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The substrate specificity of a glucoamylase with steroidal saponin-rhamnosidase activity from *Curvularia lunata*

Bing Feng, Li-ping Kang, Bai-ping Ma,* Bo Quan, Wen-bin Zhou, Yong-ze Wang, Yu Zhao, Yi-xun Liu and Sheng-qi Wang*

Beijing Institute of Radiation Medicine, Beijing 100850, China

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Abstract—In previous work, we studied and reported that an enzyme from *Curvularia lunata* 3.4381 had the novel specificity to hydrolyze the terminal rhamnosyl at C-3 position of steroidal saponin and obtained four transformed products; the enzyme was purified and ascertained as glucoamylase (EC 3.2.1.3 GA). In this work, the enzyme exhibiting steroidal saponin-rhamnosidase activity was systematically studied on 21 steroidal saponins and 6 ginsenosides. The results showed that the α-1,2-linked end-rhamnosyl residues at C-3 position of steroidal saponins could be hydrolyzed to corresponding secondary steroidal saponins, among which 18 compounds were isolated and identified, including 3 new secondary compounds. For the furostanosides having glucosyl residues at the C-26 position, hydrolysis occurred first at end-rhamnosyl at C-3 position to produce secondary furostanosides. The reaction of hydrolyzing glucosyl at C-26 position depended considerably on longer reaction times yielding the corresponding secondary spirostanosides (without rhamnosyl and glucosyl residues). The enzyme had the strict specificity for the terminal α-1,2-linked rhamnosyl residues of linear chain, or the terminal α-1,2-linked rhamnosyl residues with branched chain of 1,4-linked glycosyl residues of sugar chain at C-3 position of steroidal saponins, it was not specific for different aglycones, different glycons, and the number of glycon of sugar chain of steroidal saponin. The end-rhamnosyl of ginsenosides and *p*-nitrophenyl-α-L-rhamnopyranoside (*p*NPR) could not be hydrolyzed by the enzyme from *C. lunata*.

1. Introduction

Steroidal saponin is a kind of oligoglycosides derivative of steroid, it is divided into two major groups, spirostanoside and furostanoside (Fig. 1). The spirostanoside contains one sugar chain generally at C-3 position, and furostanoside bears two sugar chains always at C-3 and C-26 positions.[1] Steroidal saponin is a kind of main active substance in plant, as well as the lead compound in new medicines.[2] It is widely distributed in plants, such as *Paridis polyphylla* Smith var. yunnanensis (Franch) Hand Mazz, *Polygonatum kingianum* Coll. et hemsl, *Anemarrhena asphodeloides* Bunge, *Allium ascalonicum* L., *Tribulus terrestris* L., *Dioscorea nipponica* Makino, etc. Steroidal saponins have been known to improve cardiovascular function, anti-platelet aggregation, anti-tumor, anti-diabetic, etc.[3]

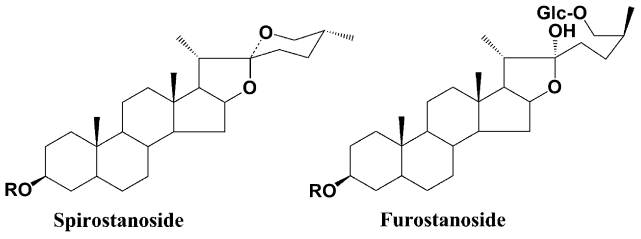

Figure 1. The structure of steroidal saponin.

Curvularia lunata is a deuteromycete best known in chemistry for 11β-hydroxylation of steroids, and production of hydrocortisone.[4,5] Glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3, GA) is an *exo*-acting enzyme capable of hydrolyzing α-1,4-linked glucose residue consecutively from the non-reducing ends of amylose, amylopectin, and glycogen producing β-glucose. Thus, glucoamylase is industrially an important biocatalyst.[6–8]

In previous work, we found that the glycosyl, which is at C-3 position of steroidal saponins, was hardly hydrolyzed by some glycosidases, such as commercial glucosidase, hemicellulase. However, the glucosyl residue at C-26 position of furostanosides could be hydrolyzed easily by some enzymes and microorganisms to give corresponding spirostanosides (Fig. 2).

In our studies, we first discovered and reported that *C. lunata* 3.4381 had the ability to hydrolyze selectively the terminal

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* Corresponding authors. Tel.: +86 10 66887429/930265; fax: +86 10 66887429/932211; e-mail: istas1101@163.com

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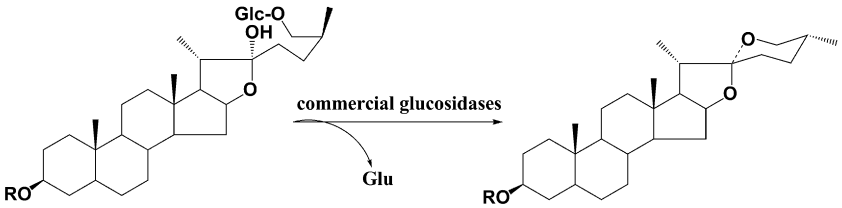


Figure 2. The transformation pathway of furostanoside to spirostanoside.

α-1,2-linked rhamnosyl residues of sugar chain at C-3 position of spirostanosides with high activity and selectivity, and obtained four main microbiological transformed products (compounds **10**–**13**).[9] At present, the enzymes secreted by *C. lunata* have been purified and determined. Fragments of its amino acid sequences were designated as 1,4-α-D-glucan glucohydrolase (EC 3.2.1.3, GA) (the paper has been submitted to Applied Microbiology and Biotechnology).

To understand the enzymatic specificity for rhamnosyl of saponins, 27 steroidal saponins and triterpenoid saponins, which have different aglycones, different glycosyls, and sugar chains with linear chain or branched chain, were chosen to investigate the substrate specificity by the purified enzyme. The results were summarized as follows: for spirostanoside, the enzyme was able to hydrolyze the terminal α-1,2-linked rhamnosyl residues of sugar chain at C-3 position to give secondary spirostanosides; for furostanoside, the terminal α-1,2-linked rhamnosyl residues of sugar chain at C-3 position was selectively hydrolyzed, and the glucosyl residues at C-26 position was retained to get secondary furostanosides, the reaction of hydrolyzing glucosyl at C-26 position depended considerably on longer reaction times and got corresponding secondary spirostanosides (without rhamnosyl and glucosyl residues). For ginsenosides, the enzyme, however, could not hydrolyze the α-1,2-linked end-rhamnosyl residues of sugar chain at C-6 position of ginsenosides, and the rhamnosyl residue of *p*-nitrophenyl-α-L-rhamnopyranoside (*p*NPR). Here we report on the substrate specificity by the purification of glucoamylase from *C. lunata* 3.4381.

2. Results

2.1. TLC analysis

2.1.1. Furostanosides. Eight furostanosides (compounds **1a**–**8a**) with the terminal α-1,2-linked rhamnosyl residues and one furostanoside (compound **9**) having the terminal 1,4-linked rhamnosyl residues of sugar chain at C-3 position were selected to culture with purified glucoamylase from *C. lunata* 3.4381 under the same reaction conditions. The results of analysis by TLC indicated the presence of converted products of compounds **1**–**7** after visualization by Ehrlich reagent (the color reaction using the Ehrlich reagent was applied in order to detect the furostanosides in the reaction. The furostanoside gave the stable bright-crimson coloration while the spirostanoside has not developed any staining.) and 10% H_2SO_4–EtOH spray reagent (Fig. 3). After visualization by Ehrlich reagent, one new spot appeared with a higher R_f value than that of substrates, and it suggested that the glycosyl residues of sugar chain at C-3 position were hydrolyzed, and the glucosyl residues at C-26 position were retained to give secondary furostanosides. After visualization by 10% H_2SO_4–EtOH reagent, more than one spot were found, it implied that they were new secondary spirostanosides, which hydrolyzed the glucosyls at C-26 position of substrates. But the terminal α-1,2-linked rhamnosyl residues with a branched chain of a 1,3-linked glucosyl residues (**8a**), and the terminal 1,4-linked rhamnosyl residues (**9a**) did not find new products after visualization by Ehrlich reagent. After visualization by 10% H_2SO_4–EtOH reagent, one new spot was found, it

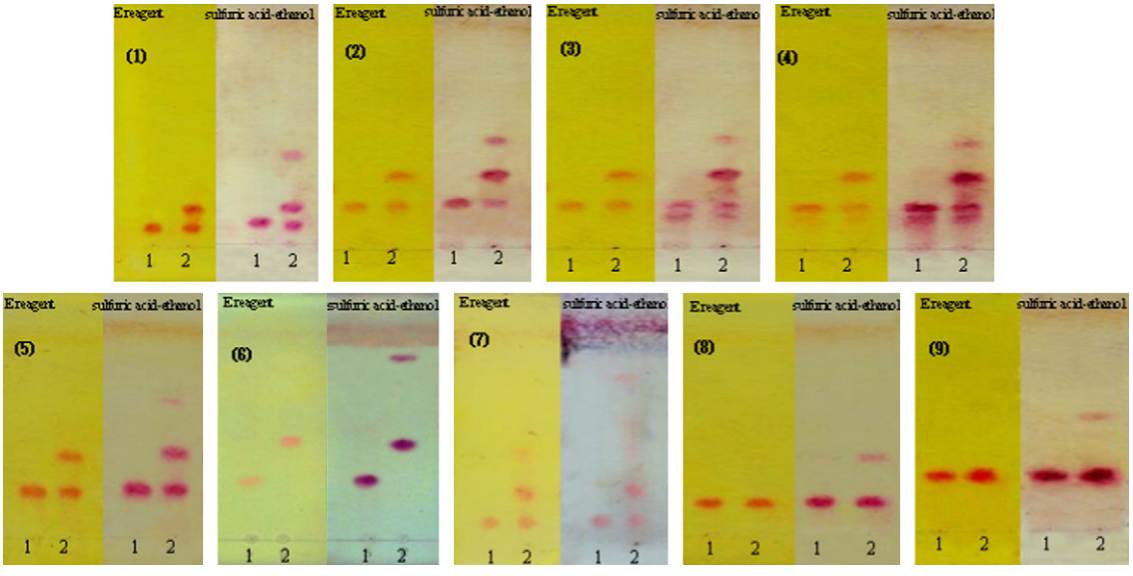


Figure 3. Analysis of furostanosides bioconverted by TLC. 1: Substrate; 2: the mixture of substrates and transformed products; (1)–(9): compounds **1**–**9**.

implied that they were the corresponding spirostanosides, which hydrolyzed the glucosyls at C-26 position of substrates.

2.1.2. Spirostanosides. Eleven spirostanosides (compounds **10a**–**20a**) having the terminal α-1,2-linked rhamnosyl residues and one with the terminal α-1,4-linked rhamnosyl (**21a**) of sugar chain at C-3 position were chosen for investigation by the purified enzyme under the same reaction conditions. The results of analysis by TLC showed the presence of converted products of compounds **10a**–**18a** after visualization by 10% H_2SO_4–EtOH spray reagent (Fig. 4). New spots (arrowhead) appear with a higher R_f value than that of substrates. This means that the glycosyl residues of sugar chain at C-3 position of spirostanosides were hydrolyzed. The products were secondary spirostanosides of substrates. The enzyme, however, was inactive against neither the terminal α-1,2-linked rhamnosyl residues with branched chain of a 1,3-linked glycosyl residues (**19a** and **20a**), nor terminal α-1,4-linked rhamnosyl residues (**21a**).

