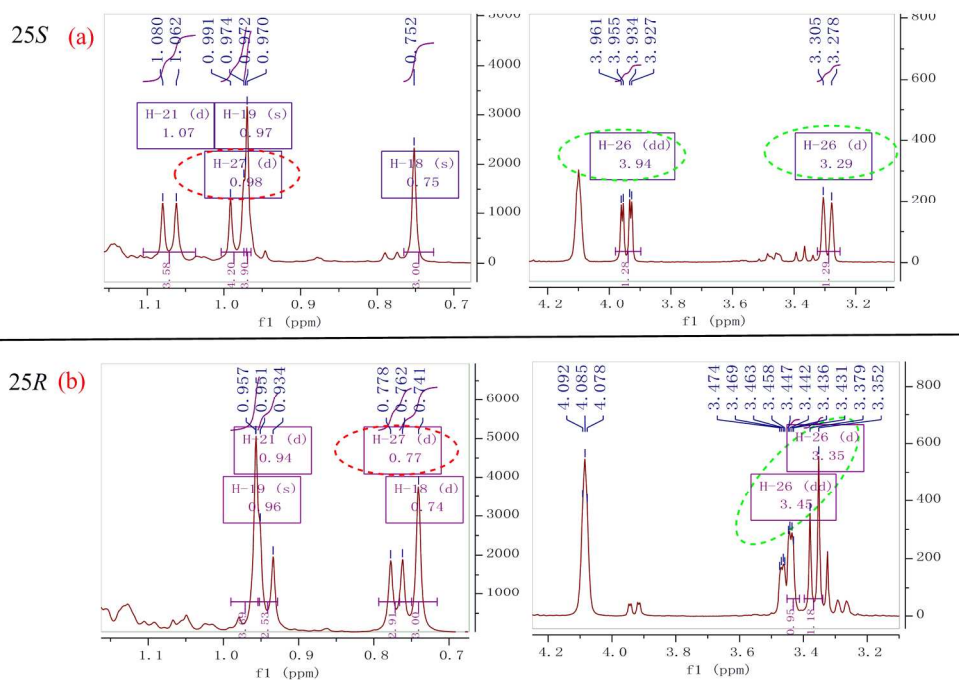


Fig. 2. ^1H -NMR (400 MHz) spectra (pyridine- d_5) (δ 0.6-1.2 ppm and δ 3.1-4.2 ppm) of compounds with 25S (a) and 25R (b): as for 25S configuration, the chemical shifts of H-27 were in the lower field than that of 25R (0.98 ppm, $J=6.4$ Hz for 25S while 0.77 ppm, $J=6.2$ Hz for 25R) and the difference between two protons of H-26 was more distinct ($\Delta 26\text{-H}=0.65$ ppm for 25S while $\Delta 26\text{-H}=0.10$ ppm for 25R). 177x120mm (300 x 300 DPI)

