Bioorganic Chemistry 87 (2019) 373–379

Contents lists available at ScienceDirect

Bioorganic Chemistry

journal homepage: www.elsevier.com/locate/bioorg

Cytotoxic polyhydroxy serratene triterpenoids from *Lycopodium complanatum*

Qiuzhu Dong[a], Zhenxing Zou[a], Xiaohui Jia[c], Xia Yu[a], Jing Li[a], Wenhao Zhou[a], Huihui Sun[a], Wei Wu[a], Guishan Tan[a,b], Kangping Xu[a,*]

[a] Xiangya School of Pharmaceutical Sciences, Central South University, Changsha 410013, PR China
[b] Xiangya Hospital of Central South University, Changsha 410008, PR China
[c] School of Chemistry and Chemical Engineering, University of South China, Hengyang 421001, PR China

ARTICLE INFO

Keywords:
Lycopodium complanatum
Polyhydroxy serratene triterpenoids
Cytotoxic activities

ABSTRACT

Phytochemical investigation of the 70% aqueous EtOH extract of *Lycopodium complanatum* led to six new polyhydroxy serratene triterpenoids (serrat A-F, **1–6**), along with a known analogue (**7**). Their structures and configurations were elucidated by data analysis of HRESIMS, 1D and 2D NMR, in combination with comparisons of reported experimental spectroscopic data. All the isolates were evaluated cytotoxic activities against HepG2 cells, MCF-7 cells and series human lung cancer cell lines A549, Calu-6, NCI-H441, NCI-H226 and NCI-H1975. The results indicated that certain compounds inhibited proliferation of human cancer cells. Moreover, all compounds possessed selective cytotoxic activities on MCF-7 cells. Further, possible biosynthesis pathways of these compounds were proposed.

1. Introduction

Lycopodium complanatum, a member of the Lycopodiaceae family, is a folk medicinal herb to treat contusions, arthrophlogosis and quadriplegia [1]. It is mainly distributed in southern China such as Guizhou, Hunan and Yunnan province. Over the past decades, researches on *Lycopodium complanatum* have been mainly focused on the lycopodium alkaloids and their anti-acetylcholinesterase (AChE) [2–6], however, with only a small amount of serratene triterpenoids and abietane diterpenoids [7–10].

Serratene triterpenoids belong to the family of pentacyclic triterpenoids with a central seven-membered ring C, usually isolated from Lycopodiaceae, Pinaceae and Huperziaceae [11–14]. It has been reported that serratene triterpenoids possess various activities such as anti-tumour, cytotoxicity, cholinesterase inhibition, β-secretase 1 inhibition, antiproliferative activity and secreted aspartic proteases inhibition [15–21]. The structure of serratene triterpenoids previously obtained generally contains a carbon-carbon double bond at C-14/C-15, hydroxy groups at C-3 and C-21 [17,22–25]. Besides, there also exist a small amount of serratene triterpenoids which are oxygenated to a carbonyl group at C-16 and a hydroxy group at C-20 [18,26]. However, there are rarely reports on serratene triterpenoids comprising an α, β-unsaturated ketone (C-14/15/16) having hydroxy substitution other than C-3, C-20 and C-21 [27]. In order to search for new and potentially active natural products, we investigated the high polarity fraction of *Lycopodium complanatum*, from which six new polyhydroxy serratene triterpenoids, serrat A-F (**1–6**), together with one known analogue (**7**) were obtained (Fig. 1). Herein, we reported the structure elucidation and cytotoxic activities of these compounds.

2. Materials and methods

2.1. General methods

Column chromatography (CC) was performed on Macroporous resin HPD-100 (Zhengzhou Qin Shi Co. Ltd., Henan, China), Sephadex LH-20 gel (GE Healthcare, Uppsala, Sweden) and Toyopearl HW-40C (TOYOPEARL TOSOH, Tokyo, Japan). Analytical HPLC experiments were conducted with a YMC Pack ODS-A column (5 μm, 250 mm × 4.6 mm i.d.; Tokyo, Japan) in Agilent 1100 (Agilent Technologies, Ltd) equipped with a diode array detector (DAD) under reversed-phase. And semipreparative HPLC separations were carried out on an Agilent 1200 with YMC Pack ODS-A column (5 μm, 250 × 10 mm, YMC Co. Ltd., Kyoto, Japan). UV spectrum was detected with Waters Acquity UPLC equipped 2998 PDA Detector (America). Optical rotations were tested on a Jasco model 1020 polarimeter (Horiba, Tokyo, Japan). The

* Corresponding author.
E-mail address: xukp395@csu.edu.cn (K. Xu).

https://doi.org/10.1016/j.bioorg.2019.03.011
Received 23 January 2019; Received in revised form 27 February 2019; Accepted 5 March 2019
Available online 06 March 2019

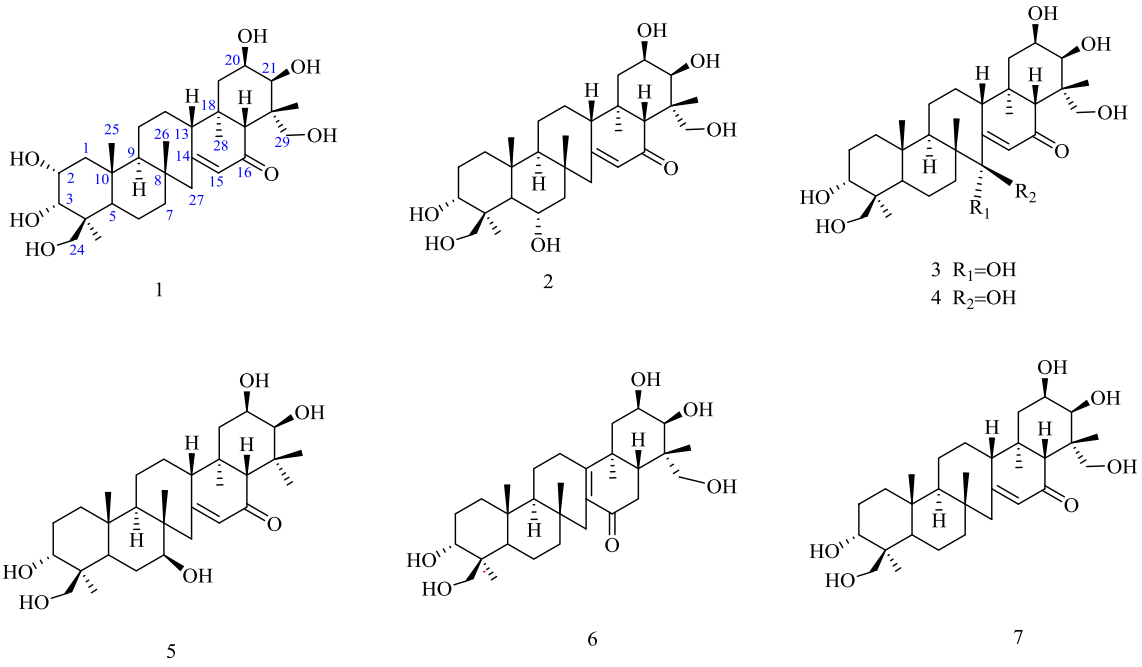

Fig. 1. Structures of compounds **1–7** from *Lycopodium complanatum*.

