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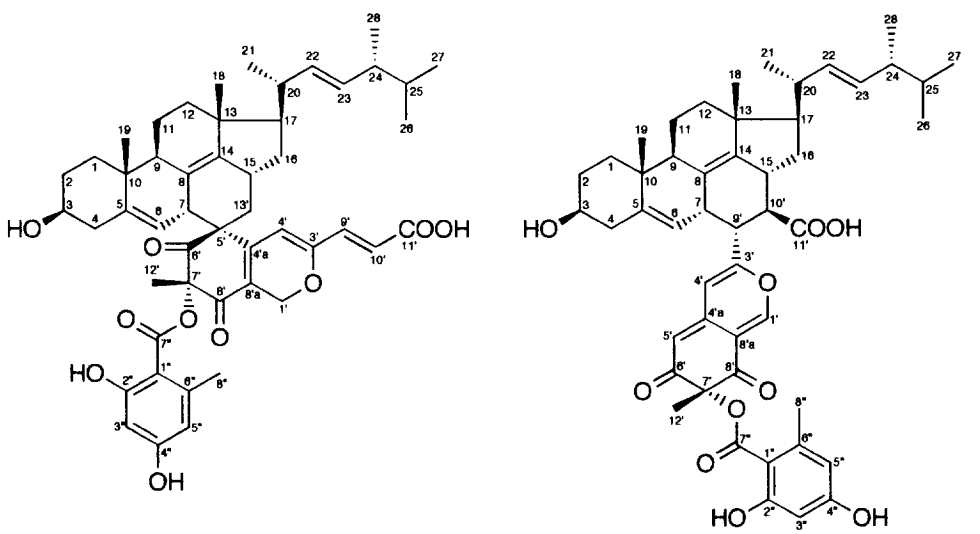
Structures of Phospholipase A_2 Inhibitors, Ergophilones A and B

Satoshi Hyodo[a*], Ken-ichi Fujita[a], Osamu Kasuya[a], Isamu Takahashi[a], Jun Uzawa[b] and Hiroyuki Koshino[b*]

a) Biological Science Laboratories, Kaken Pharmaceutical Co., Ltd., 301-Gensuke, Fujieda, Shizuoka, 426, Japan.
b) Division of Molecular Characterization, The Institute of Physical and Chemical Research (RIKEN), 2-1, Hirosawa, Wako, Saitama, 351-01, Japan.

Abstract: Two novel fungal metabolites, ergophilones A and B, were isolated as phospholipase A_2 inhibitors. The planar structures were determined on the bases of UV, MS and NMR analyses, and the stereochemistries were elucidated by the NOE experiments, coupling constant values and CD analyses. Ergophilones A and B contain three structural moieties, *i.e.*, ergostane skeleton, azaphilone chromophore and *o*-orsellinic acid, respectively.

Phospholipase A_2 (PLA2) specifically hydrolyzes the *sn*-2 position of phospholipids in the cell membranes liberating fatty acid, including arachidonic acid, and lysophospholipids. Subsequent metabolism of arachidonic acid results eicosanoid mediators that are implicated in inflammation and allergy[1]. In our screening program for new PLA2 inhibitors, we isolated novel inhibitors named ergophilones A(**1**) and B(**2**) from culture broth of *Penicillium* sp. BM-99. In this paper, we describe the structure elucidation and chemical properties of ergophilones A and B.



Ergophilone A (**1**) Ergophilone B (**2**)

Fig. 1. Chemical structures of ergophilones A and B.

RESULTS AND DISCUSSION

The culture broth of *Penicillium* sp. BM-99 was filtered, and the mycelium was extracted with 80% aqueous acetone. The crude extract was concentrated *in vacuo*; and the residual solution was extracted with EtOAc. The EtOAc layer was chromatographed on the SiO_2, Sephadex LH-20 column and ODS-HPLC, then two novel active compounds named ergophilones A(**1**) and B(**2**) were isolated.