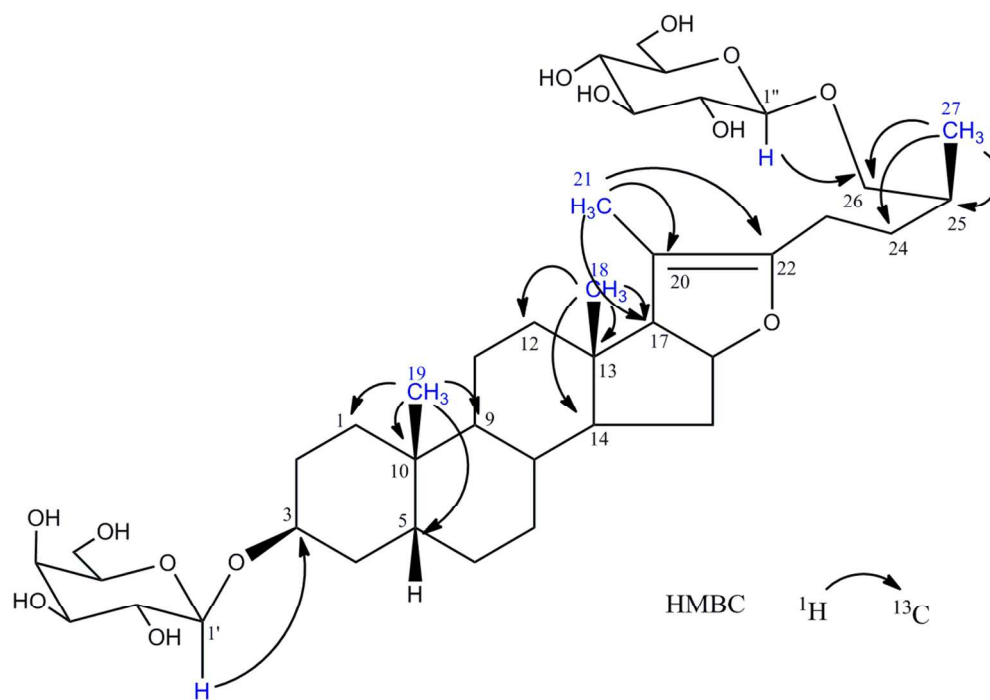


Fig. 3. Key HMBC (¹H → ¹³C) correlations of compound 5
108x75mm (300 x 300 DPI)

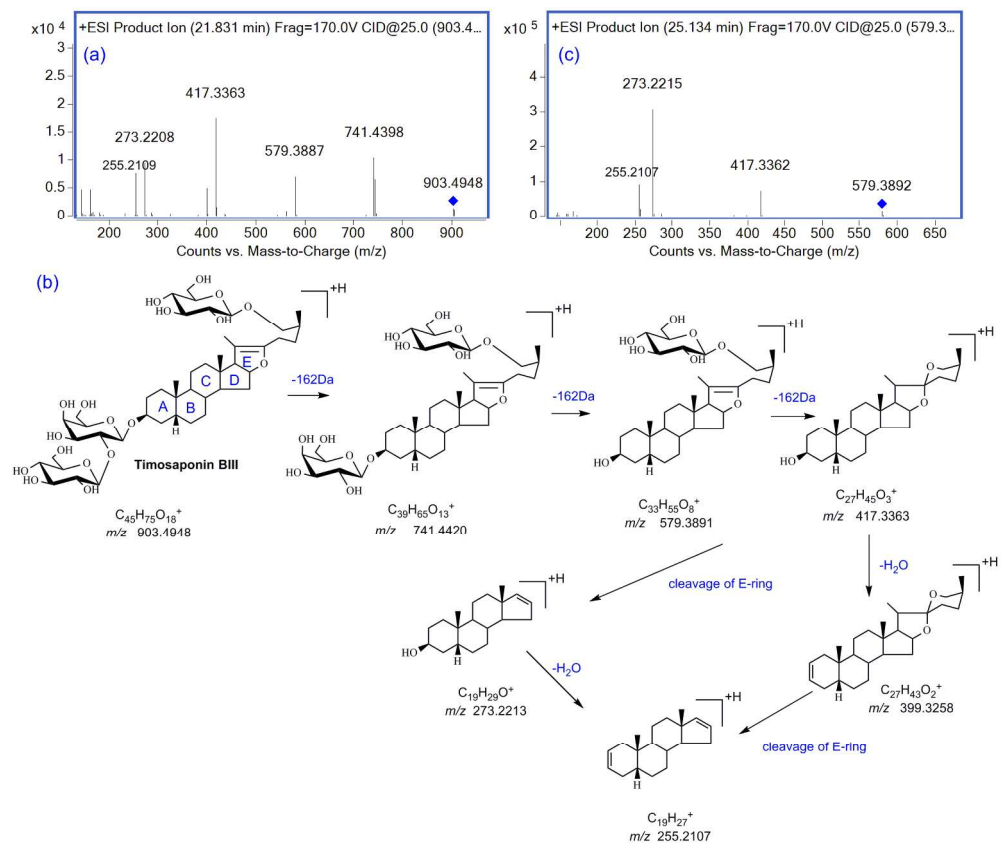


Fig. 4. Representative MS/MS spectrum of timosaponin BIII (a) and its proposed fragmentation pathway (b); the representative MS/MS spectrum of isotimosaponin A-I 191x161mm (300 x 300 DPI)