2.1.3. Ginsenosides. Two ginsenosides with the terminal α-1,2-linked rhamnosyl residues of sugar chain at C-6 position (compounds **22a** and **23a**) and four ginsenosides with terminal glucosyl residues of sugar chain at C-3 or C-20 position (compounds **24a**–**27a**) were selected for investigation by the purified enzyme under the same reaction conditions. Analysis of extracts by TLC indicated that the purified enzyme was all inactive against the ginsenosides Re (**22a**) and Rg_2 (**23a**) (with terminal rhamnosyl residues at C-6), and the ginsenosides C-K (**24a**), Rd (**25a**), F_2 (**26a**), and Rg_3 (**27a**) (with terminal glucosyl residues at C-3 or C-20 position).

2.1.4. PNP-Rha. α-L-rhamnosidase activity was determined using PNP-Rha (**28a**) as substrate. The results showed that the purified enzyme had hardly any activity for the rhamnosyl residue of PNP-Rha.

2.2. Isolation and purification of the products

Four secondary furostanosides (**1b**, **2b**, **5b**, and **6b**) and six corresponding spirostanosides (**1c**, **2c**, **5c**, **6c**, **8c**, and **9c**) of furostanosides were isolated and ascertained, three secondary furostanosides (**1b**, **2b**, and **6b**) of which were new compounds.

Eight converted products (**10b**–**16b** and **18b**) of spirostanosides were isolated and ascertained (see Table 1).

Table 1. Steroidal saponins and the isolated products

Substrates (a)	Products (b)	Products (c)
1a	**1b***	**1c** (**10b**)
2a	**2b***	**2c** (**16b**)
5a	**5b**	**5c** (**9c**, **11b**)
6a	**6b***	**6c** (**15b**)
8a		**8c**
9a		**9c** (**5c**, **11b**)
10a	**10b** (**1c**)	
11a	**11b** (**5c**, **9c**)	
12a	**12b**	
13a	**13b**	
14a	**14b**	
15a	**15b** (**6c**)	
16a	**16b** (**2c**)	
18a	**18b**	

The reaction mixture was extracted with *n*-butanol for four times. The extract (*n*-butanol layer) was isolated on silica gel C_{18} column chromatography and precipitation thin-layer chromatography (PTLC), respectively, to afford single compounds **1b** (63.2 mg), **1c** (20.3 mg), **2b** (45.8 mg), **2c** (15.6 mg), **10b** (35.6 mg), **11b** (25.9 mg), **12b** (12.0 mg), **13b** (16.0 mg), and on precipitation thin-layer chromatography (PTLC) to get compounds **5b** (68.4 mg), **5c** (21.2 mg), **6b** (9.21 mg), **6c** (11.98 mg), **8c** (17.3 mg), **9c** (13.8 mg), **14b** (87.6 mg), **15b** (112.7 mg), **16b** (31.0 mg), **18b** (184.0 mg).

2.3. Structural characterization of the products

The structures are shown in Table 2.

2.3.1. Compound 1b. Compound **1b** was obtained as white amorphous powder, which was soluble in ethanol, methanol, and water. It gave a positive Liebermann–Burchard, Molish test, and Ehrlich test. The result implied that the compound was a furostanoside. Mp: 219–222 °C. IR (KBr) ν_{max} cm^{-1}: 3600–3100 (OH), 1632 (double bond). The molecular formula of $C_{44}H_{72}O_{18}$ based on HR-ESI-MS (*m*/*z*): 911.4611 [$C_{44}H_{72}O_{18}$+Na]) and NMR studies. FABMS (*m*/*z*): 911.3 $(M+Na)^+$, 871.2 $(M+H-H_2O)^+$, 709.3 $(M+H-H_2O-162)^+$, 577.3 $(M+H-H_2O-162-132)^+$, 415.3 $(M+H-H_2O-162-132-162)^+$ implied that the compound contained three sugars, among which one was pentose and the others were hexoses. The ^1H NMR (C_5D_5N, 600 MHz) spectra display the following representative signals: four steroid methyl proton signals at δ: 0.89 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 0.98 (3H, d, J=6.6 Hz, 27-CH_3), and 1.33 (3H, d, J=7.2 Hz, 21-CH_3); one olefinic proton signal at δ 5.29 (1H, br s, H-6) ascribable to the double bond between C-5 and C-6; three

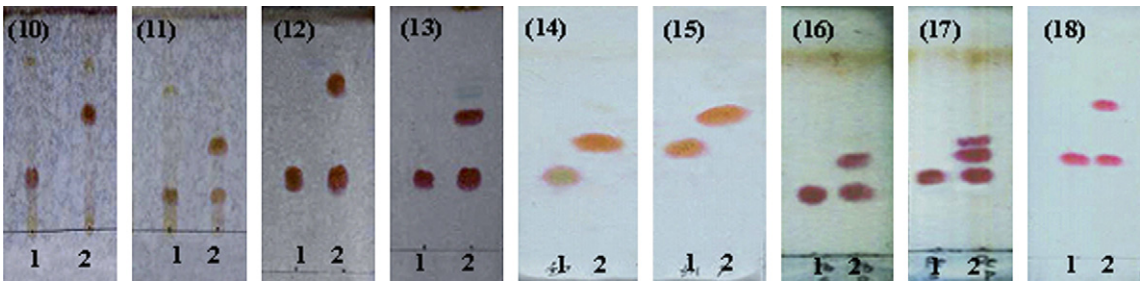


Figure 4. Analysis of spirostanosides bioconverted by TLC. 1: Substrate; 2: transformed products; (10)–(18): compounds **10**–**18**.

Table 2. The structures of substrates and converted products

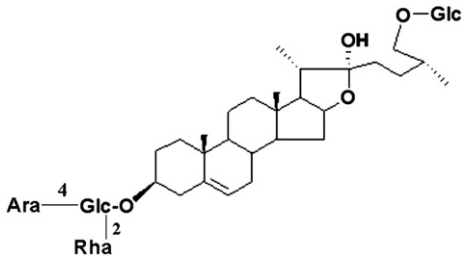
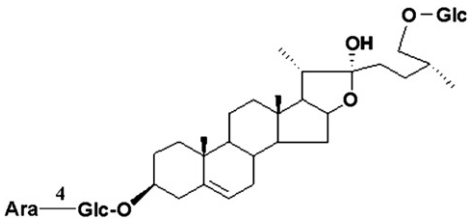
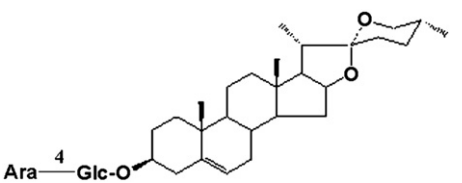
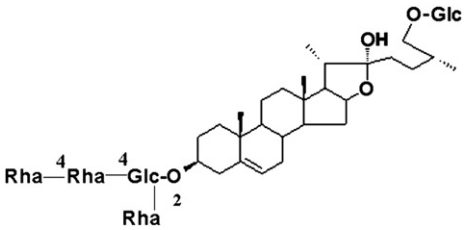
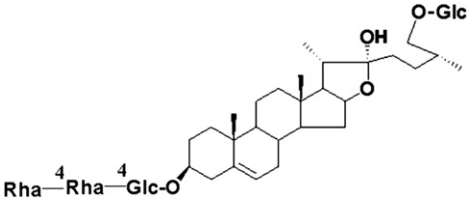
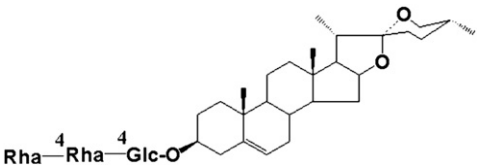
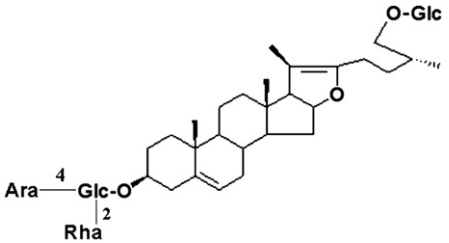
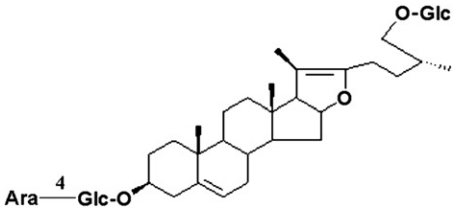
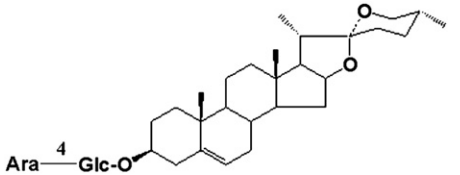
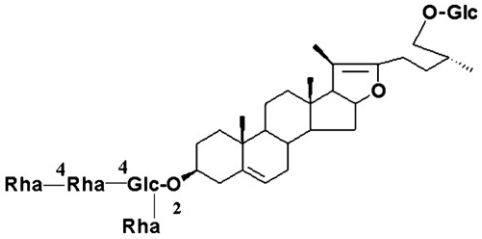
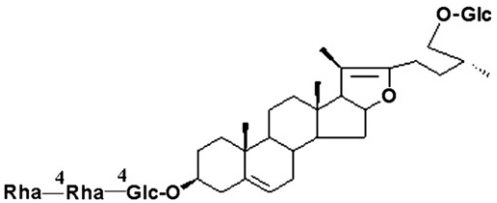
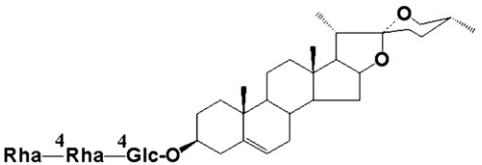
No.	Substrates	Structures of substrate (a)	Hydrolyzed glycon	Structure of products (b)	Structure of products (c)
1[a]	Proto-Pa		3-α-1,2-Rha (b) 26-Glc (c)		
2[a]	Proto-Pb		α-1,2-Rha (b) 26-Glc (c)		
3	Pseudoproto-Pa		α-1,2-Rha (b) 26-Glc (c)		
4	Pseudoproto-Pb		α-1,2-Rha (b) 26-Glc (c)		