Ergophilones A and B have the characteristic UV spectrum, respectively. The UV absorption of ergophilone B (219, 336 nm) was close similar to that of azaphilone[2]; furthermore the ^1H- and ^{13}C-NMR data (Table 1) suggested the presence of mitorubrinic acid[3] moiety. The difference between the UV spectrum of ergophilone A and that of ergophilone B indicated that ergophilone A possessed modified azaphilone chromophore. Therefore, at first we describe the structural elucidation of ergophilone B possessing usual azaphilone chromophore.

Ergophilone B(**2**) is a yellow amorphous, $[\alpha]^{25}_D$ -503° (c = 1.0, MeOH). The molecular formula of ergophilone B was determined as $C_{49}H_{58}O_{10}$ by high resolution fast-atom bombardment mass spectrum (HR-FABMS) [found m/z 807.4117 $(MH)^+$; calcd. 807.4108]. The azaphilone moiety, related to mitorubrinic acid, was confirmed by detailed NMR analyses. Remaining signals were assigned as ergosta-5,8(14),22-trien-3-ol by pulsed field gradient[4] (PFG) HMBC (Fig. 2, Fig. 3) and other NMR experiments. The PFG-DQFCOSY,

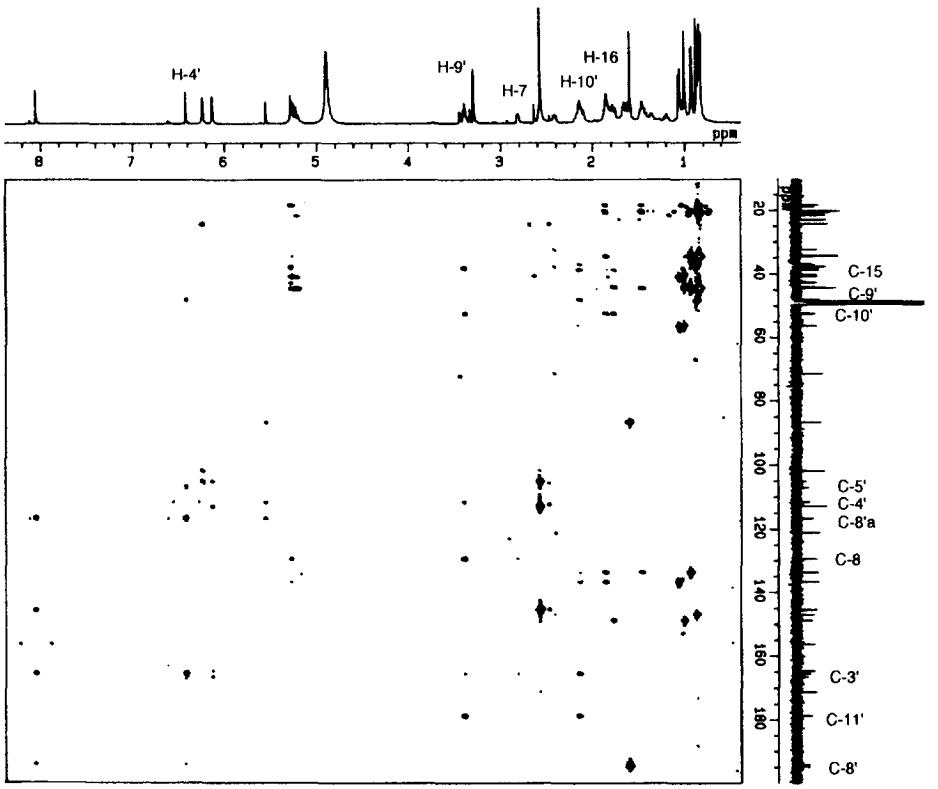


Fig. 2. PFG-HMBC spectrum of ergophilone B.

