J. Microbiol. Biotechnol. (2011), **21**(6), 582–589
doi: 10.4014/jmb.1101.12041
First published online 12 April 2011

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Stereoselective Biotransformation of Timosaponin A-III by *Saccharomyces cerevisiae*

Hu, Yong-Mei, Zhi-Ling Yu, and Wang-Fun Fong*

Centre for Cancer and Inflammation Research, School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China

Received: January 3, 2011 / Revised: February 24, 2011 / Accepted: March 15, 2011

Bioconversion of timosaponin A-III (TA-III), one of the major steroidal saponins isolated from the rhizomes of *Anemarrhenae asphodeloides* Bunge (Liliaceae), was investigated in *Saccharomyces cerevisiae*. Five bioconversion products, denoted compounds 2–6, were obtained. Biotransformation metabolite 2 was a stereoisomer of TA-III with a specific isotype F-ring and β-ranged CH_3-21, which rarely occurs in nature. The structure of 2 was elucidated by extensive spectroscopic analysis (H-H COSY, HSQC, HMBC), as well as by high-resolution mass spectral analysis. The growth inhibitory activity of compounds 1–6 was assayed against four human cancer cell lines, HepG2, H-1299, HT-29, and HCT-116. Compounds 1 and 2 obviously inhibited the growth of the four types of cancer cells with IC_{50} values being less than 19 μM. A structure–activity relationship is discussed, and the spirostane-ring F in compounds 1 and 2 appears to be the critical bioactive moiety for the cell growth inhibitory property.

Keywords: Steroidal saponins, timosaponin A-III, biotransformation, *Saccharomyces cerevisiae*, growth inhibitory effect

Timosaponin A-III (TA-III) is a steroidal saponin isolated from the dried rhizome of *Anemarrhenae asphodeloides* Bunge (*Zhi Mu* in Chinese), a Chinese medicinal herb commonly prescribed for the treatment of respiratory disorders, diabetes, constipation, and cancer [19, 20, 32]. Previous reports on TA-III reveal that it possesses an obvious apoptotic effect in cultured human cancer cells [15, 25]. Recent studies indicate that TA-III treatment can greatly improve learning and memory deficits in scopolamine-induced memory-deficient mice [4]. Acid hydrolysis of TA-III can produce its aglycone sarsasapogenin, which has been proved to have a wide range of bioactivities [5, 14, 18, 26]. The spirostane-ring **F** appears to be the major bioactive radical in this steroidal molecule. It is well known that a naturally occurring steroidal saponin usually has a normal F-ring and an α-oriented CH_3-21 group [8]. Several investigators have reported that acid hydrolysis of certain precursors (*e.g.*, sarsasapogenin) can produce their corresponding isomers with an isotype F-ring [28–30]. However, Tobari *et al.* [27]. proved that the isotype spirostanols could only be obtained through a two-step chemical treatment (5% KOH followed by acidification) of the pseudo-saponin peracetates. However, the reaction conditions are difficult to control and several stereoisomers will be produced during the reaction [27].

In an attempt to overcome the difficulties of traditional organic chemical methods, researchers have intensively investigated biological systems in the last few years [13, 17, 22]. Biotransformation has been found to be a useful and economical tool to modify bioactive derivatives with high stereo- and regioselectivity. With the development of numerous enzymatic or microbial bioconversions, biodegradations, and fermentations, biotransformation has been considered a panacea that would ultimately displace "traditional organic chemistry" [3, 11]. Feng *et al*. [12] have successfully obtained an isotype F-ring sterol saponin from TA-III using the fungal strain *Rhizopus nigricans* 3.2050 (SO1) as the biocatalyst. The Baker's yeast *Saccharomyces cerevisiae* was earliest applied to the fermentation of wine and food. Recently, *S. cerevisiae* was also selected by screening as a whole cell catalyst for the biotransformation and biosynthesis of many bioactive chemicals. Through repeated trials, *S. cerevisiae* was found to possess a multiple catalytic ability even under mild conditions in aqueous as well as organic media [6]. Because of its high bioavailability and ease of use, *S. cerevisiae* has become one of the most frequently employed microorganisms in whole cell form. It has been proposed that the transformation of the aromatic aldehydes to the corresponding acyloin product by *S. cerevisiae* first occurred *via* the glycolytic

*Corresponding author
Phone: +852 3411 2928; Fax: +852 3411 5571;
E-mail: wffong@hkbu.edu.hk

pathway [33]. The $NADH+H^+$ produced in this pathway usually act as the hydrogen sources for the reduction of ketone catalyzed by the dehydrogenase (ADH) [16, 24]. We proposed that the pyruvic acid thus produced can supply a strong acidic environment for acid hydrolysis. To date, the biotransformation of steroids using *S. cerevisiae* as a biocatalyst has not yet been reported.

In the present study, we used *S. cerevisiae* as the biotransformation tool for the structural modification of TA-III for the first time. Five bioconversion metabolites, **2–6**, were obtained after 96 h of co-incubation with yeast. Growth inhibitory effects of compounds **1–6** against the four human tumor cell lines HepG2, H-1299, HT-29, and HCT-116 were assayed using the SRB method. Their structure–activity relationships and the biotransformation mechanism are discussed.

