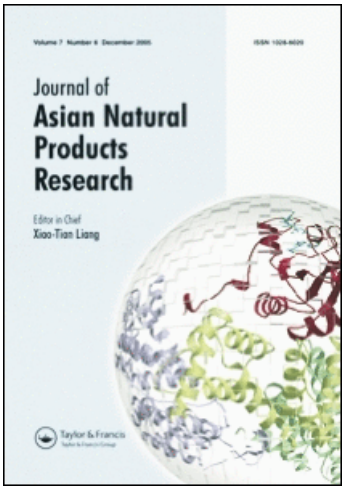

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Timosaponin BII (**1**), a steroidal saponin showing potential anti-dementia activity, was regioselectively hydrolyzed into its deglycosyl derivatives by the crude enzyme from *Aspergillus niger* AS 3.0739. Three biotransformation products, timosaponin BII-a (**2**), timosaponin BII-b (**3**), and timosaponin BII-c (**4**), were purified and their structures were elucidated on the basis of 1D NMR, 2D NMR, FAB-MS, and HR-ESI-MS spectral data. Compounds **2** and **3** are new compounds.

Keywords: biotransformation; steroidal saponin; timosaponin BII; *Aspergillus niger*; hydrolysis

1. Introduction

Steroidal saponin, one kind of glycoside derivatives of steroid, is divided into two major groups, spirostanoside and furostanoside. Spirostanoside commonly contains one sugar chain generally at the C-3 position, whereas furostanoside bears two sugar chains always at the C-3 and C-26 positions. Steroidal saponin is the main active substance in traditional Chinese medicine, as well as the lead compound for new drugs [1–3]. Timosaponin BII (**1**), a furostanoside from *Rhizoma Anemarrhenae*, was reported to be able to improve the learning and memorizing abilities [4], and has been studied as a new drug candidate for anti-vascular dementia (VD) [5].

Aspergillus niger AS 3.0739 (*A. niger*), a very important industrial strain, can produce amylase, acid proteinase, cellulase, pectinase, glucose oxidase, etc. Some strains can even catalyze the hydroxylation and are used for 11β-hydroxylation of steroids and the production of hydrocortisone [6,7].

The C-3 sugar chain of steroidal saponins plays an important role in pharmacological and biological activities. We have investigated various specific enzymes for modifying the strict glycosyl groups of the C-3 sugar chain of steroidal saponins and found some specific micro-organisms or enzymes. For example, the enzyme from *Curvularia lunata* had the specificity to hydrolyze the terminal α-1, 2-rhamnosyl residue at the C-3 position [8–10]. The enzyme preparation, Klerzyme 150, had the ability to hydrolyze the terminal α-1, 4-glycosyl residue at the C-3 position. For furostanosides, we found that the C-26 sugar chain was easily hydrolyzed by some enzyme preparations and micro-organisms,

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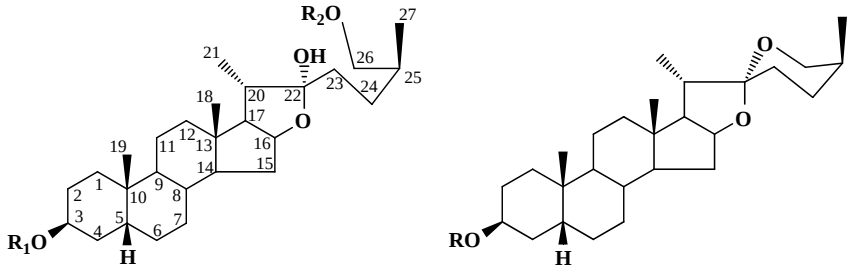
such as glucosidase, hemicellulase, amylase, etc. However, it is very difficult to hydrolyze sugar residues at C-3 first while the C-26 glucosyl remains. In this paper, the crude enzyme solution of a microorganism, *A. niger* AS 3.0739, was able to selectively hydrolyze the sugar residues at C-3 of **1** into its derivatives, timosaponin BII-a (**2**) and timosaponin BII-b (**3**), and hydrolyze the C-26 glucosyl of **1** into timosaponin BII-c (**4**, timosaponin AIII, a known compound). The three products were isolated and their structures were elucidated on the basis of 1D and 2D NMR, FAB-MS, and HR-ESI-MS spectral data. Compounds **2** and **3** are new compounds (Figure 1).

