HISPIDOSPERMIDIN, A NOVEL PHOSPHOLIPASE C INHIBITOR PRODUCED BY *Chaetosphaeronema hispidulum* (Cda) Moesz NR 7127

II. ISOLATION, CHARACTERIZATION AND STRUCTURAL ELUCIDATION

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Hispidospermidin (**1**) is a novel phospholipase C inhibitor produced by *Chaetosphaeronema hispidulum* (Cda) Moesz NR 7127. Its structure ($C_{25}H_{47}N_3O$) has been elucidated as a cage compound with a trimethylspermidine side chain based on various NMR studies, including ^1H-^1H COSY, ^{13}C-^1H COSY, HOHAHA, HMBC, COLOC and long range J C-H resolved 2D spectroscopy. The absolute configuration of **1** has been elucidated by modified MOSHER's method on the (*R*)- and (*S*)-MTPA amides of a derivative of **1**.

Phospholipase C (PLC), an enzyme that hydrolyzes phosphatidylinositol diphosphate to produce diglyceride and inositol triphosphate, plays an important role in signal transduction. Diglyceride activates protein kinase C, and inositol triphosphate increases intracellular calcium concentration, resulting in the induction of cell proliferation. Since PLC inhibitors are expected to be novel cell growth inhibitors, we screened microbial metabolites to obtain structurally unique and specific PLC inhibitors. From this screening program, we discovered a novel PLC inhibitor, hispidospermidin (**1**), from the culture broth of *Chaetosphaeronema hispidulum* (Cda) Moesz NR 7127 and determined its structure. The screening, taxonomy and fermentation of **1** have been reported in the preceding paper[1]. In the present paper, we describe its isolation, physico-chemical characterization, structural elucidation and the determination of the absolute stereochemistry. We also discuss the structural requirements for **1** to exhibit PLC inhibition.

Results

Isolation

Isolation of **1** was carried out by monitoring the inhibitory activity against rat brain PLC[1]. The isolation procedure is outlined in Fig. 1. Hispidospermidin (2.6 g) was isolated as a colorless oil from the broth filtrate (17 liters) by chromatography on Amberlite IRC-50 and Diaion HP-21 and by ethyl acetate extraction. Details of the isolation procedure are described in the experimental section.

Physico-chemical Properties

The physico-chemical properties of **1** are summarized in Table 1. Hispidospermidin (**1**) was soluble

Fig. 1. Isolation procedure of hispidospermidin (**1**).

Broth filtrate (17 liters)

↓ adjusted to pH 7 with HCl
adsorbed on Amberlite IRC-50 (Na-H, 7:3)
eluted with 0.5 N HCl

Eluate

↓ adjusted to pH 7 with 3 N NaOH
chromatographed on Diaion HP-21
eluted with H_2O and 10% aqueous acetone

Active fractions

↓ concd to remove acetone under reduce pressure
extracted with EtOAc at pH 9
evaporated under reduced pressure

Hispidospermidin (2.6 g)

Table 1. Physico-chemical properties of hispidospermidin (**1**).

Appearance	Colorless oil
Molecular formula	$C_{25}H_{47}N_3O$
HREI-MS	405.3730 (M^+) Calcd: 405.3720
UV λ_{max}^{MeOH} nm (ε)	End
IR ν_{max} (neat) cm^{-1}	3600~3100, 2940, 2860, 2810, 2780, 2760, 1460, 1370
$[\alpha]_D^{24}$	$-60°$ (c 1.45, $CHCl_3$)
Rf (Silica gel 60 F_{254})	0.69 ($CHCl_3$-MeOH-28% aqueous ammonia, 4:4:1)
Color reactions	I_2(+), Ninhydrin (+)
Solubility	Soluble in DMSO, MeOH, CH_2Cl_2 and acidic water Insoluble in H_2O

Table 2. NMR spectral data for hispidospermidin (**1**) in $CDCl_3$.

