Two new Lycopodium alkaloids from *Phlegmariurus phlegmaria* (L.) Holub

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Two new Lycopodium alkaloids, 4β-hydroxynankakurine B (1) and Δ\textsuperscript{13,14}N\textsubscript{α}-methylphlegmarine-N\textbeta-oxide (2), together with three known analogues, lycoposerramine E (3), nankakurine B (4) and lobscurinol (5), were isolated from *Phlegmariurus phlegmaria*. Their structures were established by mass spectrometry and 1D and 2D NMR techniques.

Keywords: *Phlegmariurus phlegmaria*; Huperziaceae; Lycopodium alkaloids

1. Introduction

The Huperziaceae is comprised of two genera, *Huperzia* and *Phlegmariurus* (Zhang & Zhang 2004). Since huperzine A, a potent, reversible and selective acetylcholinesterase inhibitor and a promising drug for the treatment of symptoms of Alzheimer’s disease, was discovered from *Huperzia serrata* (Thunb. Ex Murray) Trev. (White et al. 2013; Ding et al. 2014), numerous efforts on the isolation of new potent alkaloids from *H. serrata* and related plants have been carried out by many research groups, which led to the isolation of a large family of plant constituents, Lycopodium alkaloids with diverse structures including many unusual skeletons of interest from biogenetic and biological points of view and challenging targets for total synthesis (Liu et al. 1986; Ma & Gang 2004; Ma et al. 2006; Sizemore & Rychnovsky 2014). *Phlegmariurus phlegmaria* (L.) Holub (= *Lycopodium phlegmaria* L.), a medium-sized

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epiphytic fern, distributed in south China and other countries, is a traditional Chinese medicinal plant for the treatment of rheumatic pain, arthritis, traumatic injury, sore throat, edema and urticaria (Wu 1990). Previous investigations have shown that this plant is a rich source of Lycopodium alkaloids possessing diverse structures of unprecedented skeletons (Inubushi & Harayama 1982; Hirasawa et al. 2008, 2013). As a part of our research of structurally unique and biologically active compounds from medicinal plants of Yunnan, China, we have isolated and identified two new Lycopodium alkaloids, 4β-hydroxynankakurine B (1) and Δ13,14,Nα-methylphlegmarine-Νβ-oxide (2), together with three known analogues, lycoposerramine E (3), nankakurine B (4) and lobscurinol (5) from P. phlegmaria (Figure 1).

2. Results and discussion

Compound 1 was obtained as colourless gums. The ESI-MS analysis gave \( m/z \) 293 [M + H]⁺, while HR-ESI-MS analysis gave \( m/z \) 293.2596 [M + H]⁺ and established the molecular formula as C₁₈H₃₂N₂O, implying four degrees of unsaturation. The UV spectrum showed absorption at 206.6 nm. The IR spectrum was indicative of the presence of hydroxyl group (3426 cm⁻¹). The \(^1\)H and \(^13\)C data displayed three methyls, eight sp³ methylenes, six sp³ methines and one sp³ quaternary carbon. Among them, two methyls (\( \delta_C \) 42.7, \( \delta_H \) 2.06; \( \delta_C \) 40.0, \( \delta_H \) 2.57), two methylenes (\( \delta_C \) 59.7, \( \delta_H \) 3.05 and 2.17; \( \delta_C \) 48.1, \( \delta_H \) 3.00 and 2.49), one methine (\( \delta_C \) 63.6, \( \delta_H \) 2.03) and one quaternary carbon (\( \delta_C \) 62.5) were ascribed to link with N atom, and the methine (\( \delta_C \) 66.2, \( \delta_H \) 3.89) was determined to be O-bearing. Careful inspection in the \(^1\)H and \(^13\)C NMR spectra revealed that 1 was similar to nankakurine B (4) also isolated in this study. 1 differs from 4 only in the existence of an additional hydroxyl group. \(^1\)H–\(^1\)H COSY and HSQC analyses revealed the existence of four fragments, a–d (Figure S1). HMBC correlations from H-4 to C-2, C-3 and C-6 (Figure S1) suggested that the hydroxyl group was located at C-4. The ROESY correlations (Figure S1) between H-4 and H-7 indicated the hydroxyl group was β-oriented. Stereochemistry of the spiro carbon C-5 was elucidated to be R by the ROESY correlations between H-18 and H-9α (Hirasawa et al. 2006). Therefore, the structure of 1 was elucidated to be 4β-hydroxynankakurine B.

