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Purification and Structure Determination of Antifungal Phospholipids from a Marine *Streptomyces*

CHO, KI WOONG, YOUNGWAN SEO, TAEMI YOON[1], AND JONGHEON SHIN*

Marine Natural Products Laboratory, Korea Ocean Research and Development Institute, Ansan P.O. Box 29. Kyunggi-Do 425-600, Korea
[1]Department of Biochemistry, Hanyang University, Ansan, Kyunggi-Do 425-791, Korea

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Abstract A series of antifungal compounds were obtained from the methanol extract of the mycelium from marine actinomycetes M428 which was identified as a *Stereptomyces* species by fatty acid composition and biochemical characteristics. These compounds were purified by combined chromatographic techniques and the structures were characterized with spectroscopic methods including 1D and 2D NMR, and mass spectrometry as sn-1 lysophosphatidyl inositols. The side chains were established by chemical degradation followed by GC analysis to be 14-methyl pentadecanoic acid (iso-palmitic acid, i-C16:0, compound A) and 13-methyl tetradecanoic acid (iso-pentadecanoic acid, i-C15:0, compound B). These compounds displayed highly selective antifungal activity against *C. albicans* with MIC values of 5 μg/ml (compound A) and 2.5 μg/ml (compound B), while it had almost negligible antibiotic activity against *E. coli* and *P. aerogenosa* with MIC value higher than 50 μg/ml and no cytotoxic activities against human myeloma leukemia K562 (IC_{50}>100 μg/ml).

Key words: Antifungal phospholipids, lysophosphatidyl inositol, marine *Streptomyces*

Various kinds of diseases caused by fungal infection have been reported for human, animals, and plants [10, 11]. These fungal infections are particularly serious for human beings weakened by an immunosuppresive treatment for a clinical condition such as organ transplantation or with a lowered cellular immune response due to pregnancy or acquired immune deficiency syndrome (AIDS) [11]. In recent years, a marked improvement in drug development has been achieved through increased knowledge in fungal metabolism and architecture that has resulted in the rational design and development of diverse and effective compounds. Because of the eukaryotic properties of fungi, however, many antifungal compounds exhibit a potent cytotoxic effect which is a significant limitation for the application of these antifungal compounds for a practical drug [11]. In search of new antifungal compounds with low side effects such as cytotoxicity, recent efforts have focused on natural products from living organisms. So far, many compounds have been developed from terrestrial sources, especially from plants and microorganisms including actinomycetes and fungi. However, the reports of novel compounds from terrestrial sources have been decreasing very rapidly. Consequently, many leading countries have started to employ marine organisms to obtain novel bioactive substances [4]. Although it has a relatively short history, over 8,000 novel compounds have been isolated from marine benthic invertebrates such as sponges and soft corals [4].

However, the difficulty of obtaining large quantity of these unculturable animals as well as the low concentration of most bioactive substances in them are serious hindrances to the development of bioactive agents from marine sources. Marine microorganisms have recently emerged as an alternative and ultimate solution for this problem. Although less than 1% of marine-derived compounds have been isolated from these organisms, the structural diversity and potent bioactivity of secondary metabolites made marine microbes, including actinomycetes, eubacteria, and fungi, widely recognized as the promising sources for the development of new drugs for various diseases in future [9].

As part of the search for biologically active substances from marine organisms, we have screened for antifungal activity from marine actinomycetes collected from marine animals and various marine environments such as high-salt sea water, deep sea sediment, hydrothermal vent, and Antarctic sediment. In this paper, we describe the isolation, chemical characterization, and biological properties of antifungal metabolites isolated from the marine *Streptomyces* strain M428.

*Corresponding author
Phone: 82-345-400-6170; Fax: 82-345-408-4493;
E-mail: jhshin@sari.kordi.re.kr

MATERIALS AND METHODS

Chemicals

Fatty acids, fatty acid methyl esters, phospholipids, and amino acid standards were purchased from Sigma Chemical Co. (U.S.A.). Silica gel and ODS reversed phase gel were the products of Merck Co. (Germany). Media and agar were obtained from Difco Co. (U.S.A.). All other chemicals and solvents used were of either analytical grade or redistilled prior to use.