Materials and Methods

General Experimental Procedures

IR spectra were obtained on a Fourier transform near-infrared spectrophotometer (Perkin Elmer). Optical rotation was measured on a JASCO P-1020 polarimeter. 1D and 2D NMR experiments were performed on a Bruker-400 instrument with TMS as the internal standard. HR-ESI–MS was measured on Agilent 6540 series LC/MS trap mass spectrometers. ODS (C_{18}, 500 mesh; Sigma, USA), Sephadex LH-20 (Sigma) for column chromatography were used to obtain total crude saponins. Preparative HPLC was used for further purification of compounds **1–6**.

Human HepG2, H-1299, HT-29, and HCT-116 cell lines, which were originally purchased from the American Type Culture Collection (ATCC, USA), were kindly provided by Prof. K. P. Fung, the Chinese University of Hong Kong. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Invitrogen Corporation (USA). Phosphate-buffered saline (PBS) was obtained from Oxoid Ltd. (UK). Trypsin-EDTA, sulforhodamine B (SRB) stain, and Tris base were purchased from Sigma. Trichloroacetic acid (TCA) was purchased from Sigma-Aldrich Hong Kong Holding Limited (Hong Kong). TA-III was purchased from Hong Kong Jockey Club Institute of Chinese Medicine Ltd., Hong Kong, China. Acetonitrile was of HPLC grade.

Microorganisms and Culture Media

Saccharomyces cerevisiae was purchased from Sigma-Aldrich, Hong Kong, China. The yeast strain was maintained on a solid medium containing 0.5% yeast extract, 0.5% peptone, 2% glucose, and 2% agar, and was cultivated for 48 h at 35°C, stored at 4°C, and transferred every 3 weeks. For cultivation and biotransformation, potato medium was prepared, the pH of which was adjusted to 4.5–5.5 using ammonium acetate, and then sterilized in an autoclave for 30 min at 115°C.

Bioconversion of TA-III by *S. cerevisiae*

The yeast strain from the agar slant was transferred to a 250 ml Erlenmeyer flask containing 100 ml of potato medium, and incubated on an orbital shaker (240 rpm) at 35°C for 24 h to obtain stock incubation medium. The batch fermentation phase and the fed-batch bioconversion phase were performed in 10 flasks (1 L) containing 250 ml of potato medium by adding 8 ml of the stock medium to each new flask. After 24 h of incubation, a total amount of 150 mg TA-III dissolved in ethanol was evenly distributed among the incubation flasks. The fermentation was allowed to continue for 96 additional hours on the orbital shaker (240 rpm) at 35°C. The reaction was stopped by cooling the medium at 4°C.

Isolation and Purification of Bioconversion Compounds

The incubation cultures were collected and filtered to obtain the supernatant. After concentration under vacuum to 250 ml, the concentrated liquid was then extracted with EtOAc three times. The upper organic solvent was recovered, and the residue (332.6 mg) was subjected to Sephadex LH-20 column chromatography (CC) and eluted with a gradient solvent system of MeOH:H_2O (0–90%) to obtain crude saponins (86.3 mg). After an analytical HPLC inspection, the crude saponins were then subjected to prep-HPLC, flashed with gradient acetonitrile–water (32–38%) to give compounds **1** (4.2 mg), **2** (12.6 mg), **3** (18.6 mg), **4** (14.8 mg), **5** (8.1 mg), and **6** (12.4 mg).

LC–ESI/MS Analysis

An Agilent 6540 UHD Accurate-Mass Q-TOF LC/MS mass spectrometer (Agilent Technologies) was connected to the UHPLC instrument (Agilent 1290 Infinity) *via* an ESI ion source with JetStream technology. Ultrapure nitrogen (N_2) was used as the collision gas and N_2 was used as the nebulizing and sheath gas. The UHPLC conditions for LC–MS analysis were as follows: an Agilent Eclipse plus C_{18} RRHD column (2.1 mm×150 mm, 1.8 μm) was used at 40°C. A gradient elution of solvent A (Milli-Q water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid) was applied as follows: 0–2 min, 10–32% B; 2–14.5 min, 32–38% B; 14.5–16 min, 38–85% B; 16–18 min, 85% B. A pre-equilibration period of 6 min was used between individual runs. The flow rate was 0.25 ml/min, and the injection volume was 2 μl. The mass spectrometric system was operated in positive mode. The BPC chromatograms of compounds **1–6** before and after biotransformation are shown in Fig. 4.

Cell Growth Inhibitory Assay by SRB Method

Cancer cells were maintained in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, 0.1 μg/ml of streptomycin, 1 mM HEPES buffer, and 2 mM glutamine in a humidified atmosphere of 5% CO_2 at 37°C. Drugs were dissolved in DMSO and the final DMSO concentration was 0.1%. Control cells were treated with 0.1% DMSO in this experiment.

Cell number was estimated by SRB protein staining in a 96-well format. Cells were seeded at 5×10^3 cells per well and incubated overnight at 37°C. Fresh medium with drugs at different concentrations (2 to 100 μM) were added to individual wells and incubated for another 24 and 48 h. After incubation, 60 μl of 10% ice-cold TCA was added to fix the cells. The plates were chilled at 4°C for 1 h, then washed with tap water 5 times, air dried, and then stained with 100 μl of 0.4% SRB (in 1% acetic acid) for 15 min. Excess stain was removed by washing in 1% acetic acid and air drying. The stained precipitate was dissolved in 100 μl of Tris base (10 mM, pH 10.5). The optical density at 515 nm, which is proportional to cell number, was determined with a BioRad Microculture Plate Spectrophotometer. Each experiment was repeated three times. Drug toxicity was expressed in terms of the concentration inhibiting 50% growth (IC_{50}).

