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Five new *Lycopodium* alkaloids from the aerial parts of *Phlegmariurus henryi*

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A series of new *Lycopodium* alkaloids, namely 1-*epi*-malycorin A (**1**), 1-*epi*-17*S*-hydroxymalycorin A (**2**), 6α-hydroxyphlegmariurine A (**3**), 2*S*,4*R*-dihydroxyfawcettimine (**4**), and 16-hydroxylycodine (**5**), together with 24 known ones, have been isolated from the club moss *Phlegmariurus henryi*. The structures of the new compounds were determined by extensive spectroscopic analysis, including 1D and 2D NMR, as well as X-ray crystallographic analysis. Among them, the absolute configurations of **1**, **2**, and **4** and the structure of **3** were confirmed on the basis of the single-crystal X-ray diffraction analysis. 1-*Epi*-17*S*-hydroxymalycorin A (**2**) was a unique C_{19}N-type *Lycopodium* alkaloid consisting of a serratinine skeleton with 1,2-propanediol unit. 2*S*,4*R*-dihydroxyfawcettimine (**4**) was a 2,4-dihydroxy derivative of fawcettimine. 16-Hydroxylycodine (**5**) was the oxidative product of lycodine with an unusual hydroxymethyl group at C-15. All new compounds were evaluated for in vitro acetylcholinesterase (AChE) inhibitory activity and cytotoxicity against four human cancer cell lines.
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1. Introduction

The Huperziaceae family, comprised of the genus *Huperzia* and *Phlegmariurus*, was a rich source of *Lycopodium* alkaloids with fascinating skeletons and wide-range of biological activities [1]. Huperzine A, a highly potent, specific, reversible, and selective acetylcholinesterase inhibitor as well as a potential drug for the treatment of Alzheimer's disease [2] and myasthenia gravis [3], has been isolated from the club moss *Huperzia serratum* [4,5]. This finding has spurred the discovery of numerous structurally diverse and complex *Lycopodium* alkaloids, some of which have proven to be challenging targets for total synthesis [6,7].

Phlegmariurus henryi, one of club moss belonging to the Huperziaceae family, is mainly distributed in Guangxi and Yunnan Provinces of China. As part of an ongoing program aimed at discovering structurally interesting and bioactive *Lycopodium* alkaloids, the alkaloidal extract of the aerial parts of *P. henryi* was investigated. This led to the isolation of five new *Lycopodium* alkaloids, including two C_{19}N-type *Lycopodium* alkaloids, 1-*epi*-malycorin A (**1**) and 1-*epi*-17*S*-hydroxymalycorin A (**2**) consisting of a serratinine skeleton [8,9], two fawcettimine-related alkaloids 6α-hydroxyphlegmariurine A (**3**) and 2*S*,4*R*-dihydroxyfawcettimine (**4**), one lycodine-type alkaloid, 16-hydroxylycodine (**5**) (Fig. 1), together with 24 known alkaloids. Compared with the literature data, the known compounds were identified as anhydrolycodoline (**6**) [10], 8-deoxyserratinine (**7**) [8], 12-*epi*-lycodoline *N*-oxide (**8**) [11], gnidioidine (**9**) [12], huperzine A (**10**) [4,5], 6-hydroxy-6,7-dehydro-8-deoxy-13-dehydroserratinine (**11**) [13], lycoposerramine U (**12**) [14], phlegmariurine B (**13**) [15], des-*N*-methyl-β-obscurine (**14**) [16], 11α-hydroxyphlegmariurine (**15**) [17], lycofoline (**16**) [10,18], huperzine C (**17**) [19], lycoposerramine D (**18**) [14], des-*N*-methyl-huperzinine (**19**) [20], lycoflexine (**20**) [14], lycoposerramine E (**21**) [14], lycopodine (**22**) [21,22], huperzine B (**23**) [4,5], cernupaldine A (**24**) [23], lycodine (**25**) [24], malycorin A (**26**) [25], lycothunine (**27**) [26], *N*-methylhuperzine A (**28**) [4,5], and carnatine A (**29**) [27]. Their structures were determined by extensive spectroscopic analysis and the single X-ray crystallographic analysis. Herein, we report the extraction, isolation, structure elucidation, as well as *anti*-AChE activity and cytotoxicity against four human cancer cell lines of the new compounds.

2. Experimental

2.1. Gernal experimental procedures

Melting points were obtained on an X-4 micro melting point apparatus. IR spectra were obtained on a Bruker Tensor 27 spectrometer with KBr pellets. UV spectra were recorded using a Shimadzu UV-2401A spectrophotometer. Optical rotations were measured on a JASCO-20C

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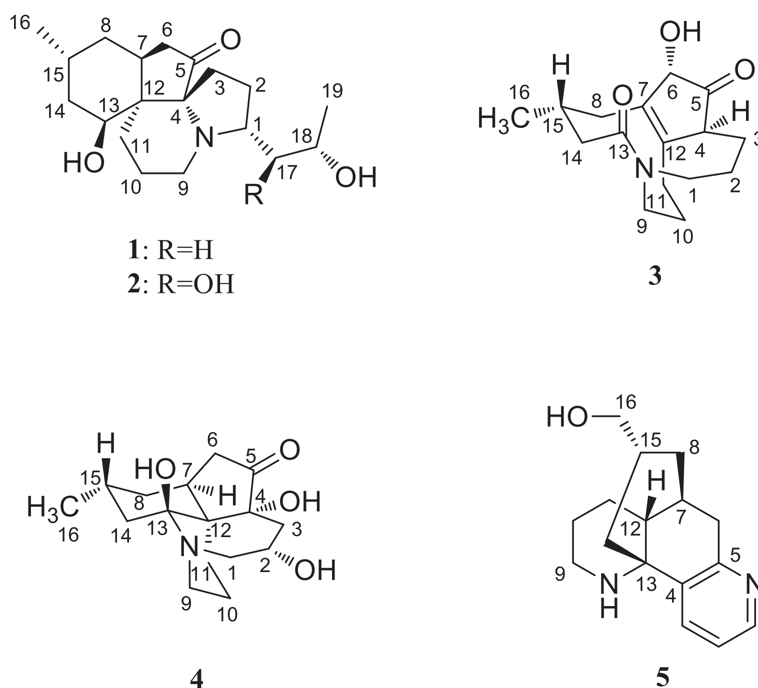