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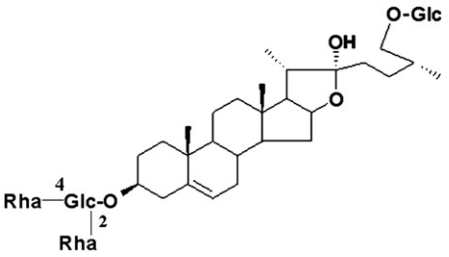
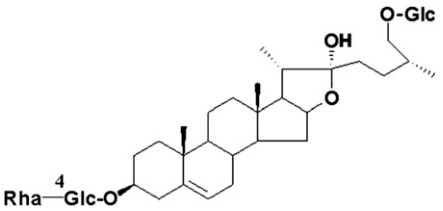
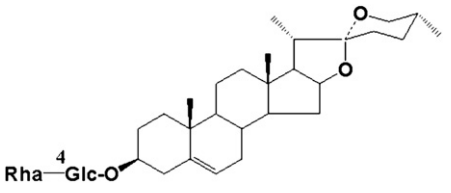
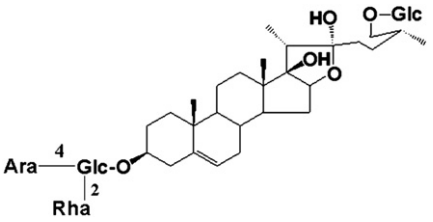
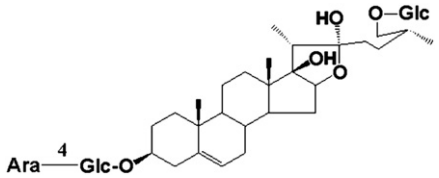
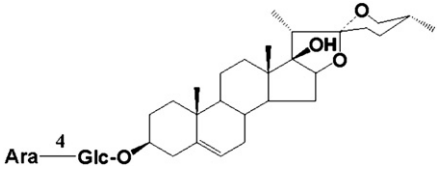
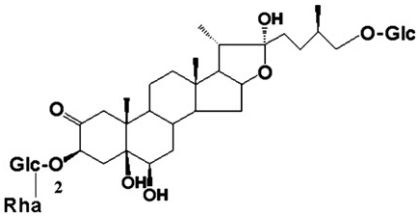
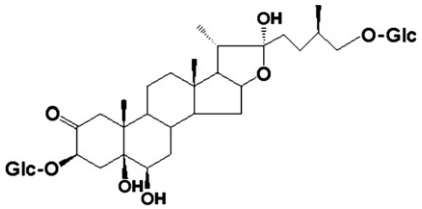
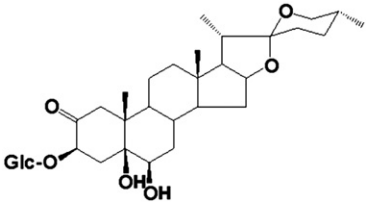
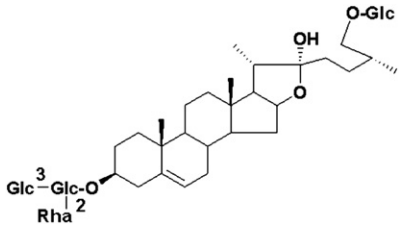
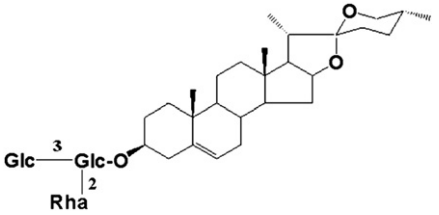
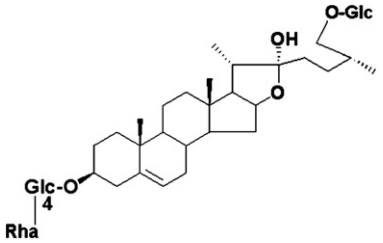
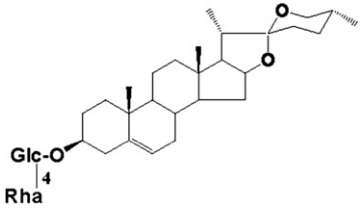
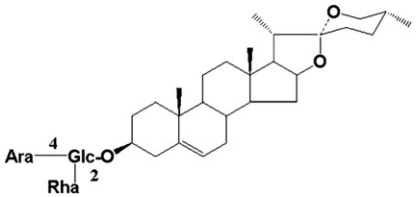
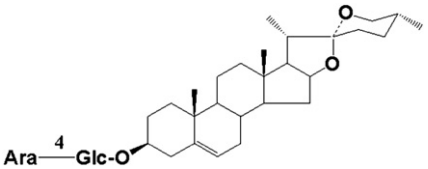
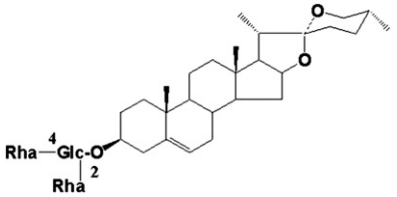
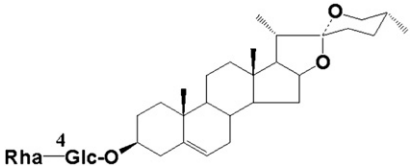
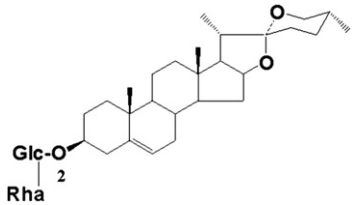
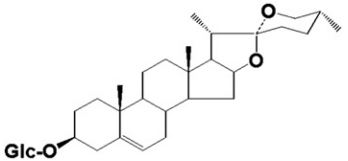
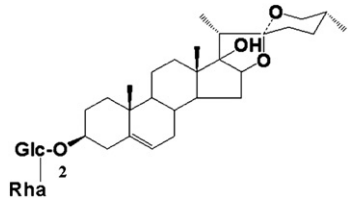
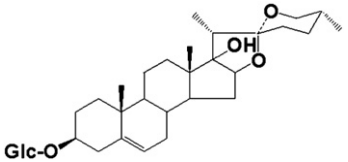
Table 2. (*continued*)

No.	Substrates	Structures of substrate (a)	Hydrolyzed glycon	Structure of products (b)	Structure of products (c)
5[a]	Protodioscin		α-1,2-Rha (b) 26-Glc (c)		
6[a]	26-*O*-β-D-Glucopyranosyl-(25*R*)-22-hydroxy-5-ene-furostane-3β,17α,26-triol-3-*O*-α-L-arabinofuranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside		α-1,2-Rha (b) 26-Glc (c)		
7	Ascalonicumside A		α-1,2-Rha (b) 26-Glc (c)		
8[a]	Protogracillin		26-Glc (c)		

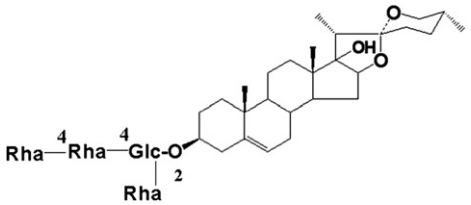
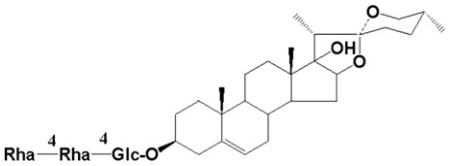
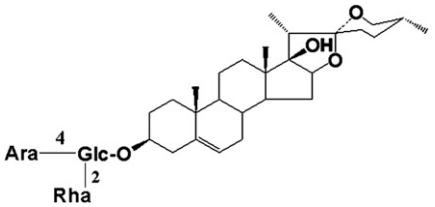
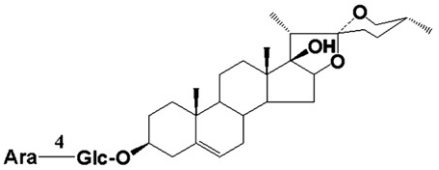
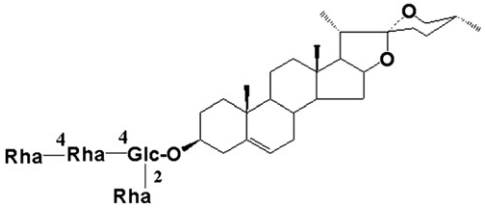
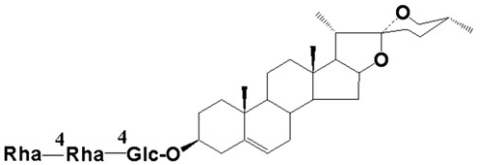
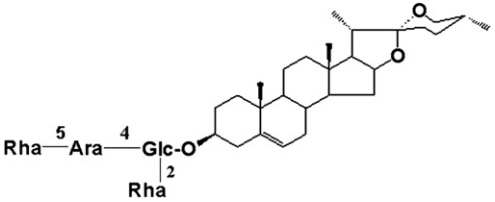
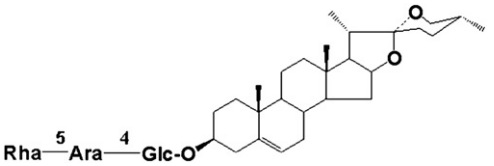
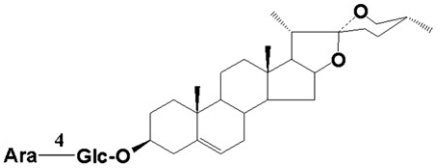
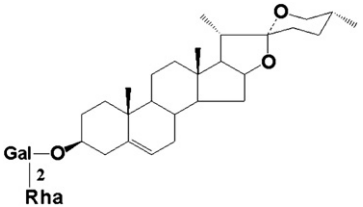
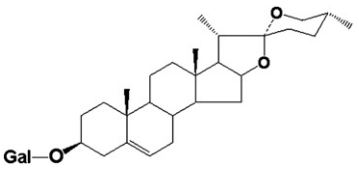
(*continued*)

Table 2. (*continued*)

No. Substrates	Structures of substrate (a)	Hydrolyzed glycon	Structure of products (b)	Structure of products (c)
9[a] Protoprogenin II		26-Glc (c)		
10[a] Polyphillin I		α-1,2-Rha		
11[a] Polyphillin III		α-1,2-Rha		
12[a] Polyphillin V		α-1,2-Rha		
13[a] Polyphillin VI		α-1,2-Rha		

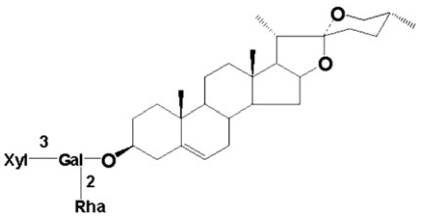
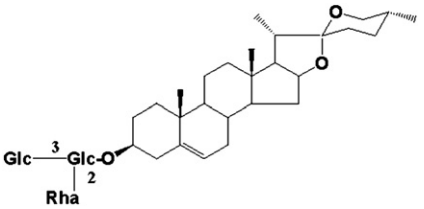
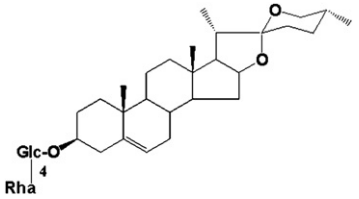
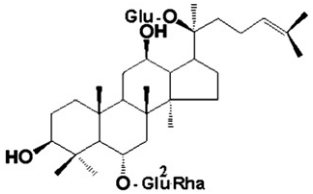
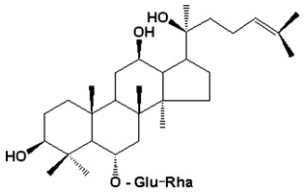
(*continued*)

Table 2. (*continued*)

No. Substrates	Structures of substrate (a)	Hydrolyzed glycon	Structure of products (b)	Structure of products (c)
14[a] Polyphillin VII		α-1,2-Rha		
15[a] Polyphillin H		α-1,2-Rha		
16[a] Pb		α-1,2-Rha		
17 Reclinatoside		α-1,2-Rha α-1,5-Rha		
18[a] Diosgenin-3-*O*-α-L-rhamno-pyranosyl (1→2)-β-D-galacto-pyranoside		α-1,2-Rha		