HRESIMS were measured on a LC-LTQ Orbitrap Velos Pro ETD (Thermo Fisher, MA, USA) in positive ion mode. NMR spectra data were collected in pyridine-d_5 on a Bruker AV-500 MHz and a Bruker AV-400 MHz (Bruker, Karlsruhe, Germany) with TMS as the internal standard. Chemical shifts (δ) were described in ppm and the coupling constants (J) in Hz. All solvents were of analytical grade.

2.2. Plant material

The whole herbs of *Lycopodium complanatum* were collected in July 2015 from ShaoYang, Hunan Province, China, identified by Prof. KangPing Xu (Xiangya School of Pharmaceutical Sciences, Central South University). The voucher specimen (20150923) was deposited at Xiangya School of Pharmaceutical Sciences, Central South University.

2.3. Extraction and isolation

The whole herbs of *Lycopodium complanatum* (15 kg) were exhaustively extracted with 70% EtOH under reflux three times to get combining extracts, which were dried under reduced pressure afterwards. Then the residue was dispersed in water and partitioned with petroleum ether (PE), ethyl acetate (EA) and *n*-butyl alcohol (*n*-BuOH) successively. The *n*-BuOH extract (300 g) was submitted to macroporous resin (HPD-100) and fractionated by ethanol-water system to afford six streams, which were H_2O, 20%, 40%, 50%, 70% and 95% respectively. The 40% EtOH portion (48 g) was subsequently subjected to HW-40C column chromatography with gradient elution of H_2O/MeOH (v/v, 100:0 to 0:100) to obtain 5 fractions (Fr. A-Fr. E) according to Analytical HPLC. Fr. D was subjected to HW-40C repeatedly to yield 8 subfractions (Fr. D1-Fr. D8). Compound **7** (3.8 mg) was yield from Fr. D7 by means of semipreparative HPLC with ACN/H_2O (0 ~ 30 min, 25% ~ 35%). Fr. D6 was subjected to HW-40C with 30% MeOH/H_2O and then purified by semipreparative HPLC with 20% ACN/H_2O isometric elution to obtain compound **5** (6.6 mg). Fr. D2-Fr. D5 were merged together and then separated by Sephadex LH-20 with 25% MeOH/H_2O to get 3 fractions (Fr. 1-Fr. 3). Further separation of Fr. 2 was conducted on HW-40C with gradient elution of MeOH/H_2O (v/v, 10:100 to 30:100), from which 9 fractions (Fr. 2.1-Fr. 2.9) were obtained. Fr. 2.3 was purified by semipreparative HPLC using ACN/H_2O (0 ~ 30 min, 15% ~ 25%) to afford compound **2** (3.4 mg) and compound **4** (0.6 mg). Compound **1** (3.0 mg) and compound **3** (10.9 mg) were produced from Fr. 2.4 with the help of semipreparative HPLC eluted with ACN/H_2O (0 ~ 40 min, 20% ~ 30%). Fr. 2.6 was separated with MeOH-H_2O (v/v, 10:100 to 30:100) on HW-40C and then further gradient eluted with ACN/H_2O (0 ~ 30 min, 20% ~ 30%) to yield compound **6** (4.8 mg).

2.3.1. Serrat A (1)

White amorphous powder; $[\alpha]_D^{25}$–83.0 (*c* 0.20, MeOH); UV (ACN-H_2O) λmax 248.7 nm; ^{1}H NMR (400 MHz in pyridine-d_5) data see Table 1, and ^{13}C NMR (100 MHz in pyridine-d_5) data see Table 2. HRESIMS *m/z* 521.3461 $[M + H]^+$ (calcd for $C_{30}H_{49}O_7$ 521.3478).

2.3.2. Serrat B (2)

White amorphous powder; $[\alpha]_D^{25}$–29.0 (*c* 0.20, MeOH); UV (ACN-H_2O) λmax 248.7 nm; ^{1}H NMR (500 MHz in pyridine-d_5) data see Table 1, and ^{13}C NMR (125 MHz in pyridine-d_5) data see Table 2. HRESIMS *m/z* 521.3464 $[M + H]^+$ (calcd for $C_{30}H_{49}O_7$ 521.3478).

2.3.3. Serrat C (3)

White amorphous powder; $[\alpha]_D^{25}$–44.0 (*c* 0.20, MeOH); (ACN-H_2O) λmax 249.9 nm; ^{1}H NMR (500 MHz in pyridine-d_5) data see Table 1, and ^{13}C NMR (125 MHz in pyridine-d_5) data see Table 2. HRESIMS *m/z* 521.3463 $[M + H]^+$ (calcd for $C_{30}H_{49}O_7$ 521.3478).

2.3.4. Serrat D (4)

White amorphous powder; $[\alpha]_D^{25}$–42.2 (*c* 0.20, MeOH); UV (ACN-H_2O) λmax 249.9 nm; ^{1}H NMR (500 MHz in pyridine-d_5) data see Table 1, and ^{13}C NMR (125 MHz in pyridine-d_5) data see Table 2. HRESIMS *m/z* 521.3463 $[M + H]^+$ (calcd for $C_{30}H_{49}O_7$ 521.3478).

2.3.5. Serrat E (5)

White amorphous powder; $[\alpha]_D^{25}$–63.4 (*c* 0.20, MeOH); UV (ACN-H_2O) λmax 248.7 nm; ^{1}H NMR (500 MHz in pyridine-d_5) data see Table 1, and ^{13}C NMR (125 MHz in pyridine-d_5) data see Table 2. HRESIMS *m/z* 505.3512 $[M + H]^+$ (calcd for $C_{30}H_{49}O_6$ 505.3529).

2.3.6. Serrat F (6)

White amorphous powder; $[\alpha]_D^{25}$–73.4 (*c* 0.20, MeOH); UV (ACN-H_2O) λmax 261.8 nm; ^{1}H NMR (500 MHz in pyridine-d_5) data see

Table 1
^{1}H NMR data of 1–6 (δ in ppm, J in Hz).

