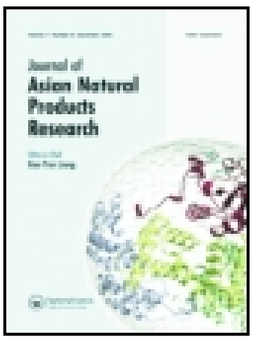

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Cycloartane triterpenes from *Beesia calthaefolia* and their anticomplement structure–activity relationship study

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ABSTRACT

Fifteen cycloartane triterpenes were isolated from *Beesia calthaefolia* and among them one was new cycloartane triterpenoid. The structure of new compound was determined by the application of spectroscopic analyses and chemical methods. The fifteen compounds were evaluated for their anticomplement activity by classic pathway. The structure–activity relationship analysis indicated that the configurations of 12-OH is preferable to be α than β, and 18-OH can decrease while 15-OH can increase the anticomplement activity, but saponin with both 15-OH and 18-OH lost most of its activity. The glycosyl moiety of most isolated cycloartane triterpenes is xylosyl. When xylosyl was substituted by glucosyl or galactosyl, their anticomplement activities were decreased or increased, respectively. Further structure–activity relationship (SAR) studies must be carried out to achieve general conclusions regarding the effect of further functionalizations on the anticomplement saponins.

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KEYWORDS
Beesia calthaefolia; cycloartane glycoside; anticomplement activity; structure–activity relationship

1. Introduction

Saponins are natural surfactants, found in many plants. Chemically, saponins are glycosides with a distinctive foaming characteristic. They consist of a polycyclic aglycone that is either a steroid or triterpenoid attached at C-3 with a sugar side chain. Saponins possess a wide range of structural diversity and versatile biological activities including anticancer, hypocholesterolemic, immunomodulatory, antiviral, hypoglycemic, and antioxidant activity [1–3]. The diverse activity of saponins could be explained by its complexity of chemical natures. Cycloartane-type triterpene glycosides are a kind of triterpenoid saponins with 9,19-cycloartane. A number of cycloartane-type triterpene glycosides have been reported to show immunomodulatory effects, including anticomplement activity [4, 5]. *Beesia calthaefolia* (Maxim.) Ulbr. (Ranunculaceae) is widely distributed in the southwest and northwest of China. Its rhizomes or the whole plant is used to treat colds, rheumatic arthritis, dysentery, sore throats, and headaches [6]. We recently isolated 15 cycloartane triterpenes, including 14 known cycloartane triterpenes (**1**–**14**) [7–9] and 1 new cycloartane triterpenoid (**15**) from

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[1]This author contributed equally to this work.

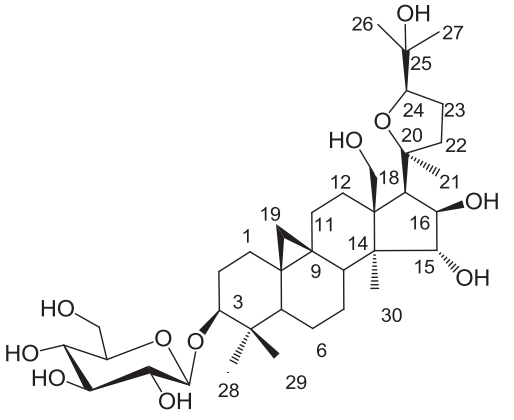


Figure 1. Structure of compound **15**.

the whole plant of *Beesia calthaefolia* (Figure 1). Structurally, cycloartane-type triterpene glycosides have a general backbone structure. However, a complexity of carbohydrate and various hydroxyls attached on the aglycone make them show different bioactivities. As part of our ongoing study on discovery of new immunoregulative compounds from natural source, we report here the isolation and structure identification of the new cycloartane triterpenoid from *Beesia calthaefolia*. In addition, the *in vitro* immunosuppressive activities of all the saponins **1–15** were assayed and the structure–activity relationship was concluded by comparison of the structure and anticomplement activities between these compounds.