Position	δ_C[a]	δ_H[a]	^1H-^1H coupling[b]	^{13}C-^1H long range coupling[c]
2	80.0			3-H (δ 1.71), 13-Hβ, 2-CH_3
3	32.2	1.48	4-Hβ	4-Hα, 2-CH_3
		1.71	4-Hβ	
4	18.1	α, 1.40		3-H (δ 1.48), 3-H (δ 1.71), 9-H, 13-Hβ
		β, 1.22[d]	3-H (δ 1.48), 3-H (δ 1.71)	
5	43.2			7-Hβ, 9-H, 12-H, 13-Hα, 6-CH_3
6	43.4	1.52 (m)	6-CH_3, 7-Hα, 7-Hβ	4-Hβ, 13-Hβ, 6-CH_3
7	29.4	α, 1.13 (m)[d]	6-H, 8-Hα, 8-Hβ	6-CH_3
		β, 1.73 (m)	6-H, 8-Hβ	
8	20.3	α, 1.57 (m)[e]	7-Hα, 9-H	7-Hα, 9-H
		β, 1.42 (m)[e]	7-Hα, 7-Hβ, 9-H	
9	51.9	1.77 (dd, J=7.5, 13 Hz)	8-Hα, 8-Hβ	4-Hβ, 8-Hα, 11-H, 13-Hα, 10-CH_3
10	81.7			11-H, 12-H, 10-CH_3
11	66.4	2.81 (d, J=5 Hz)	12-H	13-Hα, 10-CH_3
12	44.5	2.03 (t, J=5 Hz)	11-H, 13-Hα	3-H (δ 1.71), 11-H, 13-Hα, 2-CH_3
13	27.3	α, 1.45 (dd, J=5, 12.5 Hz)[f]	12-H	11-H
		β, 1.28 (d, J=12.5 Hz)[f]		
2′	48.6	2.60 (t, J=7 Hz)	3′-H	11-H
3′	28.6	1.50 (m)	2′-H	2′-H, 5′-H
4′	25.1	1.51 (m)	5′-H	2′-H, 3′-H, 5′-H
5′	57.8	2.34	4′-H	6′-NCH_3
7′	55.9	2.35	8′-H	5′-H, 8′-H, 9′-H, 6′-NCH_3
8′	25.6	1.64 (m)	7′-H, 9′-H	
9′	58.0	2.28 (2H, br t, J=7 Hz)	8′-H	8′-H, 10′-$N(CH_3)_2$
2-CH_3	28.9	1.22 (s)		3-H (1.71), 12-H
6-CH_3	14.1	0.82 (d, J=7 Hz)	6-H	7-Hα
10-CH_3	21.0	1.12 (s)		11-H
6′-NCH_3	42.3	2.22 (s)		5′-H
10′-$N(CH_3)_2$	45.6	2.22 (s)		9′-H, 10′-$H(CH_3)_2$

[a] Proton-carbon connectivities were determined by ^{13}C-^1H COSY experiments.
[b] ^{13}C-^1H long range couplings obtained by HMBC and COLOC experiments.
[c] Vicinal couplings obtained by ^1H-^1H COSY experiments.
[d] NOEs were observed between these protons and 6-CH_3 protons.
[e] The orientations were determined by comparing these chemical shifts for **1** with those of **5** and **6**.
[f] The orientation was determined by considering the dihedral angles.

in DMSO, MeOH, CH_2Cl_2 and acidic water but insoluble in alkaline water. Hispidospermidin (**1**) was positive in the iodine and Ninhydrin color tests. The molecular formula was determined to be $C_{25}H_{47}N_3O$ based on HREI-MS data [*m/z* 405.3730, calcd 405.3720 (M^+)]. The molecular formula was also supported by the ^1H NMR (Fig. 2) and ^{13}C NMR (Fig. 3) spectral data, which are summarized in Table 2. The analysis of the DEPT spectrum indicated that **1** consisted of six methyl, twelve methylene, four methine and three quaternary carbons. This finding ment that one of the 47 protons in the molecule was attached to a heteroatom. The IR spectrum of **1** (Fig. 4) showed characteristic absorption bands at $3600 \sim 3100\,cm^{-1}$,

Fig. 2. 400 MHz ^1H NMR spectrum of hispidospermidin (**1**) in $CDCl_3$.

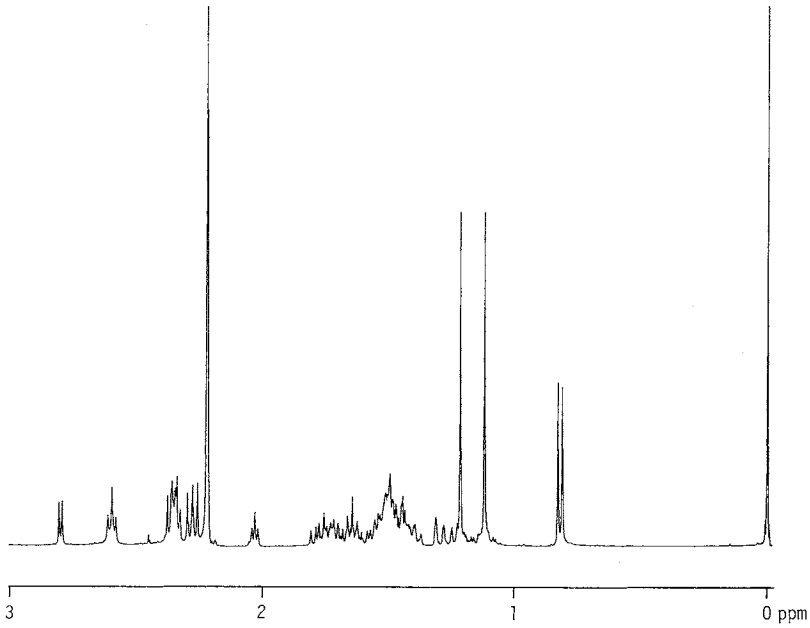


Fig. 3. 100 MHz ^{13}C NMR spectrum of hispidospermidin (**1**) in $CDCl_3$.