Microorganisms, Growth, and Screening Process

Isolated strains of marine origin were grown in SYP media (75% sea water, 10 g soluble starch, 4 g yeast extract, 2 g bactopeptone, 10 mM TrisHCl buffer, pH 7.5) at 27°C with shaking (150 rpm) for 7 days. Strains of *Candida albicans* (KCTC1940), *E. coli* (KCTC1039), and *Pseudomonas aerogenosa* (KCTC1711) were obtained from KCTC, Korea.

Taxonomic Studies of Strain M428 Producing Antifungal Reagent

For the identification of this strain, its biochemical and physiological characteristics were examined according to the "*Bergey's Manual of Determinative Bacteriology*" [6] and the physiological characteristics including utilization of carbon sources were examined by following the method of Shirling and Gottlieb [10]. For the fatty acid composition, mycellium of M428 (dry weight 0.1 g) was harvested with centrifugation. Whole cell lipids were extracted with 3×2 ml of Folch solution and saponified [5]. Fatty acid methyl esters were prepared with 5% HCl in MeOH at 80°C for 1 h and extracted with n-hexane, concentrated, and analyzed by gas chromatography (HP5890-IIplus, Hewlett-Packard Co., U.S.A.) equipped with Omegawax-320 capillary column (0.32 mm×30 m, Supelco, U.S.A.) and FID as detector. Temperature of injector and detector was 300°C and oven temperature was 180°C. Peaks were recorded through the Chromate data interface program (Interface Co., Korea) and analyzed with their equivalent chain length (ECL) value, and compared with those of authentic fatty acid methyl esters [1, 2].

Purification of Antifungal Materials from M428

Grown mycellium was harvested from culture broth using the Minikross tangential membrane filter system with a 0.2 μm membrane filter (3,500 cm^2 filtering area, Microgon Co., U.S.A.) and high speed centrifugation (8,500 rpm for 10 min). Harvested mycellium was then extracted with 10 volumes of MeOH. The extracts were concentrated *in vacuo* and then partitioned between isobutanol and water. The isobutanol layer was dried under vacuum and repartitioned between n-hexane and 10% aqueous methanol. The aqueous methanol layer was separated by C_{18} reversed-phase vacuum flash chromatography (YMC ODS A 60-I25 gel, Japan) by using sequential mixtures of MeOH and water as eluents (50, 40, 30, 20, 10% aqueous MeOH, and MeOH). The 30% aqueous MeOH fraction which showed the highest antifungal activity was separated by C_{18} reversed phase HPLC (semi-preparative YMC ODS-AQ column, 10×250 mm, solvent: 40% aqueous MeOH) to give rise to active compounds, and the purity was confirmed by TLC and HPLC.

Analysis of the Chemical Structure of Purified Antifungal Materials from M428

NMR spectra were recorded in CD_3OD solutions at 500 MHz for ^1H and 125 MHz for ^{13}C, respectively, on a Varian Unity 500 spectrometer. All of the chemical shifts were recorded in δ (ppm) based on MeOH (3.30/49.50 ppm) using TMS as the internal standard. IR spectra were recorded on a Matterson Galaxy spectrophotometer. Mass spectra were obtained by a Jeol JMS-HX 110 high-resolution mass spectrometer, provided by Korea Basic Science Institute, Taejeon, Korea. Gas chromatographic analysis for the identification of fatty acid in the purified compound was performed by the same procedure described for total fatty acid composition determination. All solvents used were either of spectral grade or redistilled from glass prior to use.

Antibiotic, Antifungal, and Cytotoxic Activity Assay

Antifungal activity was primarily determined with the paper disk method using 6-mm disks against *C. albicans* and the activity was determined with the diameter of clear zone. For the determination of antifungal activity in MIC (minimal inhibitory concentration) value for the mycelial phase of *C. albicans*, a series of solutions of compounds A and B in the range of 0.25-100 μg/ml were prepared in a YM medium and 0.1 ml of each solution were added into 0.1 ml of *C. albicans* suspension (containing about 100 spores). The mixtures were then incubated in a CO_2 incubator under 5% atmospheric CO_2 at 28°C for 24 h. For the measurement of the MIC values against bacteria, *E. coli* and *P. aerogenosa* were grown in LB (Lurina-Bertani) broth at 37°C overnight and diluted in a basal medium of 1% bactopeptone to a final bacterial cell density of about 5×10^6 CFU (colony forming unit). To 0.1 ml of each bacterial suspension, 0.1 ml of compound A and B solutions prepared in a same basal medium were added and then the suspensions were incubated at 37°C for 24 h. The MIC was defined as the minimal concentration showing no growth of the bacteria ($OD_{660\,nm}$) after incubation. Human chronic myelogeneous leukemia K562 cell was used for the assay of general cytotoxicity. Thus, cells were maintained in RPMI-1640 medium (Life Technologies, U.S.A.) containing 10% fetal bovine serum (FBS) and 20 μg/ml kanamycin. The cytotoxicity was analyzed by

MTT assay [6] and the results were measured at 540 nm with a microplate reader (Bio-Rad Model 3550, U.S.A.).