Fig. 1. Structures of the new compounds **1**–**5**.

digital polarimeter. Circular Dichroismspectra were measured on an Agilent Applied Photophysics. ESIMS were recorded on a Waters Xevo TQ-S spectrometer. HRESIMS were measured using Agilent 1290 UPLC/6540 Q-TOF-MS instrument. 1D and 2D NMR were performed on Bruker AM-400, DRX-500, or AVANCE III-600 spectrometers. X-ray diffraction was performed on a Bruker APEX DUO diffractometer using graphite-monochromated Cu Kα radiation. The HSCCC instrument used was a TBE-300B high-speed counter-current chromatography (Tauto Biotechnique Company, Shanghai, China). Column chromatography (CC) was performed over silica gel (100–200 or 200–300 mesh, Qingdao Marine Chemical Co. Ltd., Qingdao, China), MCI gel (CHP 20P, 75–150 μm, Mitsubishi Chemical Corporation, Japan), and Sephadex LH-20 (Amersham Pharmacia Biotech, Sweden). Thin-layer chromatography (TLC) was carried out on silica gel GF_{254} on glass plates (Qingdao Marine Chemical Inc.) using various solvent systems and spots were visualized by spraying improved Dragendorff's reagent to the silica gel plates.

2.2. Plant material

The club moss *P. henryi* in this study was collected in Malipo Prefecture of Wenshan, Yunnan Province, P. R. China in June 2013 and identified by Prof. Xiao Cheng of Kunming Institute of Botany, Chinese Academy of Sciences. A voucher specimen (201307120P1) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences.

2.3. Extraction and isolation

The air-dried powder of the aerial parts of the club moss *P. henryi* (12 kg) was extracted three times with 75% EtOH (each 24 h), and the crude extract was adjusted to pH 2 with 10% HCl/H_2O and then partitioned three times with EtOAc (6 L). Water-soluble portions, after being adjusted to pH 10 with 17% ammonia solution, were than partitioned three times with $CHCl_3$ (6 L). $CHCl_3$-soluble parts (39.0 g) were subjected to Sephadex LH-20 CC (MeOH) and then HSCCC (petroleum ether/n-butanol/MeOH/H_2O, 4:1:2:4) to give an alkaloidal extract (35.0 g). The later was chromatographed on reverse-phase MPLC (MCI) (MeOH/H_2O, 5% → 100%) to give fractions I–V. Fr. I (2.0 g) was chromatographed over silica gel CC (EtOAc/MeOH, 20:1 → 1:1) to get two subfractions (Fr. I-I to Fr. I-II). Fr. I-I (1.2 g) was subjected to silica gel CC ($CHCl_3$/MeOH, 25:1 → 25:25) to afford **10** (843.0 mg). Fr. I-II (0.6 g) was subjected to silica gel CC (petroleumether/acetone/diethylamine, 85:15:1 → 20:80:1) to yield **7** (2.0 mg). Fr. II (12.0 g) was subjected to Sephadex LH-20 CC (MeOH) to give three subfractions (Fr. II-I to Fr. II-III). Fr. II-I (4.0 g) was separated by silica gel CC ($CHCl_3$/MeOH, 50:1 → 50:50) to afford **3** (8.0 mg), **11** (7.2 mg), and **13** (48.8 mg). Fr. II–II (5.0 g) was subjected to silica gel CC ($CHCl_3$/MeOH, 30:1 → 50:50) to obtain **4** (4.2 mg), **22** (28.3 mg), and **23** (54.6 mg). Fr. II-III (2.5 g) was subjected to silica gel CC ($CHCl_3$/MeOH, 20:1 → 50:50) to get **12** (35.0 mg), **14** (6.1 mg), and **27** (4.4 mg). Fr. III (8.0 g) was purified by Sephadex LH-20 CC (MeOH) to get three subfractions (Fr. III-I to Fr. III-III). Fr. III-I (2.3 g) was subjected to repeated silica gel CC (petroleum ether/acetone/diethylamine, 90:10:1 → 50:50:1 and then EtOAc/MeOH, 50:10 → 50:50) to yield **6** (34.9 mg) and **26** (2.4 mg). Fr. III-II (3.6 g) was chromatographed over repeated silica gel CC ($CHCl_3$/MeOH, 90:10 → 10:90 and then petroleum ether/acetone/diethylamine, 85:15:1 → 50:50:1) to afford **5** (24.1 mg) and **9** (267.0 mg). Fr. III-III (1.7 g) was separated by repeated silica gel CC (petroleum ether/acetone/diethylamine, 75:25:1 → 50:50:1 and then petroleum ether/EtOAc/diethylamine, 80:20:1 → 50:50:1) to yield crude crystals, which recrystallized from MeOH to give **2** (7.7 mg). Fr. IV (6.0 g) was chromatographed over silica gel CC ($CHCl_3$/acetone, 15:1 → 10:90) and Sephadex LH-20 CC (MeOH) to obtain **17** (56.5 mg), **18** (52.1 mg), **21** (60.6 mg) and a subfraction Fr. IV-I. Fr. IV-I was subjected to silica gel CC (EtOAc/MeOH, 88:12 → 50:50) to afford **15** (13.3 mg) and **24** (2.3 mg). Purification of Fr. V (7.0 g) over silica gel CC ($CHCl_3$/MeOH, 90:10 → 10:90) to give three fractions: Fr. V-I to Fr. V-III. Fr. V-I (3.0 g) was performed on silica gel CC (EtOAc/MeOH, 75:25 → 50:50) to afford **19** (33.0 mg), **20** (18.1 mg), and **25** (2.3 mg). Fr. V-II (1.6 g) was chromatographed over silica gel CC (EtOAc/MeOH, 60:1 → 60:60) and Sephadex LH-20 CC (MeOH) to yield **1** (69.8 mg), **28** (14.4 mg), and **29** (1.3 mg). Compounds **8** (2.9 mg) and **16** (6.6 mg) were obtained from Fr. V-III through repeated silica gel CC ($CHCl_3$/MeOH, 20:1 → 20:20 and then petroleumether/EtOAc/diethylamine, 80:20:1 → 10:90:1).