2. Results and discussion

The biotransformation of **1** by the crude enzyme from *A. niger* was performed under pH 8.0 conditions and three products were obtained. According to the TLC and HPLC analyses, these transformed products exhibited less polarity than compound **1**, which were two secondary furostanosides and one secondary spirostanoside.

Timosaponin BII-a (**2**) was isolated as a white amorphous powder, and the positive reactions to the Liebermann–Burchard and Ehrlich reagents suggested **2** to be a furostanol saponin. Its HR-ESI-MS spectrum showed the $[M - H]^-$ ion at *m/z* 757.4406, indicating the molecular formula $C_{39}H_{66}O_{14}$, and this was supported by the ^{13}C NMR spectrum. Furthermore, the FAB-MS (positive-ion mode) of **2** showed the characteristic ion peaks at *m/z* 759.5 $[M + H]^+$, 597.4 $[M + H - 162]^+$, and 417.1 $[M + H - 162 - 162 - 18]^+$, suggesting the presence of two hexose units. Moreover, comparing with compound **1**, the FAB-MS also showed the absence of one hexose unit in compound **2**. Moreover, the ^1H and ^{13}C NMR spectral data of **2** were almost identical to those of compound **1**, possessing the characteristic signals of furostanol saponin such as the methyl singlets at δ 0.87 (s, Me-18), 0.82 (s, Me-19) and the methyl doublets at δ 1.02 (d, $J = 6.6$ Hz, Me-27), 1.32 (d, $J = 6.6$ Hz, Me-21), except for the disappearance of one set of glucose unit. Besides, only two anomeric proton signals and two corresponding carbon signals were observed at δ_H 4.85 (d, $J = 7.2$ Hz), 4.80 (d, $J = 7.8$ Hz) and δ_C 103.9 and 105.2, and these two sugar units were confirmed as galactose and glucose, respectively. According to the J values (>7 Hz) of two anomeric protons, the β-orientation of the anomeric centers for both hexoses was confirmed [11, 12]. In addition, the HMBC correlations between the anomeric proton signal at δ 4.85 (H-1′) and the carbon signal at δ 75.6 (C-3), between the anomeric proton signal at δ 4.80 (H-1″) and the carbon signal at δ 75.4 (C-26) displayed the galactose and the



1 R_1 = β-*D*-glc-(1→2)-*O*-β-*D*-gal- R_2 = β-*D*-glc

2 R_1 = β-*D*-gal R_2 = β-*D*-glc

3 R_1 = H R_2 = β-*D*-glc

4 R = β-*D*-glc-(1→2)-*O*-β-*D*-gal-

Figure 1. Chemical structures of compounds **1**–**4**.

glucose that were respectively linked to the C-3 and C-26 positions (Figure 2). Thus, the structure of **2** was determined to be (25*S*)-26-*O*-β-D-glucopyranosyl-22-hydroxy-5β-furost-3β,26-diol-3-*O*-β-D-galactopyranoside, namely, the terminal glucopyranosyl residue at C-3 sugar chain of compound **1** was enzymically hydrolyzed to give compound **2**. The complete assignment of the ^{1}H and ^{13}C NMR signals of **2** was performed by $^{1}H-^{1}H$ COSY, HSQC, and HMBC experiments (Table 1).