Position	1[b]	2[a]	3[a]	4[a]	5[a]	6[a]
1	2.12(1H, m)	1.67(1H, m)	1.95(1H, m)	1.83(1H, m)	1.80(1H, m)	1.77(1H, m)
	1.83(1H, m)	1.58(1H, m)	1.73(1H, m)	1.64(1H, m)	1.54(1H, m)	1.53(1H, m)
2	4.47(1H, d, 11.3)	2.00(1H, m)	2.22(1H, m)	2.17(1H, m)	2.15(1H, m)	2.15(1H, m)
		1.99(1H, m)	1.91(1H, m)	1.92(1H, m)	1.89(1H, m)	1.91(1H, m)
3	4.60(1H, overlapped)	4.20(1H, m)	4.49(1H, br. s)	4.48(1H, br. s)	4.41(1H, m)	4.46(1H, m)
5	1.83(1H, m)	1.78(1H, m)	2.07(1H, m)	1.98(1H, m)	2.07(1H, m)	1.88(1H, m)
6	1.58(1H, m)	4.41(1H, m)	1.79(1H, m)	1.86(1H, m)	2.20(1H, m)	1.64(1H, m)
	1.52 (1H, m)		1.69 (1H, m)	1.60 (1H, m)	1.98 (1H, m)	1.53 (1H, m)
7	1.34(1H, m)	1.97(1H, m)	2.37(1H, m)	2.30(1H, m)	3.48(1H, m)	1.60(1H, m)
	1.21(1H, m)	1.63(1H, m)	1.41(1H, m)	1.77(1H, m)		1.37 (1H, m)
9	1.04(1H, m)	1.00(1H, m)	2.30(1H, m)	1.31(1H, m)	1.09(1H, m)	1.22(1H, m)
11	2.02(1H, m)	1.97(1H, m)	2.09(overlapped)	2.08(1H, m)	2.07(1H, m)	1.84(1H, m)
	1.09(1H, m)	1.13(1H, m)	1.27 (1H, m)	1.22 (1H, m)	1.27(1H, m)	1.15(1H, m)
12	1.80(1H, m)	1.90(1H, m)	2.19(1H, m)	1.34(1H, m)	1.84(1H, m)	2.67(1H, m)
	1.06(1H, m)	1.13(1H, m)	1.89(1H, m)	1.20(1H, m)	1.21(1H, m)	2.02(1H, m)
13	2.55(1H, br. d, 9.7)	2.60(1H, br. d, 9.4)	2.83(1H, br. d, 11.4)	2.75(1H, br. d, 9.3)	2.58(1H, br. d, 9.4)	
15	5.93(1H, s)	5.95(1H, s)	6.11(1H, s)	7.10(1H, s)	6.09(1H, s)	
16						2.93(1H, m)
						2.85(1H, m)
17	3.25 (1H, s)	3.28 (1H, s)	3.37 (1H, s)	3.40 (1H, s)	3.02 (1H, s)	2.84 (1H, m)
19	2.40(1H, m)	2.46(1H, m)	2.51(1H, m)	2.47(1H, m)	2.34(1H, m)	2.42(1H, m)
	2.09(1H, m)	2.15(1H, m)	2.20(1H, m)	2.15(1H, m)	2.10(1H, m)	2.34(1H, m)
20	4.60(1H, overlapped)	4.63(1H, m)	4.65(1H, m)	4.64(1H, m)	4.39(1H, m)	4.63(1H, m)
21	4.74(1H, br. s)	4.78(1H, br. d, 1.0)	4.80(1H, br. s)	4.78(1H, br. s)	3.74(1H, br. d, 1.0)	4.52(1H, br. d, 1.0)
23	1.64(3H, s)	2.01(3H, s)	1.61(3H, s)	1.62(3H, s)	1.59(3H, s)	1.57(3H, s)
24	4.11 (1H, d, 10.9)	4.50(1H, d, 10.1)	4.17(1H, d, 10.8)	4.14(1H, d, 10.8)	4.08(1H, 10.9)	4.05(1H, 10.7)
	3.83(1H, d,10.5)	4.21(1H, d, 10.3)	3.93(1H, d, 10.9)	3.91(1H, d, 10.7)	3.90(1H, 10.8)	3.82(1H, 10.8)
25	0.91(3H, s)	1.03(3H, s)	1.03(3H, s)	0.98(3H, s)	0.95(3H, s)	0.77(3H, s)
26	0.68(3H, s)	0.82(3H, s)	0.85(3H, s)	0.91(3H, s)	0.97(3H, s)	0.78(3H, s)
27	2.27, 1.87	2.39, 2.03	3.84(1H, d, 4.0)	4.32(1H, d, 4.0)	3.25, 2.22	3.15, 1.81
	(each 1H, 14.6)	(each 1H, 15.2)			(each 1H, 14.5)	(each 1H, 14.2)
28	0.93(3H, s)	0.98(3H, s)	1.14(3H, s)	0.99(3H, s)	0.94(3H, s)	1.22(3H, s)
29	4.81(1H, d, 10.8)	4.85(1H, d, 10.7)	4.84(1H, d, 10.8)	4.84(1H, d, 10.9)	1.39(3H, s)	4.12(1H, 10.9)
	4.20 (1H, d, 10.5)	4.23(1H, d, 10.6)	4.25 (1H, d, 10.8)	4.23 (1H, d, 10.6)		4.00(1H, 11.0)
30	2.05(3H, s)	2.08(3H, s)	2.09(3H, s)	2.12(3H, s)	1.72(3H, s)	1.61(3H, s)

[a] Measured in pyridine-d_5 at 500 MHz.
[b] Measured in pyridine-d_5 at 400 MHz.

Table 1, and ^{13}C NMR (125 MHz in pyridine-d_5) data see Table 2. HRESIMS m/z 505.3516 $[M + H]^+$ (calcd for $C_{30}H_{49}O_6$ 505.3529).

2.3.7. 16-oxolyclanitin (7)

White amorphous powder; $[\alpha]_D^{25}$–24.7 (c 0.20, MeOH); UV (ACN-H_2O) λmax 248 nm; ^{1}H NMR (500 MHz in pyridine-d_5) δ_H 1.78(H-1, 1H, m), 1.52(H-1, 1H, m), 2.12(H-2, 1H, m), 1.99(H-2, 1H, m), 4.42(H-3, 1H, m), 1.83(H-5, 1H, m), 1.55(H-6, 1H, m), 1.51(H-6, 1H, m), 1.33(H-7, 1H, m), 1.22(H-7, 1H, m), 1.02(H-9, 1H, m), 1.85(H-11, 1H, m), 1.08(H-11, 1H, m), 1.88(H-12, 1H, m), 1.10(H-12, 1H, m), 2.58(H-13, 1H, br. d, 9.4), 5.94(H-15, 1H, s), 3.25 (H-17, 1H, s), 2.41(H-19, 1H, m), 2.09(H-19, 1H, m), 4.59(H-20, 1H, m), 4.73(H-21, 1H, br. d, 1.0), 4.06, 3.84(H-24, each 1H, 10.8), 4.81, 4.19(H-29, each 1H, d, 10.8), 2.31, 1.85(H-27, each 1H, 14.6), 1.58(CH_3-23, 3H, s), 0.87(CH_3-25, 3H, s), 0.71(CH_3-26, 3H, s), 0.94(CH_3-28, 3H, s), 2.04(CH_3-30, 3H, s); ^{13}C NMR (125 MHz in pyridine-d_5) δ_C 34.2 (C-1), 26.8 (C-2), 69.9 (C-3), 44.2 (C-4), 50.2 (C-5), 19.5 (C-6), 45.8 (C-7), 38.3 (C-8), 62.7 (C-9), 38.6 (C-10), 25.5 (C-11), 26.7 (C-12), 59.5 (C-13), 164.2 (C-14), 129.1 (C-15), 201.5 (C-16), 59.7 (C-17), 46.0 (C-18), 41.3 (C-19), 65.9 (C-20), 74.7 (C-21), 45.4 (C-22), 65.8 (C-24), 63.8 (C-29), 55.9 (C-27), 23.6 (C-23), 16.6 (C-25), 20.0 (C-26), 16.8 (C-28), 23.2 (C-30).