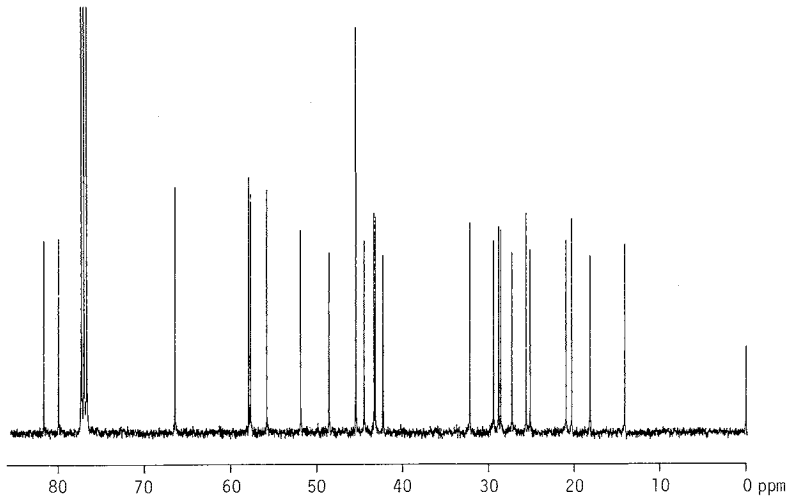


Fig. 4. IR spectrum (neat) of hispidospermidin (**1**).

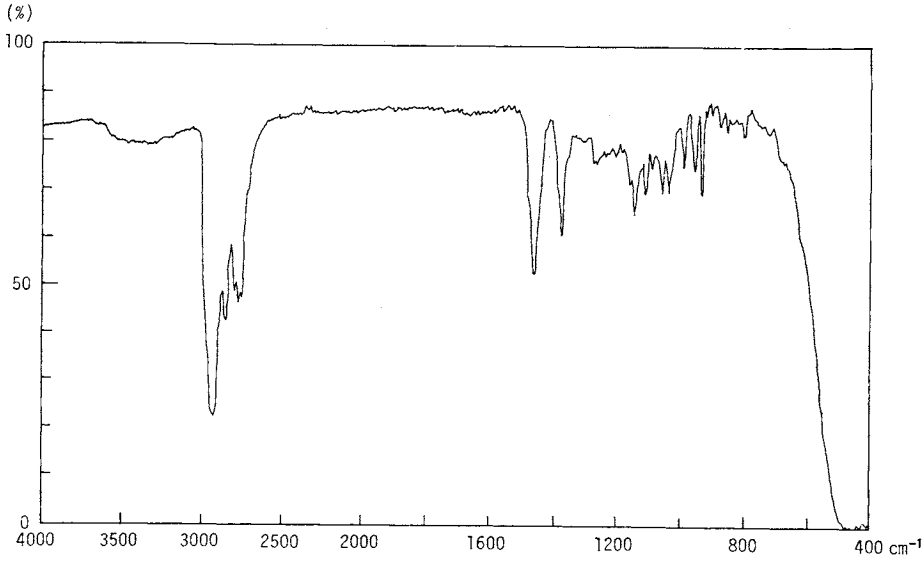
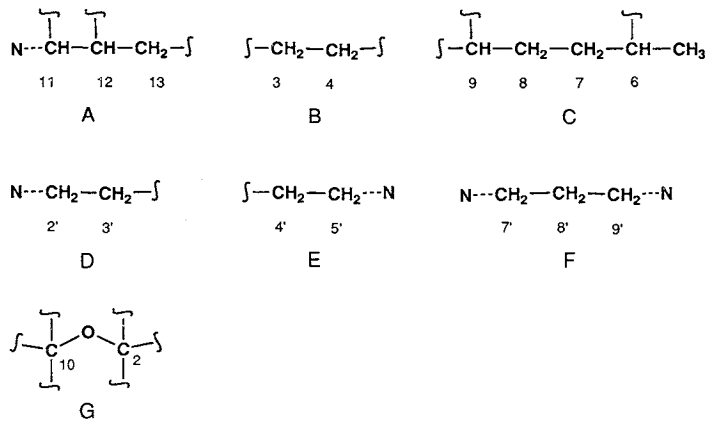


Fig. 5. Substructures of hispidospermidin (**1**) revealed by the interpretation of ^{1}H-^{1}H COSY spectral data and ^{13}C chemical shifts.



