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Lycoparins A–C, new alkaloids from *Lycopodium casuarinoides* inhibiting acetylcholinesterase

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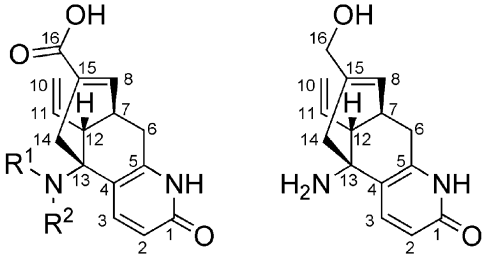
Abstract—Three new *Lycopodium* alkaloids, lycoparins A–C (**1**–**3**), have been isolated from the club moss *Lycopodium casuarinoides*. Structures and stereochemistry of **1**–**3** were elucidated on the basis of 2D NMR correlations. Lycoparins C (**3**) exhibited an inhibitory activity against acetylcholinesterase, while lycoparins A (**1**) and B (**2**) did not show activity.

1. Introduction

The *Lycopodium* alkaloids represent a large family of plant constituents obtained from the club moss belonging to Lycopodiaceae.[1] These structurally diverse alkaloids often possess unusual skeletons, and many of them continue to be of interest from biogenetic[2] and biological points of view[3] as well as providing challenging targets for total synthesis.[4] In recent 10 years, much efforts have been devoted to preparation of structurally simplified analogues and derivatives with the tricyclic skeleton of huperzine A as a promising lead compound of acetylcholinesterase inhibitors.[5]

Our interest has been focused on isolation of structurally interesting *Lycopodium* alkaloids and biosynthetic intermediates to clarify the biogenetic pathway.[6–18] In our search for new bioactive *Lycopodium* alkaloids, lycoparins A–C (**1**– **3**) were isolated from the club moss *L. casuarinoides*. This paper describes the isolation and structure elucidation of **1**–**3**, of which **3** showed inhibition of acetylcholinesterase.



1 : $R^1 = R^2 = CH_3$
2 : $R^1 = CH_3$, $R^2 = H$
3

1.1. Structures of lycoparins A–C (1–3)

The club moss *L. casuarinoides* was extracted with MeOH, and the extract was partitioned between EtOAc and 3% tartaric acid. Water-soluble materials, which were adjusted to pH 10 with sat. Na_2CO_3, were extracted with $CHCl_3$, and then *n*-BuOH. The aqueous layer was subjected to an HP-20 column (H_2O/MeOH, 1:0 → 0:1), and then an ODS column (10% MeOH → MeOH), in which a fraction eluted with 30% MeOH was purified by an amino silica gel column ($CHCl_3$/MeOH, 10:1) and then an ODS HPLC (13% CH_3CN/0.1% TFA) to afford lycoparins A (**1**, 3.0 mg, 0.0002% yield), B (**2**, 3.0 mg, 0.0002% yield), and C (**3**, 7.6 mg, 0.0005%) together with huperzines B,[19] C,[20] and D.[20]

Keywords: Alkaloids; *Lycopodium casuarinoides*; Lycoparin A; Lycoparin B; Lycoparin C; Acetylcholinesterase.
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Table 1. ^{1}H NMR data [δ_H (J, Hz)] of lycoparins A–C (**1**–**3**) in CD_3OD at 300 K

	1	2	3
2	6.56 (1H, d, 9.7)	6.54 (1H, d, 9.5)	6.41 (1H, d, 9.5)
3	7.53 (1H, d, 9.7)	7.60 (1H, d, 9.5)	7.87 (1H, d, 9.5)
6a	2.55 (1H, d, 18.2)	2.58 (1H, d, 18.6)	2.40 (1H, d, 17.7)
6b	3.13 (1H, dd, 18.2, 5.5)	3.05 (1H, m)	2.94 (1H, dd, 17.7, 5.5)
7	2.91 (1H, m)	2.97 (1H, m)	2.66 (1H, m)
8	7.03 (1H, br d, 3.8)	7.04 (1H, br d, 4.1)	5.79 (1H, m)
10a	5.46 (1H, d, 10.0)	5.49 (1H, br d, 10.4)	5.24 (1H, dd, 10.2, 2.0)
10b	5.58 (1H, d, 16.9)	5.61 (1H, d, 16.9)	5.34 (1H, dd, 16.9, 2.0)
11	6.16 (1H, ddd, 16.9 10.3, 10.0)	5.87 (1H, ddd, 16.9, 10.4, 10.0)	5.77 (1H, ddd, 16.9, 10.2, 9.9)
12	3.20 (1H, m)	3.06 (1H, m)	2.55 (1H, dd, 9.9, 3.8)
14a	2.64 (1H, d, 17.2)	2.57 (1H, m)	2.15 (1H, d, 17.1)
14b	3.22 (1H, d, 17.2)	3.01 (1H, m)	2.32 (1H, br d, 17.1)
16a			3.83 (1H, d, 30.0)
16b			3.86 (1H, d, 30.0)
17	3.02 (3H, br s)	2.81 (3H, s)	
18	3.02 (3H, br s)		