RESULTS

Structure Elucidation of Metabolites 2–6

Metabolite **2** was obtained as a white amorphous powder with positive Liberman–Burchard reaction. The quasi-molecular ion peak (positive HR-ESI–MS) at *m/z* 763.4431 $[M + Na]^+$ (calcd. 763.4895), in combination with the NMR data, suggests the molecular formula $C_{39}H_{64}O_{13}$, which is the same as the parent compound TA-III. The ^{13}C NMR and HSQC spectrum of **2** gave 39 carbon signals, 27 of which were assigned to a C_{27} steroidal skeleton for the aglycone moiety. Twelve were assigned to two sugar moieties. The ^1H NMR signals indicate the presence of two tertiary methyl groups (δ 1.04 and 0.96), assignable to H-18 and H-19, and two secondary methyl groups (δ 0.74 and 1.22), ascribable to H-27 and H-21, respectively. The A/B *cis*-ring fusion form with a 5β configuration hydrogen was confirmed by the chemical shifts of C-19 (δ 24.5) and C-5 (δ 37.4) and the NOESY correlation between H-5 and H-19 [1]. Through a careful comparison, the NMR spectroscopic data of metabolite **2** are similar to those of TA-III, except for the obvious differences in the chemical shifts of C-20 to C-27 and H-21, H-26, and H-27. In the ^1H NMR spectrum of **2**, the H-21 proton signal shifts to a lower field by about 0.2 ppm (δ 1.22) and the H-27 signal moves to a higher field by about 0.4 ppm (δ 0.74) compared with TA-III. Through the above analyses in combination with the chemical shifts of C-20 (δ 46.1) and C-21 (δ 10.5), the chiral C-20 in metabolite **2** was deduced to be in the "*R*" configuration [2, 27]. The β-orientated CH_3-21 was further confirmed by the observed NOE correlation between H-18 and H-21 (Fig. 2). A specific oxygenated multiple proton signal was observed at δ 3.59–3.66 (H-26, 2H, m) for metabolite **2**, whereas the parent compound TA-III showed two typical proton signals of H-26 at δ 3.38 (d, *J*= 10.9 Hz) and δ 4.07 (dd, *J*=10.8, 2.8 Hz). According to the reported literature [27], the F-ring in metabolite **2** was deduced to be isotype spirostan. Furthermore, the carbon resonances of **2** at C-26 (δ 66.6) and C-27 (δ 16.8) suggest a 25"*S*" configuration in the F-ring of spirostan [2, 7, 27]. Other carbon signals in ring F of metabolite **2** all shifted to a lower field by about 1–9 ppm.

Table 1. NMR spectroscopic data for metabolite **2**.

C	Aglycone of compound **2**						
	δ_C	δ_H (mult, *J* H_Z)	HMBC (H-C)	C	δ_C	δ_H (mult, *J* H_Z)	HMBC (H-C)
1	31.2	1.82, 1.49 (2H, m)	C-3, 10, 19	22	107.1		
2	27.3	1.87, 1.44 (2H, m)	C-10	23	34.9	2.11, 1.52 (2H, m)	C-20, 22
3	75.9	4.37 (1H, m)	C-5, 10, 19	24	29.7	2.16, 1.36 (2H, m)	C-26, 27
4	31.4	1.87, 1.47 (2H, m)	C-10, 19	25	28.8	2.44 (1H, m)	C-24, 27
5	37.4	2.18 (1H, m)	C-9, 19	26	67.6	3.59-3.66 (2H, m)	C-22, 24, 27
6	27.5	1.79 (1H, m) 1.15 (1H, m)	C-8, 10	27	17.1	0.74 (3H, d, 4.6)	C-24, 25, 26
7	27.1	1.30 (1H, m) 0.95 (1H, m)	C-5, 9				
				Sugar units of compound **2**			
8	36.0	1.51 (1H, m)	C-5	Gala	δ_C	δ_H (mult, *J* H_Z)	HMBC
9	42.9	1.70 (1H, m)	C-5, 19	1'	102.9	4.94 (1H, d, 7.6)	C-3
10	35.5			2'	82.3	4.59 (1H, dt,6.7, 14.2)	C-1"
11	21.7	1.32, 1.23 (2H, m)	C-9, 12	3'	75.7	4.30 (1H, dd,8.5, 1.7)	C-2', 4'
12	40.9	1.27, 1.18 (2H, m)	C-9, 11, 14	4'	70.4	4.59 (1H, m)	C-5', 6'
13	41.5			5'	77.1	4.06 (1H, m)	C-4'
14	56.9	1.06 (1H, m)	C-17, 18	6'	62.7	4.46 (2H, m)	C-4', 5'
15	34.2	2.15, 1.46 (2H, m)	C-8, 13	Glu			
16	82.9	4.61 (1H, m)	C-13, 14, 20	1"	106.5	5.30 (1H, d, 7.58)	C-2'
17	60.2	1.87 (1H, m)	C-12, 18, 20, 21	2"	77.5	4.13 (1H, m)	C-3", 4"
18	16.4	1.04 (3H, s)	C-12,13,14,17, 19	3"	78.7	4.24 (1H, t,7.8)	C-5"
19	24.5	0.96 (3H, s)	C-4, 5, 8, 9, 18	4"	72.2	4.36 (1H, m)	C-3", 5"
20	46.1	1.95 (1H, m)	C-13, 21, 23	5"	79.1	3.87 (1H, m)	C-4"
21	10.5	1.22 (3H, d, 7.4)	C-17, 20, 22	6"	64.6	4.55 (1H, dd, 9.5, 4.3) 4.59 (1H, dd, 11.5, 4.3)	C-4", 5"

All spectra were recorded in D-pyridine, 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR. The proton coupling constant (*J*) values in Hz are given in parentheses. The carbon and proton signals were assigned by ^1H-^1H COSY, HSQC, and HMBC spectroscopic data analyses.