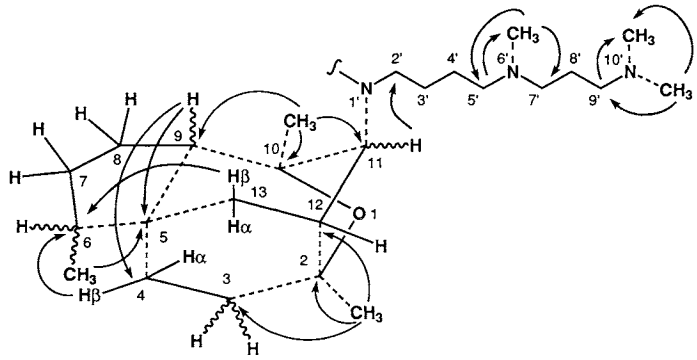
assignable to an N-H group, and at 2780 and 2760 cm^{-1}, assignable to N-CH_3 groups. The UV spectrum of **1** showed no characteristic absorption.

Structural Elucidation

Skeletal Structure

Substructures A, B, C, D, E and F were deduced from ^{1}H-^{1}H couplings in the ^{1}H-^{1}H COSY spectrum of **1** (Fig. 5). The methine at position 11 and the methylenes at positions 2′, 5′, 7′ and 9′ were assigned to be adjacent to N atoms because of their ^{13}C (δ 66.4, 48.6, 57.8, 55.9 and 58.0, respectively) and ^{1}H (δ 2.81, 2.60, 2.34, 2.35 and 2.28, respectively) chemical shifts. Substructure G was elucidated based on the ^{13}C chemical shifts of C-2 (δ 80.0) and C-10 (δ 81.7), indicating ethereal bonds between these carbons (Fig. 5). The relayed correlation from 2′-H (δ 2.60) to 5′-H (δ 2.34) through 3′-H (δ 1.50) and 4′-H (δ 1.51)

Fig. 6. ^{13}C-^{1}H long range couplings (arrows) obtained from the COLOC and HMBC experiments on hispidospermidin (**1**).



observed in the HOHAHA spectrum overcame the difficulty in connecting two overlapping methylenes, 3′-CH_2 in the substructure D and the 4′-CH_2 in the substructure E.

Further linkages among the substructures A~G and five methyl groups were established by the interpretation of ^{13}C-^{1}H long range couplings obtained by HMBC and COLOC experiments on **1** (Fig. 6). The ^{13}C-^{1}H long range couplings between the methyl protons at δ 1.22 (s, 2-CH_3) and the carbons C-2, C-3 and C-12 indicated that the methyl group 2-CH_3 was attached to the quaternary carbon C-2 and that C-2 was located between C-3 and C-12. The ^{13}C-^{1}H long range couplings between the methyl protons at δ 1.12 (s, 10-CH_3) and the carbons C-9, C-10 and C-11 indicated that the methyl group 10-CH_3 was attached to quaternary carbon C-10 and that C-10 was connected to C-9 and C-11. The linkage between C-5 and C-6 was also revealed by the ^{13}C-^{1}H long range coupling between the 6-CH_3 protons (δ 0.82) and C-5 (δ 43.2). C-5 was in turn connected to C-4, C-9 and C-13 based on the ^{13}C-^{1}H long range couplings between 13-Hβ and C-6, between 9-H and the carbons C-4 and C-5, and between 4-Hβ and C-6. A trimethylspermidine structure was deduced from the ^{13}C-^{1}H long range couplings between the *N*-methyl protons at δ 2.22 (9H, s, 6′-NCH_3 and 10′-$N(CH_3)_2$) and the carbons C-5′, C-7′, C-9′ and 10′-$(CH_3)_2$ (Fig. 6). This polyamine residue was connected to C-11 through N-1′ because of the ^{13}C-^{1}H long range coupling between 11-H and C-2′.

The presence of an N-H group suggested by the IR spectrum was confirmed by the ^{1}H NMR spectral data for the acetamide derivative **2** obtained by treatment of **1** with acetyl chloride in the presence of 4-dimethylaminopyridine in pyridine. In the ^{1}H NMR spectrum of **2**, 11-H (δ 3.84, d, J=5 Hz), 12-H (δ 2.95, m) and 2′-H (δ 3.32 and 3.42) were deshielded to a greater extent than those of **1** (δ 2.81, 2.03 and 2.60, respectively), indicating the linkage of an acetyl group to N-1′. N-1′ in **1** was thus revealed to be a secondary amino group.

Relative Stereochemistry

The ring juncture between the cyclopentane and cyclohexane (C-5, C-9, C-10, C-11, C-12 and C-13) rings was established to be *trans* by comparing the ^{13}C-^{1}H long range coupling constant between 9-H and C-13 (δ 27.3) with that between 9-H and C-4 (δ 18.1), which were obtained by the long range J C-H resolved 2D spectroscopy[2,3] on **1**. The proton 9-H had a large coupling constant with C-4 ($^{3}J_{CH}$=5 Hz), but not with C-13, indicating a *trans* relation between 9-H and C-4. Although the value was somewhat

smaller than is typical (6~9 Hz)[4], this reduction was explained by the γ-substituent effects that were also observed in admantane[5]. The relative stereochemistry between 6-CH_3 and C-4 was established to be *cis* by the NOE between 6-CH_3 and 4-Hβ. The equatorial orientation of 11-H was also established from the NOE between 11-H and 2-CH_3 protons and was supported by the coupling constant (5 Hz) between 11-H and 12-H. This was consistent with the calculated value (J=4.6 Hz) from the Karplus equation[6] (dihedral angle=45°). The relative configuration of **1** was thus elucidated as shown in Fig. 7.

