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Huperphlegmines A and B, two novel *Lycopodium* alkaloids with an unprecedented skeleton from *Huperzia phlegmaria*, and their acetylcholinesterase inhibitory activities

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ABSTRACT

Two novel *Lycopodium* alkaloids, huperphlegmines A and B (1 and 2), were isolated from the aerial parts of *Huperzia phlegmaria* collected in Vietnam, together with the five known compounds lycophlegmariol A (3), phlegmariurine B (4), 5-hydroxymethyl-2-furaldehyde (5), rhemanone C (6), and loliolide (7). The chemical structures of the present compounds were elucidated by means of 1D and 2D NMR and HRESIMS spectroscopy, and by comparisons to the reported data in the literature. Compounds 1 and 2 showed moderate acetylcholinesterase inhibitory activities, with IC_{50} values of 25.95 ± 0.67 and 29.14 ± 0.77 μg/mL, respectively.

1. Introduction

Huperzia phlegmaria (L.) Rothm. (Lycopodiaceae) [synonym: *Lycopodium phlegmaria* L.] is an epiphytic club moss found on tree trunks and rocks in forests located at heights of 100–2400 m in China, Cambodia, India, Japan, Laos, Nepal, Thailand, Vietnam, Pacific islands, Paleotropics, and South America [1]. All parts of this plant have been used in traditional Chinese medicine for the treatment of rheumatic pain, arthritis, traumatic injury, sore throat, edema, and urticaria for many years [2]. Furthermore, this plant is also used as a potential medicinal herb for the treatment of several brain diseases, such as Alzheimer's disease [3]. Previous phytochemical investigations of *H. phlegmaria* revealed that its main constituents are serratane-type triterpenes [4–6], *Lycopodium* alkaloids [7–9], and abietane diterpenes [6]. Huperzine A, the *Lycopodium* alkaloid being tested as an anti-Alzheimer's disease drug candidate [10, 11], has also been isolated from *H. phlegmaria*, as in the case of the other *Huperzia* species. Remarkably, *H. phlegmaria* reportedly contains high concentrations of huperzine A, ranging from 44 to 345 μg g^{-1} [12], which may account for the effectiveness of this plant in the treatment of several brain diseases. However, the presence of other constituents that are also effective for brain diseases in this plant has not been excluded, since the efficacy of the crude drug is generally associated with multiple components.

In our previous search for secondary metabolites with acetylcholinesterase (AChE) inhibitory activity in Vietnamese *H. phlegmaria*, we isolated several terpenoids and a known alkaloid with fawcettimine-related structures [13]. Furthermore, we recently identified two *Lycopodium* alkaloids, huperphlegmines A and B (1 − 2), with an unprecedented skeleton and a 2,5-substituted dihydrofuran moiety, together with five known compounds, lycophlegmariol A (3) [6], phlegmariurine B (4) [14], 5-hydroxymethyl-2-furaldehyde (5) [15], rhemanone C (6) [16], and loliolide (7) [17], from this plant (Fig. 1). Herein, we describe the isolation and structural elucidation of 1 and 2, as well as the AChE inhibitory activities of the isolates, except for 4. Remarkably, the new compounds 1 and 2 exhibited moderate AChE inhibitory activities.

2. Experimental

2.1. General experimental procedures

UV spectra were recorded on a Shimadzu UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Infrared spectra were recorded on an IR Prestige-21 spectrometer (Shimadzu, Kyoto, Japan). Optical