Acid hydrolysis of **2** with 2 M TFA in dioxane-H_2O yielded D-galactose and D-glucose by TLC analysis using authentic samples as references. All proton and carbon signals were fully assigned by H-H COSY, HMBC, and HSQC spectroscopic analyses (Table 1). Thus, **2** was elucidated as (20*R*, 25*S*)-5β-spirostane-3β-ol-3-*O*-β-D-glucopyranosyl-(1→2)-β-D-galacopyanoside.

Metabolites **3** and **4** were elucidated as timsaponin B-II (TB-II) and timsaponin B-III (TB-III), respectively, by comparison of ESI–MS values and NMR spectroscopic data with those reported in the literature [9, 10, 21, 32]. Metabolites **5** and **6** were characterized as the 15-hydroxylation products of TB-II and TB-III, respectively, by NMR and LC/HR-ESI analysis and further comparison with those of *Anemarrhenae* saponin I. Metabolite **5** and **6** showed superimposable NMR spectra with metabolites **3** and **4**, except for the obvious differences of the chemical shifts at C-15, C-16 and H-15, H-16 (Table 2).

(20*R*, 25*S*)-5β-spirostane-3β-ol-3-O-β-D-glucopyranosyl-(1→2)-β-D-galacopyanoside (**2**): white amorphous powder; $C_{39}H_{64}O_{13}$; HR-ESI–MS *m/z* 763.4431 $[M + Na]^+$ (calcd. 763.4895); IR (KBr)υ_{max}: 3,412 (OH), 2,928, 985, 920, 885 cm^{-1}. ^1H (*d*-pyridine, 400 MHz) and ^{13}C NMR (*d*-pyridine, 100 MHz) spectroscopic data are shown in Table 1.

Timosaponin B-II (**3**): white amorphous powder; $C_{45}H_{76}O_{19}$; HR-ESI-MS *m/z* 943.5025 $[M + Na]^+$ (calcd. 943.5075). ^1H (*d*-pyridine, 400 MHz) and ^{13}C NMR (*d*-pyridine, 100 MHz) spectroscopic data are shown in Table 2.

Timosaponin B-III (**4**): white amorphous powder; $C_{45}H_{74}O_{18}$; HR-ESI–MS *m/z* 925.4921 $[M + Na]^+$ (calcd. 925.5011). ^1H (*d*-pyridine, 400 MHz) and ^{13}C NMR (*d*-pyridine, 100 MHz) spectroscopic data are shown in Table 2.

15-Hydroxyl-timosaponin B-II (**5**): white amorphous powder; $C_{45}H_{76}O_{20}$; HR-ESI–MS *m/z* 959.5302 $[M + Na]^+$ (calcd. 959.5312). ^1H (*d*-pyridine, 400 MHz) and ^{13}C NMR (*d*-pyridine, 100 MHz) spectroscopic data are shown in Table 2.

15-Hydroxyl-timosaponin B-III (**6**): white amorphous powder; $C_{45}H_{74}O_{19}$; HR-ESI–MS *m/z* 941.4939 $[M + Na]^+$ (calcd. 941.4970). ^1H (*d*-pyridine, 400 MHz) and ^{13}C NMR (*d*-pyridine, 100 MHz) spectroscopic data are shown in Table 2.

Table 2. H^1 NMR and C^{13} NMR spectroscopic data for aglycone of metabolites **3–6**.