2.4. Cytotoxicity assay

HepG2 cells, MCF-7 cells, MCF-10A cells and series human lung cancer cell lines A549/Calu-6/NCI-H441/NCI-H226/NCI-H1975 were all gained from ATCC (Manassas, VA, USA). Cytotoxic activities of all isolates (**1**–**7**) was tested by CCK-8 assay with positive control of staurosporine (STS). All human cancer cells were suspended at 3×10^3/well in 96 well-plates in RPMI-1640 medium with 10% fetal bovine serum (FBS) and incubated for 24 h under a 5% CO_2 atmosphere at 37 °C. MCF-10A cells were cultured in MEBM medium with 100 ng/ml cholera toxin under a 5% CO_2 atmosphere at 37 °C for 24 h. Test samples were then added while a negative control without compound plus cells was set. After 72 h cultivation, fresh medium (100 μL) containing CCK8 reagent was added to each well and the cells were cultured for an additional 2 h. Finally, the optical density (OD) of the solution was measured and IC_{50} values were calculated accordingly using Graph Pad Prism.

3. Result and discussion

Compound **1** was a white amorphous powder with a molecular formula of $C_{30}H_{48}O_7$ according to the positive HRESIMS data at m/z 521.3461 $[M + H]^+$ (calcd for $C_{30}H_{49}O_7$ 521.3478), suggesting seven degrees of unsaturation. The ^{1}H NMR spectrum (Table 1) exhibited five methyl groups at δ_H 2.05 (3H, s, CH_3-30), 1.64 (3H, s, CH_3-23), 0.93(3H, s, CH_3-28), 0.91 (3H, s, CH_3-25), 0.68(3H, s, CH_3-26), four oxygenated methine groups at δ_H 4.74 (1H, br s), 4.60 (1H, m), 4.60 (1H, m), 4.47 (1H, d, J = 11.3 Hz), two separated hydroxymethyl groups at δ_H 4.81(1H, d, J = 10.8 Hz, Ha-29), 4.20 (1H, d, J = 10.5 Hz, Hb-29), 4.11 (1H, d, J = 10.9 Hz, Ha-24), 3.83 (1H, d, J = 10.5 Hz, Hb-24), in addition, an olefinic proton at δ_H 5.93(1H, br s, H-15). The ^{13}C NMR (Table 2) and DEPT spectrum showed a carbonyl group at δ_C 201.4 (C-16), a C=C bond signals at δ_C 164.1(C-14) and 129.1(C-15), four oxygenated methine groups at δ_C 74.6 (C-21), 74.2 (C-3), 65.9 (C-20) and 66.5, two oxygenated methylenes at δ_C 65.2 (C-24), 63.7 (C-29), four methines, seven methylenes [a characteristic methylene signal

Table 2
^{13}C NMR data of **1–6** (δ in ppm).

Positions	1[b]	2[a]	3[a]	4[a]	5[a]	6[a]
1	43.3	37.1	34.4	34.5	33.9	33.7
2	66.5	27.1	26.7	26.9	26.5	26.6
3	74.2	71.3	69.8	69.9	69.7	69.9
4	45.3	44.4	44.1	44.2	44.3	44.3
5	49.5	57.0	49.6	50.2	47.3	50.1
6	19.3	66.8	19.2	19.6	28.6	19.6
7	45.7	55.0	41.6	40.8	78.7	45.0
8	38.2	38.8	41.4	44.1	43.8	35.4
9	62.6	63.0	51.4	61.4	61.6	64.8
10	39.7	39.9	38.2	39.2	38.6	38.6
11	26.9	26.8	26.5	27.1	26.4	20.4
12	25.3	25.6	23.3	30.0	25.1	29.7
13	59.4	59.4	59.0	58.7	59.0	170.4
14	164.1	163.6	164.1	167.4	163.8	132.7
15	129.1	129.3	130.5	126.9	129.1	198.9
16	201.4	201.4	202.5	201.9	200.6	35.9
17	59.6	59.6	60.0	60.4	58.6	58.6
18	46.0	46.0	45.9	46.2	45.6	42.0
19	41.3	41.3	41.1	41.3	41.1	38.5
20	65.9	65.9	65.9	66.0	65.6	66.4
21	74.6	74.6	74.4	74.8	80.1	74.0
22	38.7	45.4	45.3	45.5	38.7	44.8
23	24.0	23.8	23.4	23.5	23.4	23.6
24	65.2	67.1	65.7	65.8	65.6	65.6
25	17.6	20.1	16.8	16.8	15.9	19.5
26	20.0	21.0	19.1	17.1	14.3	17.0
27	55.8	56.0	87.4	80.2	49.4	41.3
28	16.7	16.8	16.8	16.9	16.3	18.4
29	63.7	63.7	63.7	63.7	21.5	65.2
30	23.2	23.2	23.0	23.2	28.8	23.2

[a] Measured in pyridine-d_5 at 125 MHz.
[b] Measured in pyridine-d_5 at 100 MHz.

of serratene at δ_C 55.8 (C-27) included] [14], five methyls and five quaternary carbons. All above spectral data clearly indicated compound **1** a serratene triterpenoid [28]. Besides, its UV maximum absorption indicating an α, β-unsaturated ketone was included [14,15]. The difference of NMR data between compound **1** and 16-oxolyclanitin (compound **7**) displayed mainly in the ring A [9,26]. Detailed data analysis of compound **1** revealed that a methylene group was replaced by an oxygenated methine group [δ_C 66.5, δ_H 4.47(1H, d, J = 11.3 Hz)]. Therefore, it was estimated that the oxygenated methine group was exactly located on the ring A. In the ^{1}H–^{1}H COSY spectrum (Fig. 2), δ_H 4.47 was significantly correlated with H-1 (δ_H 2.12/1.83)/H-3 (δ_H 4.60). Thus it could be confirmed that a proton at C-2 was substituted by a hydroxy group. The orientations of the hydroxy groups at C-3, C-20, C-21, C-24 and C-29 were all determined by NOESY correlations in Fig. 3 and comparing the carbon chemical shifts with those of compound **7**. As observed in the NOESY spectrum (Fig. 3), the correlations of H-2 (δ_H 4.47) with CH_3-25(δ_H 0.91)/H-24(δ_H 4.11) indicated that the hydroxy group at C-2 was α-oriented. Thus, compound **1** was established as 2α, 3α, 20β, 21β, 24, 29-hexahydroxyserrat-14-en-16-one and named as serrat A.

Compound **2** was a white amorphous powder. Its positive HRESIMS data at m/z 521.3464 $[M + H]^+$ (calcd for $C_{30}H_{49}O_7$ 521.3478) suggested a molecular formula of $C_{30}H_{48}O_7$. The similar UV spectrum as compound **1** suggested that compound **2** was also a serratene triterpenoid including an α, β-unsaturated ketone. ^{13}C NMR (Table 2) and DEPT spectrum showed that compound **2** contained five methyl groups, two oxygenated methylene groups, four oxygenated methine groups and five quaternary carbons as compound **1**, except that the location of one oxygenated methine group was changed. Detailed analysis of HMBC and ^{1}H–^{1}H COSY data (Fig. 2) displayed that a proton at C-6 was oxygenated to a hydroxy group, which could be identified by obvious correlations of H-6 (δ_H 4.41) to H-5(δ_H 1.78)/H-7(δ_H1.63/1.97). In the NOESY spectrum (Fig. 3), the correlations of H-6 (δ_H 4.41) with CH_3-