Table 1. ^{13}C(150 MHz) and ^{1}H(600 MHz) NMR data for ergophilones A and B in CD_3OD

Position	Ergophilone A (1)		Ergophilone B (2)	
	C	H	C	H
1	37.32	1.18, 1.85	37.19	1.20, 1.76
2	32.36	1.39, 1.78	32.33	1.44, 1.81
3	71.29	3.27	71.24	3.40
4	42.56	2.12, 2.28	42.53	2.18, 2.42
5	145.34		146.76	
6	120.80	5.32	120.97	5.28
7	41.71	3.55	38.03	2.82
8	128.35		129.28	
9	48.37	2.01	48.46	1.85
10	37.49		37.57	
11	20.83	1.71, 1.78	20.62	1.66, 1.66
12	40.15	1.57, 2.20	40.13	1.47, 2.12
13	44.68		43.94	
14	152.04		148.63	
15	33.80	2.54	38.51	2.57
16	37.32	1.46, 1.85	36.88	1.70, 1.85
17	56.13	1.39	56.22	1.37
18	19.98	1.03	20.18	1.01
19	18.52	0.87	18.68	0.93
20	40.68	2.15	40.56	2.15
21	21.50	1.10	21.39	1.07
22	136.71	5.25	136.70	5.22
23	133.67	5.26	133.63	5.27
24	44.33	1.85	44.27	1.86
25	34.36	1.45	34.34	1.47
26	20.50	0.85	20.49	0.86
27	20.19	0.84	20.18	0.84
28	18.26	0.93	18.14	0.88
1'	64.60	4.72, 5.20	156.12	8.06
3'	159.61		165.40	
4'	110.70	5.72	111.44	6.43
4'a	154.36		145.24	
5'	55.85		106.90	5.56
6'	207.62		194.71	
7'	86.33		86.59	
8'	191.00		194.03	
8'a	118.82		116.56	
9'	137.21	6.96	47.95	3.40
10'	124.49	6.37	52.33	2.15
11'	169.12		178.54	
12'	25.30	1.79	22.80	1.61
13'	43.25	1.32, 2.20	-	-
1"	104.65		104.95	
2"	166.62		166.36	
3"	101.80	6.15	101.75	6.14
4"	164.72		164.54	
5"	112.95	6.26	112.81	6.24
6"	145.29		145.53	
7"	170.80		171.02	
8"	24.36	2.59	24.15	2.58

HOHAHA experiments established that the 7 and 15 positions of steroid moieties were connected with the methine of the 9' and 10' positions of mitorubrinic acid moieties, respectively. Moreover, in the HMBC spectrum the long-range H-C correlations from H-7 (2.82 ppm) to C-3' (165 ppm), from H-9' (3.40 ppm) to C-8 (129.28 ppm) and from H-16 (1.70, 1.85 ppm) to C-10' (52.33 ppm) revealed that carbons C-7, 8, 14 and 15 of steroid unit and C-9' and 10' of mitorubrinic acid unit constituted the new 6-membered ring system. Consequently, the planar structure of ergophilone B was determined as shown in Fig. 1. The pulsed field gradient NMR techniques were very useful for the structure elucidation, because of their low t1 noises and short measurement time.