2. Results and discussion

Compound **15** was obtained as a white powder. Its molecular formula ($C_{36}H_{60}O_{11}$) was deduced from the analysis of NMR and HR-ESI-MS data {*m/z*: 669.4222 $[M+H]^+$ (calc. for $C_{36}H_{61}O_{11}$, 669.4208)}. The ^{1}H NMR spectrum of **1** (Table 1) showed the presence of two characteristic cyclopropane protons signals at δ 0.52 and 0.21 (each d, J = 4.2 Hz), six methyl singlets characteristic at δ 0.98, 1.21 1.28, 1.34, 1.38 and 1.51, indicating that **15** is a cycloartane-type triterpene. A comparison of the spectroscopic data of **15** with those of beesioside E [10] showed that, structurally, **15** closely resembles beesioside E, with the main differences of the sugar unit. The sugar obtained after acid hydrolysis was identified as D-glucose by comparing with the standard. From the coupling constants of the anomeric signal (δ_H 4.95 1H, d, J = 7.8 Hz), the D-glucosyl was deduced to be β-configuration. A correlation was observed between H-1′ (δ_H 4.95 1H, d, J = 7.8 Hz) and C-3 (δ_C 88.6), suggesting that the sugar unit was attached at the C-3. In the ROESY spectrum and ChemDraw 3D modeling using MM2 (Figure 2), H-3 showed a correlation with H-5 and H-1′ suggesting an α-orientation of the H-3. Significant cross-peaks between H-17/H_3-30, H-16/H_3-30, H-8β/H-15, and H-24α/Me-21/Me-27 indicated the presence of OH-15α, OH-16β, and (20S*, 24R*) configurations. Thus, the structure of **15** was determined as (20S*, 24R*)-epoxy-9,19-cyclolanostane-3β,15α,16β,18,25-pentaol-3-*O*-β-D-glucopyranoside.

The isolated cycloartane glycosides **1–15** were evaluated for anticomplement activity by measuring their inhibitory activity on the classical complement pathway [11]. The results (IC_{50} values) are summarized in Table 2, and the aglycon of compounds **1–4** is type I, while

Table 1. ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectral data of **15** in pyridine-d_5.[a]

Position	δ_H	δ_C	Position	δ_H	δ_C
1	1.21–1.25 m,1.47–1.49 m	32.3	20	–	86.3
2	1.81–1.88 m,2.37–2.38 m	30.4	21	1.38s	26.3
3	3.49dd(11.4,4.2)	88.6	22-α	1.65–1.68 m	37.0
4	–	41.2	22-β	2.48–2.51 m	
5	1.31–1.34 m	47.7	23-α	1.92–1.98 m	24.6
6	0.63q(12.6)1.49–1.51 m	21.0	23-β	2.37–2.38 m	
7	1.25–1.29 m,2.11–2.13 m	26.7	24	3.96–3.99 m	85.4
8	2.09–2.11 m	48.4	25	–	70.6
9	–	20.4	26	1.21s	26.3
10	–	26.5	27	1.51s	28.5
11-α	2.15–2.17 m	26.6	28	1.28s	25.7
11-β	1.01–1.05 m		29	0.98s	15.4
12	1.68–1.73 m;2.01–2.05 m	29.7	30	1.34s	13.6
13	–	53.1	1′	4.95d(7.8)	106.8
14	–	49.7	2′	4.02–4.03 m	75.8
15	4.95d(3.6)	86.9	3′	4.22–4.24m[b]	78.7
16	4.72–4.75 m	82.5	4′	4.22–4.24m[b]	71.8
17	2.54d(8.4)	53.3	5′	3.95–3.97 m	78.2
18	4.32dd(4.8,12.0)4.55d(12.0)	65.9	6′	4.39–4.41 m,4.53–4.57 m	63.0
19	0.21d(4.2)0.52d(4.2)	29.9			

[a]Assignments based on TOCSY, HMQC, and HMBC experiments.
[b]Overlapped signals.

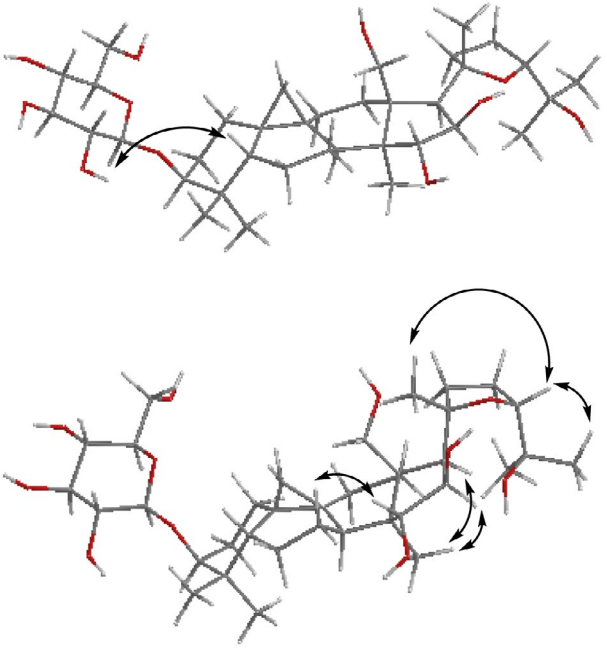
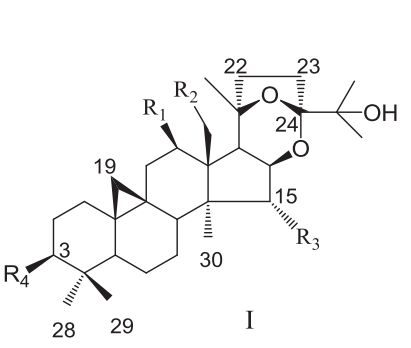


Figure 2. Selected ROESY correlations for compound **15**.