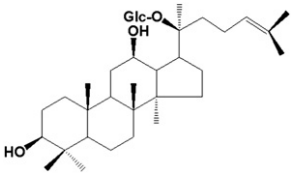
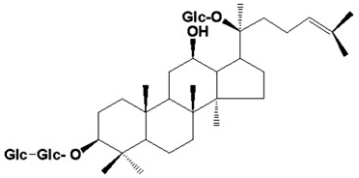
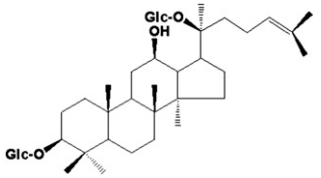
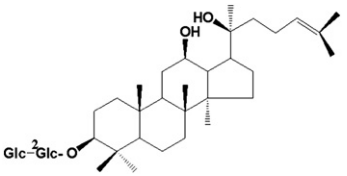
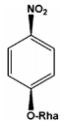
(*continued*)

Table 2. (*continued*)

No.	Substrates	Structures of substrate (a)	Hydrolyzed glycon	Structure of products (b)	Structure of products (c)
19	Diosgenin-3-*O*-α-L-rhamnopyranosyl (1→2)-[-β-D-xylopyranosyl(1→3)]-β-D-galactopyranoside				
20	Gracillin				
21	Diosgenin-3-*O*-α-L-rhamnopyranosyl (1→4)-β-D-glucopyranoside				
22	Ginsenoside Re				
23	Ginsenoside Rg_2				

(*continued*)

Table 2. (*continued*)

No. Substrates	Structures of substrate (a)	Hydrolyzed glycon	Structure of products (b)	Structure of products (c)
24 Ginsenoside C-K				
25 Ginsenoside Rd				
26 Ginsenoside F_2				
27 Ginsenoside Rg_3				
28 PNP-Rha				

[a] Products had been isolated and identified.

anomeric proton signals at δ 4.81 (1H, d, J=7.8 Hz), 4.90 (1H, d, J=7.8 Hz), and 6.05 (1H, s), respectively. With the aid of ^{1}H–^{1}H COSY, HSQC and HMBC, the ^{1}H and ^{13}C NMR data are summarized in Table 3. In the HMBC spectrum, the methyl proton signals at δ 0.89 (H-18) showed long-range correlation with carbon signals at δ 40.0 (C-12), 40.8 (C-13), 56.6 (C-14), and 63.9 (C-17), respectively. While the methyl proton signals at δ 0.91 (H-19) showed long-range correlation with carbons at δ 37.2 (C-10), 37.5 (C-1), 50.4 (C-9), and 140.9 (C-5), respectively. The HMBC experiment showed long-range correlations between the anomeric proton signal at δ 4.97 (H-1′) and the carbon signal at δ 78.3 (C-3), between the anomeric proton signal at δ 6.05 (H-1″) and the carbon signal at δ 76.0 (C-4′), and between the anomeric proton signal at δ 4.82 (H-1‴) and the carbon signal at δ 75.3 (C-26). Thus, the compound **1b** was determined to be 26-*O*-β-D-glucopyranosyl-(25*R*)-22-hydroxyl-5-ene-furostane-3β,26-diol-3-*O*-α-L-arabinofuranosyl-(1→4)-β-D-glucopyranoside. The structure is shown in Table 2 (**1b**).

2.3.2. Compound 1c. Compound **1c** was obtained as a white amorphous powder (EtOH), which was soluble in pyridine, ethanol, and methanol. It gave a positive Liebermann–Burchard, Molish test, and a negative Ehrlich test. The

Table 3. ^{1}H and ^{13}C NMR data of the compounds **1b**, **2b**, and **6b** (δ in pyridine-d_5)

C	**1b**		**2b**		**6b**	
	δ_C	δ_H (J=Hz)	δ_C	δ_H (J=Hz)	δ_C	δ_H (J=Hz)
1	37.5	0.97 (o), 1.70 (o)	37.4	0.96 (o), 1.69 (m)	37.5	0.93 (m), 1.70 (m)
2	30.2	1.74 (m), 2.08 (m)	30.2	1.69 (o), 2.06 (m)	30.2	1.70 (m), 2.06 (m)
3	78.3	3.89 (m)	78.3	3.86 (m)	78.3	3.85 (m)
4	39.3	2.44 (m), 2.68 (m)	39.3	2.44 (m), 2.69 (m)	39.3	2.44 (m), 2.66 (m)
5	140.9	—	140.9	—	140.9	—
6	121.8	5.29 (br s)	121.8	5.30 (br s)	121.8	5.28 (br s)
7	32.3	1.47, 1.86 (m)	32.3	1.47 (m), 1.84 (m)	32.4	1.88 (m), 1.51 (m)
8	31.7	1.55 (m)	31.7	1.54 (m)	32.1	1.61 (m)
9	50.4	0.87 (o)	50.4	0.86 (m)	50.3	0.95 (m)
10	37.2	—	37.2	—	37.1	—
11	21.1	1.41 (m), 1.43 (m)	21.1	1.43 (m)	21.0	1.50 (m), 1.58 (m)
12	40.0	1.12 (m), 1.75 (m)	40.0	1.11 (m), 1.74 (m)	32.3	1.9 (m), 2.17 (o)
13	40.8	—	40.8	—	45.1	—
14	56.6	1.05 (m)	56.6	1.06 (m)	53.1	2.01 (o)
15	32.5	1.43 (m), 2.00 (m)	32.5	1.43 (m), 2.00 (m)	31.9	1.47 (m), 2.15 (m)
16	81.1	4.94 (m)	81.1	4.93 (o)	90.5	4.75 (m)
17	63.9	1.93 (m)	63.9	1.92 (m)	90.8	—
18	16.5	0.89 (s)	16.5	0.89 (s)	17.5	0.99 (s)
19	19.4	0.91 (s)	19.4	0.91 (s)	19.4	0.93 (s)
20	40.7	2.23 (m)	40.7	2.23 (m)	43.6	2.49 (m)
21	16.5	1.33 (d, 6.6)	16.5	1.33 (d, 6.9)	10.5	1.37 (d, 7.0)
22	110.7	—	110.7	—	111.4	—
23	37.1	2.01 (m), 2.04 (m)	37.1	2.03 (m), 2.01 (m)	36.9	2.06 (o)
24	28.4	1.68 (m), 2.04 (o)	28.4	1.67 (m), 2.03 (m)	28.1	1.68 (o), 2.05 (o)
25	34.3	1.91 (m)	34.3	1.92 (m)	34.3	1.92 (m)
26	75.3	3.61 (dd, 6.0, 9.0), 3.93 (m)	75.2	3.61 (m, 6.0 9.5), 3.93 (o)	75.2	3.62 (m), 3.94 (m)
27	17.5	0.98 (d, 6.6)	17.5	0.98 (d, 6.7)	17.3	0.99 (d, 5.9)
3-Glc′						
1	102.5	4.97 (d, 7.8)	102.6	4.94 (d)	102.5	4.95 (d)
2	75.2	4.02 (m)	75.7	3.98 (m)	75.2	4.01 (o)
3	76.7	4.29 (o)	76.6	4.23 (o)	76.7	4.28 (m)
4	76.0	4.52 (m)	77.7	4.46 (m)	76.0	4.51 (m)
5	77.1	3.86 (m)	77.2	3.69 (m)	77.1	3.84 (m)
6	61.7	4.27 (m), 4.35 (m)	61.5	4.09 (m), 4.22 (m)	61.7	4.26 (m), 4.33 (m)
	4′-Ara″		**4′-Rha″**		**4′-Ara″**	
1	109.3	6.05 (s)	102.2	5.87 (s)	109.3	6.04 (s)
2	82.7	4.89 (m)	73.1	4.57 (m)	82.7	4.89 (o)
3	78.4	4.83 (m)	73.5	4.60 (m)	78.4	4.82 (m)
4	87.1	4.97 (m)	80.3	4.46 (m)	87.7	4.96 (o)
5	62.6	4.16 (dd 11.4, 4.2), 4.27 (m)	68.3	5.03 (o)	62.7	4.15 (dd, 11.7, 4.2), 4.27 (m)
			18.9	1.68 d (6.0)		
			4″-Rha‴			
			103.2	6.31 (s)		
			72.7	4.89 (m)		
			72.9	4.53 (m)		
			74.1	4.31 (m)		
			70.4	4.38 (m)		
			18.5	1.60 d (6.0)		
	26-Glc‴		**26-Glc⁗**		**26-Glc‴**	
1	105.0	4.82 (d, 7.8)	105.0	4.81 (d, 7.8)	105.0	4.81 (d, 7.8)
2	75.2	4.02 (m)	75.3	4.01 (m)	75.2	4.01 (o)
3	78.7	4.23 (m)	78.7	4.21 (o)	78.6	4.24 (m)
4	71.7	4.22 (m)	71.7	4.22 (o)	71.7	4.22 (m)
5	78.5	3.95 (m)	78.5	3.93 (o)	78.5	3.94 (m)
6	62.9	4.38 (dd, 11.4, 5.4), 4.54 (m)	62.9	4.37 (m), 4.53 (m)	62.9	4.38 (dd, 11.7, 5.2), 4.55 (m)

results imply that the compound is a spirostan with steroidal type skeleton. Mp 239–241 °C. IR (KBr) ν_{max} cm^{-1}: 3600—3100 (OH), 1632 (double bond). FABMS (*m/z*): 709.3 $(M+H)^+$, 577.3 (M+H−132), 415.2 (M+H−132−146) implied that the compound has a pentose and a hexose. The ^{1}H NMR (C_5D_5N, 600 MHz) spectra display the following representative signals: four steroid methyl protons at δ 0.68 (3H, d, J=4.8 Hz, 27-CH_3), 0.82 (3H, s, 18-CH_3), 0.90 (3H, s, 19-CH_3), and 1.13 (3H, d, J=7.2 Hz, 21-CH_3); the anomeric proton signals at δ 4.96 (1H, d, J=7.2 Hz) and 6.04 (1H, s) attributable to H-1 of glucose (β-linkage) and H-1 of arabinose, respectively; one olefinic proton at δ 5.30 (1H, br s, H-6) ascribable to the double bond between C-5 and C-6. The ^{13}C NMR spectral data are shown in Table 4. The compound **1c** was deduced to be diosgenin-3-*O*-α-L-arabinofuranosyl-(1→4)-β-D-glucopyranoside. The structure is shown in Table 2 (**1c** and **10b**).[10]

