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Bioorganic & Medicinal Chemistry 15 (2007) 1703–1707
Bioorganic & Medicinal Chemistry

Carinatumins A–C, new alkaloids from *Lycopodium carinatum* inhibiting acetylcholinesterase

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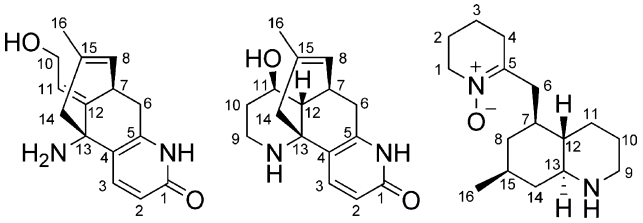
Received 10 November 2006; revised 4 December 2006; accepted 4 December 2006
Available online 15 December 2006

Abstract—Three new *Lycopodium* alkaloids, carinatumins A–C (**1**–**3**), have been isolated from the club moss *Lycopodium carinatum*. Structures and stereochemistry of **1**–**3** were elucidated on the basis of 2D NMR correlations. Carinatumins A (**1**) and B (**2**) exhibited a potent inhibitory activity against acetylcholinesterase.

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 ten 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 bioactive *Lycopodium* alkaloids, carinatumins A–C (**1**–**3**), new alkaloids showing potent inhibitory activity against acetylcholinesterase, were isolated from the club moss *L. carinatum*. This paper describes the isolation and structure elucidation of **1**–**3**, of which **1** and **2** showed inhibition of acetylcholinesterase.



1 **2** **3**

2. Structures of carinatumins A–C (1–3)

The club moss *L. carinatum* 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. *n*-BuOH-soluble materials (1.14 g) were subjected to an ODS column (10% MeOH→MeOH), in which a fraction eluted with 50% MeOH was purified by an LH-20 column ($CHCl_3$/MeOH, 1:1), a silica gel column ($CHCl_3$/MeOH/TFA, 1:0:0→5:5:1), and then an ODS HPLC (10→50% MeOH/0.01% TFA) to afford carinatumins A (**1**, 1.6 mg, 0.0002% yield) and B (**2**, 0.6 mg, 0.00007% yield) together with huperzine A. Furthermore, a fraction eluted with 70% MeOH in the ODS column was purified by

Keywords: Alkaloids; *Lycopodium*; Carinatumin A; Carinatumin B; Carinatumin C; Acetylcholinesterase.
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doi:10.1016/j.bmc.2006.12.005

Table 1. ^{1}H NMR Data [δ_H (J, Hz)] of carinatumins A–C (**1**–**3**) in CD_3OD at 300 K

	1	2	3
1			3.75 (2H, br t, 5.7)
2	6.45 (1H, d, 9.3)	6.50 (1H, d, 9.6)	1.95 (2H, m)
3	7.65 (1H, d, 9.3)	7.59 (1H, d, 9.6)	1.75 (2H, m)
4			2.53 (2H, m)
6a	2.93 (1H, dd, 17.0, 5.1)	2.96 (1H, dd, 18.6, 5.4)	2.26 (1H, dd, 13.1, 10.6)
6b	2.69 (1H, d, 17.0)	2.46 (1H, d, 18.6)	2.80 (1H, dd, 13.1, 3.7)
7	3.76 (1H, br t)	3.03 (1H, m)	1.81 (1H, m)
8a	5.51 (1H, br d, 4.8)	5.66 (1H, d, 5.4)	0.86 (1H, ddd, 12.5, 12.5, 12.5)
8b			1.55 (1H, m)
9a		2.87 (1H, br t, 12.3)	2.77 (1H, ddd, 12.7, 11.5, 2.2)
9b		3.34 (1H, m)	3.16 (1H, br d, 11.5)
10a	4.25 (2H, d, 6.2)	2.13 (1H, br d, 9.0)	1.61 (1H, m)
10b		1.81 (1H, m)	1.86 (1H, m)
11a	5.49 (1H, t, 6.2)	3.60 (1H, ddd, 10.8, 10.4, 4.4)	1.13 (1H, dddd, 12.5, 12.5, 11.4, 3.6)
11b			2.07 (1H, br d, 11.4)
12		1.94 (1H, dd, 10.4, 3.6)	1.05 (1H, m)
13			2.53 (1H, m)
14a	2.42 (1H, d, 16.2)	2.63 (1H, d, 16.2)	1.01 (1H, ddd, 12.1, 12.1, 12.1)
14b	2.58 (1H, d, 16.2)	2.24 (1H, d, 16.2)	1.81 (1H, m)
15			1.55 (1H, m)
16	1.62 (3H, s)	1.65 (3H, s)	0.94 (3H, d, 6.0)