No.	δ_C				δ_H (mult, *J* H_Z)			
	3	4	5	6	3	4	5	6
1	30.6	30.8	30.7	30.8	1.40°, 1.73°	1.42°, 1.74°	1.41°, 1.72°	1.39°, 1.71°
2	27.0	26.9	26.6	26.9	1.43, 1.78°	1.41°, 1.76 m	1.42, 1.77°	1.41°, 1.77 m
3	75.0	75.5	75.4	75.5	4.29°	4.32°	4.28° m	4.30°
4	31.0	31.0	31.0	31.1	1.65°	1.67°	1.66°	1.65°
5	36.1	36.9	35.8	36.6	2.15 m	2.14 m	2.14 m	2.16 m
6	26.8	27.0	27.1	27.1	1.71 m	1.73 m	1.69 m	1.72° m
					1.78 (brd, 13.1)	1.78 (brd, 12.9)	1.76 (brd, 12.6)	1.78 (brd, 12.9)
7	26.6	27.0	26.9	27.0	1.07, 1.43 m	1.06°, 1.42 °	1.02°, 1.40°	1.02°, 1.41°
8	35.9	35.3	36.2	35.6	1.35 m	1.36 m	1.38 m	1.34 m
9	41.5	40.4	40.3	40.4	1.15 m	1.13 m	1.16 m	1.11° m
10	35.3	35.4	35.1	35.4				
11	21.2	21.3	21.1	21.3	1.11 m, 1.22°	1.10 m, 1.21°	1.12, 1.24° m	1.10 m, 1.21 m
12	41.1	40.2	38.5	40.2	1.01, 1.64 m	1.02°, 1.66 m	0.98°, 1.66 m	1.01°, 1.66 m
13	40.6	43.9	41.2	43.6				
14	56.8	55.1	59.6	55.1	0.94 (d, 5.4)	0.94 (d, 5.4)	1.06 (d, 5.6)	1.05 (d, 5.6)
15	32.6	34.5	77.6	75.7	1.33, 1.91 m	1.33, 1.92 m	4.16 (t, 9.7)	4.21 (t, 11.4)
16	82.1	84.6	90.6	88.6	4.90 (ddd, 9.2, 7.6, 5.3)	4.64 (ddd, 9.8, 7.8, 5.4)	4.88 (dd, 9.7, 4.2)	4.57 (dd, 11.4, 3.8)
17	63.1	65.1	61.4	63.4	1.90 m	2.52 (d, 10.2)	1.92 m	2.55 (d, 10.1)
18	16.2	14.4	17.8	15.2	0.73 s	0.72 s	0.76 s	0.77 s
19	23.5	24.3	24.5	24.6	0.84 s	1.03 s	0.88 s	1.07 s
20	40.3	103.8	40.7	104.1	2.17 m		2.14 m	
21	15.9	11.8	15.5	12.1	1.22 (d, 6.8)	1.70 (s)	1.24 (d, 6.9)	1.69 (s)
22	112.9	152.5	111.6	150.4				
23	40.4	24.0	40.4	24.3	1.96, 2.06 m	2.12–2.14 m	1.94, 2.05 m	2.13–2.16 m
24	28.0	31.5	28.1	31.2	1.60, 1.95° m	1.47, 1.99 m	1.60, 1.94°	1.49, 2.06 m
25	33.6	34.1	33.4	33.9	1.84° m	1.91 m	1.83°	1.95° m
26	75.3	75.5	75.1	75.8	3.42(dd, 11,6.5)	3.95 (dd, 10.4, 6.5),	3.45 (dd, 10.7, 6.7)	3.94 (dd, 10.6, 6.6)
					4.01(dd, 11,5.6)	3.89 (dd, 10.4, 4.6)	4.06 (dd, 10.7, 6.1)	3.87 (dd, 10.6, 5.1)
27	17.0	17.4	16.8	17.3	0.96 (d, 6.5)	1.12 (d, 6.7)	0.98 (d, 6.5)	1.13 (d, 6.6)

All spectra were recorded in D-pyridine, 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR. Proton coupling constant (*J*) values in Hz are given in parentheses; °, overlapped with other signals.

Table 3. Growth inhibitory effects of compounds **1–6** on HepG2, HT-29, HCT-116, and H-1299 cells (n=3) after 24 and 48 h.

Compound	IC_{50}±SD (%) (μM)			
	HepG2 24 h 48 h	HT-29 24 h 48 h	HCT-116 24 h 48 h	H-1299 24 h 48 h
1	18.6±0.5 14.2±0.4	16.8±2.1 12.8±1.1	15.9±0.8 17.4±0.2	18.2±0.2 16.6±0.3
2	16.9±0.6 14.7±0.3	18.5±0.9 13.4±2.3	18.8±1.9 16.3±2.0	18.8±1.7 18.2±1.4
3	60.5±8.2 54.3±5.1	>100 46.3±5.8	55.2±6.2 50.8±3.9	61.3±2.6 46.8±4.3
4	41.3±5.1 43.6±3.6	52.2±1.4 46.9±2.6	66.2±1.5 52.9±2.4	61.0±2.9 55.8±3.2
5	>100 54.2±6.3	>100 52.8±5.5	>100 61.6±4.2	>100 64.9±4.6
6	56.9±6.6 24.7±1.2	66.5±3.2 43.6±2.6	68.6±2.2 58.1±3.6	58.2±3.1 52.2±1.9
Oridonin	25.8±0.8 16.4±0.2	25.6±1.1 13.4±1.3	22.2±0.2 20.6±0.6	21.9±1.1 18.2±1.6

Results presented are the mean values of three independent experiments.