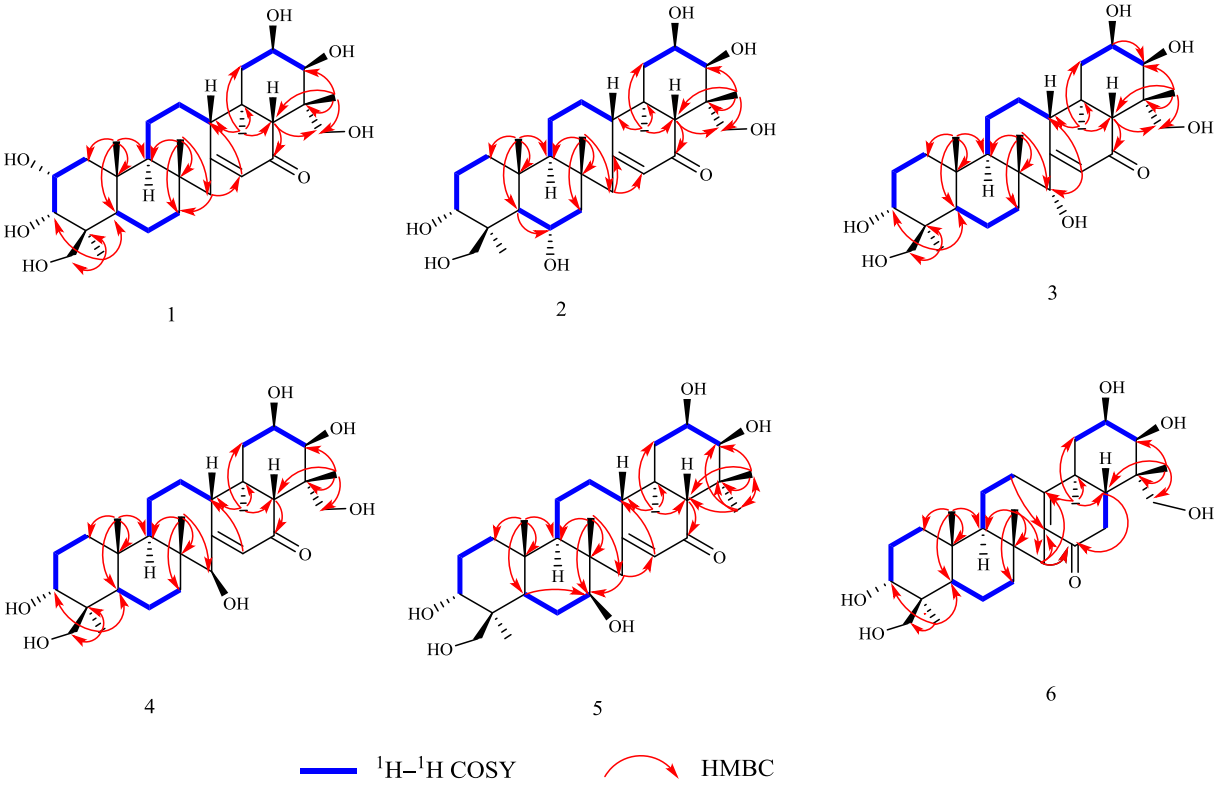

Fig. 2. Key HMBC and ^{1}H–^{1}H COSY correlations of compounds **1–6.**

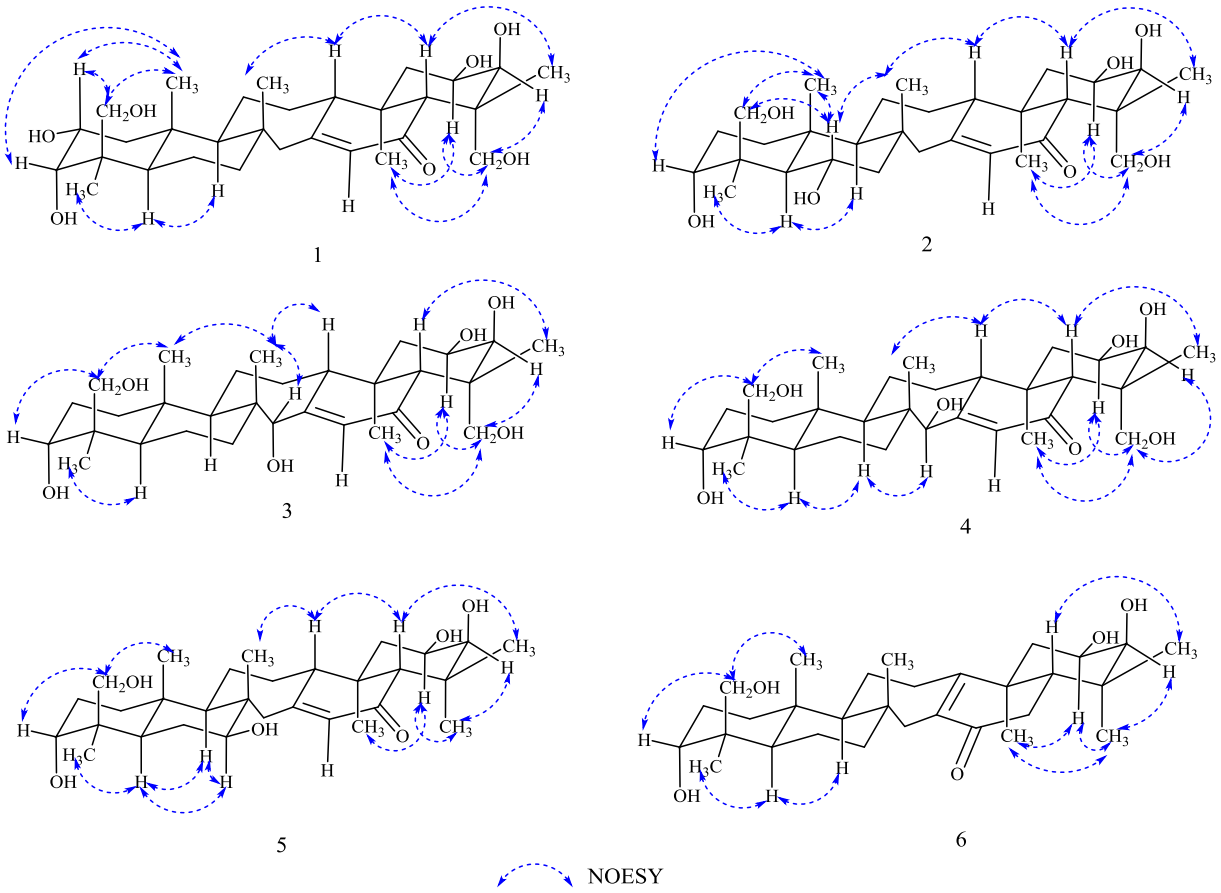

Fig. 3. Key NOESY correlations of compounds **1**–**6**.

25(δ_H 1.03)/CH_3-26(δ_H 0.82)/H-24(δ_H 4.21) indicated that the hydroxy group at C-6 was α-oriented. Therefore, compound **2** was finally established as 3α, 6α, 20β, 21β, 24, 29-hexahydroxyserrat-14-en-16-one and named as serrat B.