Absolute Stereochemistry

The absolute configuration of **1** was elucidated by applying modified MOSHER's method to the derivatives **5** and **6**. They were prepared by the reactions as outlined in Fig. 8. The trimethylspermidine side chain of **1** was cleaved by oxidation with hydrogen peroxide in the presence of sodium tungstate[7] to give nitro compound **3a** as the main product and its epimer **3b**. The nitro group in **3a** was reduced by lithium aluminum hydride to give a primary amine **4**, which was then separately treated with (*S*)- and

Fig. 7. Structures of hispidospermidin (**1**) and its acetamide derivative **2** and NOEs (arrows) obtained from the NOESY experiments on hispidospermidin (**1**).

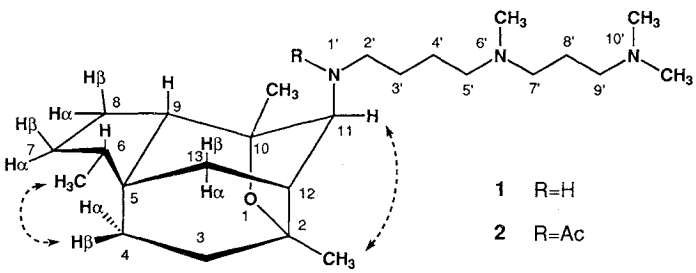


Fig. 8. Conversion of hispidospermidin (**1**) to MTPA derivatives **5** and **6**.

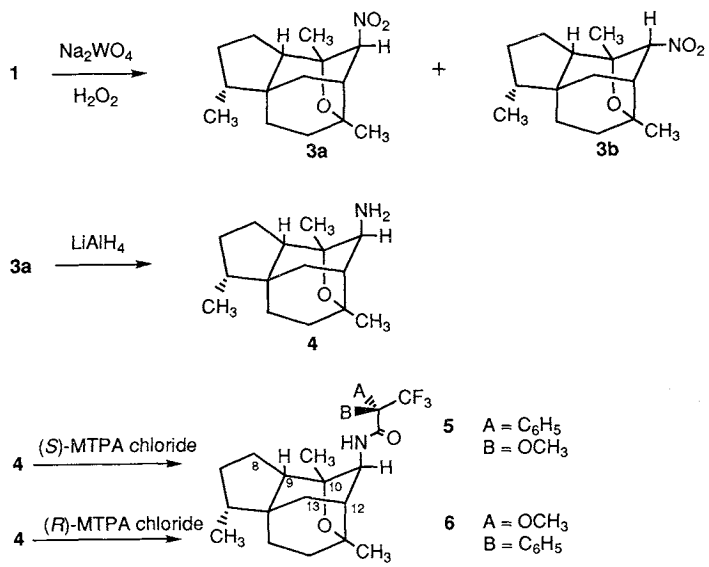


Table 3. ^{1}H NMR spectral data[a] for the MTPA derivatives, **5** and **6**, in $CDCl_3$.

	5	6	$\Delta\delta$ ($\delta_6-\delta_5$)
3-H, 4-Hα	1.42~1.52 (2H), 1.77 (1H)	1.42~1.52 (2H), 1.77 (1H)	0
4-Hβ	1.30 (1H)[b]	1.29 (1H)[b]	−0.01
6-H	1.46 (ddq, J=9, 10, 7 Hz)	1.47 (ddq, J=9, 10, 7 Hz)	+0.01
7-Hα	1.18 (dddd, J=4, 10, 12.5, 13 Hz)[b,c]	1.19 (dddd, J=4, 10, 12.5, 13 Hz)[b,c]	+0.01
7-Hβ	1.77 (dddd, J=5, 9, 10, 13 Hz)	1.80 (dddd, J=5, 9, 10, 13 Hz)	+0.03
8-Hα	1.61 (dq, J=5, 12.5 Hz)[c]	1.63 (dq, J=5, 12.5 Hz)[c]	+0.02
8-Hβ	1.44 (dddd, J=4, 7.5, 10, 12.5 Hz)	1.51 (dddd, J=4, 7.5, 10, 12.5 Hz)	+0.07
9-H	1.25 (dd, J=7.5, 12.5 Hz)	1.36 (dd, J=7.5, 12.5 Hz)	+0.11
11-H	4.21 (dd, J=5, 9 Hz)	4.24 (dd, J=5, 9 Hz)	
12-H	2.25 (t, J=5 Hz)	2.17 (t, J=5 Hz)	−0.08
13-Hα	1.70 (dd, J=5, 13 Hz)[d]	1.67 (dd, J=5, 13 Hz)[d]	−0.03
13-Hβ	1.03 (d, J=13 Hz)[d]	0.93 (d, J=13 Hz)[d]	−0.10
2-CH_3	1.29 (s)	1.28 (s)	−0.01
6-CH_3	0.87 (d, J=7 Hz)	0.85 (d, J=7 Hz)	−0.02
10-CH_3	1.10 (s)	1.17 (s)	+0.07
NH	6.90 (d, J=9 Hz)	6.69 (d, J=9 Hz)	
pH	*ca.* 7.42 (3H, m),	*ca.* 7.42 (3H, m),	
	ca. 7.54 (2H, m)	*ca.* 7.53 (2H, m)	
OCH_3	3.45 (q, J=1.2 Hz)	3.43 (q, J=1.2 Hz)	