Timosaponin BII-b (**3**) was isolated as a white amorphous powder, and the positive reactions to the Liebermann–Burchard and Ehrlich reagents suggested **3** to be a furostanol saponin. Its HR-ESI-MS spectrum showed the $[M-H]^{-}$ ion at *m/z* 595.3863, indicating the molecular formula $C_{33}H_{56}O_9$, and this was supported by the ^{13}C NMR spectrum. Furthermore, the FAB-MS (positive-ion mode) of **3** showed characteristic ion peaks at *m/z* 597.5 $[M+H]^{+}$ and 417.1 $[M+H-162-18]^{+}$, suggesting the presence of one hexose unit. Moreover, comparing with compound **1**, the FAB-MS also showed the absence of two hexose units in compound **3**. Moreover, the ^{1}H and ^{13}C NMR spectral data of **3** were almost identical to those of compound **1**, possessing the characteristic signals of furostanol saponin such as the methyl singlets at δ 0.91 (s, Me-18), 1.01 (s, Me-19) and the methyl doublets at δ 1.02 (d, $J = 6.6$ Hz, Me-27), 1.33 (d, $J = 6.6$ Hz, Me-21), except for the disappearance of two sets of hexose units. Besides, only one anomeric proton signal and one corresponding carbon signal were observed at δ_H 4.81 (d, $J = 7.8$ Hz) and δ_C 105.2, and this sugar unit was confirmed as glucose by comparing with those of compound **1** which were assigned previously. According to the *J* value (>7 Hz) of the, anomeric proton, the β-orientation of the anomeric center for the hexose was confirmed [11, 12]. In addition, the HMBC correlations between the anomeric proton signal at δ 4.81 (H-1″) and the carbon signal at δ 75.4 (C-26) displayed that the glucose was linked to the C-26 position . Thus, the structure of **3** was determined to be (25*S*)-22,3-dihydroxy-5β-furost-3β,26-diol-26-*O*-β-D-glucopyranoside, namely, the sugar moiety attached to C-3 in compound **2** was enzymically hydrolyzed to give compound **3**. The complete assignment of the ^{1}H and ^{13}C NMR signals of **3** was performed by $^{1}H-^{1}H$ COSY, HSQC, and HMBC experiments (Table 1).

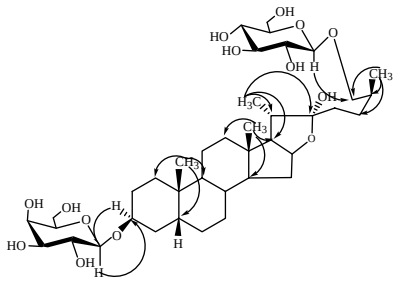


Figure 2. Key HMBC correlations of **2** (H → C).

We investigated the time course (0–84 h) catalyzed by the crude enzyme from *A. niger* AS 3.0739, and the results were analyzed by HPLC (Figure 3). The first product (**2**) was observed at 6 h, and the second product (**3**) was detected at 12 h, and the third product (**4**) was detected after 24 h. The results suggested that the terminal glucosyl group of C-3 sugar chain was first hydrolyzed, then the galactosyl residue at C-3 was removed, and finally the C-26 glucosyl moiety was hydrolyzed. The reaction path is illustrated in Figure 4.

In conclusion, this is the first report that the crude enzyme from *A. niger* could hydrolyze the sugars of timosaponin BII. This study provides some important information on the application of *A. niger* in regioselective hydrolysis of steroidal saponins, and the biological activity of these derivatives and their structure–activity relationship will be evaluated in the future.

Table 1. ^{1}H and ^{13}C NMR spectral data of **2** and **3** in pyridine-d_5 (δ in ppm, J in Hz).