2.3.1. 1-Epi-malycorin A (**1**)

Colorless columnar crystals (MeOH); mp 175–178 °C; $[\alpha]_D^{24.0}$ – 1.32 (c 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 203 (3.38), 279 (2.74) nm; IR (KBr) ν_{max} 3431, 2924, 1744, 1631, 1457, 1127, and 579 cm^{-1}; ^{1}H NMR and ^{13}C NMR data see Tables 1 and 2; ESIMS (positive) m/z 322 [M + H]$^+$; HRESIMS (positive) m/z 322.2385 [M + H]$^+$ (calcd for $C_{19}H_{32}NO_3$, 322.2377).

2.3.2. 1-Epi-17S-hydroxymalycorin A (**2**)

Colorless needles (MeOH); mp 242–245 °C; $[\alpha]_D^{21.3}$ + 8.64 (c 0.189, MeOH); UV (MeOH) λ_{max} (log ε) 203 (3.50), 280 (2.79) nm; IR (KBr) ν_{max} 3447, 2925, 1638, 1456, 1384, 1052, and 715 cm^{-1}; ^{1}H NMR and ^{13}C NMR data see Tables 1 and 2; ESIMS (positive) m/z 338 [M + H]$^+$; HRESIMS (positive) m/z 338.2324 [M + H]$^+$ (calcd for $C_{19}H_{32}NO_4$, 338.2326).

2.3.3. 6α-Hydroxyphlegmariurine A (**3**)

Colorless crystals (MeOH); mp 230–233 °C; $[\alpha]_D^{23.6}$ + 0.24 (c 0.14, MeOH); UV (MeOH) λ_{max} (log ε) 202 (3.70), 392 (1.89) nm; IR (KBr) ν_{max} 3449, 2920, 1637, 1599, 1463, 1384, 1080, and 695 cm^{-1}; ^{1}H NMR and ^{13}C NMR data see Tables 1 and 2; ESIMS (positive) m/z 278 [M + H]$^+$; HRESIMS (positive) m/z 278.1753 [M + H]$^+$ (calcd for $C_{16}H_{24}NO_3$, 278.1751).

2.3.4. 2S,4R-Dihydroxyfawcettimine (**4**)

Colorless crystrals (MeOH); mp 201–205 °C; $[\alpha]_D^{22.8}$ + 26.61 (c 1.03, MeOH); UV (MeOH) λ_{max} (log ε) 201 (3.37), 254 (3.22) nm; IR (KBr) ν_{max} 3425, 2953, 1742, 1632, 1459, 1384, 1139, 1026, 871, and 591 cm^{-1}; ^{1}H NMR and ^{13}C NMR data see Tables 1 and 2; ESIMS (positive) m/z 296 [M + H]$^+$; HRESIMS (positive) m/z 296.1859 [M + H]$^+$ (calcd for $C_{16}H_{26}NO_4$, 296.1856).

2.3.5. 16-Hydroxylycodine (**5**)

Colorless oil;$[\alpha]_D^{21.5}$ + 2.90 (c 0.46, MeOH); UV (MeOH) λ_{max} (log ε) 204 (3.70), 268 (3.52) nm; CD (c 1.79 × 10^{-4} M, MeOH) λ_{max} ($\Delta\varepsilon$) 259 (−1.39), 280 (+2.51) nm; IR (KBr) ν_{max} 3422, 2921, 1632, 1581, 1436, 1384, 1063, 807, 735, and 564 cm^{-1}; ^{1}H NMR and ^{13}C NMR data see Tables 1 and 2; ESIMS (positive) m/z 259 [M + H]$^+$; HRESIMS (positive) m/z 259.1808 [M + H]$^+$ (calcd for $C_{16}H_{23}N_2O$, 259.1805).