[a] Assignments, multiplicity and coupling constants were determined by ^{1}H-^{1}H COSY, homodecoupling, NOESY and selective COSY[14] experiments.
[b] NOEs were observed between these protons and 6-CH_3 protons.
[c] An NOE was observed between 7-Hα and 8-Hα.
[d] The orientations were determined by considering the dihedral angles.

(*R*)-2-methoxy-2-phenyl-2-(trifluoromethyl)acetic acid (MTPA) chlorides to yield the (*R*)- and (*S*)-MTPA amides **5** and **6**, respectively.

Detailed analyses of the NMR spectral data of these MTPA derivatives revealed significant and systematic differences between the proton chemical shifts of these MTPA derivatives (Table 3). When the molecular models of **5** and **6** with the 11*S* configurations were constructed, the $\Delta\delta$ ($\delta_6-\delta_5$) values for the protons oriented toward the left side of the MTPA plane[8~10] were all negative, while those oriented toward the right side of the plane were all positive. These data indicated the *S* configuration at the position 11 according to modified MOSHER's method. Therefore, the absolute configurations of **4** were determined to be 2*R*, 5*S*, 6*R*, 9*S*, 10*S*, 11*S*, 12*R*. This conclusion was confirmed by an X-ray crystallographic analysis of its (1*S*)(+)-10-camphorsulfonic acid salt[11]. The absolute structure of **1** was thus determined as shown in Fig. 7.

Discussion

We recently reported a PLC inhibitor of microbial origin, vinaxanthone[12]. Its structure is completely different from that of **1**. Hispidospermidin (**1**) is a cage compound with a trimethylspermidine side chain, whereas vinaxanthone is a polycyclic xanthone with poly acidic functional groups. Since spermine and spermidine were reported to show inhibitory activity against PLC of *Clostridium welchii*[13], we compared the inhibitory activities of **1** and the primary amine **4** with those of spermine and spermidine against rat brain PLC[1]. Hispidospermidin (**1**) inhibited the enzyme with an IC_{50} of 16 μM, whereas **4** did not show inhibitory activity at concentrations up to 7.4 mM. Spermine (IC_{50} = 59 μM) and spermidine (IC_{50} = 1.2 mM) weakly inhibited rat PLC and were found to be 4 and 75 times less potent than **1**, respectively. These results suggest that the polyamine moiety in **1** is essential for the inhibitory activity, and the hydrophobic substituents in **1** potentiate the inhibitory activity. Details of the biological activity will be reported elsewhere.

Experimental

General Procedures

UV spectra and IR spectra were recorded on a Kontron Uvikon 860 UV spectrometer, and on a Hitachi 270-30 infrared spectrophotometer, respectively. Mass spectra were obtained with a JEOL DX-300 spectrometer. ^1H and ^{13}C NMR spectra were recorded on a JEOL GSX-400 or -A-500 NMR spectrometer with TMS as the internal standard. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. The X-ray data[11] were measured on a Siemens R3m/V diffractometer and refined by full-matrix least squares with software written by Prof. G. M. SHELDRICK, University of Göttingen, Germany.

Isolation of 1

In the following procedures, fractions were monitored by the assay method described in the preceding paper[1], which observes inhibitory activity against the hydrolysis of [^3H]phosphatidylinositol by rat brain PLC. After cultivating the producing organism for three days in a 50-liter jar fermentor by the procedure described in the preceding paper[1], the mycelium was removed by filtration. The broth filtrate (17 liters) was adjusted to pH 7 with 1 N HCl and applied to a column (8 × 30 cm) of Amberlite IRC-50 (Na - H, 7 : 3) (ROHM and HAAS). The column was washed with water (5 liters), and then the active principle was eluted with 0.5 N HCl (10 liters). The eluate was adjusted to pH 7 with 3 N NaOH and applied to a column (8 × 30 cm) of Diaion HP-21 (Mitsubishi Chemical Industries). A part of the active principle was eluted with water (3.8 liters) and the remaining active principle was successively eluted with 10% aqueous acetone (8.2 liters). The combined active fractions were concentrated to remove acetone and extracted with ethyl acetate (7.5 liters × 2) at pH 9. The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure to give **1** (2.6 g) as a colorless oil.