5–**15** is type II. The structure–activity relationships were inferred by comparing of the structure and anticomplement activities. Saponins **1** and **2** have the same sapogenin parts, while **1** is one more *β*-D-glucopyranose than **2**. The activities of saponin **2** > **1** suggested that the glucosyl moiety at C-3 of xylosyl (Xyl) can decrease the anticomplement activity. Compound **2** showed much more anticomplement activity than **3**, which means both the

Table 2. Inhibitory effects of compounds **1**–**15** on the complement system.



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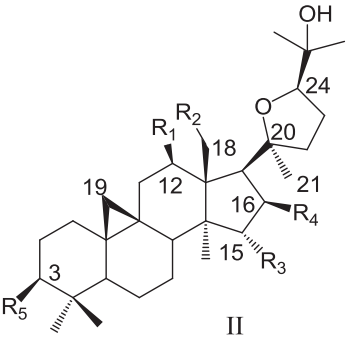

II

No.	Aglycon	R_1	R_2	R_3	R_4	R_5	IC_{50} (μM)[a]
1	I	OH	H	H	Xyl-Glu	–	395.3 ± 15.1
2	I	OH	H	H	Xyl	–	206.0 ± 21.4
3	I	H	OH	OH	Xyl	–	NA[c]
4	II	*β*-OH	H	H	OAc	Xyl	120.2 ± 11.5
5	II	*β*-OH	H	OH	OH	Xyl	209.9 ± 18.6
6	II	*β*-OH	H	OH	H	Xyl	NA[c]
7	II	*α*-OH	H	OH	OH	Xyl	200.1 ± 19.1
8	II	*α*-OH	OH	OH	OH	Xyl	315.0 ± 25.6
9	II	*β*-OH	OH	OH	OAc	Xyl	407.1 ± 30.1
10	II	*β*-OH	OH	OH	OH	Xyl	367.0 ± 34.2
11	II	*β*-OH	OH	H	OH	Gal	248.0 ± 22.5
12	II	*β*-OH	OH	H	OH	Xyl	NA[c]
13	II	*α*-OH	OH	H	OH	Xyl	343.3 ± 17.8
14	II	H	OH	OH	OH	Xyl	467.4 ± 29.2
15	II	H	OH	OH	OH	Glu	NA[c]
Rosmarinic acid[b]	–	–	–	–	–	–	181.8 ± 13.9

[a]Values are means ± SD, $n = 3$, IC_{50} in μM.
[b]Positive control.
[c]$IC_{50} > 500$ μM.

number and location of OH at C-12, C-15, and C-18 are essential for the anticomplement activities. To further infer the contributions of 12-OH, 15-OH, and 18-OH, more type I cyclorartane glycosides need to be isolated. The only differences of **8** and **10**, **12** and **13** are the configurations of 12-OH, **8** and **13** showed better anticomplement activity than **10** and **12**, respectively, suggesting the configurations of 12-OH are preferable to be *α* than *β*. Saponins **10** and **8** are one more 18-OH than **5** and **7**, respectively, but the activities of **5** > **10** and **7** > **8** suggested that 18-OH can decrease the anticomplement activity. Saponins **10** and **8** are one more 15-OH than **12** and **13**, respectively, the activities of **10** > **12** and **8** > **13** suggesting that 15-OH can increase the anticomplement activity. Comparing with the activity of **9** and **10**, **5** and **6**, the 16-OH could increase, while 16-OAc could decrease the anticomplement activities. Saponins (**11** and **12**) and saponins (**14** and **15**) with the same sapogenin part showed different activities (**11** > **12** and **14** > **15**), indicating that C-3 glycosyl moieties contribute differently to the anticomplement activities (Gal > Xyl, Xyl > Glu). It is of interest to note that **4** showed stronger inhibitory activity (IC_{50} 120.2 μM) than positive control (Rosmarinic acid, IC_{50} 181.8 μM). Comparing with **4**, compound **9** is two more OH at C-15 and C-18, but **9** almost showed no anticomplement activity. This may be ascribed to the steric hindrance of 15-OH and 18-OH groups. As a conclusion, further SAR studies must be carried out to achieve general conclusions regarding the effect of further functionalizations on the anticomplement saponins.