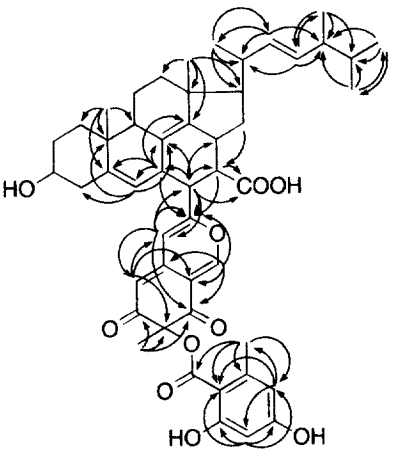


Fig. 3. H-C long-range correlations observed in HMBC spectrum of ergophilone B

The stereochemistries of the new 6-membered ring system were determined by the coupling constant values and the NOE experiments (Fig. 4). The NOEs were observed between H-19 and 7, H-7 and 15, and H-15 and 18, respectively; consequently both the H-7 and 15 were elucidated as β configuration. The configuration of H-15 and 10' was estimated *trans-diaxial* that was suggested by $^{3}J_{H\text{-}15,H\text{-}10'}$ value of 10 Hz. The NOE was observed at H-4' and 10', which indicated H-9' and 10' was *trans*, therefore, the configuration of H-7 and 9' was resulted in *cis*. The dihedral angle of H-7 and 9' was estimated approximately 20°, and that of H-9' and 10' was 140° by each coupling constant values ($^{3}J_{H\text{-}7,H9'}$ = 6 Hz, $^{3}J_{H\text{-}9',H\text{-}10'}$ = 6 Hz) and the Karplus's equation. The absolute configuration of C-7' in azaphilone unit was determined by comparison of CD spectral data to the reported values of related azaphilone compounds[2,5]. The Cotton effects of ergophilone B ($\Delta\varepsilon_{335}$ = -2.2, $\Delta\varepsilon_{303}$ = 1.0) clearly indicated the configuration at the C-7' shown in Fig. 1 (Fig. 5). The chemical shift (δ17.55) of C-28 observed in $CDCl_3$ solution was compared with

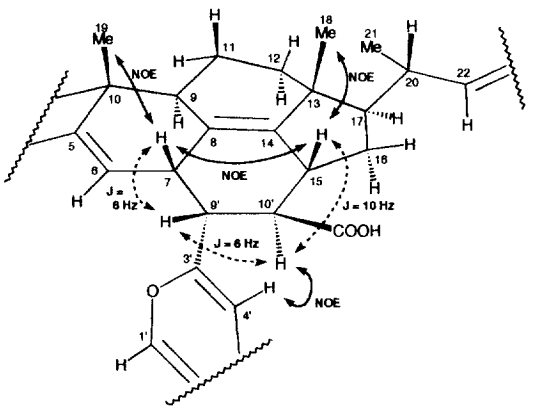


Fig. 4. Partial structures of ergophilone B.

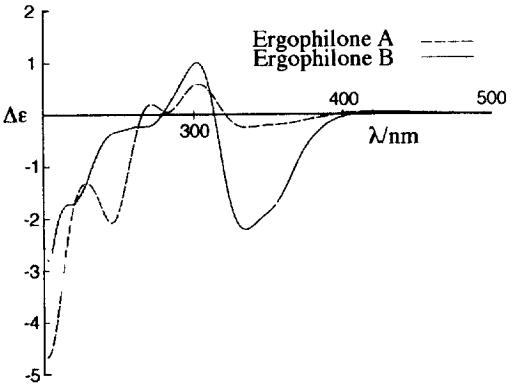


Fig. 5. CD spectra of ergophilones A and B.

that of authentic sample of ergosterol (δ17.60); thus C-24 was elucidated as R configuration[6]. Based on the above spectral data the structure of ergophilone B was determined as shown in Fig. 1.

During the NMR studies of ergophilone B, we found interesting phenomenon that the methin proton of H-5' (5.56 ppm) was exchanged for deuterium in CD_3OD during a half day.

Ergophilone A(**1**) is a yellow amorphous, $[\alpha]^{25}_D$ -222° (*c* = 1.0, MeOH). The molecular formula of ergophilone A was determined as $C_{50}H_{60}O_{10}$ by HR-FABMS [found *m/z* 821.4224 $(MH)^+$; calcd. 821.4265]. Comparison of the ^{1}H-NMR, ^{13}C-NMR (Table 1) and UV spectra of ergophilones A and B indicated that there are close similarities except azaphilone chromophore. The presence of ergosta-5,8(14),22-trien-3-ol and *o*-orsellinic acid were confirmed by NMR data. The structures of azaphilone chromophore were revealed by PFG-HMBC spectrum (Fig. 6). Namely, the H-C long-range correlations from H-1' (4.72 ppm, 5.20 ppm), H-4' (5.72 ppm) and H-12' (1.79 ppm) were observed as shown in Fig. 7. The PFG-DQFCOSY and HOHAHA spectra determined that the methylene of 13' position was connected with 15 position of steroid. In the HMBC spectrum, the H-C long-range correlations were observed from H-13' (1.32 ppm, 2.20 ppm) to C-7 (41.71 ppm), to C-14 (152.04 ppm), to C-15 (33.80 ppm), to C-5' (55.85 ppm) and to C-6' (207.62 ppm). Additionally, the H-C long-range correlations were observed from H-7 (3.55 ppm) to C-4'a (154.36 ppm) and to C-5'