RESULTS AND DISCUSSION

Screening of Antifungal Activity

The strain M428 producing antifungal agents was isolated from sea sediment (a depth of 20 m), collected by scuba diving off the coast of Geomun island in 1996 and was selected as an antifungal compound producing strain, based on the growth inhibition activity against *C. albicans* by the disc diffusion method (Fig. 1).

Taxonomical Studies of the Strain M428

The strain M428 was maintained on a yeast extract-malt extract agar containing 70% natural sea water. The colony morphology of strain M428 grown on yeast malt agar at 30°C for 3 days was yellow brown, opaque, round, regular, entire and matt type vegetative, of white aerial mycelium with spiral sporopore (Spirales). This strain was Gram-positive and immobile. The temperature for optimum growth was 30°C, and the strain did not grow at 45°C and 10°C. Chitin, gelatin, and starch were degraded. This strain utilized D-glucose, D-xylose, L-arabinose, cellobiose, and cellulose. Scanning electron micrograph of strain M428 shows straight chains tangled with numerous spores, which is a typical pattern of *Streptomyces*, and spores are

Table 1. Basic biochemical and physiological properties of *Streptomyces* sp. M428.

Properties		Sugar utilization	
Color of aerial mycellium	gray	Glucose	+
Culture at 4°C	-	Mannitol	+
at 40°C	+	Inositol	+
β-galactosidase	-	Sorbitol	+
Arginine dihydrolase	-	Rhamnose	+
Lysine decarboxylase	-	Sucrose	+
Ornithine decarboxylase	-	Melibiose	+
Urease	+	Arabinose	+
Gelatinase	+	Citrate utilization	-
Amylase	+	H_2S production	-
VP-test	+	Gram staining	+
Indole production	-	Mobility	-
Tryptophan utilization	+	Nitrate reduction	-

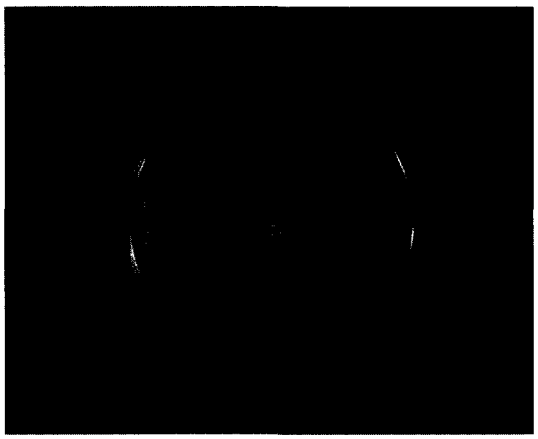

Fig. 1. Photograph of paper disk antifungal test of *Streptomyces* sp. M428.

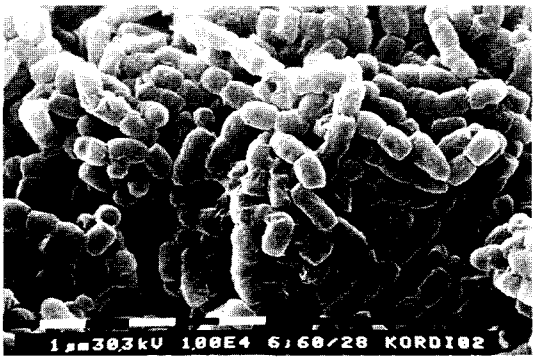

Fig. 2. SEM Photograph of *Streptomyces* sp. M428 mycellium.