Compound **3** was obtained as a white amorphous powder. Its molecular formula was $C_{30}H_{48}O_7$, the same as compound **1**, based on the positive HRESIMS data at *m/z* 521.3463 $[M + H]^+$ (calcd for $C_{30}H_{49}O_7$ 521.3478). Similarities of UV spectrum and ^{13}C NMR data (Table 2) demonstrated that compound **3** contained four oxygenated methines and two oxygenated methylenes, just a hydroxy substitution site was changed. It was the disappearance of characteristic methylene that deduced the hydroxy group probably at C-27, as proved by the correlations of CH_3-26 (δ_H 0.85) with C-8 (δ_C 44.4)/C-7 (δ_C 41.6)/C-9 (δ_C 51.4)/C-27(δ_C 87.4), H-27 (δ_H 3.84) with C-15 (δ_C 130.5) in HMBC spectrum (Fig. 2). The NOESY spectrum (Fig. 3) displayed that H-27 (δ_H 3.84) was apparently correlated with CH_3-26 (δ_H 0.85), which led to an α-oriented configuration of 27-OH. Naturally, compound **3** could be determined as 3α, 20β, 21β, 27α, 24, 29-hexahydroxyserrat-14-en-16-one and named as serrat C.

Compound **4** was obtained as a white amorphous powder with a molecular formula of $C_{30}H_{48}O_7$, which was measured by HRESIMS at *m/z* 521.3463 $[M + H]^+$ (calcd for $C_{30}H_{49}O_7$ 521.3478). Its UV spectrum was the same as compound **3**, both at 249.9 nm. A further comparison of NMR data between compound **4** and **3** revealed that the only distinction was that the chemical shift of C-27 moved to the upfield significantly, indicating an opposite configuration of the hydroxy group at C-27, which was confirmed in detailed data analysis next. The HMBC spectrum (Fig. 2) of 4 displayed that CH_3-26 (δ_H 0.91) was correlated to C-27 (δ_C 80.2), proving the existence of 27-OH. It could be concluded that the hydroxy group at C-27 was β-oriented due to the correlation between H-27 (δ_H 4.32) and H-9 (δ_H 1.31) in the NOESY spectrum (Fig. 3). Finally, compound **4** was established as 3α, 20β, 21β, 27β, 24, 29-hexahydroxyserrat-14-en-16-one and named as serrat D.

Compound **5** was obtained as a white amorphous powder. Its molecular formula was identified as $C_{30}H_{48}O_6$ by HRESIMS at *m/z* 505.3512 $[M + H]^+$ (calcd for $C_{30}H_{49}O_6$ 504.3451), suggesting seven degrees of unsaturation. Its UV spectrum still indicated an α, β-unsaturated ketone. The ^1H NMR (Table 1) showed six methyl groups at δ_H 1.72 (3H, s, CH_3-30), 1.59 (3H, s, CH_3-23), 1.39 (3H, s, CH_3-29), 0.97 (3H, s, CH_3-26), 0.95 (3H, s, CH_3-25), 0.94 (3H, s, CH_3-28), four oxygenated methine groups at δ_H 4.41 (1H, m), 4.39 (1H, m), 3.74 (1H, br s), 3.48 (1H, m), a hydroxymethyl group at δ_H 4.08(1H, d, J = 11.3 Hz, Ha-24), 3.90(1H, d, J = 11.2 Hz, Hb-24). The ^{13}C NMR (Table 2) and DEPT spectrum displayed four oxygenated methines at δ_C 80.1 (C-21), 69.7 (C-3), 65.6 (C-20) and δ_C 78.7, one oxygenated methylene at δ_C 65.6 (C-24), four methines, six methylenes, six methyls and five quaternary carbons, which illustrated that the hydroxy group of C-29 was substituted by a proton. Comparing the spectrum data of compound **5** with that of 3α, 20β, 21β–trihydroxy-16-oxoserrat-14-en-24-oic acid and 3α, 21β, 24, 29-tetrahydroxyserrat-14-en-16-one could speculate a hydroxy group locating on the ring B [9,28]. In the HMBC spectrum (Fig. 2), H-5 (δ_H 2.07)/H-27 (δ_H 2.22)/CH_3-26 (δ_H 0.97) were all in correlations with δ_C 78.7. And in the ^1H–^1H COSY spectrum (Fig. 2), δ_H 3.48 was exactly correlated with H-6 (δ_H 2.20/1.98). Thus, both the data confirmed the existing of the hydroxy group at C-7. In the NOESY spectrum (Fig. 3), the correlations of H-7 (δ_H 3.48) with H-5 (δ_H 2.07)/H-9 (δ_H 1.09) indicated that the hydroxy group of C-7 was β-oriented. Therefore, compound **5** was determined as 3α, 7β, 20β, 21β, 24-pentahydroxyserrat-14-en-16-one and named as serrat E.

Compound **6** was obtained as a white amorphous powder. Its molecular formula was identified as $C_{30}H_{48}O_6$ by HRESIMS at *m/z* 505.3516 $[M + H]^+$ (calcd for $C_{30}H_{49}O_6$ 504.3451), suggesting