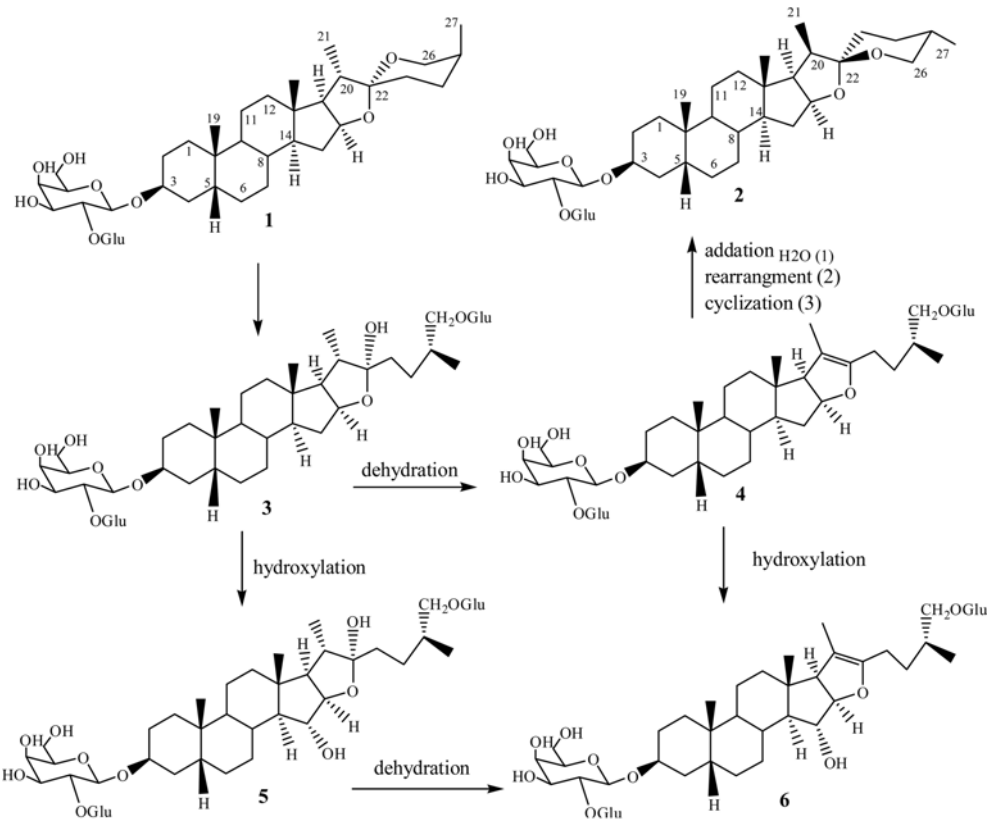

Fig. 1. Structures of compounds **1–6** and a proposed biotransformation pathway of TA-III by *S. cerevisiae.*

Bioassay of Compounds 1–6

The cell growth inhibitory activity of compounds **1–6** was evaluated in HepG2, HCT1-16, HT-29, and H1-299 cells. Oridonin (purity >98% by HPLC; Jiangsu Institute for Food and Drug Control), a known cell growth-inhibiting agent from *Rabdosia rubescens* [31], was used as the positive control. Growth inhibition at two different time points was assayed by the SRB protein staining method [24]. As shown in Table 3, compounds **1** and **2** inhibited the growth of the four types of human cancer cells in an

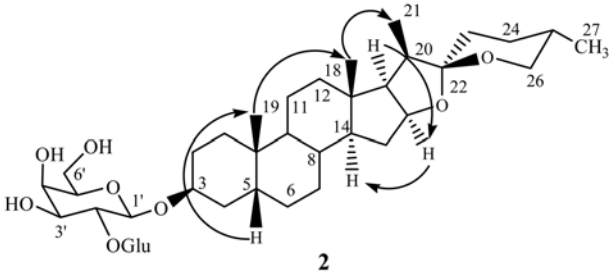

Fig. 2. Key NOE correlations of metabolite **2**.

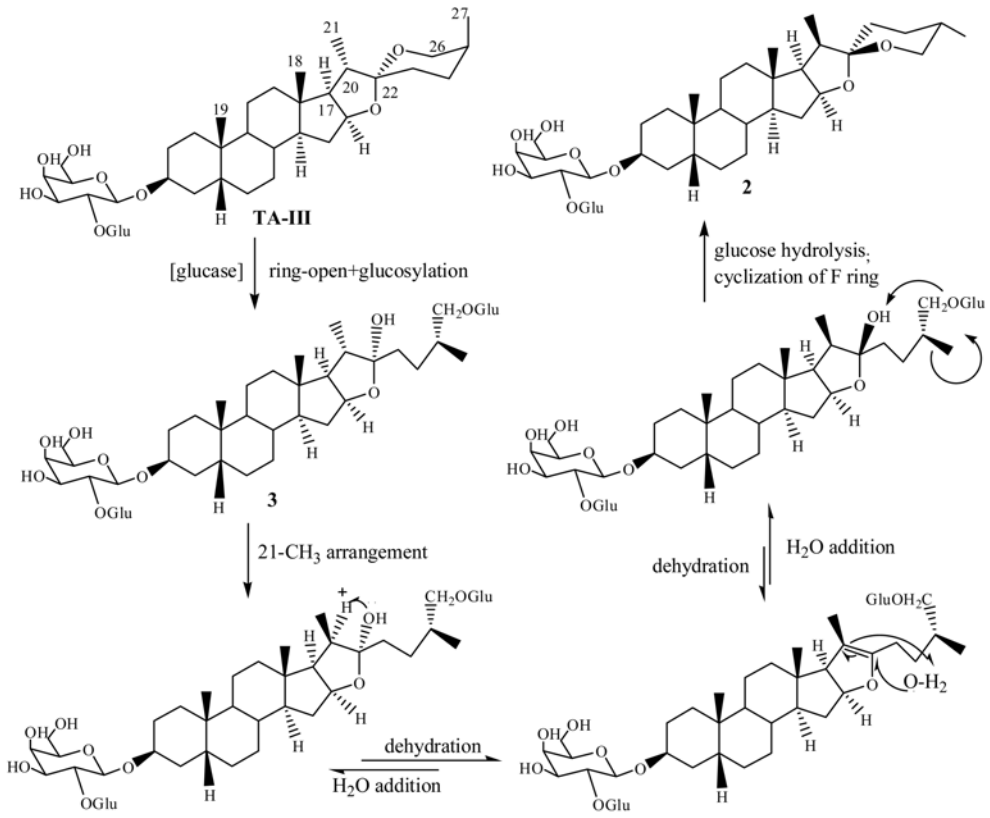

Fig. 3. The proposed mechanism for the bioconversion of metabolite **2** from TA-III.