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

No.	**1**[a]	**2**[a]	**3**[a]	**4**[b]	**5**[a]
1a	3.09 (m)	2.79(m)	2.61 (ddd, 14.0, 14.0, 2.1)	2.98 (m)	8.31 (dd, 4.8, 1.4)
1b			3.85 (ddd, 14.0, 3.4, 3.4)	4.27 (dd, 13.2, 6.0)	
2a	1.99 (m)	2.26(m)	1.47 (m)	5.28 (m)	7.29 (dd, 7.9, 4.8)
2b	2.25[c]		1.86 (m)		
3a	1.91 (ddd, 12.2, 10.5, 10.5)	1.91(m)	2.14 (m)	2.83[c]	7.92 (dd, 7.9, 1.4)
3b	2.25[c]	2.24(m)	2.35[c]	3.49 (m)	
4a			2.76 (m)		
4b					
5					
6a	2.39 (m)	2.35 (m)	4.48 (s)	2.64[c]	2.70 (d, 18.8)
6b		2.40 (m)		2.83[c]	3.17 (dd, 18.8, 7.2)
7a	2.85 (m)	2.80 (m)		2.64[c]	2.19 (m)
7b					
8a	1.31 (m)	1.29 (m)	2.27 (m)	1.40 (dd, 13.2, 4.0)	1.42 (ddd, 12.8, 12.8, 3.9)
8b	1.56 (m)	1.56 (m)	2.38 (m)	1.60 (br d, 13.2)	1.86 (d, 12.8)
9a	2.41 (m)	2.46 (m)	3.14 (d, 14.3)	2.94 (m)	2.45 (ddd, 8.4, 8.4, 1.9)
9b	2.86 (m)	2.90 (br d, 10.4)	4.25 (ddd, 14.3, 14.3, 3.2)	3.40 (t, 12.8)	2.78 (ddd, 8.4, 1.3, 1.3)
10a	1.55 (m)	1.54 (m)	1.69 (m)	1.36 (m)	1.58 (m)
10b	1.81 (m)	1.81 (m)	2.12 (m)	2.26[c]	
11a	1.11 (ddd, 14.8, 14.8 5.3)	1.12[c]	2.25 (m)	1.89 (ddd, 13.8, 13.8, 4.8)	1.16 (m)
11b	1.83 (m)	1.83 (m)	3.06 (m)	2.63 (m)	1.65 (ddd, 13.0, 4.1, 4.1)
12					1.75 (ddd, 13.0, 2.9, 2.9)
13	3.50 (m)	3.50 (m)			
14a	1.37 (m)	1.37 (m)	1.96 (ddd, 13.7, 1.8, 1.8)	1.64 (br d, 12.8)	1.36 (t, 12.1)
14b	1.54 (m)	1.53 (m)	2.90 (dd, 13.7, 4.0)	2.24[c]	1.60 (m)
15	2.02 (m)	2.02 (m)	2.34[c]	2.38 (m)	1.25 (m)
16	0.90 (d, 6.6)	0.90 (d, 6.6)	1.12 (d, 6.4)	0.95 (d, 6.7)	3.29, 3.25 (ABq, 10.7)
17a	1.32 (m)	3.01 (m)			
17b	1.45 (m)				
18	3.85 (m)	3.59 (m)			
19	1.05 (d, 6.0)	1.12 (d, 6.0)			

[a] Recorded at 600 MHz in CD_3OD.
[b] Recorded at 600 MHz in C_5D_5N.
[c] Overlapping signals.

2.4. X-ray crystal structure analysis

Crystal analysis were performed on a Bruker APEX DUO diffractometer equipped with an APEX II CCD, using Cu Kα radiation (λ = 1.54178 Å). Cell refinement and data reduction were performed with Bruker SAINT.

The structure of **1** was solved by direct methods using SHELXS-2014. Refinements were performed with SHELXL-2014 using full-matrix least-squares, with anisotropic displacement parameters for all the non-hydrogen atoms. The H-atoms were placed in calculated positions and refined using a riding model.

The structures of **2–4** were solved by direct methods using SHELXS-97. Refinements were performed with SHELXL-97 using full-matrix least-squares, with anisotropic displacement parameters for all the non-hydrogen atoms. The H-atoms were placed in calculated positions and refined using a riding model.

Crystallographic data for the structures in this paper have been deposited with the Cambridge Crystallographic Data Centre (CCDC 1410616 for **1**; CCDC 1406786 for **2**; CCDC 1406748 for **3**; CCDC 1410318 for **4**). Copies of the data can be obtained free of charge from the CCDC via www.ccdc.cam.ac.uk.

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

No.	**1**[a]	**2**[a]	**3**[a]	**4**[b]	**5**[a]
1	71.0	76.2	50.8	62.5	147.7
2	29.5	28.0	24.5	65.7	123.4
3	24.8	24.6	33.3	44.8	135.9
4	77.9	78.1	55.7	79.1	137.4
5	219.7	219.6	221.3	213.0	159.4
6	40.2	40.2	76.3	39.5	35.7
7	33.3	33.4	137.3	39.1	34.2
8	32.9	32.8	34.1	32.5	38.9
9	55.5	55.9	51.8	51.8	42.3
10	22.2	22.1	24.6	20.8	27.1
11	25.7	25.6	28.8	25.5	27.4
12	46.4	46.4	142.6	50.2	44.8
13	74.2	74.2	175.5	87.1	57.9
14	38.9	38.9	41.0	44.2	46.3
15	20.9	20.9	28.9	24.3	35.1
16	22.6	22.5	23.6	22.2	68.4
17	43.6	75.8			
18	69.7	74.0			
19	23.7	19.8			

[a] Recorded at 150 MHz in CD_3OD.
[b] Recorded at 150 MHz in C_5D_5N.