2.3.3. Compound 2b. Compound **2b** was obtained as a white amorphous powder, which was soluble in ethanol, methanol, and water. It gave a positive Liebermann–Burchard, Molish test, and Ehrlich test. The result implied that the compound was a furostanoside. Mp 198–200 °C. IR (KBr) ν_{max} cm^{-1}: 3600–3200 (OH), 1628 (double bond). The molecular formula of $C_{51}H_{82}O_{21}$ based on HR-ESI-MS (*m/z*): 1071.5406 [$C_{51}H_{82}O_{21}$+Na]) and NMR studies. FABMS (*m/z*): 1071.4 $(M+Na)^+$, 1031.4 $(M+H-H_2O)^+$, 885.4 $(M+H-H_2O-146)^+$, 739.3 $(M+H-H_2O-146-146)^+$, 577.3 $(M+H-H_2O-146-146-162)^+$, 415.2 $(M+H-H_2O-162-146-146-162)^+$ implied that the compound contained four sugars, among which two were methyl pentoses and others were hexoses. The ^{1}H NMR (C_5D_5N, 600 MHz) spectra display the following representative signals: four steroid methyl proton signals at δ: 0.86 (3H, s, 18-CH_3), 0.88 (3H, s, 19-CH_3), 0.95 (3H, d, J=6.6 Hz, 27-CH_3), and 1.30 (3H, d, J=6.6 Hz, 21-CH_3); δ 4.78 (1H, d, J=7.8 Hz), 4.91 (1H, d, J=7.8 Hz), 5.85 (1H, s) and 6.29 (1H, s) were four anomeric proton signals, respectively; δ 1.57 (3H, d, J=6.0 Hz, Rha-CH_3) and 1.66 (3H, d, J=6.6 Hz, Rha-CH_3) attributable to methyl proton signals of two methyl pentoses; one olefinic proton signal at δ 5.23 (1H, br s, H-6) attribute to the double bond between C-5 and C-6. With the aid of ^{1}H–^{1}H COSY, HSQC, and HMBC, the ^{1}H and ^{13}C NMR data are summarized in Table 3. In the HMBC spectrum, the methyl proton signals at δ 0.88 (H-18) showed long-range correlation with carbon signals at δ 40.0 (C-12), 40.8 (C-13), 56.6 (C-14), and 63.9 (C-17), respectively. While the methyl proton signals at δ 0.91 (H-19) showed long-range correlation with carbons at δ 37.2 (C-10), 37.4 (C-1), 50.4 (C-9), and 140.9 (C-5), respectively. The HMBC experiment showed long-range correlations between the anomeric proton signal at δ 4.94 (H-1′) and the carbon signal at δ 78.3 (C-3), between the anomeric proton signal at δ 5.85 (H-1″) and the carbon signal at δ 77.7 (C-4′), between the anomeric proton signal at δ 6.29 (H-1‴) and the carbon signal at δ 80.3 (C-4″), and between the anomeric proton signal at δ 4.78 (H-1⁗) and the carbon signal at δ 75.2 (C-26). Thus, the compound **2b** was deduced to be 26-*O*-β-D-glucopyranosyl-(25*R*)-22-hydroxy-5-ene-furostane-3β,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside. The structure is shown in Table 2 (**2b**).

2.3.4. Compound 2c. See compound **16b**.

2.3.5. Compound 5b. Compound **5b** was obtained as white amorphous powder, which was soluble in ethanol, methanol, and water. It gave a positive Liebermann–Burchard, Molish test, and Ehrlich test. The result implied that the compound was a furostanoside. Mp 156–160 °C. IR (KBr) ν_{max} cm^{-1}: 3600–3000 (OH), 1630 (double bond). FABMS (*m/z*): 885.4 $(M+H-H_2O)^+$, 723.3 $(M+H-H_2O-162)^+$, 577.3 $(M+H-H_2O-162-146)^+$, 415.2 $(M+H-162-146-162)^+$ implied that the compound contained three sugars, among which one was methyl pentose and the others were hexoses. ^{1}H NMR (C_5D_5N, 600 Hz): 0.89 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 0.98 (3H, d, J=6.6 Hz, 27-CH_3), and 1.32 (3H, d, J=7.0 Hz, 21-CH_3) were four steroid methyl proton signals, respectively. δ 1.65 (3H, d, J=6.5 Hz, Rha-CH_3) was methyl proton signal of methyl pentose; δ 6.30 (1H, d, J=1.2 Hz, Rha H-1), 4.95 (1H, d, J=7.5 Hz, Glc H-1), and 4.80 (1H, d, J=7.6 Hz, Glc H-1″) were three anomeric proton signals; one olefinic proton signal at δ 5.28 (1H, br

Table 4. ^{13}C NMR data of the compounds **5b**, **1c**, **5c**, **6c**, **8c** and **9c** (δ in pyridine-d_5)

C	**5b**	**1c** (**10b**)	**5c** (**9c**, **11b**)	**8c**	C	**5b**	**1c**	**5c**	**8c**
1	37.2	37.5	37.5	37.5		**3-Glc′**			
2	30.2	30.2	30.1	30.1	1	102.5	102.5	102.7	100.1
3	78.6	78.2	78.4	78.7	2	75.3	75.3	75.5	77.0
4	39.4	39.3	39.3	38.7	3	77.2	76.7	76.7	89.6
5	140.9	140.8	140.7	140.8	4	78.4	78.4	78.4	69.6
6	121.8	121.8	121.9	121.8	5	78.3	77.1	77.1	77.7
7	32.3	32.2	32.2	32.2	6	61.6	62.6	61.6	62.4
14	56.6	56.6	56.6	56.7		**4-Rha″**	**4-Ara″**	**4-Rha″**	**2-Rha″**
15	32.5	32.3	32.3	32.2	1	102.8	109.3	102.7	102.2
16	81.1	81.1	81.1	81.1	2	72.7	82.7	72.7	72.5
17	63.9	62.9	62.9	62.9	3	72.9	78.2	72.9	72.8
18	16.5	16.4	16.3	16.3	4	74.1	87.1	74.0	74.1
19	19.4	19.4	19.4	19.4	5	70.5	61.7	70.4	69.6
20	40.7	42.0	41.9	42.0	6	18.6		18.6	18.7
21	16.5	15.0	15.1	15.0		**26-Glc‴**			**3-Glc‴**
22	110.7	109.3	109.3	109.3	1	105.0			104.6
23	37.2	31.7	31.7	31.4	2	75.6			75.0
24	28.4	29.3	29.2	29.3	3	78.6			78.5
25	34.3	30.6	30.5	30.6	4	71.8			71.5
26	75.2	66.9	66.8	66.9	5	78.5			77.9
27	17.5	17.3	17.2	17.3	6	62.9			62.9

s, H-6) attribute to the double bond between C-5 and C-6. The ^{13}C NMR spectrum data are shown in Table 4 and are comparable to that of literature[11] as 26-*O*-β-D-glucopyranosyl-(25*R*)-22-hydroxyl-5-ene-furostane-3β,26-diol-3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside. The structure is shown in Table 2 (**5b**).

2.3.6. Compound 5c. Compound **5c** was obtained as a white needle crystal (EtOH), which was soluble in pyridine, ethanol, and methanol. It gave a positive Liebermann–Burchard, Molish test, and a negative Ehrlich test. The results imply that the compound is a spirostan with steroidal type skeleton. Mp 242–246 °C. IR (KBr) ν_{max} cm^{-1}: 3600–3200 (OH), 1628 (double bond). FABMS (*m/z*): 815.4 $(M+H+C_3H_8O_3)^+$, 723.4 (M+H), 577.3 (M+H−146), 415.3 (M+H−146−162) indicates that the compound has two hexoses. The ^1H NMR spectra display the following representative signals: four steroid methyl protons at δ 0.68 (3H, d, *J*=5.4 Hz, 27-CH_3), 0.82 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), and 1.13 (3H, d, *J*=7.20 Hz, 21-CH_3); δ 4.95 (1H, d, *J*=7.8 Hz) and 5.84 (1H, s) attributable to H-1 of glucose (β-linkage) and H-1 of rhamnose; one olefinic proton at δ 5.30 (1H, br s, H-6) attribute to the double bond between C-5 and C-6. The ^{13}C NMR spectral data are shown in Table 4. The compound **5c** was deduced to be diosgenin-3-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside. The structure is shown in Table 2 (**5c**, **9c**, **11b** and **21a**).[12]

2.3.7. Compound 6b. Compound **6b** was obtained as white amorphous powder, which was soluble in ethanol, methanol, and water. It gave a positive Liebermann–Burchard, Molish test, and Ehrlich test. The result implied that the compound was a furostanoside. Mp 222–224 °C. IR (KBr) ν_{max} cm^{-1}: 3600–3200 (OH), 1630 (double bond). The molecular formula of $C_{44}H_{72}O_{19}$ based on HR-ESI-MS (*m/z*): 927.4560 [$C_{44}H_{72}O_{19}$+Na]) and NMR studies. FABMS (*m/z*): 927.4 $(M+Na)^+$, 887.4 $(M+H-H_2O)^+$, 869.4 (M+H−H_2O−H_2O), 707.3 (M+H−H_2O−H_2O−162)$^+$, 575.3 (M+H−H_2O−H_2O−162−132)$^+$, 413.2 (M+H−H_2O−H_2O−162−132−162)$^+$ implied that the compound contained three sugars, among which one was pentose and others were hexoses. ^1H NMR (C_5D_5N, 600 Hz): δ 0.93 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 1.02 (3H, d, *J*=6.6 Hz, 27-CH_3), and 1.37 (3H, d, *J*=7.2 Hz, 21-CH_3) were four steroid methyl proton signals, respectively; one olefinic proton signal at δ 5.28 (1H, br s, H-6) ascribable to the double bond between C-5 and C-6; three anomeric proton signals at δ 4.90 (1H, d, *J*=7.8 Hz), 4.81 (1H, d, *J*=7.8 Hz), and 6.04 (1H, s), respectively. With the aid of ^1H–^1H COSY, HSQC, and HMBC, the ^1H and ^{13}C NMR data are summarized in Table 3. In the HMBC spectrum, the methyl proton signals at δ 0.90 (H-18) showed long-range correlation with carbon signals at δ 32.1 (C-12), 45.1 (C-13), 53.1 (C-14), and 90.8 (C-17), respectively. While the methyl proton signals at δ 0.93 (H-19) showed long-range correlation with carbons at δ 37.1 (C-10), 37.5 (C-1), 50.3 (C-9), and 140.9 (C-5), respectively. The HMBC experiment showed long-range correlations between the anomeric proton signal at δ 4.95 (H-1′) and the carbon signal at δ 78.3 (C-3), between the anomeric proton signal at δ 6.05 (H-1″) and the carbon signal at δ 76.0 (C-4′), and between the anomeric proton signal at δ 4.81 (H-1‴) and the carbon signal at δ 75.2 (C-26). Thus, compound **6b** was deduced to be 26-*O*-β-D-glucopyranosyl-(25*R*)-22-hydroxyl-5-ene-furostane-3β,17α,26-triol-3-*O*-α-L-arabinofuranosyl-(1→4)-β-D-glucopyranoside. The structure is shown in Table 2 (**6b**).

2.3.8. Compound 6c. See compound **15b**.

2.3.9. Compound 8c. Compound **8c** was obtained as a white needle crystal (EtOH), which was soluble in ethanol and methanol. It gave a positive Liebermann–Burchard, Molish test, and a negative Ehrlich test. The results imply that the compound was a spirostanoside. Mp 216–220 °C. IR (KBr) ν_{max} cm^{-1}: 3400–3000 (OH), 1628 (double bond). FABMS (*m/z*): 907.5 $(M+Na)^+$, 885.6 $(M+H)^+$, 577.5 (M+H−146−162)$^+$, 415.5 (M+H−146−162−162)$^+$ implied that the compound contained three sugars, among which one was methyl pentose and others were hexoses. ^1H NMR (C_5D_5N, 600 Hz): δ 0.68 (3H, d, *J*=5.7 Hz, 27-CH_3), 0.82 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), and 1.13 (3H, d, *J*=7.0 Hz, 21-CH_3) were four steroid methyl proton signals, respectively; one olefinic proton signal at δ 5.32 (1H, br s, H-6) ascribable to the double bond between C-5 and C-6; δ 1.56 (3H, d, *J*=4.7 Hz, Rha-CH_3) attributable to methyl proton signal of methyl pentose; δ 4.94 (1H, d, *J*=7.0 Hz), 5.11 (1H, d, *J*=7.7 Hz), and 6.39 (1H, s) were three anomeric proton signals, respectively. The ^{13}C NMR spectrum data (Table 4) were comparable to that of literature[13] as diosgenin-3-*O*-β-D-glucopyranosyl-(1→3)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside. The structure is shown in Table 2 (**8c** and **20a**).