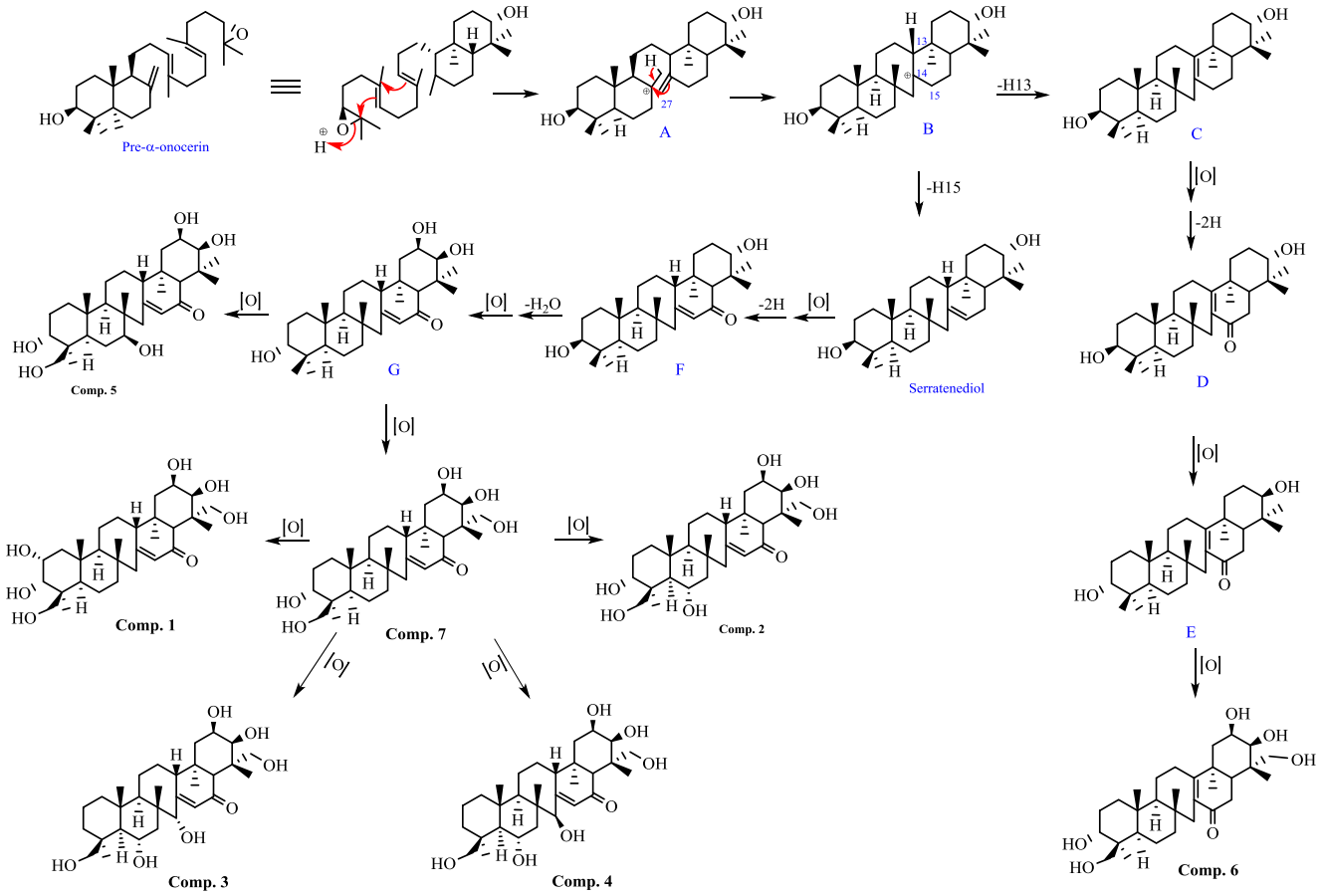

Fig. 4. Potential biosynthesis pathway of compounds **1–7**.

Table 3
Cytotoxicities of compounds **1–7**.

No.	Cytotoxicity IC_{50} (μM)							
	A549	calu-6	NCI-H226	NCI-H441	NCI-H1975	HepG2	MCF-7	MCF-10A
1	NA	NA	21.78	NA	NA	NA	29.65	NA
2	NA	NA	NA	NA	NA	35.25	24.79	NA
3	NA	53.84	NA	43.18	58.56	NA	16.90	69.76
4	NA	NA	NA	NA	NA	NA	18.78	NA
5	NA	NA	58.91	NA	NA	59.19	29.82	NA
6	NA	NA	NA	NA	NA	NA	21.36	NA
7	NA	NA	NA	NA	NA	73.04	23.66	NA
STS	0.03	0.005	0.17	0.38	0.01	0.05	0.03	2.32

NA: No active; STS: Staurosporine (positive control).

seven degrees of unsaturation. The NMR data of compound **5** was resembled to **7** generally, except for the D ring. Since the chemical shifts of C=C bond and carbonyl group, as well as their surrounding carbons (C-12, C-16, C-27), were quite different from those of **7**, it could be inferred that the positions of C=C bond and carbonyl group may change. Coincidentally, the NMR data (Table 2) of ring D was of great resemblance with that of 21α-methoxyserrat-13-ene-3, 15-dione, which was identified to comprise an α, β-unsaturated ketone at C-13/14/15 [29]. Furthermore, the correlations of H-27 (δ_H 1.81/3.15) to C-13 (δ_C 170.4)/C-14 (δ_C 132.7)/C-15 (δ_C 198.9), CH_3-28 (δ_H 1.22, s) to C-13 (δ_C 170.4), H-17 (δ_H 2.84, s) to C-15 (δ_C 198.9), H-12 (δ_H 2.02/2.67) to C-14 (δ_C 132.7) were all observed in HMBC spectrum (Fig. 2), proving the fact that C=C bond was at C-13/C-14 and carbonyl group at C-15. Its NOESY spectrum (Fig. 3) showed that 3-OH was α-oriented, 20-OH was β-oriented and 21-OH was β-oriented, the same as those of compounds **1–5**. Therefore, compound **6** was finally established as 3α, 20β, 21β, 24-pentahydroxyserrat-13-en-15-one and named as serrat F.

In addition, a potential biosynthesis pathway of compounds **1–7** was deduced as shown in Fig. 4. Serratene triterpenoids were not biosynthesized from α-onocerin as previously assumed but directly from pre-α-onocerin [30]. Pre-α-onocerin might be protonated and then converted to Intermediate B through cyclization reaction. B was dehydrogenated at C-13, followed by series oxidation and dehydration to give compound 6. In addition, serratenediol (3β-OH, 21α-OH), a key precursor, was produced by the dehydrogenation at C-15 of intermediate B. And then intermediate G (3α-OH, 21β-OH) was yield after sequential dehydration and oxidation of serratenediol. Compound **1**, **2**,

3, **4**, **5** and **7** were all produced by a series of oxidation reactions at different sites of G.

The inhibitory effects of compounds**1-7** against human cancer cells (A549, Calu-6, NCI-H441, NCI-H226, NCI-H1975, HepG2 and MCF-7) were evaluated by the CCK-8 assay with positive control of STS (Table3). Compounds **1**, **3** and **5** exhibited moderate cytotoxicities against lung cancer cell lines. Compounds **2**, **5** and **7** inhibited HepG2 cells moderately. Especially, all compounds displayed inhibitory activities on MCF-7 cells with IC_{50} values < 30 μM, among which compound **3** with the lowest IC_{50} value 16.90 μM.

4. Conclusion

Six new together with a known serratene triterpenoids were isolated from *Lycopodium complanatum*. Herein, we reported the structure identification of them for the first time. It is worth mentioning that the structure of these polyhydroxy triterpenoids is quite novel compared to conventional serratene triterpenoids. Additionally, compounds **1**, **3** and **5** displayed proliferation inhibition effects of human lung cancer cell lines in varying extent. Compounds **2**, **5** and **7** exhibited moderate inhibitory activities to HepG2 cells. Particularly, all compounds presented selective cytotoxicities against MCF-7 cells with IC_{50} values < 30 μM, where compounds **3** and **4** had IC_{50} values < 20 μM. To summarise, discovery of these isolates may provide some supports for further investigation of bioactive chemical constituents and biosynthesis pathway of *Lycopodium complanatum*.

Acknowledgements

This research was financially supported by Postgraduates innovation subject of Central South University (No. 2018zzts872) and Key Project of Changsha Science and Technology Plan (No. kq1801072). We are appreciated support of Prof. GuoGen Liu for the NMR measurement and Prof. ShaoGang Liu for the HRESIMS measurement.

Conflict of interest

There are no conflicts of interest to declare.