Compound 2 was obtained as colourless powder. The ESI-MS analysis gave \( m/z \) 279 [M + H]⁺, while HR-ESI-MS analysis gave \( m/z \) 279.2435 [M + H]⁺ and established the molecular formula as C₁₇H₂₉N₂O, implying four degrees of unsaturation. The UV spectrum displayed absorption at 239.8 nm. The IR spectrum showed the presence of a C=N group (1635 cm⁻¹). The \(^1\)H and \(^13\)C data revealed 17 carbon signals due to two methyls, ten sp³ methylenes, four sp³ methines and one sp² quaternary carbon. \(^1\)H–\(^1\)H COSY and HSQC spectra disclosed the presence of three fragments, a–c (Figure S2). Detailed inspection in the \(^1\)H and \(^13\)C
NMR spectra disclosed that the NMR data of 2 exhibited similarities with those of \( N_\alpha \)-methylphlegmarine, a phlegmarine-skeleton alkaloid (Wolfe et al. 2010). The only sp\(^2\) quaternary carbon appeared at \( \delta_C 150.5 \) indicated the presence of a nitrene moiety, which was confirmed by downfield shift of H-9 (\( \delta_H 3.84, 3.77 \)) (Gao et al. 2008). The downfield shift of C-9 (\( \delta_C 58.3 \)) suggested that \( N_\beta \) atom was oxidized, which was verified by the HMBC correlations from H-9, H-11, H-12 and H-14 to C-13 (Figure S2). ROESY correlations (Figure S2) between H-5 and H-7 indicated that both H-5 and H-7 took \( \alpha \) orientation. ROESY correlations among H-8b, H-12 and H-14b, and among H-8a, H-14a and H-15 suggested that H-12 was \( \alpha \)-oriented, whereas H-15 was \( \beta \)-oriented. The structure of 2 was thus established as \( \Delta^{13,N,N_\alpha}-methylphlegmarine \( N_\beta \)-oxide.

By comparison of the obtained spectroscopic data with those reported in the literature, the chemical structures of known compounds were determined as lycoposerramine E (3) (Takayama et al. 2002), nankakurine B (4) (Morita et al. 2006) and lobscurinol (5) (Ayer & Kasitu 1989). 3 and 4 were isolated from the genus Phlegmariurus for the first time and 5 was originally obtained from P. phlegmaria.

3. Experimental

3.1. Apparatus and reagents

ESI-MS and HR-ESI-MS spectra were obtained from a VG Auto Spec-3000 spectrometer. IR spectra were recorded on a Bio-Rad FTS-135 polarimeter. UV spectra were measured on a UV-24021PC spectrometer. Optical rotations were determined on a Horiba SEAP-300 spectropolarimeter. NMR spectra were acquired using a Bruker Avance-500 spectrometer, using TMS as an internal standard. Column chromatography was carried out on silica gel H (Qingdao Haiyang Chemical Factory, Qingdao, China). TLC was performed on silica gel GF\(_{254}\) (Yantai Jiangyou Silica Gel Co. Ltd, Yantai, China). Solvents were of industrial purity and distilled prior to use.

3.2. Plant material

P. phlegmaria were collected from a mountain (103 \(^{0}\)50' East longitude and 22 \(^{0}\)52' North Latitude, 1200 meters above sea level) in Wenshan of Yunnan Province, China in January, 2014 and identified by Prof. Shugang Lu, School of Life Science, Yunnan University, Kunming, China, where a voucher specimen (No. 1401003) is deposited.

3.3. Extraction and isolation

The whole plants of P. phlegmaria (1.0 kg) were extracted with MeOH (10 L \times 4) at room temperature, and the extract was concentrated, added with 1% aq. H\(_2\)SO\(_4\) to about 100 mL, and then partitioned with EtOAc (Wu et al. 2014). The acidic aqueous phase was basified with aq. Na\(_2\)CO\(_3\) to pH 10 and then extracted with CHCl\(_3\) (500 mL \times 3). The CHCl\(_3\)-soluble phase (8 g) was subjected to silica gel column (6 \times 50 cm, CHCl\(_3\)/MeOH, 1:0→5:1) to provide fractions A–C. Fr. A (1.5 g) was further chromatographed on silica gel column (3 \times 30 cm, CHCl\(_3\)/MeOH, 10:1) to afford 3 (50 mg). Fr. B (3.5 g) was separated by silica gel column (3 \times 30 cm, petroleum ether/acetone/diethylamine, 10:1:1) to yield 1 (120 mg) and 4 (40 mg). Fr. C (1.0 g) was chromatographed on silica gel column (3 \times 30 cm, petroleum ether/acetone/diethylamine, 5:1:1) to obtain 2 (30 mg) and 5 (35 mg).

3.3.1. 4\( \beta \)-Hydroxynankakurine B (1)