Lycoparin A {**1**, $[\alpha]_D^{27}$ + 1° (*c*, 1.0, MeOH)} was revealed to have the molecular formula $C_{17}H_{20}N_2O_3$, by HRESITOFMS [*m*/*z* 301.1541 $(M+H)^+$, Δ−1.2 mmu]. UV absorptions at 230 nm and 310 nm indicated the presence of α-pyridone ring. IR absorptions implied the presence of an α,β-unsaturated ketone (1660 cm^{-1}) and a hydroxyl (3440 cm^{-1}) groups. ^{1}H and ^{13}C NMR data (Tables 1 and 2, respectively) suggested the presence of one exomethylene, four sp^2 methines, three sp^2 quaternary carbons, two sp^3 methylenes, two sp^3 methines, one sp^3 quaternary carbon, two ketones, and two sp^3 methyl groups. Among them, one carbonyl (δ_C 164.9) and one sp^2 quaternary carbon (δ_C 146.3) were attached to the nitrogen atom in the α-pyridone ring. Another nitrogen atom was ascribed to dimethylamino group attached to the sp^3 quaternary carbon (δ_C 69.4).

Partial structures **a** (C-6–C-8 and C-10–C-12) and **b** (C-2 and C-3) were deduced from detailed analyses of ^{1}H-^{1}H COSY spectrum of **1** (Fig. 1). The structure of the α-pyridone ring (C-1–C-5 and N) was deduced from the HMBC correlations. The HMBC cross-peaks of H-3, H-11, H_3-17, and H_3-18 to C-13, and H-14 to C-4 indicated the connection among C-4, C-12, C-14, and N-2 through C-13. The connection between C-4 and C-5 was deduced from HMBC correlations of H_2-6 to C-4 and C-5. And the connection among C-8, C-14, and C-16 through quatenary carbon C-15 was elucidated by HMBC correlations for H-8 to C-15 and C-16, and H_2-14 to C-8 and C-16. Thus, the gross structure of lycoparin A (**1**) was elucidated to be **1** possessing a $C_{15}N_2$ type skeleton such as huperzine C with a carboxylic acid at C-15, instead of methyl group. The relative stereochemistry of **1** was elucidated by NOESY correlations as shown in computer-generated 3D drawing (Fig. 2). The vinyl group at C-12 was elucidated to be α-configuration by the NOESY correlation of H-6a/H-11. Thus, the relative stereochemistry of **1** was assigned as shown in Figure 2. The absolute structure was deduced to be in Figure 3 by use of Flack parameter[21] of the X-ray crystal structure of lycoparin A (**1**) TFA salt.

Table 2. ^{13}C NMR data (δ_C) of lycoparins A–C (**1**–**3**) in CD_3OD at 300 K

	1	2	3
1	164.9	165.0	164.4
2	120.8	120.5	118.5
3	139.7	139.0	142.6
4	111.8	113.4	122.2
5	146.3	145.4	143.3
6	28.5	28.6	30.0
7	39.5	37.9	37.1
8	141.7	141.8	126.7
10	122.2	123.5	120.1
11	136.5	134.5	137.7
12	43.7	43.2	51.1
13	69.4	61.4	52.1
14	37.4	35.7	44.0
15	129.5	129.1	138.4
16	168.5	169.1	66.0
17	41.0	28.3	
18	41.0		

HRESITOFMS data [*m*/*z* 287.1396, $(M+H)^+$, Δ±0.0 mmu] of lycoparin B (**2**) established the molecular formula to be $C_{16}H_{18}N_2O_3$, which was smaller than that of lycoparin A (**1**) by a CH_2 unit. ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **2** were analogous to those of **1**, although a methyl signal (δ_H 3.02) at N-2 lacking for **2** was observed for **1**. The gross structure of **2** was elucidated by 2D NMR (^{1}H–^{1}H COSY, HMQC, and HMBC) data (Fig. 4). By analysis of NOESY spectrum

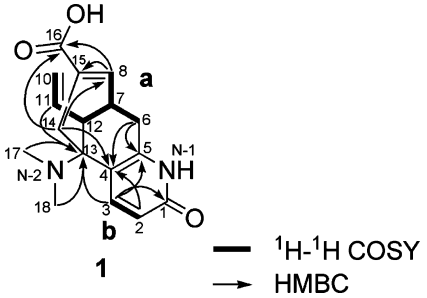

Figure 1. Selected 2D NMR correlations for lycoparin A (**1**).