Preparation of 2

To a solution of **1** (75.5 mg) and 4-dimethylaminopyridine (90.9 mg) in dry pyridine (7 ml) was added acetyl chloride (52.9 μl) at 0°C. This solution was stirred overnight at 90°C. After removal of the pyridine under reduced pressure, the residue was extracted with EtOAc at pH 9. The organic layer was washed with water and dried over anhydrous sodium sulfate. The solvent was removed and the residue was then purified by preparative TLC (Merck, Kieselgel 60F_{254}) with $CHCl_3$ - MeOH - 28% aqueous ammonia (80 : 20 : 1) as the mobile phase to give **2** (34.6 mg) as a colorless oil. $[\alpha]_D^{21}$ −58 (*c* 1.66, $CHCl_3$); HREI-MS *m/z* 477.3823 (M^+) (Calcd for $C_{27}H_{49}N_3O_2$ 447.3822); IR ν_{max} (neat): 2944, 2870, 2770, 1660, 1464, 1378, 1275, 1150, 1040 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.84 (3H, d, J=7 Hz, 6-CH_3), 1.16 (2H, m), 1.26 (3H, s, 10-CH_3 or 2-CH_3), 1.29 (3H, s, 2-CH_3 or 10-CH_3), 1.42 ~ 1.58 (8H), 1.59 ~ 1.80 (8H), 2.17 (3H, s, 6′-NCH_3 or $COCH_3$), 2.20 (3H, s, $COCH_3$ or 6′-NCH_3), 2.22 (6H, s, 10′-$N(CH_3)_2$), 2.28 (2H, t, J=7 Hz, NCH_2), 2.34 (4H, 2 × NCH_2), 2.95 (1H, m, 12-H), 3.32 (1H, m, 2′-*H*CH), 3.42 (1H, m, 2′-HC*H*), 3.84 (1H, d, J=5 Hz, 11-H); ^{13}C NMR ($CDCl_3$) δ 14.1 (6-CH_3), 18.0 (C-4), 20.4 (C-8), 22.8 (10-CH_3), 23.4 ($COCH_3$), 24.7 (C-4′), 25.5 (C-8′), 27.3 (C-13), 28.5 (C-3′), 28.8 (2-CH_3), 29.3 (C-7), 32.0 (C-3), 42.0 (6′-NCH_3), 43.6 (C-5), 43.8 (C-6), 45.1 (C-12), 45.5 (10′-$N(CH_3)_2$), 47.3 (C-2′), 52.9 (C-9), 55.7 (C-7′), 57.3 (C-5′ or C-9′), 57.9 (C-9′ or C-5′), 63.6 (C-11), 79.1 (C-2), 81.7 (C-10), 173.3 (N–CO).

Preparation of 3a and 3b

To a solution of **1** (10 g) in dry methanol (650 ml) were added $Na_2WO_4 \cdot 2H_2O$ (814 mg) and 30% hydrogen peroxide (30.8 ml). This mixture was stirred at 40°C overnight. The reaction mixture was concentrated under reduced pressure and partitioned between water (150 ml) and ethyl acetate (200 ml). The organic layer was washed with water (100 ml), dried over anhydrous sodium sulfate, and evaporated under reduced pressure. The residue was separated by silica gel column chromatography with hexane - ethyl acetate (20 : 1) to yield **3a** and **3b** as crystals.

Recrystallization of **3a** from hexane gave 2.04 g of colorless needles, mp 134 ~ 136°C; $[\alpha]_D^{21}$ −77.5° (*c* 1.0, $CHCl_3$); EI-MS (30 eV) *m/z* 265 (M^+), 219 ($M-NO_2$)$^+$; IR ν_{max} (KBr) cm^{-1} 1548 (NO_2), 1380 (NO_2); ^1H NMR ($CDCl_3$) δ 0.83 (3H, d, J=6.5 Hz, 6-CH_3), 1.15 (1H), 1.27 (1H), 1.28 (3H, s, CH_3), 1.34 (1H), 1.46 (3H, s, CH_3), 1.46 ~ 1.7 (6H), 1.7 ~ 1.9 (3H), 2.73 (1H, t, J=5 Hz, 12-H), 4.50 (1H, d, J=5 Hz, 11-H); ^{13}C NMR ($CDCl_3$) δ 13.9 (6-CH_3), 17.7 (C-4), 20.3 (C-8), 21.6 (10-CH_3), 28.3 (2-CH_3), 29.0 (C-13),

29.5 (C-7), 31.4 (C-3), 42.8 (C-5), 43.1 (C-6), 45.0 (C-12), 52.7 (C-9), 79.8 (C-2), 80.9 (C-10), 89.3 (C-11).

Anal Calcd for $C_{15}H_{23}NO_3$: C 67.90, H 8.74, N 5.28.
Found: C 68.04, H 8.77, N 5.35.