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

	1	2	3
1	165.4	165.3	58.9
2	119.5	120.4	23.8
3	138.5	138.4	19.3
4	116.8	112.2	31.4
5	146.1	147.0	156.3
6	36.4	29.6	36.4
7	34.2	30.0	39.0
8	125.2	126.6	41.6
9		40.7	46.4
10	58.2	32.8	25.6
11	120.5	65.0	28.4
12	137.2	45.8	46.7
13	57.8	60.7	61.2
14	45.9	44.7	41.0
15	133.3	131.8	31.8
16	22.3	22.5	22.5

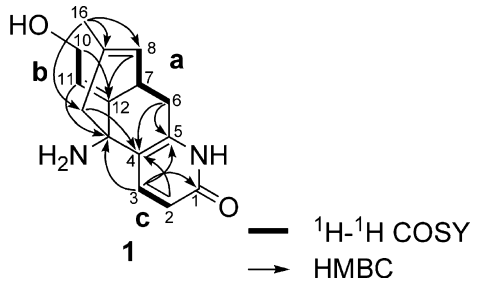

Figure 1. Selected 2D NMR correlations for carinatumin A (**1**).

an amino silica gel column (hexane/isopropanol, 9:1) to afford carinatumin C (**3**, 3.6 mg, 0.0004% yield).

Carinatumin A {**1**, $[\alpha]_D^{25}$ −30° (*c* 0.3, MeOH)} was revealed to have the molecular formula $C_{15}H_{18}N_2O_2$, by HRESITOFMS [*m*/*z* 259.1455 $(M+H)^+$, Δ+0.8 mmu]. UV absorptions at 231 and 309 nm indicated the presence of α pyridone ring. IR absorption implied the presence of αβ-unsaturated ketone (1680 cm^{-1}) and a hydroxyl (3450 cm^{-1}) group. ^{1}H and ^{13}C NMR data (Tables 1 and 2, respectively) suggested the presence of four sp^2 methines, four sp^2 quaternary carbons, three sp^3 methylenes, one sp^3 methines, one sp^3 quaternary carbon, one ketone, and one methyl group. Among them, one carbonyl (δ_C 165.4) and one sp^2 quaternary carbon (δ_C 146.1) were attached to the nitrogen atom in the α pyridone ring. Another nitrogen atom was ascribed to an amino group attached to the sp^3 quaternary carbon (δ_C 57.8).

Partial structures **a**–**c** (C-6 ∼ C-8, C-10 ∼ C-11, and C-2 and C-3) were deduced from detailed analyses of 2D NMR data (^{1}H–^{1}H COSY) of **1** (Fig. 1). The structure of the α-pyridone ring (C1–C5 and N) was deduced from the HMBC correlations. The ^{1}H signal of H_2-10 (δ_H 4.25) which was relatively observed at downfield by anisotropic effect of double bond at C-11 confirmed the position of a hydroxyl group at C-10. Connections among the partial structures as shown in Figure 1 were derived from HMBC cross-peaks for H_2-10 and H-8 to C-12, H-6 to C-5 and C-4, H-3 and H-11 to C-13, H_3-16 to C-8, C-14, and C-15, H-2 and H-14 to C-4, and H-3 to C-1 and C-5. Thus, the gross structure of carinatumin A (**1**) was elucidated to be **1** possessing a $C_{15}N_2$ type skeleton such as huperzine A with a hydroxyl at C-10, which might be produced from $C_{16}N_2$ type skeleton such as lycodine[19] and huperzine B.[20] The relative stereochemistry of **1** was elucidated by NOESY correlations as shown in computer-generated 3D drawing (Fig. 2). Geometry of the double bond at C-11 was elucidated to be *E* by the NOESY correlation of H_2-10/H-7. Thus, the relative stereochemistry of **1** was assigned as shown in Figure 2.