2.3.10. Compound 9c. See compound **5c**.

2.3.11. Compound 10b. See compound **1c**.

2.3.12. Compound 11b. See compound **5c**.

2.3.13. Compound 12b. Compound **12b** was obtained as a white needle crystal (EtOH), which was soluble in pyridine, ethanol, and methanol. It gave a positive Liebermann–Burchard, Molish test, and a negative Ehrlich test. The results imply that the compound is a spirostan with steroidal type skeleton. Mp 262–263 °C. IR (KBr) ν_{max} cm^{-1}: 3600–3200 (OH), 1630 (double bond). FABMS (*m/z*): 577.3 $(M+H)^+$, 415.2 (M+H−162), 397.2 (M+H−H_2O−162) implied that the compound has a hexose. The ^1H NMR spectra display the following representative signals: four steroid methyl protons at δ 0.68 (3H, d, *J*=5.7 Hz, 27-CH_3), 0.82 (3H, s, 18-CH_3), 1.05 (3H, s, 19-CH_3), and 1.13 (3H, d, *J*=7.0 Hz, 21-CH_3); δ 5.23 (1H, d, *J*=7.7 Hz) attributable to H-1 of glucose and indicating a β-linkage; one olefinic proton at δ 5.42 (1H, br s, H-6) ascribable to the double bond between C-5 and C-6. The ^{13}C NMR spectral data are shown in Table 5. The compound **12b** was deduced to be diosgenin-3-*O*-β-D-glucopyranoside. The structure is shown in Table 2 (**12b**).[14,15]

2.3.14. Compound 13b. Compound **13b** was obtained as a white needle crystal (EtOH), which was soluble in pyridine, ethanol, and methanol. It gave a positive Liebermann–Burchard, Molish test, and a negative Ehrlich test. The results imply that the compound is a spirostan with steroidal type skeleton. Mp 276–79 °C. IR (KBr) ν_{max} cm^{-1}: 3600–3100 (OH), 1633 (double bond). FABMS (*m/z*): 593.2 $(M+H)^+$, 575.2 (M+H−H_2O), 431.2 (M+H−162), 413.2 (M+H−H_2O−162)

Table 5. ^{13}C NMR data of the compounds **14b–16b** and **18b** (δ in pyridine-d_5)

C	12b	13b	14b	15b (6c)	16b (2c)	18b	C	12b	13b	14b	15b (6c)	16b (2c)	18b
1	37.5	37.7	37.5	37.5	37.5	371.5		**3-Glc′**	**3-Glc′**	**3-Glc′**	**3-Glc′**	**3-Glc′**	**3-Gal′**
2	30.4	30.3	30.2	30.2	30.2	30.3	1	102.5	102.6	102.5	102.5	102.5	103.2
3	78.2	78.4	78.2	78.3	78.2	78.0	2	75.5	75.4	75.7	75.2	75.6	72.7
4	39.0	39.1	39.3	39.3	39.3	39.4	3	78.2	78.4	76.6	76.7	76.6	75.5
5	140.8	141.0	140.9	140.9	140.9	141.0	4	71.8	71.7	77.7	76.0	77.7	70.4
6	121.8	121.8	121.8	121.8	121.8	121.7	5	78.2	78.1	77.2	77.1	77.2	77.0
7	32.3	32.5	32.4	32.4	32.3	32.3	6	62.7	62.9	61.8	61.7	61.5	62.6
8	31.7	31.9	31.8	31.8	31.7	31.7					**4-Ara″**		
9	50.3	50.4	50.2	50.2	50.3	50.3	1				109.3		
10	37.2	37.3	37.1	37.1	37.1	37.3	2				82.7		
11	21.1	21.1	21.0	21.0	21.1	21.1	3				78.4		
12	39.9	32.1	32.1	32.1	39.9	39.9	4				87.1		
13	40.5	45.1	45.2	45.2	40.5	40.5	5				62.6		
14	56.7	53.2	53.1	53.1	56.7	56.7				**4-Rha″**		**4-Rha″**	
15	32.2	32.5	32.3	32.3	32.2	32.2	1			102.2		102.2	
16	81.1	90.3	90.0	90.1	81.1	81.1	2			73.5		73.0	
17	62.9	90.2	90.1	90.1	62.9	62.9	3			73.0		73.4	
18	16.3	17.2	17.2	17.2	16.3	16.4	4			80.3		80.3	
19	19.4	19.5	19.5	19.3	19.4	19.4	5			68.3		68.3	
20	42.0	44.9	44.8	44.8	42.0	42.0	6			18.9		18.9	
21	15.0	9.5	9.8	9.8	15.0	15.0				**4′-Rha‴**		**4′-Rha‴**	
22	109.3	109.9	109.9	109.8	109.3	109.3	1			103.2		103.2	
23	31.7	32.1	32.1	32.3	31.8	31.8	2			72.7		72.7	
24	29.3	28.9	28.8	28.8	29.3	29.3	3			72.9		72.8	
25	30.6	30.5	30.5	30.5	30.6	30.6	4			74.0		74.0	
26	66.9	66.9	66.7	66.7	66.9	66.9	5			70.4		70.4	
27	17.3	17.3	17.3	17.3	17.3	17.3	6			18.5		18.5	

implied that the compound has a hexose and an active hydroxyl. The ^1H NMR spectra display the following representative signals: four steroid methyl protons at δ 0.67 (3H, d, J=5.7 Hz, 27-CH_3), 0.93 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), and 1.22 (3H, d, J=7.14 Hz, 21-CH_3); δ 5.03 (1H, d, J=7.69 Hz) attributable to H-1 of glucose and indicating a β-linkage; one olefinic proton at δ 5.29 (1H, br s, H-6) ascribable to the double bond between C-5 and C-6. The ^{13}C NMR spectral data are shown in Table 5. The compound **13b** was deduced to be pennogenin-3-*O*-β-D-glucopyranoside. The structure is shown in Table 2 (**13b**).[14,15]

2.3.15. Compound 14b. Compound **14b** was obtained as a white needle crystal (EtOH), which was soluble in ethanol and methanol. It gave a positive Liebermann–Burchard, Molish test, and a negative Ehrlich test. The results imply that the compound was a spirostanoside. Mp 216–220 °C. IR (KBr) ν_{max} cm^{-1}: 3600–3000 (OH), 1632 (double bond). FABMS (*m/z*): 907.5 $(M+Na)^+$, 867.5 $(M+H-H_2O)^+$, 705.5 $(M+H-H_2O-162)^+$, 559.5 $(M+H-H_2O-162-146)^+$, 413.5 $(M+H-H_2O-162-146-146)^+$ implied that the compound contained three sugars, among which one was hexose and others were methyl pentoses. ^1H NMR (C_5D_5N, 600 Hz): δ 0.68 (3H, d, J=5.4 Hz, 27-CH_3), 0.94 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), and 1.24 (3H, d, J=5.4 Hz, 21-CH_3) were four steroid methyl proton signals, respectively; one olefinic proton signal at δ 5.26 (1H, br s, H-6) ascribable to the double bond between C-5 and C-6; δ 1.62 (3H, d, J=4.8 Hz, Rha-CH_3) and 1.67 (3H, d, J=6.0 Hz, Rha-CH_3) attributable to methyl proton signals of two methyl pentoses; δ 4.97 (1H, d, J=7.2 Hz), 5.84 (1H, s), and 6.29 (1H, s) were three anomeric proton signals, respectively. The ^{13}C NMR spectrum data (see Table 5) were comparable to that of literature[16] as pennogenin-3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside. The structure is shown in Table 2 (**14b**).

2.3.16. Compound 15b. Compound **15b** was obtained as a white needle crystal (EtOH), which was soluble in ethanol and methanol. It gave a positive Liebermann–Burchard, Molish test, and a negative Ehrlich test. The results imply that the compound was a spirostanoside. Mp 168–172 °C. IR (KBr) ν_{max} cm^{-1}: 3600–3100 (OH), 1632 (double bond). FABMS (*m/z*): 747.4 $(M+Na)^+$, 707.4 $(M+H-H_2O)^+$, 545.4 $(M+H-H_2O-162)^+$, 413.4 $(M+H-H_2O-162-132)^+$ implied that the compound contained two sugars, among which one was pentose and another was hexose. The ^1H NMR (C_5D_5N, 600 MHz) spectra display the following representative signals: four steroid methyl proton signals at δ: 0.65 (3H, d, J=5.4 Hz, 27-CH_3), 0.90 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), and 1.19 (3H, d, J=7.2 Hz, 21-CH_3); one olefinic proton signal at δ 5.26 (1H, br s, H-6) was ascribable to the double bond between C-5 and C-6; two anomeric proton signals at δ 4.92 (1H, d, J=7.8 Hz) and 6.01 (1H, s), respectively. The ^{13}C NMR spectrum data (see Table 5) were comparable to that of literature[10] as pennogenin-3-*O*-α-L-arabinofuranosyl-(1→4)-β-D-glucopyranoside. The structure is shown in Table 2 (**15b** and **6c**).

2.3.17. Compound 16b. Compound **16b** was obtained as a white needle crystal (EtOH), which was soluble in ethanol and methanol. It gave a positive Liebermann–Burchard, Molish test, and a negative Ehrlich test. The results imply that the compound was a spirostanoside. Mp 120–124 °C. IR (KBr) ν_{max} cm^{-1}: 3600–3200 (OH), 1632 (double bond). FABMS (*m/z*): 891.4 $(M+Na)^+$, 869.4 $(M+1)^+$, 723.4 $(M+H-146)^+$, 577.4 $(M+H-146-146)^+$, 415.4 $(M+H-146-146-162)^+$ implied that the compound contained three sugars, among which one was hexose and others were methyl pentoses. ^1H NMR (C_5D_5N, 600 Hz): δ 0.69 (3H, d, J=5.7 Hz, 27-CH_3), 0.83 (3H, s, 18-CH_3), 1.04 (3H, s, 19-CH_3), and 1.13 (3H, d, J=6.9 Hz, 21-CH_3) were

four steroid methyl proton signals, respectively; one olefinic proton signal at δ 5.28 (1H, br s, H-6) ascribable to the double bond between C-5 and C-6; δ 1.58 (3H, d, J=6.0 Hz, Rha-CH_3) and 1.59 (3H, d, J=6.4 Hz, Rha-CH_3) attributable to methyl proton signals of two methyl pentoses; δ 4.94 (1H, d, J=7.2 Hz), 5.83 (1H, s), and 6.28 (1H, s) were three anomeric proton signals, respectively. The ^{13}C NMR spectrum data (see Table 5) were comparable to that of literature[17] as diosgenin-3-*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranoside. The structure is shown in Table 2 (**16b** and **2c**).