Recrystallization of **3b** from hexane gave 603 mg of colorless needles, mp 136~139°C; $[\alpha]_D^{21}$ −140° (*c* 1.0, $CHCl_3$); EI-MS (30 eV) *m/z* 265 (M^+), 219 $(M-NO_2)^+$; IR ν_{max} (KBr) cm^{-1} 1558 (NO_2), 1378 (NO_2); ^1H NMR ($CDCl_3$) δ 0.83 (3H, d, J=6.5 Hz, 6-CH_3), 1.17 (1H, d, J=13.5 Hz), 1.23~1.43 (2H), 1.36 (3H, s, CH_3), 1.39 (3H, s, CH_3), 1.46~1.74 (6H), 1.75~1.90 (2H,), 1.94 (1H, dd, J=5, 13.5 Hz), 2.95 (1H, d, J=5 Hz, 12-H), 4.45 (1H, s, 11-H); ^{13}C NMR ($CDCl_3$) δ 14.0 (6-CH_3), 17.6 (C-4), 17.9 (10-CH_3), 20.5 (C-8), 27.8 (2-CH_3), 29.9 (C-7), 32.5 (C-3), 34.7 (C-13), 42.6 (C-6), 43.7 (C-5), 48.0 (C-12), 60.8 (C-9), 83.4 (C-2 or C-10), 84.2 (C-10 or C-2), 98.8 (C-11).

Anal Calcd for $C_{15}H_{23}NO_3$: C 67.90, H 8.74, N 5.28.
Found: C 68.05, H 8.78, N 5.12.

Preparation of **4**

To a suspension of lithium aluminum hydride (1 g) in dry ether (100 ml) was added a solution of **3a** (1.75 g) dissolved in dry ether (30 ml) with stirring. The mixture was refluxed for 23 hours. Then water (3 ml) was added to the cooled reaction mixture. When gray suspension became white, anhydrous sodium sulfate (*ca.* 20 g) was added. Inorganic salt was filtered with suction and the filtrate was evaporated to dryness. The residue was purified on a silica gel column with CH_2Cl_2-isopropyl alcohol (10:1) to give **4** (1.16 g) as a crystalline solid. An analytical sample was prepared by recrystallization from hexane. Colorless needles, mp 67.5~68.0°C; $[\alpha]_D^{21}$ −141° (*c* 1.0, $CHCl_3$); EI-MS (30 eV) *m/z* 235 (M^+); IR ν_{max} (KBr) cm^{-1} 3420 (NH), 3350 (NH), 1630 (NH); ^1H NMR ($CDCl_3$) δ 0.84 (3H, d, J=6.5 Hz, 6-CH_3), 1.11 (3H, s, 10-CH_3), 1.15 (1H, m), 1.22 (3H, s, 2-CH_3), 1.23 (2H, m), 1.35~1.85 (11H), 1.95 (1H, t, J=5 Hz, 12-H), 3.07 (1H, d, J=5 Hz, 11-H); ^{13}C NMR ($CDCl_3$) δ 14.1 (6-CH_3), 18.2 (C-4), 20.3 (C-8 and 10-CH_3), 27.2 (C-13), 28.7 (2-CH_3), 29.4 (C-7), 32.1 (C-3), 43.1 (C-5), 43.5 (C-6), 46.9 (C-12), 50.9 (C-9), 59.6 (C-11), 79.6 (C-2), 81.6 (C-10).

Anal Calcd for $C_{15}H_{25}NO$: C 76.55, H 10.71, N 5.95.
Found: C 76.47, H 10.74, N 6.05.

Preparation of **5**

A solution of (*R*)-MTPA (47 mg) in $SOCl_2$ (2 ml) was refluxed for 2 hours. The reaction mixture was evaporated to dryness. To the residue was added dry pyridine (2 ml), 4-dimethylaminopyridine (1.2 mg) and **4** at 0°C. This solution was stirred at 90°C overnight, diluted with ethyl acetate (50 ml), and was washed with diluted hydrochloric acid (pH 4.5), water, saturated $NaHCO_3$ solution and again water. The organic layer was dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was purified on a silica gel column with hexane-ethyl acetate (10:1) to give **5** as a colorless oil (41 mg), $[\alpha]_D^{21}$ −74° (*c* 1.64, $CHCl_3$); IR ν_{max} (KBr) cm^{-1} 3448 (NH), 1708 (amide), 1516 (amide); HREI-MS (30 eV) *m/z* calcd for $C_{25}H_{32}NO_3F_3$: 451.2333, found: 451.2321 (M^+).

Preparation of **6**

The (*S*)-MTPA amide **6** (42 mg) was obtained as a colorless oil from **4** (24.3 mg) and (*S*)-MTPA (39 mg) by a similar procedure for **5**, $[\alpha]_D^{21}$ −38° (*c* 1.64, $CHCl_3$); IR ν_{max} (KBr) cm^{-1} 3448 (NH), 1708 (amide), 1516 (amide); HREI-MS (30 eV) *m/z* calcd for $C_{25}H_{32}NO_3F_3$: 451.2333, found: 451.2317 (M^+).

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