	2		**3**	
Position	δ_C	δ_H	δ_C	δ_H
1	31.0	1.47 (o) 1.43 (m)	28.7	1.73 (o) 1.61 (o)
2	27.6	1.86 (m) 1.09 (m)	27.2	1.88 (m) 1.14 (m)
3	75.6	4.39 (m)	66.1	4.38 (m)
4	30.6	1.97 (m) 1.81 (o)	30.7	1.85 (m) 1.54 (m)
5	37.0	1.94 (m)	37.1	1.96 (m)
6	26.8	1.26 (o)	26.9	1.54 (m)
7	26.8	0.94 (m)	26.9	1.33 (o)
8	35.4	1.50 (m)	35.7	1.04 (o)
9	40.3	1.29 (m)	40.2	1.35 (m)
10	35.3		35.7	
11	21.2	1.33 (m) 1.20 (m)	21.3	1.42 (m) 1.29 (m)
12	40.4	1.72 (m) 1.08 (m)	40.5	1.79 (m) 1.18 (m)
13	41.3		41.3	
14	56.4	1.06 (m)	56.6	1.14 (m)
15	32.5	2.02 (m) 1.40 (m)	32.5	2.01 (m) 1.41 (m)
16	81.3	4.97 (m)	81.3	5.00 (m)
17	64.1	1.91 (m)	64.1	2.01 (m)
18	16.8	0.87 (s)	16.8	0.91 (s)
19	23.9	0.82 (s)	24.3	1.01 (s)
20	40.7	2.23 (m)	40.7	2.25 (m)
21	16.5	1.32 (d, $J = 6.6$)	16.5	1.33 (d, $J = 6.6$)
22	110.7		110.7	
23	37.2	2.07 (m)	37.2	2.09 (m)
24	28.4	2.04 (m) 1.68 (m)	28.4	2.07 (m) 1.69 (m)
25	34.5	1.92 (m)	34.5	1.92 (m)
26	75.4	4.08 (m) 3.48 (m)	75.4	4.08 (o) 3.48 (m)
27	17.5	1.02 (d, $J = 6.6$)	17.5	1.02 (d, $J = 6.6$)
3-*O*-β-Gal-				
1′	103.9	4.85 (d, $J = 7.2$)		
2′	72.8	4.47 (m)		
3′	76.9	4.09 (o)		
4′	70.4	4.59 (o)		
5′	75.6	4.17 (m)		
6′	62.5	4.47 (m) 4.44 (o)		
26-*O*-β-Glu-				
1″	105.2	4.80 (d, $J = 7.8$)	105.2	4.81 (d, $J = 7.8$)
2″	75.3	4.01 (m)	75.3	4.01 (m)
3″	78.6	4.23 (o)	78.6	4.23 (o)
4″	71.7	4.22 (o)	71.7	4.23 (o)
5″	78.5	3.93 (m)	78.5	3.94 (m)
6″	62.9	4.53 (m) 4.36 (o)	62.9	4.55 (o) 4.39 (o)

Note: (o) Overlapped with other signals.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured with a Perkin-Elmer 343 polarimeter. NMR spectra were recorded with a Varian UNITY INOVA 600 (at 599.8 (^{1}H) and 150.8 MHz (^{13}C), Palo Alto, CA, USA), and the chemical shifts were given in δ (ppm) with TMS as the internal standard. FAB-MS were recorded on a Micromass Zabspec (Manchester, UK). HR-ESI-MS were recorded on a Synapt MS System (Waters Corporation, Milford, MA, USA), the

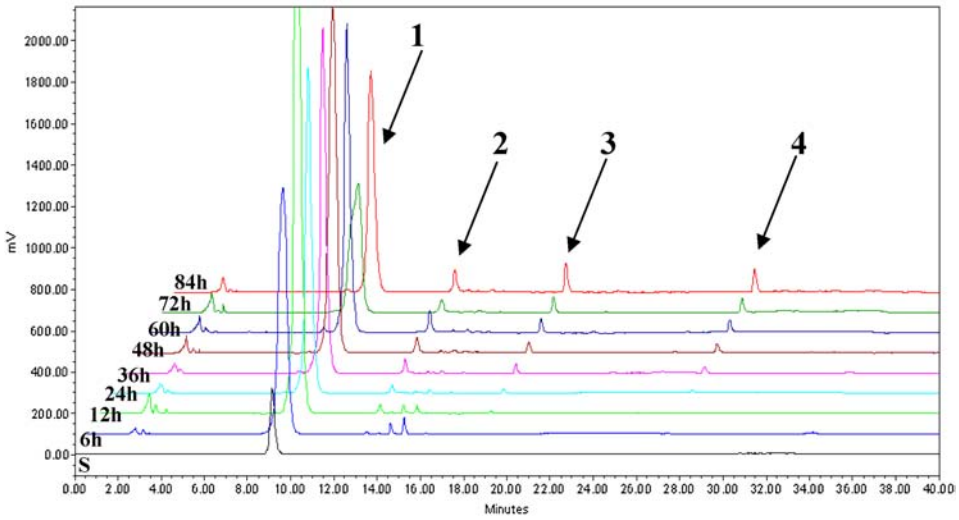


Figure 3. HPLC analyses of the time course (6–84 h) for the biotransformation of timosaponin BII. **S**, timosaponin B-II; 6–84 h. The reaction mixture of the biotransformation of timosaponin BII on every detection time point.