Carinatumin B {**2**, $[\alpha]_D^{25}$ −16° (*c* 0.3, MeOH)} displayed a pseudomolecular ion peak at *m*/*z* 273 $(M+H)^+$ and the

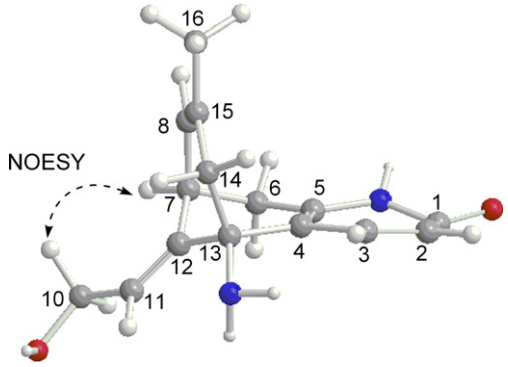

Figure 2. Selected NOESY correlation and relative stereochemistry for carinatumin A (**1**).

molecular formula, $C_{16}H_{20}N_2O_2$, was established by HRESITOFMS [*m*/*z* 273.1599, Δ−0.4 mmu]. IR absorptions implied the presence of one hydroxyl (3440 cm^{-1}) and one carbonyl (1680 cm^{-1}) functionalities. The absorptions at 231 and 309 nm in the UV spectra indicated the presence of an α-pyridone ring. ^1H and ^{13}C NMR data (Tables 1 and 2, respectively) revealed sixteen carbon signals due to one carbonyl, three sp^2 quaternary carbons, one sp^3 quaternary carbon, three sp^2 methines, three sp^3 methines, four sp^3 methylenes, and one methyl group. Partial structures **a** (C-6 ∼ C-8 and C-9 ∼ C-12) and **b** (C-2 ∼ C-3) were deduced from detailed analyses of the ^1H–^1H COSY spectrum of **2** (Fig. 3). The presence of a secondary hydroxyl group at C-11 was deduced from its ^1H and ^{13}C chemical shifts (δ_H 3.60; δ_C 65.0). Connections among the partial structures and the α-pyridone ring (C1∼C5 and N) were shown by HMBC cross-peaks for H_3-16 to C-8, C-14, and C-15, H-9 and H-3 to C-13, H-3 to C-1 and C-5, H-2 to C-4, H-6 to C-4 and C-5, and H-14 to C-4 and C-12 as shown in Figure 3. These data suggested that **2** possessed lycodine type skeleton ($C_{16}N_2$ type) such as huperzine B[20] with a hydroxyl at C-11. The relative stereochemistry of **2** was elucidated by NOESY correlations as shown in computer-generated 3D drawing (Fig. 4). NOESY correlations of H-11/H-6b and H-9a, and H-12/H-10b and H-14a indicated a β configuration of H-12 and an α configuration of H-11. Thus, the relative stereochemistry of carinatumin B (**2**) was assigned as shown.

Carinatumin C {**3**, $[\alpha]_D^{25}$ +35° (*c* 0.3, MeOH)} was revealed to have the molecular formula $C_{16}H_{28}N_2O$, by

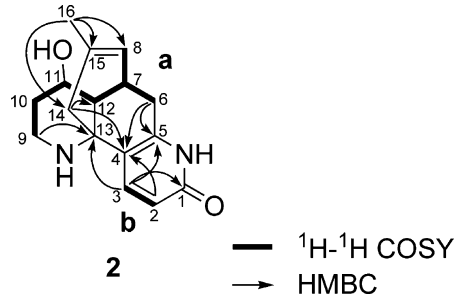

Figure 3. Selected 2D NMR correlations for carinatumin A (**2**).