Colourless gum; \([\alpha]_D^{19} + 4.81 \) (c 0.0038, CHCl\(_3\)); ESI-MS \( m/z \): 293 [M + H]\(^+\); HR-ESI-MS \( m/z \): 293.2596 [M + H]\(^+\) (calcd for C\(_{18}\)H\(_{33}\)N\(_2\)O: 293.2587); UV (MeOH): \( \lambda_{\text{max}} \) 206.6 nm; IR (KBr):
3426, 2945, 2867, 2772, 1636, 1455; $^1$H-NMR (CDCl$_3$, 500 MHz): $\delta$ 3.89 (1H, m, H-4), 3.05 (1H, d, $J$ = 12.0 Hz, H-9a), 3.01 (1H, td, $J$ = 12.5, 5.0 Hz, H-1a), 2.94 (1H, d, $J$ = 12.5 Hz, H-6a), 2.57 (3H, s, H-18), 2.49 (1H, dd, $J$ = 12.0, 6.0 Hz, H-1b), 2.37 (1H, m, H-10), 2.17 (1H, dd, $J$ = 12.0, 3.0 Hz, H-9b), 2.06 (3H, s, H-17), 2.03 (1H, m, H-13), 2.01 (1H, overlapped, H-15), 1.99 (1H, overlapped, H-14a), 1.95 (1H, overlapped, H-7), 1.92 (1H, m, H-2a), 1.69 (1H, ddd, $J$ = 13.5, 5.5, 2.0 Hz, H-3a), 1.65 (1H, m, H-11a), 1.58 (1H, m, H-3b), 1.55 (1H, m, H-6b), 1.52 (1H, overlapped, H-8a), 1.52 (1H, overlapped, H-12), 1.38 (1H, overlapped, H-11b), 1.38 (1H, overlapped, H-2b), 1.16 (1H, td, $J$ = 12.5, 5.0 Hz, H-8b), 0.88 (1H, dt, $J$ = 12.5, 1.5 Hz, H-14b), 0.85 (3H, d, $J$ = 6.5 Hz, H-16); $^{13}$C-NMR (CDCl$_3$, 125 MHz): $\delta$ 66.2 (d, C-4), 63.6 (d, C-13), 62.5 (s, C-5), 59.7 (t, C-9), 48.1 (t, C-14), 40.9 (t, C-8), 40.0 (q, C-18), 39.0 (t, C-12), 35.7 (d, C-11), 32.4 (d, C-7), 32.1 (t, C-11), 31.7 (t, C-6), 31.0 (t, C-10), 25.3 (t, C-3), 22.7 (q, C-16), 21.0 (d, C-15), 19.6 (t, C-2).  

3.3.2. $\Delta^{13,N}$, $N_a$-Methylphlegmarine-$N^\beta$-oxide (2)

Colourless powder; [$\alpha$]$^\text{D}$ + 79.49 (c 0.0026, MeOH); ESI-MS $m/z$: 279 [M + H]$^+$; HR-ESI-MS $m/z$: 279.2435 [M + H]$^+$ (calcd for C$_{17}$H$_{31}$N$_2$O: 279.2431); UV (MeOH): $\lambda_{\text{max}}$ 239.8 nm; IR (KBr): 3430, 2929, 2866, 2779, 2722, 1708, 1635, 1446, 1170; $^1$H-NMR (CDCl$_3$, 500 MHz): $\delta$ 3.84 (1H, overlapped, H-9a), 3.83 (1H, overlapped, H-14a), 3.77 (1H, overlapped, H-9b), 2.94 (1H, d, $J$ = 12.0 Hz, H-1a), 2.36 (3H, s, H-17), 2.33 (1H, overlapped, H-1b), 2.20 (1H, overlapped, H-11a), 2.20 (1H, overlapped, H-5), 2.07 (1H, m, H-6a), 2.00 (1H, m, H-12), 1.97 (1H, m, H-10a), 1.90 (1H, dd, $J$ = 13.0, 2.5 Hz, H-8a), 1.79 (1H, m, H-10b), 1.74 (1H, m, H-2a), 1.67 (1H, overlapped, H-4a), 1.66 (1H, overlapped, H-15), 1.63 (1H, overlapped, H-3a), 1.63 (1H, overlapped, H-2b), 1.43 (1H, m, H-4b), 1.40 (1H, overlapped, H-14b), 1.35 (1H, overlapped, H-7), 1.35 (1H, overlapped, H-3b), 1.34 (1H, overlapped, H-11b), 1.05 (1H, ddd, $J$ = 17.0, 7.5, 5.5 Hz, H-6b), 1.02 (3H, d, $J$ = 6.5 Hz, H-16), 0.84 (1H, dd, $J$ = 25.0, 12.0 Hz, H-8b); $^{13}$C-NMR (CDCl$_3$, 125 MHz): $\delta$ 150.5 (s, C-13), 62.3 (d, C-5), 58.3 (t, C-9), 56.1 (t, C-1), 43.3 (d, C-12), 42.4 (d, C-7), 41.2 (q, C-17), 41.0 (t, C-8), 37.9 (t, C-6), 34.1 (t, C-14), 31.7 (t, C-4), 30.8 (d, C-15), 24.3 (t, C-11), 23.8 (t, C-3), 23.7 (t, C-2), 22.2 (q, C-16), 21.6 (t, C-10).

4. Conclusion

In our study, five Lycopodium alkaloids were isolated from P. phlegmaria and their structures were elucidated as 4$\beta$-hydroxynankakurine B (1), $\Delta^{13,N}$, $N_a$-methylphlegmarine-$N^\beta$-oxide (2), lycoserramine E (3), nankakurine B (4) and lobscurinol (5). 1 and 2 were two new alkaloids, whereas 3 and 4 were isolated from the genus Phlegmariurus for the first time and 5 was originally obtained from P. phlegmaria.

Supplementary material

Supplementary material relating to this article is available online.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Note
1. Zhichong Wang and Jichun Wu contributed equally to this work.

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