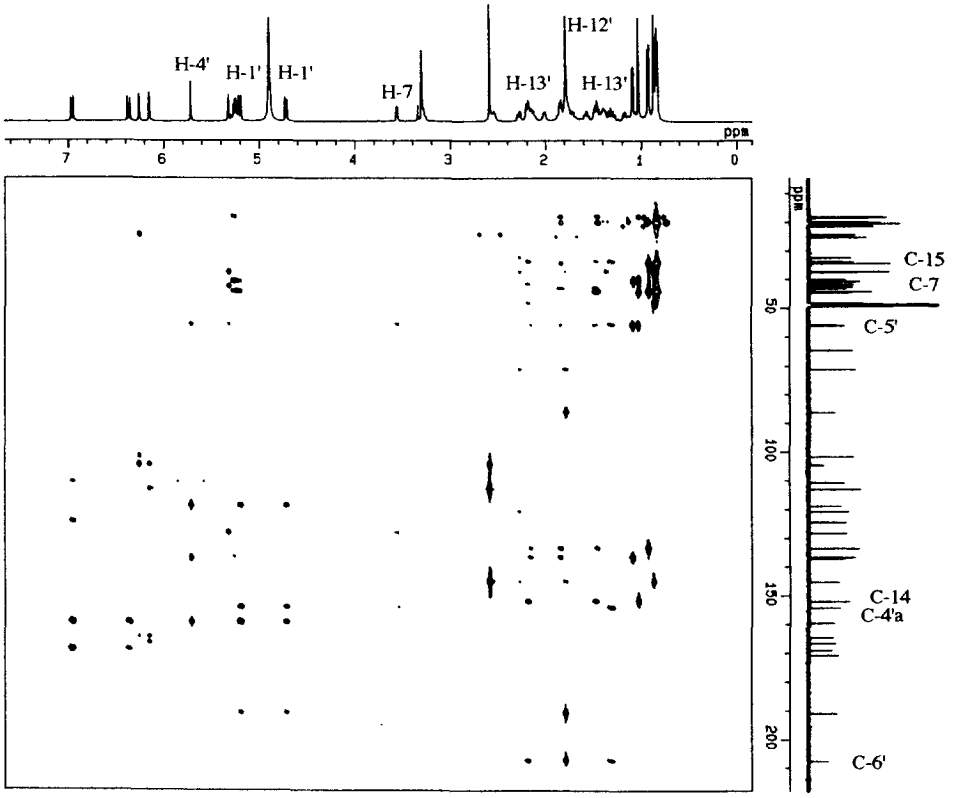


Fig. 6. PFG-HMBC spectrum of ergophilone A.

(55.85 ppm). Therefore, the planar structure of ergophilone A was determined as shown in Fig. 1.

The stereochemistry at C-3, 7, 9, 10, 13, 17, 20 and 24 of ergophilone A were determined as same configuration for ergophilone B by the NOE experiments and coupling constant values. The NOEs were observed between H-19 and 7, and H-15 and 18, respectively; thus both H-7 and 15 were elucidated as β configuration. The NOE observed at H-4' (5.72 ppm) and H-9 (2.01 ppm) established the stereochemistry of C-5' spiro carbon; H-4' proton located at α side of the B ring or C ring of the steroid, and the downfield shift of H-7 (3.55 ppm) was interpreted that the effect of the deshielding by C-6' carbonyl. The stereochemistry of C-7' of ergophilone A was speculated to be the same configuration as that of ergophilone B, based on the similarity of the Cotton effects in the CD spectra of both compounds (Fig. 5), although there are some differences in chromophore moieties, and the biogenetical speculation. Hence based on above spectral data the structure of ergophilone A was determined as shown in Fig. 1.