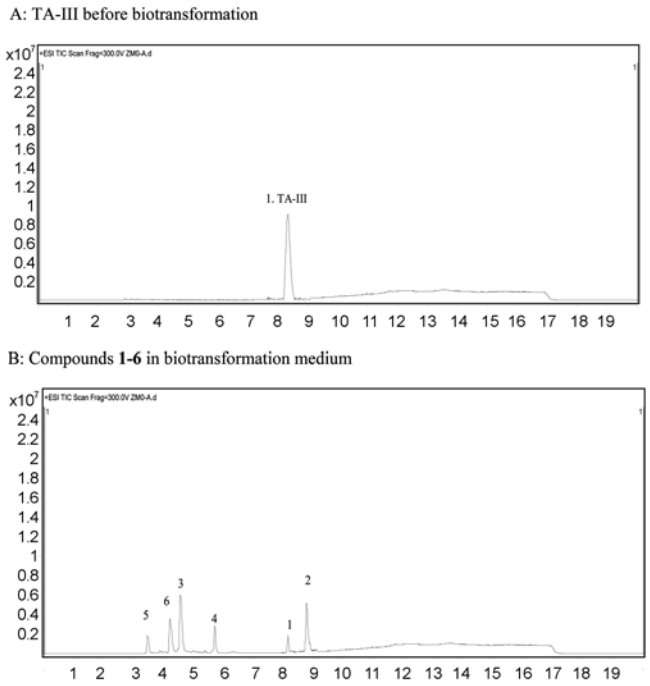

Fig. 4. Specific LC/MS chromatograms of compounds **1–6** before (**A**) and after (**B**) biotransformation.

obvious dose- and time-dependent manner, whereas compounds **3–6** showed no specific cytotoxicity against human tumor cells.

Proposed Stereoselective Biotransformation Mechanism of Metabolite 2

The structures of compounds **1–6** and the proposed biotransformation pathway of metabolites **2–6** are shown in Fig. 1. The bioconversion of metabolite **2** from substrate TA-III possibly occurred through several biochemical reactions catalyzed by different enzymes contained in *S. cerevisiae*. According to the literature [17], to obtain the isotype spirostanol steroid, the F-ring should open first (hydrolysis), followed by a series of rearrangement, dehydration, glucosylation, addition, rehydrolysis, and ring-closure reactions, *etc*. (Fig. 3). The experimental results indicate that the fungal strain *S. cerevisiae* supplies certain conditions and specific enzymes for the stereoselective bioconversion of TA-III.

Discussion

For the first time, we have demonstrated the use of Baker's yeast as a biotransformation tool to produce the isotype

spirostanol. Although present in a relatively lower yield ratio, the expected isomer of TA-III with an isotype ring-F was obtained after a series of purification steps in the present study. The principle of this strategy could be useful for similar biotransformations. Through a further modification of the fungal strain *S. cerevisiae* and optimization of the biotransformation conditions, a higher yield radio of metabolite **2** is expected to be obtained in the future.

Acknowledgments

We are grateful to the Department of Chemistry, Hong Kong Baptist University, for measuring the NMR spectra, and to Mr. C. L. Chan, Research & Development Division, School of Chinese Medicine, Hong Kong Baptist University, for performing the HR-ESI–MS experiment.