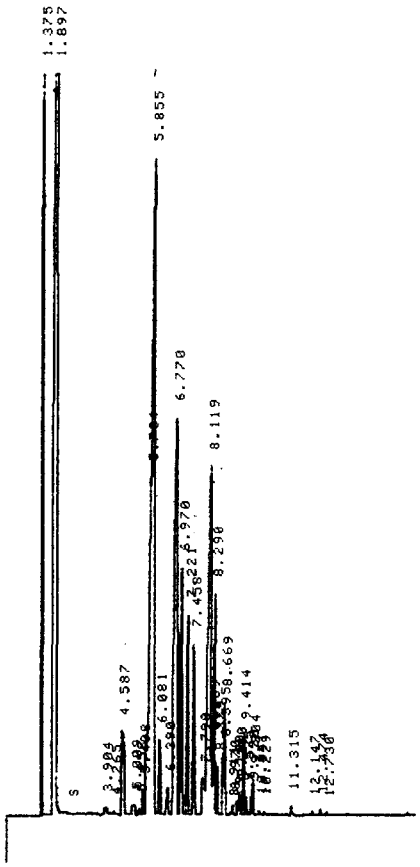

Fig. 3. Gas chromatogram of fatty acid composition of *Streptomyces* sp. M428.

cylindrical with a smooth surface (Fig. 2). For the analysis of cell wall materials, diaminopimelic acid analysis showed only LL-form and cellular sugar analysis with TLC showed only arabinose. The basic biochemical and physiological properties of the strain M428 were summarized in Table 1. Cellular fatty acid profile revealed iso-pentadecanoic acid (i-C15:0, 30.34% of total fatty acid) and iso-palmitic acid (i-C16:0, 24.56% of total fatty acid) as the major fatty acids (Fig. 3). These results indicate that this strain should be assigned to the genus of *Streptomyces* [5], and was designated as *Streptomyces* sp. M428.

Isolation and Purification

The general purification process is shown in Fig. 4. The culture broth (335 l) was concentrated with tangential filtration (filter pore size: 0.2 μm) to 10 l, and then centrifuged at 8,500 rpm for 10 min to separate broth filtrate and mycelial cake. After removing the supernatant, the mycelial cake (wet weight 3.633 kg) was extracted with methanol (3×6 l). The extracts were concentrated *in vacuo* and then partitioned between isobutanol and water. The isobutanol layer (69.48 g) was dried under vacuum and repartitioned between n-hexane and 10% aqueous methanol. The aqueous methanol layer (56.16 g) was separated by C_{18} reversed-phase vacuum flash chromatography (YMC ODS A 60-I25 gel, YMC) using sequential mixtures of MeOH and water as eluents (50, 40, 30, 20, 10% aqueous MeOH, and MeOH). The fraction eluted with 30% aqueous MeOH which showed the highest antifungal activity was separated by C_{18} reversed-phase HPLC (semi-preparative YMC ODS-AQ column, 10×300 mm, solvent: 40% aqueous MeOH) to yield active compounds A (89.6 mg) and B (19.3 mg).

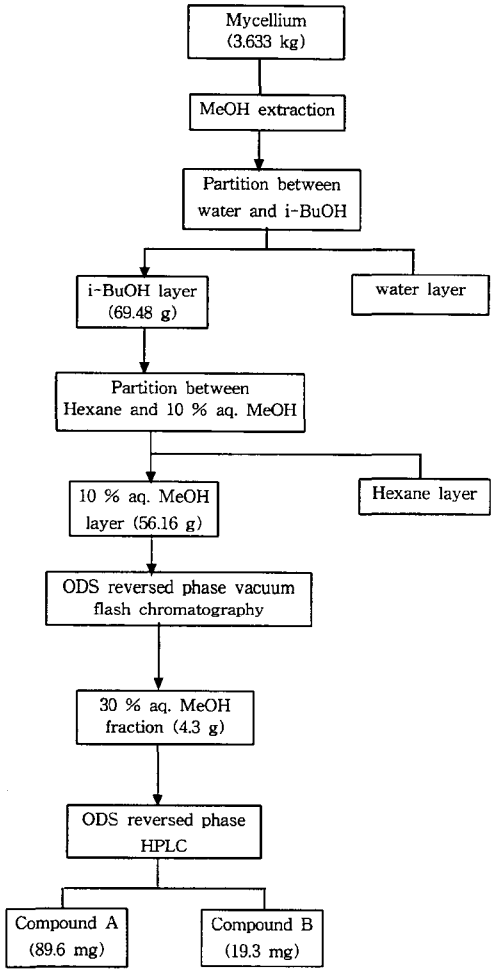

Fig. 4. Schematic diagram for the purification of lysophosphatidyl inositols.