*2.4.1. Crystal data for **1***

$C_{19}H_{31}NO_3$, $M = 321.45$, $a = 9.5904(3)$ Å, $b = 13.2775(4)$ Å, $c = 14.1768(5)$ Å, $\alpha = \beta = \gamma = 90°$, $V = 1805.22(10)$ Å^3, $T = 100(2)$ K, space group $P212121$, $Z = 4$, μ (Cu Kα) = 0.623 mm^{-1}, 10,433 reflections measured, 3203 independent reflections ($R_{int} = 0.0243$). The final R_1 values were 0.0463 ($I > 2\sigma(I)$). The final wR (F^2) values were 0.1464 ($I > 2\sigma(I)$). The final R_1 values were 0.0463 (all data). The final wR (F^2) values were 0.1465 (all data). The goodness of fit on F^2 was 1.242. Flack parameter = 0.12(4).

*2.4.2. Crystal data for **2***

$C_{19}H_{31}NO_4$, $M = 337.45$, orthorhombic, $a = 9.2445(5)$ Å, $b = 13.6708(7)$ Å, $c = 14.3114(8)$ Å, $\alpha = \beta = \gamma = 90.00°$, $V = 1808.67(17)$ Å^3, $T = 100(2)$ K, space group $P212121$, $Z = 4$, μ (Cu Kα) = 0.689 mm^{-1}, 8811 reflections measured, 3081 independent reflections ($R_{int} = 0.0382$). The final R_1 values were 0.0339 ($I > 2\sigma(I)$). The final wR (F^2) values were 0.0899 ($I > 2\sigma(I)$). The final R_1 values were 0.0339 (all data). The final wR (F^2) values were 0.0899 (all data). The goodness of fit on F^2 was 1.099. Flack parameter = 0.17(17).

*2.4.3. Crystal data for **3***

$C_{16}H_{23}NO_3$, $M = 277.35$, orthorhombic, $a = 7.8065(3)$ Å, $b = 8.4274(4)$ Å, $c = 20.9588(8)$ Å, $\alpha = \beta = \gamma = 90.00°$, $V = 1378.85(10)$ Å^3, $T = 100(2)$ K, space group $P212121$, $Z = 4$, μ (Cu Kα) = 0.736 mm^{-1}, 9505 reflections measured, 2378 independent reflections ($R_{int} = 0.2174$). The final R_1 values were 0.1648 ($I > 2\sigma(I)$). The final wR (F^2) values were 0.3715 ($I > 2\sigma(I)$). The final R_1 values were 0.3189 (all data). The final wR (F^2) values were 0.4698 (all data). The goodness of fit on F^2 was 1.424.

*2.4.4. Crystal data for **4***

$C_{17}H_{29}NO_5$, $M = 327.41$, monoclinic, $a = 9.9283(4)$ Å, $b = 7.6972(4)$ Å, $c = 11.4894(5)$ Å, $\alpha = 90.00°$, $\beta = 107.239(2)°$, $\gamma = 90.00°$, $V = 838.58(7)$ Å^3, $T = 100(2)$ K, space group $P21$, $Z = 2$, μ (Cu Kα) = 0.773 mm^{-1}, 6195 reflections measured, 2551 independent reflections ($R_{int} = 0.0476$). The final R_1 values were 0.0530 ($I > 2\sigma(I)$). The final wR (F^2) values were 0.3715 ($I > 2\sigma(I)$). The final R_1 values were 0.3189 (all data). The final wR (F^2) values were 0.1511 (all data). The goodness of fit on F^2 was 1.092. The Hooft parameter is 0.09(11) for 985 Bijvoet pairs.

2.5. Anti-AChE assay

Acetylcholinesterase (AChE) inhibitory activities of the new compounds were assayed by Amplex acetlycholinestrase assay kit (Life Technologies, Carlsbad, CA, USA) using 10-acetyl-3,7-dihydroxyphenoxazine (Amplex® Red reagent) as specified [28]. The fluorescence of each well was measured by BioTek Synergy HT microplate reader with excitation and emission wavelengths of 535 nm and 590 nm, respectively.

Tacrine was used as positive control with final concentration of 0.333 μM. All the reactions were performed in triplicate. The percentage inhibition was calculated as follows: % inhibition = (E − S) / E × 100 (E is the activity of the enzyme without test compound and S is the activity of enzyme with test compounds).

2.6. Cytotoxicity assay

Cytotoxicity of new compounds against HepG2, HCT116, A549 and AGS cell lines was assessed using the MTT method [29]. Cells were plated in 96-well plates for 12 h before treatment and continuously exposed to different concentrations of compounds. After 48 h, 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution were added to each well, which were incubated for another 4 h. Then 20% SDS (100 μL) were added to each well. After 12 h at room temperature, the OD value of each well was recorded at 595 nm. The IC_{50} value of each compound was calculated by the Reed and Muench method [30].

3. Results and discussion

3.1. Chemistry

Compound **1** was obtained as colorless columnar crystals (MeOH). The positive ion at m/z 322.2385 [M + H]$^+$ in HRESIMS established the molecular formula of **1** as $C_{19}H_{31}NO_3$, with five degrees of unsaturation. The IR spectrum was indicative of the presence of a ketone (1744 cm^{-1}) and a hydroxy (3431 cm^{-1}) groups. The ^{1}H and ^{13}C NMR (Tables 1 and 2) and DEPT spectra of **1** displayed 19 carbon signals due to one sp^2 carbonyl carbon (δ_C 219.7), two sp^3 quaternary carbons (δ_C 77.9 and 46.4), five sp^3 methines (δ_C 74.2, 71.0, 69.7, 33.3 and 20.9), nine sp^3 methylenes (δ_C 55.5, 43.6, 40.2, 38.9, 32.9, 29.5, 25.7, 24.8 and 22.2), and two methyl groups (δ_C 22.6 and 23.7). The above data indicated that **1** was *Lycopodium* alkaloid possessing fawcettimine-related skeleton [31–33], which have similar spectral properties with those of malycorin A [25]. Since the carbonyl group accounted for only one degree of unsaturation, the remaining four degrees of unsaturation required **1** to possess a tetracyclic ring system.