2.3.18. Compound 18b. Compound **18b** was obtained as a white needle crystal (EtOH), which was soluble in ethanol and methanol. It gave a positive Liebermann–Burchard, Molish test, and a negative Ehrlich test. The results imply that the compound was a spirostanoside. Mp 240–242 °C. IR (KBr) ν_{max} cm^{-1}: 3600–3100 (OH), 1632 (double bond). FABMS (*m*/*z*): 599.0 $(M+Na)^+$, 577.1 $(M+H)^+$, 415.1 $(M+H-162)^+$, 397.1 $(M+H-162-H_2O)$ implied that the compound contained one hexose.^{1}H NMR (C_5D_5N, 600 Hz): δ 0.68 (3H, d, J=6.0 Hz, 27-CH_3), 0.85 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), and 1.13 (3H, d, J=7.0 Hz, 21-CH_3) were four steroid methyl proton signals, respectively; one olefinic proton signal at δ 5.25 (1H, br s, H-6) ascribable to the double bond between C-5 and C-6; δ 4.90 (1H, d, J=7.2 Hz) was one anomeric proton signal. The ^{13}C NMR spectrum data are shown in Table 5, the compound **18b** was deduced to be diosgenin-3-*O*-β-D-galactopyranoside.[18] The structure is shown in Table 2 (**18b**).

3. Discussion

It is known that glucoamylase hydrolyzes α-1,4-linked glucose residues consecutively from the non-reducing end of amylose, amylopectin, and glycogen producing β-glucose. In previous work, hydrolyzation of the end-rhamnosyl residues of steroidal saponin with glucoamylase from *C. lunata* and purification of the enzyme by chromatography had been reported by us, but the definite substrate specificity of the enzyme for saponins did not be confirmed by large amounts of saponins. In this work, 12 spirostanosides, 9 furostanosides, and 6 triterpenosides, which had different aglycones, different glycosyl residues, and number of glycon, the sugar chain with linear chain or branched chain, were selected to make a systematic investigation by the purified glucoamylase from *C. lunata*. Analysis of converted products indicated that hydrolysis, as for furostanosides, occurred chiefly at the α-1,2-linked end-rhamnosyl of sugar chain at C-3 position, rather than at the glucosyl residues at C-26 position to produce corresponding secondary furostanoside (compounds **1b**–**7b**); as for spirostanosides, the hydrolysis occurred all at the α-1,2-linked end-rhamnosyl residues to give secondary spirostanosides (compounds **10b**–**18b**). *The results ascertain that the glucoamylase was strictly specific for α-1,2-linked end-rhamnosyl of steroidal saponins.* The products **1b**, **1c**, **2b**, **2c**, **5b**, **5c**, **6b**, **6c**, **8c**, **9c**, **10b**–**16b**, and **18b** were isolated and ascertained from reaction mixtures, among which products **1b**, **2b**, and **6b** were new compounds. Other products **3b**, **3c**, **4b**, **4c**, **7b**, **7c**, and **17b** were not isolated in sufficient amounts to be identified.

The substrate specificity of the glucoamylase from *C. lunata* toward steroidal saponins was summarized as follows: (1) the glucoamylase was able to hydrolyze the terminal α-1,2-linked rhamnosyl residues at C-3 position of steroidal saponin. (2) It was not strictly specific for different aglycones of steroidal saponins, such as diosgenin (compounds **1a**, **2a**, **5a**, **10a**–**12a**, and **16a**–**18a**), pennogenin (compounds **6a** and **13a**–**15a**), or other (compounds **3a**, **4a**, and **7a**). (3) As for furostanosides, hydrolysis occurred chiefly at α-1,2 end-rhamnosyl at C-3 position to produce secondary furostanosides (**1b**–**7b**); the reaction of hydrolyzing glucosyl at C-26 position depended considerably on longer reaction times to give corresponding spirostanoside with removed rhamnosyl and glucosyl residues (removing rhamnosyl and glucosyl residues, **1c**–**7c**). (4) As for spirostanosides, it was more active for the substrates with more than three glycosyl residues and pennogenin (**14a** and **15a**), and a half active for the substrates having two glycosyl residues and diosgenin (**10a**–**13a** and **16a**–**18a**) of sugar chain at C-3 position. (5) The sugar chain of steroidal saponin at C-3 position contains linear or branched chains. The glucoamylase was only inactive against the terminal α-1,2-linked rhamnosyl residues with the branched chain of 1,3-linked glycosyl residues (**8a**, **19a**, and **20a**). The terminal α-1,2-linked rhamnosyl residues with the branched chain of 1,4- (**1a**–**6a**, **10a**, **11a**, and **14a**–**16a**) or 1,5-linked (17a) glycosyl residues, however, was hydrolyzed without difficulty. The result suggested that the steric hindrance of 1,3-linked branch-glycosyl residues effects considerably on the enzymatic activity hydrolyzing terminal α-1,2-linked rhamnosyl residues. (6) The glucoamylase was also inactive against the terminal 1,4-linked rhamnosyl residues of sugar chain at C-3 position of steroidal saponins (**9a** and **21a**). (7) It was inactive against neither the end-rhamnosyls of ginsenosides (**22a** and **23a**) and *p*NPR (**28a**), nor the glucosyl residues of ginsenosides (**24a**–**27a**).

The structures of hydrolysis of steroidal saponins and ginsenosides by the purified enzyme are presented in Table 2.

In conclusion, the glucoamylase from *C. lunata* had the strict specificity that hydrolyzed the terminal α-1,2-linked rhamnosyl residues, or the 1→2 linked rhamnosyl residues with branched chain of 1,4- or 1,5-linked rather than 1,3-linked glycosyl residues of sugar chain at C-3 position of steroidal saponins; it was not specific for the different aglycones, different sugars, and the number of glycosyl residues of sugar chain.

In addition, the results of analysis by TLC showed two new converted products of compound **17** (spirostanoside) having a terminal α-1,2- and a terminal α-1,5-linked rhamnosyl residues, among which one product was determined as diosgenin-3-*O*-α-L-arabinofuranosyl-(1→4)-β-D-glucopyranoside (compounds **1c**, **10b**, and **17c**), removing the terminal α-1,2- and terminal α-1,5-linked rhamnosyl residues, but another product was not diosgenin-3-*O*-α-L-arabinofuranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (compound **10a**) retaining the terminal 1,2 rhamnosyl residue by TLC and HPLC analysis, it was supposed to be the product removing the terminal 1,2-linked rhamnosyl residue while retaining the 1,5-linked rhamnosyl residue (compound **17b**). The results suggest that the purified enzyme

hydrolyzed chiefly the α-1,2-linked end-rhamnosyl residue, then hydrolyzed α-1,5 end-rhamnosyl residue.

To understand the specificity for steroidal saponin and why the glucoamylase from *C. lunata* had it, the view of 'moonlighting' (to serve additional functions that are generally not enzymatic, but rather structural or regulatory) and 'catalytic promiscuity' (capable of catalyzing secondary reactions at an active site that are specialized to catalyze a primary reaction) may add new explanation.[19] To utilize the moonlighting and promiscuity functions of glucoamylase from *C. lunata* for attempts to manipulate gene expression for investigative purposes, the protein engineering efforts will be designed to build upon the specific activity.

4. Experimental

4.1. Substrates, chemicals, and general

The steroidal saponins: proto-Pa (**1a**), proto-Pb (**2a**), pseudoproto-Pa (**3a**), pseudoproto-Pb (**4a**), protodioscin (**5a**), 26-*O*-β-D-glucopyranosyl-(25*R*)-22-hydroxy-5-ene-furostane-3β,17α,26-triol-3-*O*-α-L-arabinofuranosyl-(1→4)-[α-L-rhamnopyranosyl-(1→2)]-β-D-glucopyranoside (**6a**), ascalonicumside A (**7a**), protogracillin (**8a**), protoprogenin II (**9a**), polyphillin I (**10a**), polyphillin III (**11a**), polyphillin V (**12a**), polyphillin VI (**13a**), polyphillin VII (**14a**), polyphillin H (**15a**), Pb (**16a**), reclinatoside (**17a**), gracillin (**20a**), and diosgenin-3-*O*-α-L-rhamnopyranosyl (1→4)-β-D-glucopyranoside (**21a**) were prepared in our laboratory as substrates. Their purity was determined by HPLC-ELSD and was over 95% pure; ginsenoside Re (**22a**), ginsenoside C–K (**24a**), ginsenoside Rd (**25a**), ginsenoside F2 (**26a**), and ginsenoside Rg_3 (**27a**) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP). Diosgenin-3-*O*-α-L-rhamnopyranosyl (1→2)-β-D-galactopyranoside (**18a**), diosgenin-3-*O*-α-L-rhamnopyranosyl (1→2)-[β-D-xylopyranosyl-(1→3)]-β-D-galactopyranoside (**19a**), and ginsenoside Rg_2 (**23a**) were gifts from State Key Laboratory of Natural and Biomimetic Drugs, Peking University School of Pharmaceutical Science. *p*-Nitrophenyl-α-L-rhamnopyranoside (*p*NPR, **28a**) and bovine serum albumin were purchased from Sigma Chemical Co.(St. Louis, MO). Molecular mass markers (protein test mixture 6, i.e., rabbit phosphorylase b 97,400 kDa, bovine serum albumin 66,200 kDa, rabbit actin 43,000 kDa, bovine carbonic anhydrase 31,000 kDa, trypsin inhibitor 20,100 kDa, and hen egg white lysozyme 14,400 kDa) for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie Brilliant Blue R-250 were obtained from Serva (Heidelberg, Germany). S-Sepharose Fast Flow, Q-Sepharose Fast Flow, Sephacyl S-200 were obtained from Pharmacia Biotech (Uppsala, Sweden). TLC analysis and preparation were carried out on pre-coated silica gel GF_{254} plates (0.25 mm thick, 1.2 mm thick, 25 cm×25 cm, Qingdao Haiyang Chemical Group Co., China). Visualization of the TLC plates was performed by Ehrlich reagent and 10% H_2SO_4–EtOH spray reagent, followed by heating. HPLC analysis was carried out on Agilent 1100 unit with an Alltech Evaporative Light Scattering Detector 2000 using an Alltech-Apollo-C18 column (5 mm, 250×4.6 mm). ^{1}H NMR spectra were recorded in pyridine-d_5 solutions at 600 Hz with Varian UNITY INOVA 600 spectrometer. ^{13}C NMR spectrophotometry was run in pyridine-d_5 at 150.79 Hz with Varian UNITY INOVA 600 spectrometer. Chemical shifts are given in parts per million (δ). HR-ESI-MS spectra were taken on Brucker 9.4 TQ-FT-MS Apex Qe instruments. IR spectra were measured on a Bio-Rad FTS-135 spectrometer with KBr pellets. FABMS spectra were recorded on a Micromass Zabspec EFAB, 1000 spectrophotometer. All chemicals used were of analytical grade.

4.2. Fungal strains, medium, and culture conditions

C. lunata 3.4381 (The Institute of Microbiology, Chinese Academy of Sciences, AS, Beijing, China) was rejuvenated on a potato dextrose agar medium (PDA) slant and then was grown in medium containing 1.2 g of corn steep liquor/liter, 1.0 g of glucose/liter, 0.2 g of yeast extract/liter, and 0.5 g of $(NH_4)_2SO_4$/liter. The medium was adjusted to pH 6, and incubated at 29±1 °C in a constant temperature shaker at 150–180 rpm/min for 5 days.