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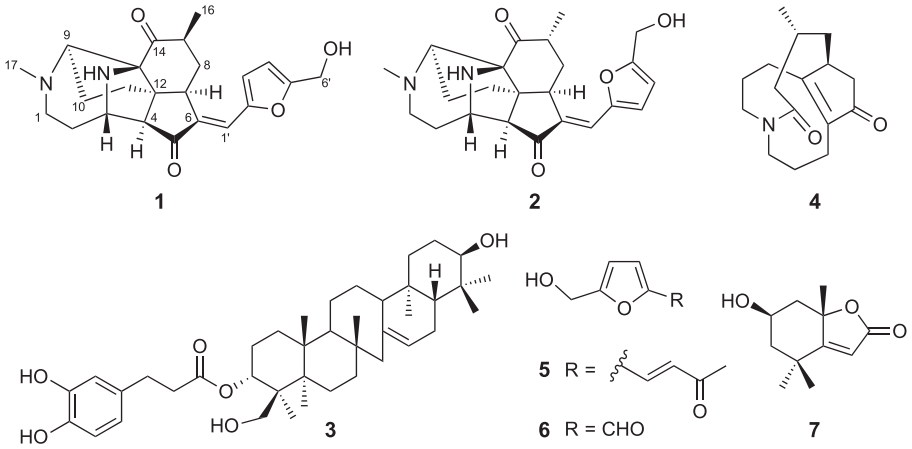

Fig. 1. Structures of compounds 1–7 isolated from the aerial parts of *H. phlegmaria*.

rotations were measured on a JASCO P-2100 polarimeter (Hachioji, Tokyo, Japan). High-resolution electron spray ionization mass spectrometry (HRESIMS) data were acquired, using a micrOTOF-Q 10187 mass spectrometer (Bruker, Massachusetts, USA). NMR spectra were recorded using a Bruker Avance 500 spectrometer (^{1}H NMR for 500 MHz, ^{13}C NMR for 125 MHz) (Bruker, MA, USA), with TMS as an internal reference. Column chromatography was performed using silica gel (60 N, spherical, neutral, 40–50 µm, Kanto Chemical Co., Inc., Tokyo, Japan), Cosmosil 75C18-OPN (Nacalai Tesque Inc., Kyoto, Japan), YMC RP-18 (Fuji Silysia Chemical Ltd., Kasugai, Aichi, Japan), and Sephadex LH-20 (Dowex® 50WX2–100, Sigma–Aldrich, USA). Analytical TLC was performed on pre-coated silica gel 60F_{254} and RP-18 F_{254} plates (0.25 or 0.50 mm thickness, Merck KGaA, Darmstadt, Germany). AChE, acetylthiocholine (ACTI), 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB), and galanthamine hydrobromide were purchased from Sigma-Aldrich (USA). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). All chemicals used were of the highest grade available.

2.2. Plant material

The aerial parts of *Huperzia phlegmaria* (L.) Rothm. were collected from Quang Tri province, Vietnam, 16°55′42.5"N 106°36′30.3"E; 16°56′40.4"N 106°33′49.9"E, in June 2016, and were identified by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, VAST, Vietnam. A voucher specimen (No. HP01) was deposited at the Faculty of Pharmacy, Hue University of Medicine and Pharmacy, Vietnam.

2.3. Extraction and isolation

The dried aerial parts of *H. phlegmaria* (1.5 kg) were extracted with MeOH (3 times, 5.0 L each) at room temperature to yield 75 g of a dark solid extract. This extract was then dissolved in 3% tartaric acid (1.0 L) and filtered to separate the solid residues (solid A). The remaining acidic solution was adjusted to pH 10 with saturated aqueous Na_2CO_3, and partitioned with CH_2Cl_2 (3 times, 2.0 L each) to obtain the total alkaloidal fraction (HC, 8 g), after removal of the solvent *in vacuo*.