References

[1] P.G. Xiao, Modern Chinese Material Medical, Chemistry and Industry Press, Beijing, 2002, pp. 135–139.
[2] Z.J. Zhang, Y. Nian, Q.F. Zhu, X.N. Li, J. Su, X.D. Wu, J. Yang, Q.S. Zhao, Lycoplanine A, a $C_{16}N$ lycopodium alkaloid with a 6/9/5 tricyclic skeleton from *Lycopodium complanatum*, Org. Lett. 19 (2017) 4668–4671.
[3] J.T. Cheng, F. Liu, X.N. Li, X.D. Wu, L.B. Dong, L.Y. Peng, S.X. Huang, J. He, Q.S. Zhao, Lycospidine A, a new type of lycopodium alkaloid from *Lycopodium complanatum*, Org. Lett. 15 (2013) 2438–2441.
[4] K. Ishiuchi, T. Kubota, H. Ishiyama, S. Hayashi, T. Shibata, K. Mori, Y. Obara, N. Nakahata, J. Kobayashi, Lyconadins D and E, and complanadine E, new lycopodium alkaloids from *Lycopodium complanatum*, Bioorg. Med. Chem. 19 (2011) 749–753.
[5] K. Ishiuchi, T. Kubota, S. Hayashi, T. Shibata, J. Kobayashi, Lycopladines F and G, new $C_{16}N_2$-type alkaloids with an additional C_4N unit from *Lycopodium complanatum*, Tetrahedron Lett. 50 (2009) 4221–4224.
[6] G.A. Czapski, W. Szypuła, M. Kudlik, B. Wileńska, M. Kania, W. Danikiewicz, A. Adamczyk, Assessment of antioxidative activity of alkaloids from *Huperzia selago* and *Diphasiastrum complanatum* using in vitro systems, Folia Neuropathol. 52 (2013) 394–406.
[7] V.T. Nguyen, B.T. Zhao, S.H. Seong, J.A. Kim, M.H. Woo, J.S. Choi, B.S. Min, Inhibitory effects of serratene-type triterpenoids from lycopodium complanatum on cholinesterases and β-secretase 1, Chem.-Biol. Interact. 274 (2017) 150–157.
[8] Y. Zhang, P. Yi, Y. Chen, Z.N. Mei, X. Hu, G.Z. Yang, Lycojaponicuminol A-F: cytotoxic serratene triterpenoids from *Lycopodium japonicum*, Fitoterapia 96 (2014) 95–102.
[9] J. Yan, P. Yi, B. Chen, L. Lu, Z.R. Li, X.M. Zhang, L. Zhou, M.H. Qiu, Polyhydroxyserratane triterpenoids from *Diphasiastrum complanatum*, Phytochemistry 69 (2008) 506–510.
[10] Y.M. Weng, X. Yu, J. Li, Q.Z. Dong, F.S. Li, F. Cheng, Y. Zhang, C.P. Yao, Z.X. Zou, W.H. Zhou, G.S. Tan, K.P. Xu, Abietane diterpenoids from *Lycopodium complanatum*, Fitoterapia 128 (2018) 135–141.
[11] X.T. Tong, C.H. Tan, H. Zhou, S.H. Jiang, X.Q. Ma, D.Y. Zhu, Triterpenoid constituents of *Huperzia miyoshiana*, Chin. J. Chem. 21 (2003) 1364–1368.
[12] R. Tanaka, K. Ohmori, A. Katsuhiko Minoura, S. Matsunaga, Two new epoxyserratanes from the cuticle of *Picea jezoensis*, J. Nat. Prod. 59 (1996) 237–241.
[13] J.J. Wei, W.Q. Wang, W.B. Song, L.J. Xuan, Serratene-type triterpenoids from *Palhinhaea cernua*, Fitoterapia 127 (2018) 151–158.
[14] J. Li, P.S. Xu, L.H. Tan, Z.X. Zou, Y.K. Wang, H.P. Long, G. Zhou, G. Li, K.P. Xu, G.S. Tan, Neolignans and serratane triterpenoids with inhibitory effects on xanthine oxidase from *Palhinhaea cernua*, Fitoterapia 119 (2017) 45–50.
[15] V.T. Nguyen, D.C. To, M.H. Tran, S.H. Oh, J.A. Kim, M.Y. Ali, M.H. Woo, J.S. Choi, B.S. Mina, Isolation of cholinesterase and β-secretase 1 inhibiting compounds from *Lycopodiella cernua*, Bioorg. Med. Chem. 23 (2015) 3126–3134.
[16] S. Wittayalai, S. Sathalalai, S. Thorroad, P. Worawittayanon, S. Ruchirawat, N. Thasana, Lycophlegmariols A-D: cytotoxic serratene triterpenoids from the club moss *Lycopodium phlegmaria* L, Phytochemistry 76 (2012) 117–123.
[17] Z.Z. Zhang, M.R. ElSohly, H.N. Jacob, D.S. Pasco, L.A. Walker, A.M. Clark, Natural products inhibiting Candida albicans secreted aspartic proteases from *Lycopodium cernuum*, J. Nat. Prod. 65 (2002) 979–985.
[18] X.L. Li, Y. Zhao, X. Cheng, L. Tu, L.Y. Peng, G. Xu, Q.S. Zhao, Japonicumins A-D: four new compounds from *Lycopodium japonicum*, Helv. Chim. Acta. 89 (2006) 1467–1473.
[19] J. Yan, X.M. Zhang, Z.R. Li, L. Zhou, J.C. Chen, L.R. Sun, M.H. Qiu, Three new triterpenoids from *Lycopodium japonicum* Thunb, Helv. Chim. Acta. 88 (2005) 240–244.
[20] J. Otaka, M. Komatsu, Y. Miyazaki, Y. Futamura, H. Osada, Two new triterpenoids from the roots of *Pinus densiflora*, Biosci. Biotechnol. Biochem. 81 (2016) 449–452.
[21] R. Tanaka, K. Tsujimoto, Y. In, T. Ishida, S. Matsunaga, H. Tokuda, O. Muraoka, Jezananals A and B: two novel skeletal triterpene aldehydes from the stem bark of *Picea jezoensis var. jezoensis*, Tetrahedron 58 (2002) 2505–2512.
[22] J. Yan, B.H. Cheng, X.M. Zhang, M.H. Qiu, Three new serratane triterpenoids from *Phlegmariurus squarrosus*, Helv. Chim. Acta. 89 (2007) 2975–2980.
[23] N. Miller, C. Hootele, J.C. Braekman, Triterpenoids of *Lycopodium megastachyum*, Phytochemistry 12 (1973) 1759–1761.
[24] H. Zhou, C.H. Tan, S.H. Jiang, H.Q. Liu, S.H. Jiang, D.Y. Zhu, Serratene-type triterpenoids from *Huperzia serrata*, J. Nat. Prod. 66 (2003) 1328–1332.
[25] H. Zhou, Y.S. Li, X.T. Tong, H.Q. Liu, S.H. Jiang, D.Y. Zhu, Serratane-type triterpenoids from *Huperzia serrata*, Nat. Prod. Res. 18 (2004) 453–459.
[26] Y. Tsuda, T. Fujimoto, K. Isobe, K. Sano, T. Kobayashi Masako, Chemotaxonomical studies on the triterpenoids of Lycopodium plants, Yakugaku Zasshi 94 (1974) 970–990.
[27] X.L. Li, Y. Zhao, X. Cheng, L. Tu, L.Y. Peng, G. Xu, Q.S. Zhao, Japonicumins A-D: four new compounds from *Lycopodium japonicum*, Helv. Chim. Acta. 89 (2010) 1467–1473.
[28] J. Yan, L. Sun, X. Zhang, Z.R. Li, L. Zhou, M.H. Qiu, Serratene triterpenoids from *Palhinhaea cernua var. sikkimensis*, Chem. Pharm. Bull. 57 (2009) 1381–1384.
[29] R. Tanaka, K. Tsujimoto, A. Yasuko In, S. Matsunaga, New methoxytriterpene dione from the cuticle of *Picea jezoensis var. jezoensis*, J. Nat. Prod. 60 (1997) 319–322.
[30] Y. Saga, T. Araki, H. Araya, K. Saito, M. Yamazaki, H. Suzuki, T. Kushiro, Identification of serratane synthase gene from the fern *Lycopodium clavatum*, Org. Lett. 19 (2017) 496–499.