3. Experimental

3.1. General experimental procedures

Optical rotations were determined on a PolAAr-3005 digital polarimeter (OA Co., Ltd, Cambridgeshire, UK). IR spectra were obtained on a Vertex 70 FT-IR (by a KBr disk method) spectrometer (Bruker, Karlsruhe, Germany). NMR spectra were measured in pyridine-d_5 on a Varian INOVA 600 spectrometer (Palo Alto, CA, USA). HRESI mass spectra were recorded using a Waters SYNAPT (Q-TOF) mass spectrometer. Silica gel (200–300 mesh, from Haiyang Chemical Group Co., Qingdao, China) and RI-102 ODS-A-HG (Ymc Co., Kyoto, Japan) were used. Compounds were finally isolated with the help of a Hanbon Sci. & Tech NP7000 preparative HPLC system equipped with a *Shodex* detector using an ODS column (YMC-ODS, 20 × 250 mm, 5 μm). TLC was carried out on silica gel GF254 (0.15–0.20 mm) (from Jiangyou silica gel Group Co., Yantai, China) and the spots were visualized by spraying with 10% H_2SO_4 and heating.

3.2. Plant material

The whole plant of *Beesia calthaefolia* was collected at Wudang, Guiyang City, Guizhou Province, China in 2009 and identified by Dr. Jianxin Zhang, key laboratory of Chemistry for Natural Products of Guizhou Province and Chinese Academy of Sciences. The voucher specimen (collection No 196) is deposited in the Department of Clinical Pharmacology, PLA General Hospital.

3.3. Extraction and isolation

The air-dried and pulverized whole plant of *B. calthaefolia* (6.8 kg) was extracted two times with 95% EtOH for 2 h under reflux (80 L × 2) and then extracted with 50% EtOH for 2 h under reflux (80 L × 2). After removal of solvent, the residue (1.0 kg) obtained by 50% EtOH was suspended in water (1000 ml) and partitioned successively with petroleum ether (1000 ml × 6), $CHCl_3$ (1000 ml × 6), EtOAc (1000 ml × 6), and *n*-BuOH (1000 ml × 6).

The $CHCl_3$-soluble fraction (170 g) was subjected to low-pressure column chromatography on silica gel (200–300 mesh). Gradient elution with petroleum ether–EtOAc (1:4, 1:6, 1:9,), EtOAc, EtOAc–MeOH (20:1, 15:1, 10:1), and MeOH gave nine fractions, A (8 g), B (9 g), C (10 g), D (12 g), E (10 g), F (15 g), G (12 g), H (15 g), and I (9 g). The fraction D further separated by Silica gel (200–300 mesh) eluting with $CHCl_3$–MeOH (40:1–8:1) and preparative HPLC at a flow rate of 15.0 ml/min to afford **2** (300 mg, t_R = 19.8 min, 75% MeOH). The fraction E was further separated by silica gel (200–300 mesh) eluting with $CHCl_3$–MeOH (30:1–12:1) and preparative HPLC at a flow rate of 15.0 ml/min to afford **13** (11 mg, t_R = 7.5 min, 75% MeOH). Fraction F was fractionated by LPLC over Silica gel H to give six smaller fractions, eluting with $CHCl_3$–MeOH (40:1–8:1). The fifth was further separated by ODS column chromatography [MeOH:H_2O(65:35→68:32→70:30→75:25→80:20)] to afford **9** (9.9 mg) and by preparative HPLC at a flow rate of 15.0 ml/min to afford **4** (105 mg, t_R = 21.4 min, 75% MeOH) and **3** (45 mg, t_R = 6.8 min, 75% MeOH). Fraction H was isolated by LPLC over Silica gel H, eluting with $CHCl_3$–MeOH (40:1–9:1) to give four small fractions. The fourth fraction was further separated by preparative HPLC at a flow rate of

15.0 ml/min to afford **10** (36.7 mg, t_R = 4.5 min, 75% MeOH), **12** (24.7 mg, t_R = 12.75 min, 75% MeOH), and **14** (149 mg, t_R = 11.5 min, 75% MeOH), respectively.