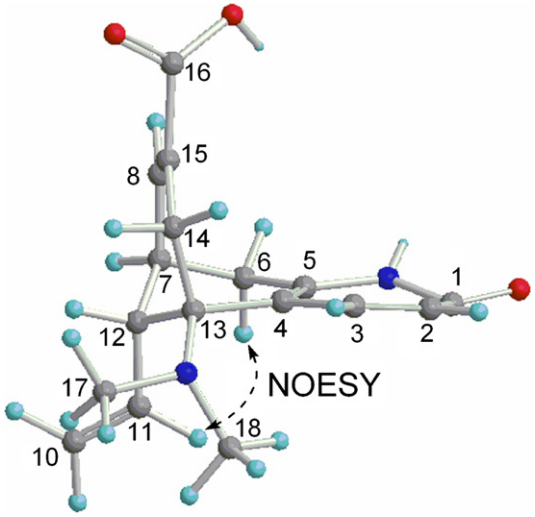

Figure 2. Selected NOESY correlation and relative stereochemistry for lycoparin A (**1**).

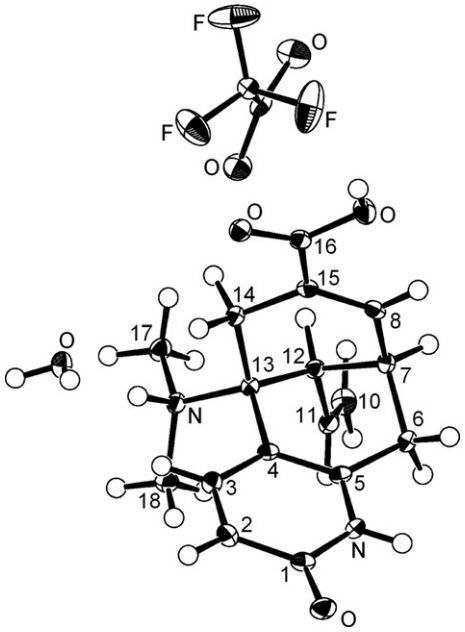

Figure 3. X-ray structure of lycoparin A (**1**) one H_2O and one TFA molecules are contained in the crystal.

as shown in Figure 5, the relative stereochemistry of **2** was assigned to be an *N*-demethyl form of lycoparin A (**1**).

Lycoparin C (**3**), colorless amorphous solid, $[\alpha]_D^{27} - 12$ (*c*, 1.0, MeOH), was shown to have the molecular formula of $C_{15}H_{18}N_2O_2$ by HRESITOFMS [*m*/*z* 259.1429, $(M+H)^+$, $\Delta -1.7$ mmu], which was smaller than that of huperzine D by 28 mmu. ^1H and ^{13}C NMR data of **3** were analogous to those of huperzine D, although two *N*-methyl signals lacking for **3** was observed for huperzine D.[20] The gross structure of **3** was elucidated by 2D NMR (^1H–^1H COSY, HMQC, and HMBC) data, and relative stereochemistry of **3** was

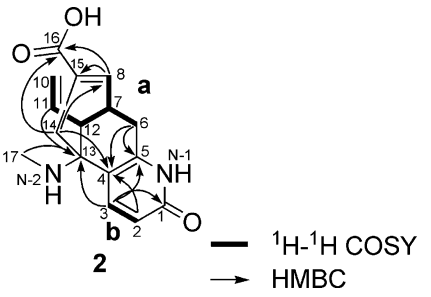

Figure 4. Selected 2D NMR correlations for lycoparin **B (2).**

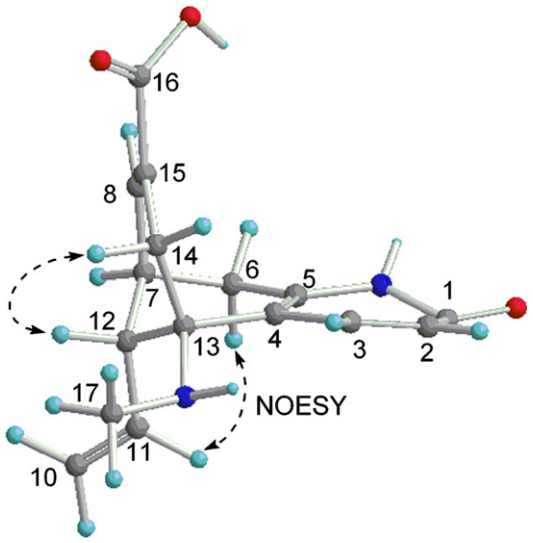

Figure 5. Selected NOESY correlations and relative stereochemistry for lycoparin B (**2**).

assigned as des-*N*-methyl form of huperzine D by NOESY correlation of H-6b/H-11 (Figs. 6 and 7).