The alkaloidal fraction was chromatographed on a normal phase silica gel column, eluted with a stepwise gradient of CH_2Cl_2–MeOH (40:1 to 10:1, *v*/v) to obtain 6 sub-fractions, Fr.1–Fr.6. Fr.5 (2.87 g) was subjected to Sephadex LH-20 column chromatography, eluted with MeOH to obtain 5 sub-fractions, Fr.5.1–Fr.5.5. Fr.5.3 (1.6 g) was then applied to a YMC RP-18 column, eluted with acetone–MeOH–water (1:3:1, *v*/v) to yield 7 fractions, Fr.5.3.1–Fr.5.3.7. Fr.5.3.4 (789 mg) was chromatographed on a normal phase silica gel column, eluted with CH_2Cl_2–acetonitrile-ethylamine (10:1:0.1, v/v), followed by a Sephadex LH-20 column eluted with MeOH–water (4:1, *v*/v), and then subjected to preparative reversed phase HPLC (Zorbax SB–C18 5 µm, 9.4 × 250 mm) using MeOH-water (60:40, flow rate 2 mL/min) as the eluent, to afford 1 (38.0 mg) and 2 (21.0 mg). Fr.3 (1.32 g) was separated on a Sephadex LH-20 column eluted with CH_2Cl_2-MeOH (9:1, v/v) to obtain 4 sub-fractions, Fr.3.1–Fr.3.4, and further purification of HC3.3 (660 mg) yielded compound 4 (10.2 mg).

The solid A (57 g) was washed with distilled water until neutral, and extracted with EtOAc to yield the EtOAc fraction (HE, 38 g). The EtOAc fraction was chromatographed on a normal phase silica gel column, eluted with a gradient of CH_2Cl_2–MeOH (100:0, 50:50, 0:100, *v*/v), to obtain 3 fractions (HE1–HE3), according to their TLC profiles. The fraction HE2 (4.92 g) was subjected to Sephadex LH-20 column chromatography, eluted with CH_2Cl_2-MeOH (9:1, *v*/v), to obtain 5 sub-fractions, HE2.1-HE2.5. The purification of HE2.2 (1.81 g) afforded 5 (3.4 mg), 6 (4.5 mg), and 7 (5.7 mg). A portion (7.5 g) of HE3 was chromatographed on a normal phase silica gel column, eluted with a gradient of CH_2Cl_2–acetone (60:0 to 1:1, v/v), to obtain 12 sub-fractions, HE3.1–HE3.12, and the purification of HE3.8 (1.02 g) yielded 3 (15.0 mg).

Huperphlegmine A (1): Yellow oil; $[\alpha]_D^{22}$–65 (*c* 0.1, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3439, 2926, 1701, 1612, 1570, 1456, 1169, 1020, 901, 795; UV (MeOH) λ_{max} (log ε): 347 (4.55); ^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD): see Table 1; HRESIMS *m/z* 397.2122 $[M + H]^+$ (calcd. For $C_{23}H_{29}O_4N_2$, 397.2127).

Huperphlegmine B (2): Yellow oil; $[\alpha]_D^{22}$ -162 (*c* 0.1, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3402, 2930, 1699, 1609, 1570, 1448, 1169, 1020, 901, 795; UV (MeOH) λ_{max} (log ε): 349 (4.57); ^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD): see Table 1; HRESIMS *m/z* 397.2122 $[M + H]^+$ (calcd. For $C_{23}H_{29}O_4N_2$, 397.2127).

2.4. AChE inhibition assay

The acetylcholinesterase inhibitory assay was performed in triplicate in 96-well microplates, and the absorbance was recorded using an ELISA microplate reader (EMR-500, LABOMED INC., CA, USA). The AChE inhibitory assay was performed by the colorimetric method reported by Ellman, with slight modifications [18]. ACTI was used as the substrate to examine the inhibitory effect of the samples on the AChE activities. The reaction mixture, containing 140 µL Tris-HCl buffer (pH 8.0), 20 µL of the tested sample solution, and 20 µL of the AChE solution (0.25 units/mL), was incubated at room temperature for 15 min, and the reaction was then initiated by adding 10 µL of 0.24 mM ACTI, along with 10 µL of 0.24 mM Ellman's reagent (DTNB), which produces a yellow 5-thio-2-nitrobenzoate anion. The mixture was incubated further at room temperature for 15 min, and the optical density was measured at 405 nm to calculate the percentage inhibition.