REFERENCES

1. Agrawa, P. K., D. C. Jain, P. K. Gupta, and R. S. Thakur. 1985. Carbon-13 NMR spectroscopy of steroidal sapogenin and steroidal saponins. *Phytochemistry* **24:** 2479–2496.
2. Agrawa, P. K., D. C. Jain, and A. K. Pathak. 1995. NMR spectroscopy of steroidal sapogenins and steroidal saponins: An update. *Magn. Reson. Chem*. **33:** 923–953.
3. Aharonowitz, Y. and G. Cohen. 1981. The microbiological production of pharmaceuticals. *Sci. Am*. **245:** 140–152.
4. Bomi, L., K. Jun, and D. H. Kim. 2009. Timosaponin AIII, a saponin isolated from *Anemarrhena asphodeloides*, ameliorates learning and memory deficits in mice. *Pharmacol. Biochem. Behav*. **93:** 121–127.
5. Bao, W. N., H. F. Pan, M. Lu, Y. Ni, R. Zhang, and X. G. Gong. 2007. The apoptotic effect of sarsasapogenin from *Anemarrhena asphodeloides* on HepG2 human hepatoma cells. *Cell Biol. Int*. **31:** 887–892.
6. Csuk, R. and B. I. Glanzer. 1991. Baker's yeast mediated transformations in organic chemistry. *Chem. Rev*. **91:** 49–97.
7. Debella, A., E. Haslinger, Q. Kunert, G. Michl, and D. Abebe. 1999. Steroidal saponins from *Asparagus africanus*. *Phytochemistry* **51:** 1069–1075.
8. Devon, T. K. and A. I. Scott. 1972. *Handbook of Naturally Occurring Compounds*, *Vol. II*, *Terpenes*, pp. 404–411. Academic Press, NY. 1972.
9. Dong, J. X. and G. Y. Han 1991. A new active steroidal saponin from *Anemarrhena asphodeloides*. *Planta Med*. **57:** 460–462.
10. Dong, J. X. and G. Y. Han. 1992. Studies on the active constituents of *Anemarrhena asphodeloides* Bunge. *Yao Xue Xue Bao* **27:** 26–32.
11. Eveleigh, D. E. 1981. The microbiological production of industrial chemicals. *Sci. Am*. **245:** 154–178.
12. Feng, B., S. Q. Wang, and B. P. Ma. 2005. Study on biotransformation of steroidal saponins and its specific glycosidase. Dissertation of Doctor Degree of China Military Medical Science Institute, pp. 29–40.
13. Hamada, H., Y. Miyamoto, N. Nakajima, and T. Furuya. 1998. Lipase-mediated asymmetric acetylation of prochiral diols directed towards total syntheses of biologically active molecules. *J. Mol. Catal. B Enzym*. **5:** 187–189.
14. Hu, Y. E., Z. Q. Xia, Q. X. Sun, A. Orisi, and D. Rees. 2005. A new approach to the pharmacological regulation of memory: Sarsasapogenin improves memory by elevating the low muscarinic acetylcholine receptor density in brains of memory-deficit rat models. *Brain Res*. **1060:** 26–39.
15. King, F. W., S. Fong, C. Griffin, M. Shoemaker, R. Staub, Y. L. Zhang, I. Cohen, and E. Shtivelman. 2009. Timosaponin AIII is preferentially cytotoxic to tumor cells through inhibition of mTOR and induction of ER stress. *PLoS ONE* **4**: 7283.
16. Kaoru, N., I. Kiyoko, U. Kazutoshi, O. Shinzaburo, and O. Atsuyoshi. 1988. Stereochemical control on yeast reduction of α-keto esters. Reduction by immobilized Bakers' yeast in hexane. *J. Org. Chem.* **53:** 2589–2593.
17. Miyazawa, M. and M. Ohsawa. 2002. Biotransformation of α-terpineol by the larvae of common cutworm (*Spodoptera litura*). *J. Agric. Food Chem*. **50:** 4916.
18. Ni, Y., X. G. Gong, M. Lu, H. M. Chen, and Y. Wang. 2008. Mitochondrial ROS burst as an early sign in sarsasapogenin-induced apoptosis in HepG2 cells. *Cell Biol. Int*. **32:** 337–343.
19. Nian, H., L. P. Qin, W. S. Chen, Q. Y. Zhang, H. C. Zheng, and Y. Wang. 2006. Protective effect of steroidal saponins from rhizomes of *Anemarrhena asphodeloides* on ovariectomy-induced bone loss in rats. *Acta Pharmacol. Sin*. **27:** 728–734.
20. Niwa, A., O. Takeda, M. Ishimaru, Y. Nakamoto, K. Yamasaki, H. Kohda, *et al*. 1988. Screening test for platelet aggregation inhibitor in natural products. The active principle of *Anemarrhenae* rhizoma. *Yakugaku Zasshi* **108:** 555–561.
21. Saito, S., S. Nagase, and K. Ichinose. 1994. New steroidal saponins from the rhizomes of *Anemarrhena asphodeloides* Bunge (Liliaceae). *Chem. Pharm. Bull.* **42:** 2342–2345.
22. Shimoda, K., S. Y. Yamane, H. Hirakawa, S. Ohta, and T. Hirata. 2002. Biotransformation of phenolic compounds by the cultured cells of *Catharanthus roseus*. *J. Mol. Catal. B Enzym*. **16:** 275–279.
23. Tomas, H., T. Toshiya, and G. G. Kumar. 1991. Yeast-mediated resolution of β-keto esters of prochiral alcohols. *J. Org. Chem.* **56:** 3619–3623.
24. Skenhan, P., R. Storeng, D. Scudiero, A. Monks, J. McMahon, D. Vistica, *et al*. 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst*. **82:** 1107–1112.
25. Sy, L. K., S. C. Yan, C. N. Lok, R. Y. Man, and C. M. Che. 2008. Timosaponin A-III induces autophagy preceding mitochondria-mediated apoptosis in HeLa cancer cells. *Cancer Res*. **68:** 10229–10237.
26. Takahashi, M., C. Komo, and H. Hikino. 1985. Isolation and hypoglycemic activity of anemarans A, B, C, and D, glycans of *Anemarrhena asphodeloides* rhizomes. *Planta Med*. **51:** 100–102.
27. Tobari, A., M. Teshima, J. Koyanagi, M. Kawase, H. Miyamae, K. Yoza, A. Takasaki, Y. Nagamura, and S. Saito, 2000. Spirostanols obtained by cyclization of pseudosaponin derivatives and

comparison of anti-platelet agglutination activities of spirostanol glycosides. *Eur. J. Med. Chem*. **35:** 511–527.
28. Wall, M. E., C. R. Eddy, and S. Serota. 1954. Steroidal sapogenins. XIX. Stereochemistry of sapogenins and cholesterol at carbon 20. *J. Am. Chem. Soc*. **76:** 2849–2850.
29. Wall, M. E. and S. Serota. 1954. Steroidal sapogenins. XX. Configuration of spiroketal side chain at carbon 22. *J. Am. Chem. Soc*. **76:** 2850–2852.
30. Wall, M. E., C. R. Eddy, and S. Serota. 1955. Steroidal sapogenins. XVIII. Partial hydrolysis of steroidal saponins of *Yucca schidigera*. *J. Am. Chem. Soc*. **77:** 1230.
31. Zhang, Y. H., Y. L. Wu, S. I. Tashiro, S. Onodera, and T. Ikejima. 2009. Involvement of PKC signal pathways in oridonin-induced autophagy in HeLa cells: A protective mechanism against apoptosis. *Biochem. Biophys. Res. Commun*. **378:** 273–278.
32. Zhang, J. Y., Z. Y. Meng, M. Y. Zhang, D. S. Ma, S. X. Xu, and H. Kodama. 1999. Effect of six steroidal saponins isolated from *Anemarrhenae* rhizoma on platelet aggregation and hemolysis in human blood. *Clin. Chim. Acta* **289:** 79–88.
33. Zubay, G. 1989. *Biochemistry*, 2nd Ed. Macmillan Publishing Company, New York.