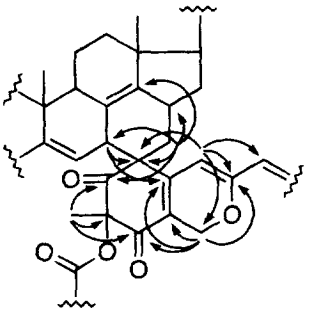


Fig. 7. H-C long-range correlations of ergophilone A.

Both ergophilones A and B have the ergostane skeleton and the azaphilone chromophore moieties. According to their stereochemistries, we suppose that above connections are formed by the biological Diels-Alder reaction (Fig. 8). Ergophilone A possessed one more carbon at C-13' position compared to ergophilone B. The C-13' carbon was probably derived from methionine and incorporated into azaphilone unit before Diels-Alder reaction. In the biosynthetic studies of citrinin[7] and austdiol[8], the origin of the C_1 unit of the same position is established and derived from methionine. The stereochemistries of new 6-membered ring of the connection suggested that our supposed presumable transition state of the biogenesis was reasonable for the end-rule of the Diels-Alder reaction.

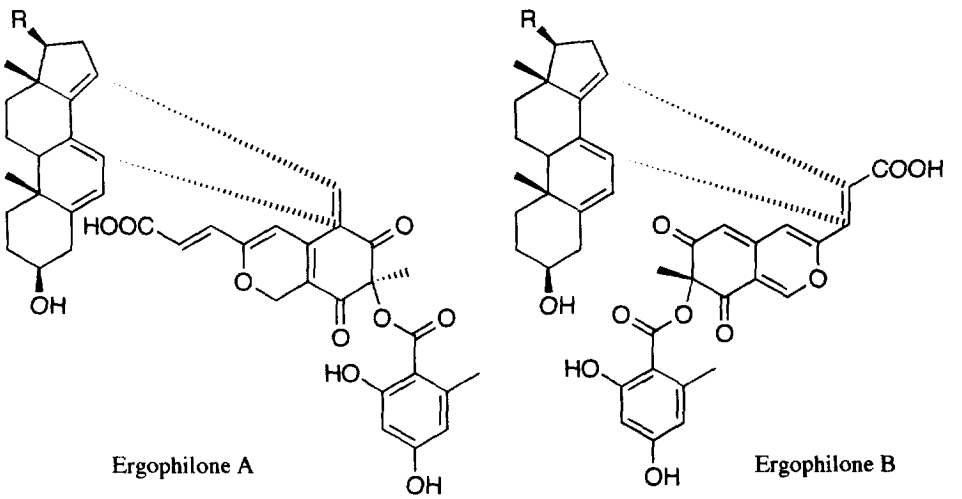


Fig. 8. Presumable transition states of ergophilones A and B.

Ergophilones A and B showed inhibitory activities against the rat platelet PLA2 with IC_{50} value of 0.44 μM and 0.56 μM, respectively. However, ergophilones A and B showed weak inhibition against the porcine pancreatic PLA2 with IC_{50} value of 51.7 μM and 39.0 μM, respectively.

Screrotiorin, related azaphilone, was previously reported as phospholipase A_2 inhibitor[9]. And several azaphilones have already been reported previously, for examples that the chlamydospore-like cell-inducing substances mitorubrinic acid, screrotiorin, lunatoic acid and so on[10], the monoamine oxidase inhibitor (8*R*)-7-deacetyl-*O*^{8},8-dihydro-7-*epi*-screrotiorin and its (*Z*)-isomer[11]. And some azaphilones were reported as pigments and/or toxic substances[12]. To the best of our knowledge, this is the first report on the unique compounds that an azaphilone connected with a steroid.

EXPERIMENTAL

General methods UV spectra were measured with a Shimadzu UV-240 spectrophotometer in MeOH solution. CD spectra were measured on a JASCO J-720 spectropolarimeter in MeOH solution. Optical rotations were measured on a JASCO DIP-140 polarimeter in MeOH solution at 25°C. FABMS spectra were performed on a JEOL SX102 instrument. NMR spectra were recorded on a JEOL JNM-A500 spectrometer or a JEOL JNM-A600 spectrometer. High performance liquid chromatography for purification was performed on Shimadzu LC-6AD and for PLA2 assay was Shimadzu LC-6A and Shimadzu RF-535 fluorescence HPLC monitor.