The detailed 2D analysis established the planar structure of **1**. Analysis of the ^{1}H-^{1}H COSY and HSQC experiments revealed the presence of three isolated spin systems: **a** (C-3/C-2/C-1/C-17/C-18/C-19), **b** (C-6/C-7/C-8/C/15/C-14/C-13, and C-15/C-16), **c** (C-9/C-10/C-11), as shown in Fig. 2. The two oxygenated carbons were assigned at C-13 (δ_C 74.2) and C-18 (δ_C 69.7), which were confirmed by the HMBC correlations of H-13 (δ_H 3.50) with C-14 (δ_C 38.9) and C-15 (δ_C 20.9), and H-18 (δ_H 3.85) with C-17 (δ_C 43.6) and C-19 (δ_C 23.7), respectively. On the basis of the HMBC cross-peaks of H-1 (δ_H 3.09) with C-4 (δ_C 77.9) and C-9 (δ_C 55.5), and H_2-9 (δ_H 2.41, 2.86) with C-1 (δ_C 71.0), C-4, the connections of C-1, C-4 and C-9 through a nitrogen atom were established. The HMBC correlations from H-6 (δ_H 2.39) and H-7 (δ_H 2.85) to C-5 (δ_C 219.7) indicated that the carbonyl carbon (δ_C 219.7) were located at C-5, which connected to unit **a** through C-4 as evidenced by the HMBC correlations from H_2-3 (δ_H 1.91, 2.25) and H-6 to C-4. Meanwhile, the key HMBC networks from H_2-3 to C-12 (δ_C 46.4), and H_2-11 (δ_H 1.11, 1.83) to C-4 and C-12 suggested that units **a** and **c** was connected through C-4 and C-12. Furthermore, a cyclopentanone ring was

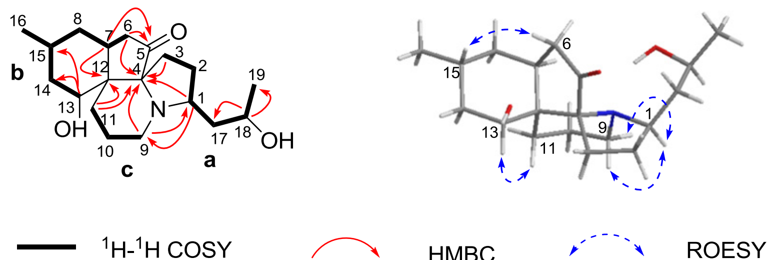

Fig. 2. The key 2D–NMR correlations of compound **1**.

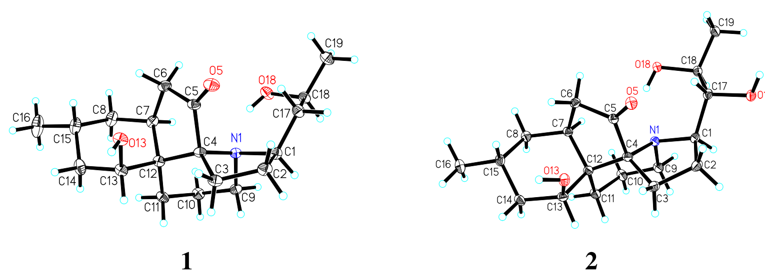

Fig. 3. The X-ray structures of compounds **1** and **2**.

constructed by the HMBC cross-peak of H-7 with C-12. Finally, the HMBC networks of H-13 with C-11 (δ_C 25.7) and C-12 established the connectivity of units **b** and **c** though C-12. These data led to the assignment of the planar structure of **1** as serratinine skeleton with 2-propanol unit similar to those of malycorin A [25]. Compared to the 1D NMR spectroscopic data of malycorin A, **1** presented deshielded shifts of C-1 ($\Delta\delta_C + 11.4$) and C-9 ($\Delta\delta_C + 11.6$) indicating the α-orientation of 2-propanol unit at C-1, which was confirmed by the ROESY correlations of H-9a and H-9b with H-1. The single-crystal X-ray crystallography by employing graphite-monochromated Cu Kα radiation of **1** confirmed the above deduction. The Flack parameter of 0.12(4) [34] allowed the assignment of the absolute configuration of **1** as 1*R*, 4*R*, 7*S*, 12*S*, 13*S*, 15*R*, 18*S* (Fig. 3). Hence, the structure of **1** was elucidated as 1-*epi*-malycorin A.