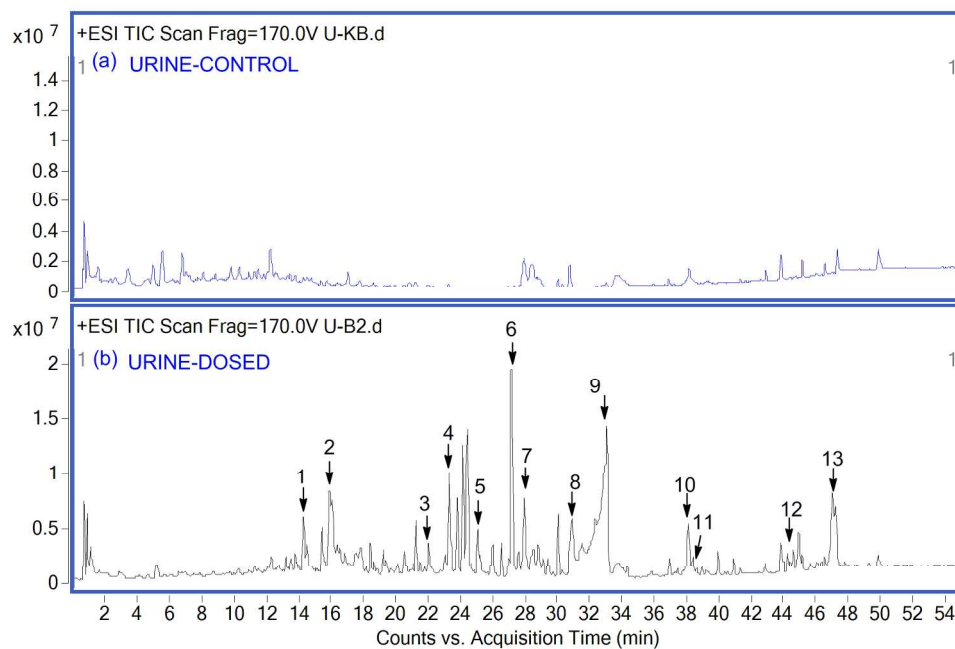


Fig. 5. Total ion chromatograms (TICs) for rat blank urine (a) and urine after oral administration of TB-II (b) in positive mode
186x121mm (300 x 300 DPI)

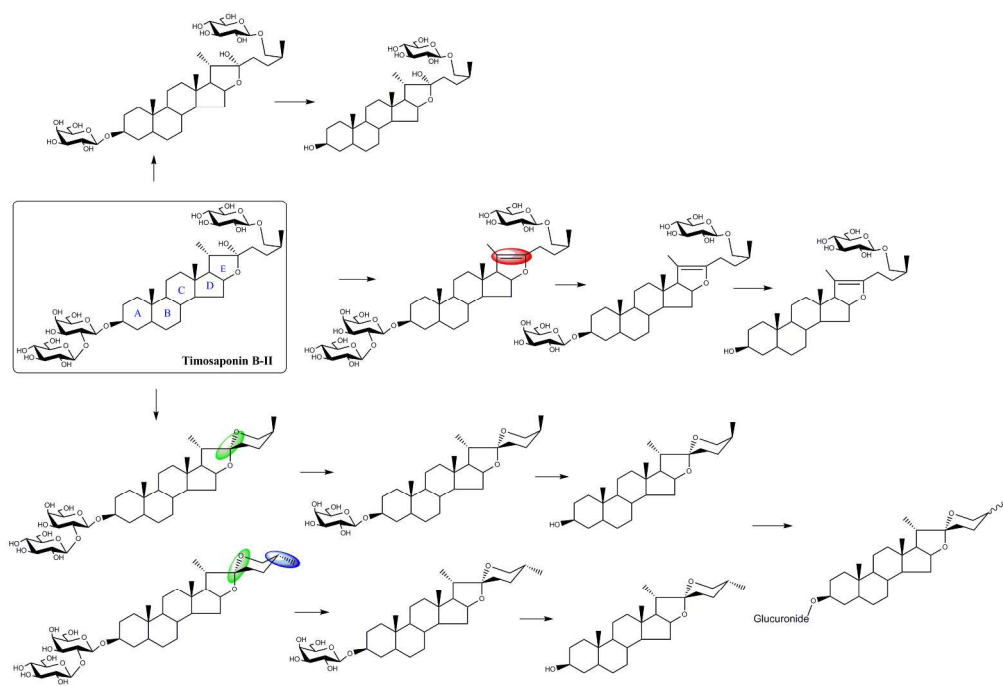


Fig. 6. Proposed metabolic pathways of TB-II in rat.
239x162mm (300 x 300 DPI)