Cultivation The producing organism, *Penicillium* sp. BM-99 was isolated from a soil obtained from Ogasa town Shizuoka pref., Japan. *Penicillium* sp. BM-99 was cultured at the slant medium of the potato dextrose agar, then the mycelium was scrapped off and used to inoculate 500 ml flask with 80 ml of medium containing, glucose 5%, soybean meal 0.5%, malt extract 0.5%, yeast extract 0.1%, polypeptone 0.5%, $FeSO_4 \cdot 7H_2O$ 0.05%, $MnSO_4 \cdot nH_2O$ 0.05% and $MgSO_4 \cdot 7H_2O$ 0.05%. The pH was adjusted to 6.5 before sterilization. The flask was shaken at 210 rpm and 25 °C for 30 hours. This seed culture was inoculated with 1 % to medium mentioned above. The flask was shaken at 210 rpm and 25 °C for 5 days.

Isolation The mycelium of 40 liters of culture broth was extracted with 80% aqueous acetone. After removal of acetone *in vacuo*, the residual solution was extracted with EtOAc. EtOAc layer was concentrated to dryness *in vacuo*, and chromatographed on a silica gel (Wakogel C-200) column eluting with a gradient mixture of $CHCl_3$-MeOH (100:0; 50:1; 10:1; 5:1). The active fractions were eluted with $CHCl_3$-MeOH 10:1 and 5:1, and these fractions were mixed and concentrated to dryness and applied to a silica gel (Wakogel C-200) column chromatography eluting with a gradient mixture of hexane-EtOAc (1:1; 1:2). The eluate of hexane-EtOAc 1:2 was concentrated *in vacuo* and chromatographed on the Sephadex LH-20 column in MeOH. The active fraction mixture was confirmed by characteristic UV absorption. The final purification was achieved by preparative HPLC on a ODS column (Shiseido CAPCELL PAK C_{18} AG120, φ20x250) with 95% acetonitrile, affording 60 mg of ergophilone A and 200 mg of ergophilone B.

Ergophilone A(1) $[\alpha]^{25}_D$ = -222° (*c* = 1.0, MeOH); HR-FABMS [found *m/z* 821.4224 $(MH)^+$; calcd. 821.4265]; UVλ_{max}nm (log ε) 216(4.7), 240(4.5)sh, 267(4.5), 302(4.2), 393(4.3); CD$\lambda_{extreme}$nm (Δε) 203(-4.7), 227(-1.3), 245(-2.1), 272(0.2), 283(0.05), 305(0.6), 335(0.25); ^1H and ^{13}C NMR spectra see Table 1.

Ergophilone B(2) $[\alpha]^{25}_D$ = -503° (*c* = 1.0, MeOH); HR-FABMS [found *m/z* 807.4117 $(MH)^+$; calcd.

807.4108]; UVλ_{max}nm (log ε) 219(4.7), 269(4.4), 336(4.5); CD$\lambda_{extreme}$nm (Δε) 203(-2.8), 216(-1.7), 265(-0.25), 303(1.0), 335(-2.2); ^{1}H and ^{13}C NMR spectra see Table 1.

Assay for inhibition of phospholipase A_2 activity The reaction mixtures for standard assay were prepared as below, 1M-tris-HCl (pH 9.0) buffer 25 μl, 0.1M-$CaCl_2$ 10 μl, 1mM-substrate 100 μl, enzyme solution 50 μl, inhibitor solution 50 μl and distilled water 15 μl. The amounts of enzyme were adjusted to optimize linear kinetics for assay. PLA2 activity was measured by the method described previously[13]; namely 1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine was used as substrate, and the resulting freely fatty acid was quantitated by fluorecence HPLC analyses using a C_8 column (Shiseido CAPCELL PAK C_8 AG120, φ4.6x250) after reacted with 9-anthryldiazomethane.

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