1.2. Acetylcholinesterase inhibition

Lycoparin C (**3**) inhibited acetylcholinesterase (from bovine erythrocyte) with IC_{50} 25 μM, whereas lycoparins A (**1**) and B (**2**) possessing a carboxylic acid at C-15 and one or two *N*-methyl groups did not show such activity ($IC_{50} > 200$ μM).[22]

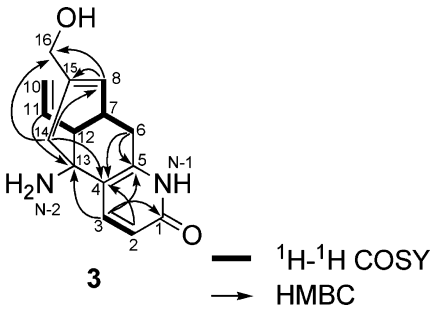

Figure 6. Selected 2D NMR correlations for lycoparin C (**3**).

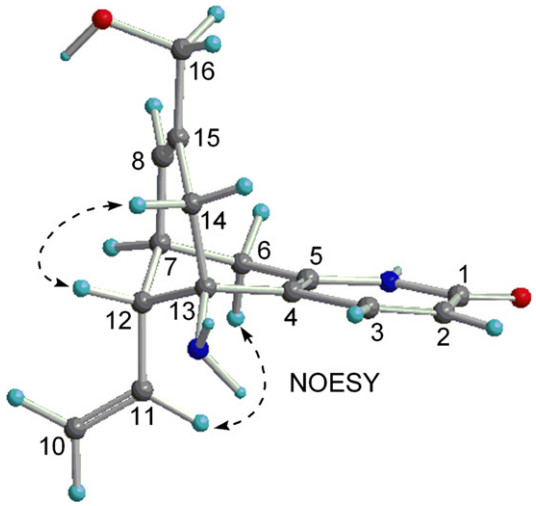

Figure 7. Selected NOESY correlations and relative stereochemistry for lycoparin C (**3**).

2. Experimental

2.1. General methods

^{1}H and 2D NMR spectra were recorded on a 600 MHz spectrometers at 300 K, while ^{13}C NMR spectra were measured on a 150 MHz spectrometer. Each NMR sample of lycoparins A–C (**1-3**) was prepared by dissolving 3.0 mg in 250 μL of CD_3OD in 5.0 mm micro cells (Shigemi Co. Ltd) and chemical shifts were reported using residual CD_3OD (δ_H 3.31 and δ_C 49.0) as an internal standard. Standard pulse sequences were employed for the 2D NMR experiments. ^{1}H–^{1}H COSY, HOHAHA, and NOESY spectra were measured with spectral widths of both dimensions of 4800 Hz, and 32 scans with two dummy scans were accumulated into 1 K data points for each of 256 t_1 increments. NOESY and HOHAHA spectra in the phase sensitive mode were measured with a mixing time of 800 and 30 ms, respectively. For HMQC spectra in the phase sensitive mode and HMBC spectra, a total of 256 increments of 1 K data points were collected. For HMBC spectra with *Z*-axis PFG, a 50 ms delay time was used for long-range C-H coupling. Zero-filling to 1 K for F_1 and multiplication with squared cosine-bell windows shifted in both dimensions were performed prior to 2D Fourier transformation.

2.2. Material

The club moss *Lycopodium casuarinoides* was collected in Kagoshima, Japan. The botanical identification was made by Mr. N. Yoshida, Health Sciences University of Hokkaido. A voucher specimen has been deposited in the herbarium of Hoshi University.

2.3. Extraction and isolation

The club moss *L. casuarinoides* (1.5 kg) was extracted with MeOH, and the extract (130 g) was partitioned between EtOAc and 3% tartaric acid. Water-soluble materials, which were adjusted to pH 10 with sat. Na_2CO_3, were extracted with $CHCl_3$, and then *n*-BuOH. The aqueous layer was subjected to an HP-20 column (H_2O/MeOH, 1:0 → 0:1), and then an ODS column (10% MeOH → MeOH), in which a fraction eluted with 30% MeOH was purified by an amino silica gel column($CHCl_3$/MeOH, 10:1) and then an ODS HPLC (13% CH_3CN/0.1% TFA) to afford lycoparins A (**1**, 3.0 mg, 0.0002% yield), B (**2**, 3.0 mg, 0.0002% yield), and C (**3**, 7.6 mg, 0.0005%) together with huperzines B, C, and D.