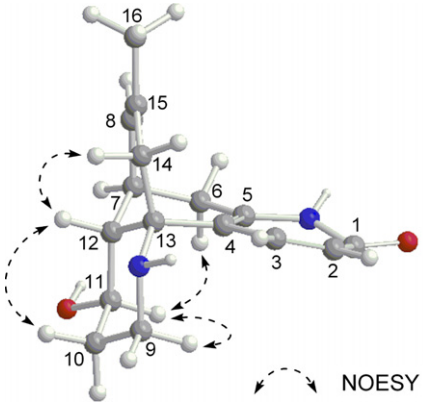

Figure 4. Selected NOESY correlations and relative stereochemistry for carinatumin B (**2**).

HRESITOFMS [*m*/*z* 265.2288 $(M+H)^+$, Δ +0.8 mmu]. ^1H and ^{13}C NMR data (Tables 1 and 2, respectively) suggested the presence of one sp^2 quaternary carbon, ten sp^3 methylenes, four sp^3 methines, and one methyl group. Among them, one sp^2 quaternary carbon, two sp^3 methylenes, and one sp^3 methine were ascribed to those bearing a nitrogen atom. The ^1H–^1H COSY spectrum in CD_3OD revealed connectivities of **a** (C-1 ∼ C-4) and **b** (C-6 ∼ C-8, C-9 ∼ C-16) as shown in Figure 5. HMBC correlations observed for H-6 to C-5 and C-4 and H-1 to C-5 revealed connectivities of C-4 to C-6, and C-1 to C-5 through a nitrogen atom. The HMBC cross-peak for H-9 to C-13 indicated that C-9 and C-13 were connected to each other through a nitrogen atom. The relative stereochemistry of **3** was eluci-

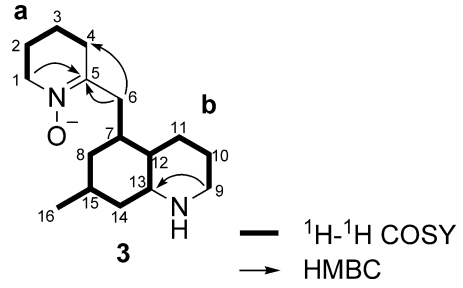

Figure 5. Selected 2D NMR correlations for carinatumin C (**3**).

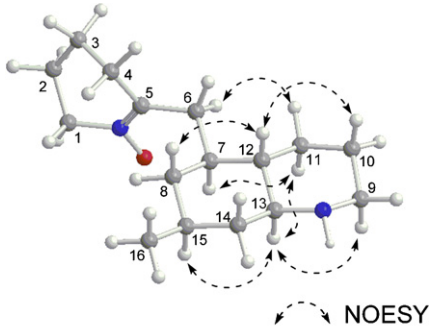

Figure 6. Selected NOESY correlations and relative stereochemistry for carinatumin C (**3**).

dated by NOESY correlations as shown in computer-generated 3D drawing (Fig. 6). The ring junction between the cyclohexane ring and the piperidine ring with chair form in the decahydroquinoline ring was elucidated to be *trans* by NOESY correlations as shown in Figure 6. The H-7 and H-15 were assigned as both α-orientation by NOESY correlations of H-7/H-11, H-13/H-15, and H-8a/H-12, and large ^{3}J coupling pattern and constants of H-8a (1H, ddd, 12.5, 12.5, 12.5). Thus, the relative stereochemistry of carinatumin C (**3**) was assigned as shown.