The *n*-BuOH-soluble fraction (106 g) was subjected to low-pressure column chromatography on Silica gel (200–300 mesh). Gradient elution with $CHCl_3$–MeOH (15:1, 10:1) and $CHCl_3$–MeOH–H_2O (8:2:0.2, 7:3:0.5) gave six fractions, A (13.0 g), B (10.0 g), C (10.4 g), D (9.9 g), E (10.4 g), and F (9.0 g). Fraction A was isolated by LPLC over Si gel (200–300 mesh) to give seven fractions, eluting with $CHCl_3$–MeOH (12:1–4:1) and $CHCl_3$–MeOH–H_2O (8:2:0.2). The third was separated by MPLC (MeOH: H_2O = 10:90–70:30) at a flow rate of 15.0 ml/min to afford **1** (168 mg). Fraction B was separated by ODS column chromatography (MeOH: H_2O = 10:90–70:30) at a rate of 15.0 ml/min to afford 100 fractions. The 77–78 fractions were further separated by preparative HPLC at a flow rate of 15.0 ml/min to afford **5** (86.7 mg, t_R=11.0 min, 75% CH_3CN), **7** (52.0 mg, t_R=15.0 min, 75% CH_3CN), and **8** (14.9 mg, t_R=12.9 min, 28% CH_3CN). Fraction C was separated by ODS column chromatography (MeOH: H_2O = 30:70–85:15) at a rate of 15.0 ml/min to afford **6** (63 mg). Fraction D was separated by ODS column chromatography (MeOH: H_2O = 15:85–70:30) at a rate of 15.0 ml/min to afford 85 fractions. The 73–79 fractions were further separated by preparative HPLC at a flow rate of 15.0 ml/min to afford **11** (66.7 mg, t_R=10.0 min, 75% MeOH) and **15** (108.0 mg, t_R=13.5 min, 75% MeOH). The purity of all compounds was assessed by HPLC as more than 95%.

3.3.1. Compound 15

White amorphous powder (MeOH). $[\alpha_D^{20}] + 30.6$ (*c* 0.16, MeOH); IR (KBr) ν_{max}: 3412, 2940, 1637, 1383, 1164 and 1051 cm^{-1}; ^{1}H-NMR (C_5D_5 N, 600 MHz) and ^{13}C-NMR (C_5D_5 N, 150 MHz) spectral data are given in Table 1. HR-ESI-MS: *m/z* 669.4222 $[M + H]^+$ (calcd for $C_{36}H_{61}O_{11}$, 669.4208).

3.4. Hydrolysis of compound 15

Compound **15** (5 mg) was heated in 2 M trifluoroacetic acid (5 ml) at 95 °C for 5 h. The reaction mixture was extracted with $CHCl_3$ (5 ml × 3). The remaining aqueous layer was concentrated to dryness to give a residue, which was dissolved in pyridine (2 ml), to which 3 mg L-cysteine methyl ester hydrochloride was added. The mixture was kept at 60 °C for 1 h. After the reaction, the residue was added with trimethylchlorosilane (0.5 ml) and hexamethyldisilane (1 ml) at for 0.5 h. Finally, the supernatant (0.5 ml) was analyzed by GC-MS (GC-MS-QP 2012, Shimadzu Corporation, kyoto, Japan) under the following conditions: capillary column, Rtx-5MS (30 m × 0.25 mm inner diameter, 0.25-mm film), detector temperature: 60 °C-300 °C, programmed increase, 20 °C/min; carrier, helium gas (1.0 ml/min); and injection volume: 1.0 µl. D-glucose was confirmed by comparing the retention time with that of standard sample.

3.5. Anticomplement bioassays

The anticomplement assay used in this study measures inhibition of sheep red blood cell hemolysis following induction of the complement binding reaction. This assay was carried out according to the method of Kabat and Mayer and Klerx *et al.* [12, 13] with modifications. The rate of hemolysis was measured spectrophotometrically and compared to the

untreated control. Primary EA (sensitized erythrocytes) were used for the complement binding reaction of the classical pathway. A diluted solution of normal guinea pig serum (complement serum, 100.0 µl) collected from healthy guinea pigs (female) was mixed with barbitol buffer solution (BBS, 200.0 µl) with and without sample. Each sample was dissolved in DMSO, which was also used as the negative control, followed by the addition of sensitized erythrocytes (sheep red blood cells, 200.0 µl). The mixture was preincubated at 37 °C for 30 min. After incubation under identical conditions, the mixture was centrifuged (4 °C, 1500 rpm), and the optical density of the supernatant (200 µl) was measured at 405 nm [14]. The anticomplement activity of each sample was determined as a mean of triple measurements and expressed as the IC_{50} values from complement-dependent hemolysis of the control [15]. Purity of the isolated compounds used in the assay was above 95% as determined by analytical HPLC/ELSD.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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