4.3. Preparation of purified enzyme

All column operations were conducted at room temperature, unless stated otherwise. The fractions collected were tested for the steroidal saponin-rhamnosidase activity.

4.3.1. Ammonium sulfate precipitation. A culture of *C. lunata* for 5 days was centrifuged at 17,500×*g* for 15 min at 4 °C to remove supernatants, and the mycelia were dissolved in citrate phosphate buffer (pH 6) to a concentration of 0.1 g/mL (wet weight) and then disrupted by sonication using a Ultrasonic Liquid Processors from SONICS (USA) The sonicated suspensions were centrifuged at 17,500×*g* for 20 min at 4 °C, and to remove cells, then the pellets of $(NH_4)SO_4$ were slowly added to the supernatant with shaking to 70%, and stored at 4 °C overnight. The mixture was centrifuged to collect the protein precipitate. This crude protein was dissolved in distilled water and dialyzed against 20 mM citrate phosphate buffer, pH 6. After removing the non-dissolved fraction by centrifugation, the supernatant was collected and lyophilized to get crude enzyme.

4.3.2. Gel filtration. Crude enzyme (2 g) was dissolved in distilled water (20 mL). The enzyme solution was loaded onto a Sephacryl S-200 column (3×20 cm) pre-equilibrated with 20 mM PBS (elemental sodium phosphate–sodium dihydrogen phosphate–sodium, pH 8). Elution was performed with the same buffer at a flow rate of 20 mL/h, and active fractions were eluted and assayed by TLC. The fractions with steroidal saponin-rhamnosidase activity were pooled, concentrated, and dialyzed overnight against 20 mM PB (elemental sodium phosphate–sodium dihydrogen phosphate, pH 8).

4.3.3. Cation-exchange chromatography. The dialyzed enzyme solution was further purified by cation-exchange chromatography on an S-Sepharose FF column (2.5×15 cm) pre-equilibrated with 20 mM sodium citrate buffer (pH 6.0). The column was washed extensively with the same buffer and proteins were eluted using a linear NaCl gradient of 0–1.0 M in the same buffer at 2 mL/min. The

fraction with steroidal saponin-rhamnosidase activity was pooled, concentrated, and dialyzed overnight against 20 mM sodium citrate buffer (pH 6).

4.3.4. Anion-exchange chromatography. The dialyzed enzyme solution was loaded onto a Q-Sepharose FF column (2.5×15 cm) equilibrated with 20 mM sodium citrate buffer (pH 6.0). The column was washed extensively with the same buffer and eluted with a linear NaCl gradient of 0–0.5 M in the same buffer. A single peak exhibiting steroidal saponin-rhamnosidase activity was eluted, dialyzed, and lyophilized. This enzyme was the purified preparation used for substrate specificity.

4.4. Preparation of substrates

4.4.1. Compounds 1a–4a, 6a, 8a, 10a, 12a–17a, and 20a. The dried roots of *Rhizoma Paridis* were sliced and then extracted with 95% EtOH. The extract was concentrated and further separated by chromatography on a porous-polymer polystyrene (SP825) eluted stepwise with 5, 15, 25, 45, 50, 70, and 80% of C_3H_6O–H_2O, the fractions of 25, 45, and 80% were collected and concentrated.

The fraction of 25% was loaded on a silica gel C_{18} column chromatography and eluted with 22% acetone–water; the eluant was subjected to silica gel column chromatography and developed with $CHCl_3$–CH_3OH–H_2O (65:35:10, lower phase) to give compound **6a**.

The fraction of 45% was loaded on a SP825 column chromatography and eluted stepwise with 20, 30, 40, 50, and 80% acetone–water. The 30, 40, and 50% fractions were collected. The fraction of 40% was subjected to a Silica gel C_{18} column chromatography, eluted stepwise with 25, 30, 32, 35, 40, and 50% C_3H_6O–H_2O to give compounds **1a**–**4a** and **8a**.

The fraction of 80% was loaded on a SP825 column chromatography and eluted stepwise with 40, 45, 55, 65, 70, and 80% C_3H_6O–H_2O. The fraction of 65% was subjected to a Silica gel column chromatography and developed with gradient $CHCl_3$–CH_3OH–H_2O (50:1:0.1→10:1:0.1→5:1:0.1→60:35:10) to give mixture of **16a** and **20a**, the mixture of **10a** and **12a**, the mixture of compounds **13a** and **14a**, respectively. Absolute alcohol was added to the mixture of **10a** and **12a** to crystallize **10a** and **12a**. The mixture of **13a** and **14a** was subjected to a silica gel column chromatography and developed with gradient $CHCl_3$–CH_3OH–H_2O (50:1:0.1→10:1:0.1→5:1:0.1→60:35:10) to give compounds **13a** and **14a**. The mixture of **16a** and **20a** was separated by HPLC using CH_3CN–H_2O (50:50) to get compounds **16a** and **20a**. The fraction of 55% was loaded on a silica gel column chromatography and developed with $CHCl_3$–CH_3OH–H_2O (72:18:4, lower phase) to obtain compound **15a**. The fraction of 70% was subjected to a silica gel column chromatography and developed with CH_3CN–H_2O (45%) to give compound **17a**.

4.4.2. Compounds 5a and 11a. The dried roots of *D. nipponica* Makino were sliced and then extracted with 60% EtOH. The extract was concentrated and further separated by chromatography on porous-polymer polystyrene (SP825) eluted stepwise with 20, 50, and 80% C_3H_6O–H_2O, the fractions of 50 and 80% were collected and concentrated.

The fraction of 50% was loaded on a SP825 column chromatography and eluted stepwise with 25, 35, 45, and 60% C_3H_6O–H_2O. The fraction of 35% was subjected to a silica gel C_{18} column chromatography and eluted stepwise with 28, 32, and 40% C_3H_6O–H_2O, 32% fraction was separated by HPLC using CH_3OH to get compound **5a**.

The fraction of 80% was subjected to a Silica gel column chromatography and developed with gradient $CHCl_3$–CH_3OH–H_2O (50:1:0.1→10:1:0.1→5:1:0.1→60:35:10→5:5:2, lower phase) to give **11a**.

4.4.3. Compound 7a. The *A. ascalonicum* L. were minced and extracted with hot water. The extract was concentrated and subjected to a SP825 column chromatography, and eluted stepwise with 10, 20, 30, 45, and 80% C_3H_6O–H_2O. The fraction of 20% of C_3H_6O–H_2O was subjected to a silica gel C_{18} column and eluted stepwise with 15, 20, 25, and 30% C_3H_6O–H_2O. The fraction of 25% was separated by HPLC using CH_3OH–H_2O to get compound **7a**.

4.4.4. Compounds 9a and 21a. Compounds **9a** and **21a** were prepared by biotransformation with *C. lunata* from **5a** and **11a**, respectively.

The reaction mixture was extracted with *n*-BuOH for four times, and the extract was evaporated under vacuum to give the crude extract. The crude extract was loaded on a silica gel C_{18} column and eluted with 32% of C_3H_6O–H_2O to give compound **9a**, and 75% of C_3H_6O–H_2O to get compound **21a**.

4.5. Rhamnosidase activity assays

4.5.1. Steroidal saponin-rhamnosidase activity. Steroidal saponin-α-L-rhamnosidase activity was measured using steroidal saponins 0.5 mg/mL in 20 mM citrated phosphate buffer (pH 4.0) as the substrate. Enzyme solution of 0.2 mL was added to the same volume of substrate solution and allowed to react at 50 °C for 6 h. Then *n*-butanol 0.5 mL was added to stop the reaction. The transformed product was removed to the *n*-butanol layer and the product was ascertained by thin-layer chromatography (TLC) on silica gel GF_{254} plate using $CHCl_3$–CH_3OH–H_2O=1:2:3 (see TLC analysis) as developing solvent, visualization of the TLC plate was performed by Ehrlich reagent (for furostanosides) and 10% H_2SO_4–EtOH spray reagent (for spirostanosides), followed by heating.

4.5.2. Ginsenosides-rhamnosidase activity. Ginsenoside-α-L-rhamnosidase activity was measured using ginsenosides 0.5 mg/mL in 20 mM citrated phosphate buffer (pH 4.0) as the substrate. Enzyme solution of 0.2 mL was added to the same volume of substrate solution and allowed to react at 50 °C for 6 h. Then *n*-butanol 0.5 mL was added to stop the reaction. The transformed product was removed to the *n*-butanol layer and the product was analyzed by thin-layer chromatography (TLC) on silica gel GF_{254} plate using $CHCl_3$–CH_3OH–H_2O=2 (see TLC analysis) as developing solvent. Visualization of the TLC plate was performed by 10% H_2SO_4–EtOH spray reagent, followed by heating.

4.5.3. Rhamnosidase activity. α-L-rhamnosidase activity was determined using *p*-nitrophenyl-α-L-rhamnopyranoside (PNP-Rha) as the substrate as described previously.[20]

4.6. Isolation and purification of converted products

After the transformation, the reaction mixture was extracted four times with *n*-BuOH; the combined *n*-BuOH layer was evaporated under reduced pressure to give the crude converted products.

The crude converted products of compounds **1**, **2**, and **10**–**15** were loaded on a silica gel C_{18} column, eluted stepwise with 20, 22, 24, and 26% C_3H_6O–H_2O (compounds **1** and **2**), 45, 55, 60, 65, 70, 75, and 85% C_3H_6O–H_2O (compounds **10**–**15**), respectively. The fractions of same compound characterized by TLC or HPLC analysis was collected and evaporated to produce compounds **1b**, **1c**, **2b**, **2c**, and **10b**–**15b**.

The reaction mixture of compounds **5**, **6**, **8**, **9**, **16**, and **18** was separated by precipitation thin-layer chromatography (PTLC) on a silica gel GF_{254} plate, developed by $CHCl_3$–CH_3OH–H_2O=1:2:3 (see TLC analysis), the same bonds were scraped and eluted with C_3H_6O–H_2O (for furostanosides) or CH_3OH (for spirostanosides) four times, evaporated, and lyophilized to produce compounds **5b**, **5c**, **6b**, **6c**, **8c**, **9c**, **16b**, and **18b**.

4.7. Analysis methods

4.7.1. TLC analysis. The homogeneity of the converted products was ascertained by thin-layer chromatography (TLC) on silica gel GF_{254} plate using (1) $CHCl_3$–CH_3OH–H_2O=70:15:2 (v/v) (for spirostanosides), (2) $CHCl_3$–CH_3OH–H_2O=70:26:6 (v/v, lower phase) (for spirostanosides, ginsenosides), and (3) $CHCl_3$–CH_3OH–H_2O=65:35:10 (v/v, (lower phase)) (for furostanosides) as developing solvents.

4.7.2. HPLC analysis. The transformed products were dissolved in analytical CH_3OH and filtered, analyzed with HPLC-ELSD. The mobile phase consisted of 35–85% C_3H_6O–H_2O. The liquid flow rate was set at 1.0 mL/min with an injection volume of 10–80 μL. The temperature of ELSD was set at 100 °C and the gas flow was set at 2.4 L/min.

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Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2007.04.076.

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