3. Bioactivities of carinatumins

Carinatumins A (**1**) and B (**2**) inhibited acetylcholinesterase (from bovine erythrocyte) with IC_{50} 4.6 and 7.0 μM, respectively, whereas carinatumin C (**3**) did not show such activity ($IC_{50} > 100$ μM).[21] Carinatumin A (**1**) possessing a hydroxyl at C-10 showed less potent inhibition than huperzine A (huperzine A, IC_{50} 0.8 μM), while carinatumin B (**2**) showed inhibition of acetylcholinesterase comparable to that of huperzine B (IC_{50} 8 μM).[22]

4. Experimental

4.1. General methods

^{1}H and 2D NMR spectra were recorded on a 600 MHz spectrometer at 300 K, while ^{13}C NMR spectra were measured on a 150 MHz spectrometer. Each NMR sample of carinatumins A–C (**1**–**3**) was prepared by dissolving 1.0 mg in 30 μL CD_3OD in 2.5 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 1K 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 1K 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 1K for F_1 and multiplication with squared cosine-bell windows shifted in both dimensions were performed prior to 2D Fourier transformation. FABMS was measured by using glycerol as a matrix.

4.2. Material

The club moss *L. carinatum* (Desv.ex Poir.) Trevis was collected in Cameron Highlands, Malaysia, in January 2006. The botanical identification was made by Professor Dato' Dr. Abdul Latiff Mohamed from Universiti Kebangsaan Malaysia, Bangi, Malaysia. The voucher specimen was classified as reference voucher FF/LC/01/06 and deposited in the Faculty of Pharmacy, Universiti Teknologi MARA, Shah Alam, Malaysia.

4.3. Extraction and isolation

The club moss *L. carinatum* (0.9 kg) 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. *n*-BuOH-soluble materials (1.14 g) were subjected to an ODS column (10% MeOH→MeOH), in which a fraction eluted with 50% MeOH was purified by an LH-20 column ($CHCl_3$/MeOH, 1:1), a silica gel column ($CHCl_3$/MeOH/TFA, 1:0:0→5:5:1), and then an ODS HPLC (10→50% MeOH/0.01% TFA) to afford carinatumins A (**1**, 1.6 mg, 0.0002% yield) and B (**2**, 0.6 mg, 0.00007% yield) together with huperzine A. Furthermore, a fraction eluted with 70% MeOH in the ODS column was purified by an amino silica gel column (hexane/isopropanol, 9:1) to afford carinatumin C (**3**, 3.6 mg, 0.0004% yield).

4.3.1. Carinatumin A (1). Colorless solid; $[\alpha]_D^{25}$ −30° (*c* 0.3, MeOH); IR (KBr) ν_{max} 3450, 2930, 1680, and 1200 cm^{-1}; ^{1}H and ^{13}C NMR data (Tables 1 and 2); FABMS *m/z* 259 $(M+H)^+$; HRESITOFMS *m/z* 259.1455 (M+H; calcd for $C_{15}H_{19}N_2O_2$, 259.1447).

4.3.2. Carinatumin B (2). Colorless solid; $[\alpha]_D^{25}$ −16° (*c* 0.3, MeOH); IR (KBr) ν_{max} 3440, 2980, 1680, 1260, and 1101 cm^{-1}; ^{1}H and ^{13}C NMR data (Tables 1 and 2); FABMS *m/z* 273 $(M+H)^+$; HRESITOFMS *m/z* 273.1599 (M+H; calcd for $C_{16}H_{21}N_2O_2$, 273.1603).

4.3.3. Carinatumin C (3). Colorless solid; $[\alpha]_D^{25}$ +35° (*c* 0.3, MeOH); IR (KBr) ν_{max} 3430, 2950, 1620, 1450, and 1160 cm^{-1}; ^{1}H and ^{13}C NMR data (Tables 1 and 2); FABMS *m/z* 265 $(M+H)^+$; HRESITOFMS *m/z* 265.2288 (M+H; calcd for $C_{16}H_{29}N_2O$, 265.2280).

4.4. Acetylcholinesterase inhibitory activity

Assay of acetylcholinesterase inhibition was carried out according to colorimetric Ellman method described in Ref. 21.

Acknowledgments

The authors thank Prof. Koichi Takeya, Tokyo University of Pharmacy and Life Sciences, for measurements of ESITOFMS. This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from the Takeda Science Foundation. The authors also wish to acknowledge Institute of Research, Development and Commercialisation (IRDC), UiTM, for partly supporting this research.

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