ionization mode was negative electrospray (ESI^-). HPLC was performed on an Agilent 1100 system (quaternary pump; Santa Clara, CA, USA.) with a YMC-Pack ODS-A C_{18} (YMC, 4.6 mm i.d. × 250 mm, and 10 mm i.d. × 250 mm, ODS, 5 μm; Kyoto, Japan; detectors were RID and ELSD (Alltech 2000 Evaporative Light Scattering Detector, temperature: 110°C, gas flow: 2.4 liters/min). Macroporous resin SP825 (Mitsubishi chemical, Tokyo, Japan), silica gel (SiO_2; Qingdao Haiyang Chemical Co. Ltd, Qingdao, China), and ODS silica gel (120 Å. 50 μm, YMC) were used for column chromatography. In order to interrupt the reaction, the reaction mixtures were

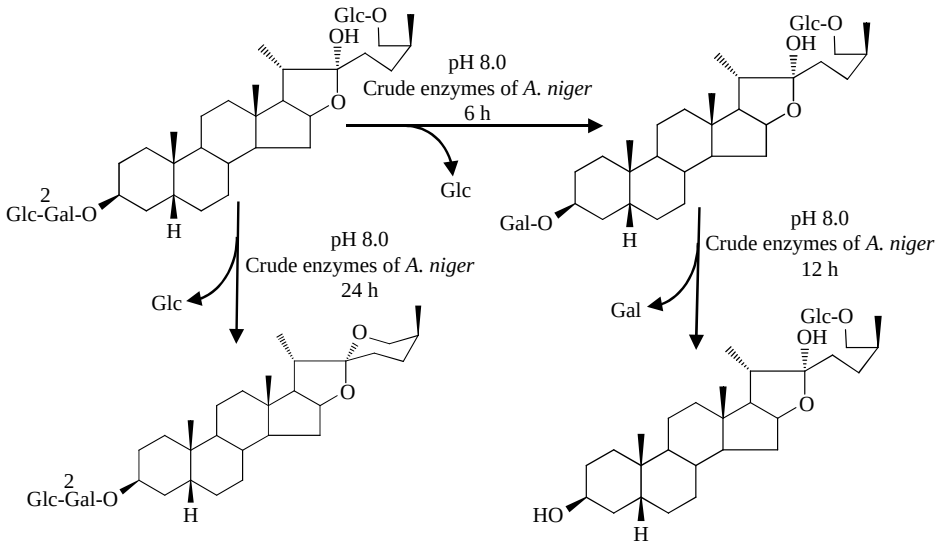


Figure 4. The reaction path for the biotransformation of timosaponin BII.

performed on Cleanert ODS-SPE (500 mg/3 ml; Agela Technologies Inc. Tianjin, China) to separate the enzyme and transglycosylation products. Timosaponin BII (**1**) was isolated from *R. Anemarrhenae* by the author, and its purity was above 99% as determined by HPLC.

3.2 Micro-organisms

A. niger AS 3.0739, *Aspergillus oryzae* AS 3.8000, *Aspergillus flavus* AS 3.2792, *Aspergillus ustus* AS 3.3534, *Aspergillus phoenicis* AS 3.4335, *A. oryzae* AS 3.4437, *Neurospora crassa* AS 3.1598, *N. crassa* AS 3.1604, *Penicillium canescens* AS 3.0224, and *Penicillium roqueforti* AS 3.4345 were purchased from the China General Microbiological Culture Collection Center in Beijing, China.

3.3 Culture medium and buffer

All cultures of filamentous fungi were performed in the M15 medium, whose composition was as follows: sucrose 30.0 g, $NaNO_3$ 3.0 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, KCl 0.5 g, $FeSO_4{\cdot}4H_2O$ 0.01 g, K_2HPO_4 1.0 g, in 1 liter distilled water.