2.3.1. Lycoparin A (1). Colorless solid; $[\alpha]_D^{27}$ + 1° (*c* 1.0, MeOH); IR (KBr) ν_{max} 3440, 1660, 1620, 1430, and 1200 cm^{-1}; UV (MeOH) λ_{max} 310 (ε 2300) and 230 (4000) nm; ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 301 $(M+H)^+$; HRESITOFMS *m/z* 301.1541 (M+H; calcd for $C_{17}H_{21}N_2O_3$, 301.1553).

2.3.2. Lycoparin B (2). Colorless solid; $[\alpha]_D^{27}$ ± 0° (*c* 1.0, MeOH); IR (KBr) ν_{max} 3440, 1680, 1610, and 1200 cm^{-1}; UV (MeOH) λ_{max} 310 (ε 2400) and 230 (4100) nm; ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 287 $(M+H)^+$; HRESITOFMS *m/z* 287.1396 (M+H; calcd for $C_{16}H_{19}N_2O_3$, 287.1396).

2.3.3. Lycoparin C (3). Colorless solid; $[\alpha]_D^{27}$ − 12° (*c* 1.0, MeOH); IR (KBr) ν_{max} 3360, 29000, 1650, 1600, and 1560 cm^{-1}; UV (MeOH) λ_{max} 310 (ε 5000) and 230 (4000) nm; ^{1}H and ^{13}C NMR data (Tables 1 and 2); ESIMS *m/z* 259 $(M+H)^+$; HRESITOFMS *m/z* 259.1429 (M+H; calcd for $C_{15}H_{19}N_2O_2$, 259.1446).

2.4. X-ray analysis of lycoparin A (1)

Lycoparin A (**1**) was crystallized from MeOH–H_2O to give colorless needles. Crystal data: $C_{19}H_{23}F_3N_2O_6$, crystal dimensions 0.32 × 0.25 × 0.09 mm, space group $P2_1$ (#4), a = 7.76827 (14), b = 15.1685 (3), c = 8.97298 (16) Å, β = 118.4745(7)°, V = 929.41(3) Å^3, Z = 2, D_{calc} = 1.545 g/cm^3. All measurements were made on a Rigaku RAXIS RAPID imaging plate area detector with graphite monochromated Cu-Kα radiation (λ = 1.54187 Å). The data were collected at a temperature of −180 ± 1°C to a maximum 2θ value of 136.5°. A total of 96 oscillation images were collected. Of the 17084 reflections that were collected, 3352 were unique (R_{int} = 0.037); equivalent reflections were merged. The linear absorption coefficient, μ, for Cu-Kα radiation is 11.617 cm^{-1}.

The structure was solved by direct methods and expanded using Fourier techniques. The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were refined using the riding model. The final cycle of full-matrix least-squares refinement on F^2 was based on 3352 observed reflections and 274 variable parameters and converged (largest parameter shift was 0.00 times its esd) with unweighted and weighted agreement factors of $R_1 = \Sigma \; | \; |F_o|-|F_c| \; |/\Sigma \; |F_o| = 0.0278$ $[I > 2.00\sigma(I)]$ and $wR_2 = [\Sigma w(F_o^2-F_c^2)^2/\Sigma w(F_o^2)^2]^{1/2} = 0.0730$. The absolute structure was deduced based on Flack parameter, −0.01(10), using 1603 Friedel pairs.[21]

Neutral atom scattering factors were taken from Cromer and Waber.[23] Anomalous dispersion effects were included in F_{calc}; the values for $\Delta f'$ and $\Delta f''$ were those of Creagh and McAuley.[24] The values for the mass attenuation coefficients are those of Creagh and Hubbell.[25] All calculations were performed using the CrystalStructure crystallographic software package[26] except for refinement, which was performed using SHELXL-97. The refined fractional atomic coordinates, bond lengths, bond angles, and thermal parameters have been deposited at the Cambridge Crystallographic Data Centre (Deposition No.: CCDC 684133).

2.5. Acetylcholinesterase inhibitory activity

Assay of acetylcholinesterase inhibition was carried out according to colorimetric Ellman method described in Ref. 22.

Acknowledgments

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