Compound **2** was isolated as colorless needles (MeOH). Its molecular formula was determined as $C_{19}H_{31}NO_4$ by the positive HRESIMS ion at *m/z* 338.2324 $[M + H]^+$ (calcd. 338.2326), requiring five degrees of unsaturation. IR absorption band at 1638 and 3447 cm^{-1} implied the presence of the carbonyl and the hydroxy groups. Comparing the 1D NMR spectroscopic data (Tables 1 and 2) of **2** with those of **1** suggested that their structures were quite similar, except for the existence of one more hydroxy group at C-17 (δ_C 75.8), as deduced from the HMBC correlations of H-17 (δ_H 3.01) with C-1 (δ_C 76.2), C-2 (δ_C 28.0), C-18 (δ_C 74.0), and C-19 (δ_C 19.8). The relative configurations at C-1, C-13 (δ_C 74.2), C-15 (δ_C 20.9) of **2** were determined to be the same as those of **1** on the basis of ROESY correlations of H-1/H-9a, H-1/H-9b, H-13/H-11a, H-15/H-6. To elucidated the absolute configuration of **2**, the single X-ray crystallography by employing graphite-monochromated Cu Kα radiation was carried out. The Flack parameter of 0.17(17) [35] and the Hooft parameter of 0.09(6) [36] assigned the absolute configuration of **2** as 1*R*, 4*R*, 7*S*, 12*S*, 13*S*, 15*R*, 17*S*, 18*S* (Fig. 3). Thus, the structure of **2** was established as 1-*epi*-17*S*-hydroxymalycorin A.

Compound **3** possessed a molecular formula of $C_{16}H_{23}NO_3$ as deduced from the HRESIMS analysis (*m/z* 278.1753 $[M + H]^+$, calcd. 278.1751), corresponding to six degrees of unsaturation. Its IR spectrum showed strong absorptions at 3449, 1637 cm^{-1}, indicated the existence of hydroxy and carbonyl groups. The ^{13}C and DEPT NMR spectroscopic data exhibited 16 carbon signals, including one methyl (δ_C 23.6), eight *sp*^{3} methylenes (δ_C 24.5, 24.6, 28.8, 33.3, 34.1, 41.0, 50.8, and 51.8), three *sp*^{3} methines (δ_C 28.9, 55.7, and 76.3), and four *sp*^{2} quaternary carbons (δ_C 137.3, 142.6, 175.5, and 221.3). The above NMR spectroscopic data of **3** were similar to those of phlegmariurine A [14], with the only difference being that **3** possessed an extra hydroxy group (δ_H 4.48, δ_C 76.3), which was located at C-6 based on HMBC correlations of H-6 with C-4 (δ_C 55.7), C-5 (δ_C 221.3), C-8 (δ_C 34.1), and H-4 (δ_H 2.76) and H-8a (δ_H 2.27) with C-6. In the ROESY experiment, the observed correlations of H-2b/H-15 and H-2b/H-6 demonstrated that these protons had identical orientations, which supported the structural assignment of **3** as 6α-hydroxyphlegmariurine A. Fortunately, a colorless crystal of **3** were obtained by recrystallization from MeOH, which allowed a successful performance of X-ray crystallography by employing graphite-monochromated Cu Kα radiation. The single-crystal X-ray diffraction study not only determined the structure of **3** as assigned by spectroscopic data analysis but also confirmed its relative configurations of C-4, C-6, C-15 (Fig. 4). Therefore, the structure of **3** was established, and it was named 6α-hydroxyphlegmariurine A.

Compound **4** was isolated as colorless crystals (MeOH). Its molecular formula, $C_{16}H_{25}NO_4$, was established on the basis of HRESIMS for the $[M + H]^+$ ion at *m/z* 296.1859 (calcd. 296.1856), indicating five degrees of unsaturation. The IR spectrum showed absorptions for a hydroxy and a carbonyl groups at 3425 and 1742 cm^{-1}, respectively. The ^{13}C NMR spectroscopic data of **4** exhibited 16 carbon signals (Table 2), which were classified from HSQC spectra as one methyl (δ_C 22.2), eight *sp*^{3} methylenes (δ_C 20.8, 25.5, 32.5, 39.5, 44.2, 44.8, 51.8, and 62.5), three *sp*^{3} methines (δ_C 24.3, 39.1, 65.7), three *sp*^{3} quaternary carbons (δ_C 50.2, 79.1, and 87.1), and one *sp*^{2} quaternary carbon (δ_C 213.0). The 1D NMR spectroscopic data (Tables 1 and 2) of **4** exhibited many similarities to those of fawcettimine [14], suggesting their structures to be closely related. The differences were the appearance of one oxygenated quaternary carbon (δ_C 79.1) and one oxygenated methine (δ_C 65.7) in **4**

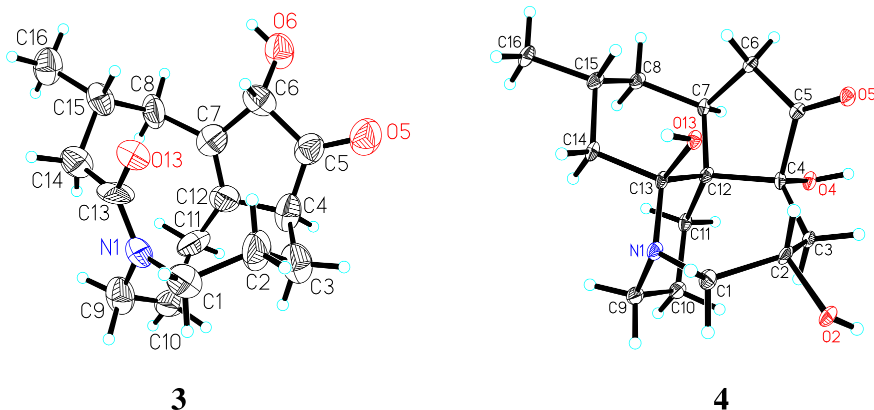

Fig. 4. The X-ray structures of compounds **3** and **4**.