The buffer of the biotransformation reaction consisted of the following composition : $Na_2HPO_4{\cdot}12H_2O$ 166.9 g, $NaH_2PO_4{\cdot}2H_2O$ 1.7 g, in 1 liter distilled water (0.2 M, phosphate buffer, pH 8.0).

3.4 The extract of the crude enzyme

The crude enzyme was extracted from all filamentous fungi incubated 3 days in the M15 medium, respectively. The flasks were incubated on a rotary shaker at 120–140 rpm at 30 ± 1°C. The cultures were filtered by filter paper, and the filtrates were used as the crude enzyme.

3.5 Biotransformation procedures

Screening-scale biotransformation of timosaponin BII was carried out in a 1.5 ml microtube with 0.5 ml buffer, 1 mg substrate, and 0.1 ml crude enzyme. The microtubes were placed on a thermostatic water bath at 50°C for 48 h. Reaction controls were used without the substrate. The controls contained the same amount of buffer but was not added to the crude enzyme under the above conditions.

Preparative-scale biotransformation of timosaponin BII by the crude enzyme from *A. niger* was carried out in 500 ml Erlenmeyer flasks, 1 g of timosaponin BII was used for the scale-up biotransformation in 270 ml, 200 mM phosphate buffer with pH 8.0, and this culture fluid was added to the 30 ml crude enzyme from *A. niger* AS 3.0739. The flask was incubated on a rotary shaker at 100–120 rpm at 50 ± 1°C for 2 days.

3.6 Extraction and isolation

The reaction mixture was isolated on a macroporous resin SP 825 and eluted with a gradient mixture of Me_2CO–H_2O (1:9, 3:7, 6:4, and 8:2) to give four fractions (Fr. A, B, C, and D). Fr. B yielded substrate **1** (785 mg). Fr. C (336 mg) was chromatographed on ODS silica gel (50 μm) with Me_2CO–H_2O (25:75, 30:70, 35:65, 40:60, 50:50, 60:40, and 80:20) to give 10 fractions (Fr. C-1–Fr. C-10). Fr. C-2 and Fr. C-3 were put together to obtain substrate **1** (124.4 mg), Fr. C-4 and Fr. C-6 were put together to obtain a mixture of **1** and **2** (68.4 mg), which was chromatographed by preparative HPLC with Me_2CO–H_2O (38:72) as the eluent to obtain **2** (33.4 mg). Fr. C-8 and Fr. C-9 were put together to obtain **3** (16.4 mg). Fr. D yielded **4** (26.6 mg).

3.6.1 *Timosaponin BII-a ((25S)-26-O-β-D-glucopyranosyl-22-hydroxy-5β-furost-3β,26-diol-3-O-β-D-galactopyranoside; **2**)*

White amorphous powder. $[\alpha]_D^{20}$ − 40.8 ($c = 0.063$, pyridine). ^1H and ^{13}C NMR spectral data: see Table 1. FAB-MS

(pos.): *m/z* 759.5 $[M + H]^+$, 597.4 $[M + H - 162]^+$ and 417.1 $[M + H - 162 - 162 - 18]^+$. HR-ESI-MS (neg.): *m/z* 757.4406 $[M - H]^-$ (calcd for $C_{39}H_{65}O_{14}$, 757.4374).

*3.6.2 Timosaponin BII-b ((25S)-22,3-dihydroxy-5β-furost-3β,26-diol-26-O-β-D-glucopyranoside; **3**)*

White amorphous powder. $[\alpha]_D^{20} -17.8$ ($c = 0.029$, pyridine). ^1H and ^{13}C NMR spectral data: see Table 1. FAB-MS (pos.): *m/z* 597.5 $[M + H]^+$ and 417.1 $[M + H - 162 - 18]^+$. HR-ESI-MS (neg.): *m/z* 595.3863 $[M - H]^-$ (calcd for $C_{33}H_{55}O_9$, 595.3846).

Acknowledgements

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