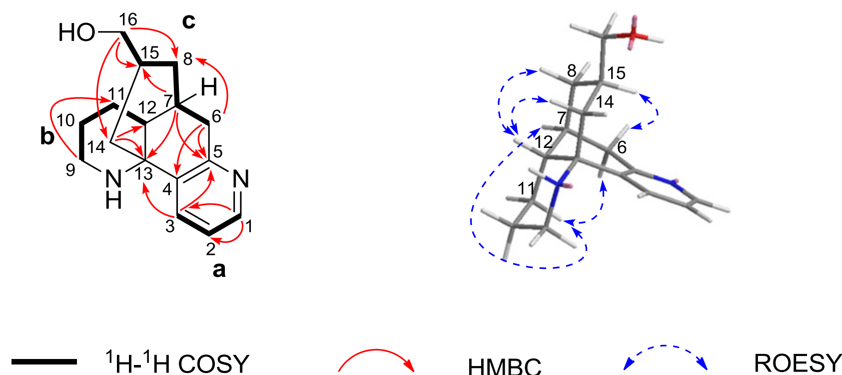

Fig. 5. The key 2D–NMR correlations of compound **5**.

and the absence of one methine and one methylene in fawcettimine, which indicated that **4** was a dihydroxy derivative of fawcettimine. The obvious downfield shifts of C-1 (δ_C 62.5), C-2 (δ_C 65.7) and C-4 (δ_C 79.1) and the key HMBC correlations from H_2-1 (δ_H 2.98, 4.27) and H-3a (δ_H 2.83) to C-2 and H-3 to C-4 indicated that two hydroxy groups were placed at C-2 and C-4, respectively. Due to some overlapping signals in the ^{1}H NMR spectrum of **4**, no solid evidences could be used to determine the configurations of C-2, C-4, and C-13. Thus, a single X-ray diffraction was necessary to assign the stereochemistry of chiral centers in **4**. Fortunately, a suitable single crystal of **4** was obtained from MeOH and the single X-ray crystallography was achieved by an experiment using Cu Kα radiation (Fig. 4). The Hooft parameter of 0.09(11) [36] allowed the assignment of the absolute configuration of **4** as 2*S*, 4*R*, 7*S*, 12*S*, 13*S*, 15*R*. Consequently, the structure of **4** was deduced as 2*S*,4*R*-dihydroxyfawcettimine.

Compound **5**, was obtained as colorless oil. The HRESIMS showed a pseudo molecular ion peak at *m/z* 259.1808 $[M + H]^+$ (calcd. 259.1805), which established a molecular formula of $C_{16}H_{22}N_2O$, indicating seven degrees of unsaturation. The IR absorption bands at 1632 and 3422 cm^{-1} suggested the existence of double bond and amino/hydroxy groups, respectively. Analysis of the 1D NMR spectroscopic data revealed the existence of 16 carbons attributed to two sp^2 quaternary carbons (δ_C 159.4, 137.4), one sp^3 quaternary (δ_C 57.9), three sp^2 methines (δ_C 147.7, 135.9, and 123.4), three sp^3 methines (δ_C 34.2, 35.1 and 44.8), seven sp^3 methylenes (δ_C 27.1, 27.4, 35.7, 38.9, 42.3, 46.3, and 68.4). Based on the above data, the structure of **5** was deduced to be a lycodine-type alkaloid, which resembled that of lycodine [24]. The sole difference was that the Me-16 in **5** was replaced by CH_2OH group as revealed by HMBC cross-peaks of H_2-16 (δ_H 3.29, 3.25) with C-8 (δ_C 38.9), C-14 (δ_C 46.3), and C-15 (δ_C 35.1). To our knowledge, compound **5** was the fifth lycodine-type alkaloid with the CH_2OH group at C-15 except the previously reported ones, casuarinines E and F [37], huperzine D [37], and 16-hydroxyhuperzine B [38]. The ROSEY correlations of H-6a/H-11b, H-7/H-11b, H-12/H-8b, H-12/H-14a indicated that H-7 and H-12 were α- and β-orientated, respectively. Furthermore, the observed correlations between H-6b and H-15 demonstrated the β-orientation of H-15 (Fig. 5). Finally, the structure of **5** was elucidated as 16-hydroxylycodine. The absolute configuration of **5** was defined by comparison of its ECD spectrum (Supplementary data, Fig. S51) with that of casuarinine *J* [37], whose absolute configuration has been unequivocally established by the observed Cotton effects. The positive Cotton effect at the corresponding UV absorption band around 278 nm and the negative value near 257 nm for both **5** and casuarinine *J* indicated that they have the same absolute configurations at C-13 (13*R*). Accordingly, the absolute configuration of **5** was assigned as 7*S*, 12*R*, 13*R*, 15*R*.

The isolated new compounds **1**–**5** were tested for in vitro AChE inhibitory activity using the Amplex® Red fluorescence method reported previously (with Tacrine as the positive control, $IC_{50} = 0.33$ μM). Unfortunately, all compounds were inactive against AChE inhibitory activity. All new compounds were also evaluated for in vitro cytotoxicity against four human tumor cell lines (HepG2, HCT116, A549 and AGS), but showed no activity with IC_{50} values of more than 40 μM.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

The 1D NMR, 2D NMR, IR, UV, and HR-ESI-MS spectra for compounds **1**–**5**, are available in the Supplementary data associated with this article can be found in the online version, at doi: 10.1016/j.fitote.2016.10.005.

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