Physicochemical Properties and Structure Elucidation

Active fractions A and B were white sticky materials. These fractions were freely soluble in a mixture of MeOH/water (1:1) while insoluble in chloroform, methylene chloride, and ethyl acetate, moderately soluble in MeOH, and slightly soluble in water.

The FAB mass spectrum of fraction A showed a prominent molecular ion peak at *m/z* 595 and a less intense peak at *m/z* 593, suggesting the presence of a mixture of homologues which were not separated further. The presence of hydroxyl and ester groups was indicated by intense absorption bands in the IR spectrum at υ_{max} 3350 and 1735 cm^{-1}, respectively. The presence of a phosphate group was also revealed by ^{31}P-NMR spectral data (δ+ 5.5, phosphoric acid as an internal standard) and the absorption band of P=O and P-O-C stretches appeared at 1225 and 1035 cm^{-1} in the IR spectrum, respectively (Table 2).

Table 2. Physicochemical properties of Antifungal lysophosphatidyl inositol.

Appearance	White sticky solid	
Molecular formula	$C_{25}H_{49}O_{12}P$ (A)	$C_{24}H47O_{12}P$ (B)
FAB-MS	595, 593 (A)	581 (B)
Solubility:		
soluble	mixture of MeOH/water (1:1)	
moderately soluble	MeOH	
slightly soluble	water	
insoluble	chloroform, methylene chloride, ethyl acetate	
Presence of		
hydroxyl group	υ_{max} 3350 cm^{-1}	
ester group	υ_{max} 1735 cm^{-1} intense bands in the IR spectrum	
phosphate group		
P=O stretches	1225 cm^{-1}	
P-O-C stretches	1035 cm^{-1}	
	^{31}P NMR (δ+5.5, internal standard phosphoric acid)	

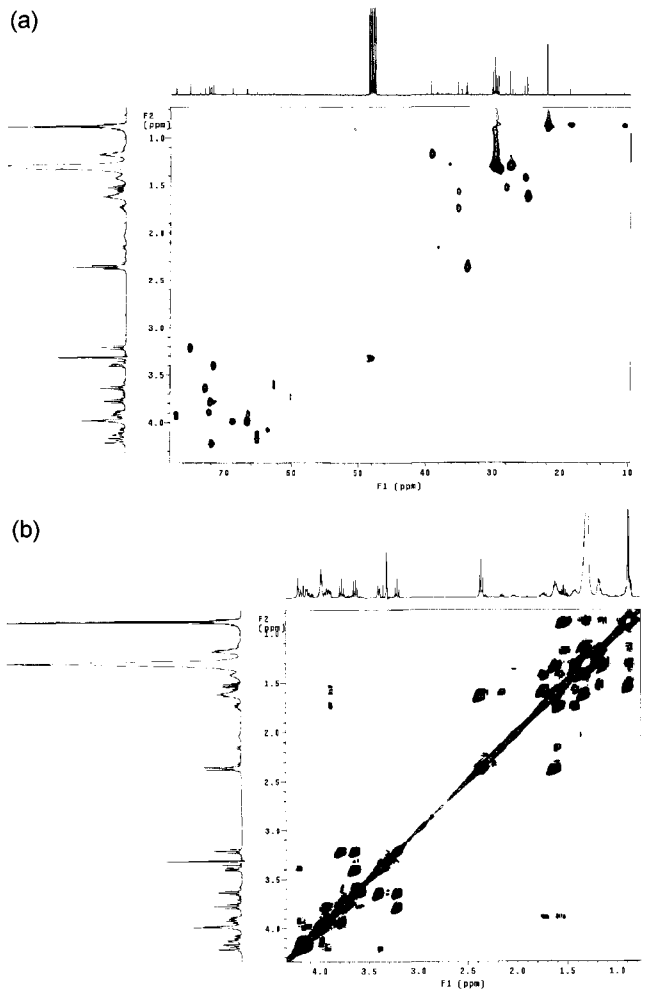

Fig. 5. (a) HMQC and (b) COSY45 spectrum of antifungal lysophosphatidyl inositol.

Preliminary structural elucidation, based on the analysis of the ^{1}H- and ^{13}C-NMR in combination with ^{1}H-COSY and HMQC experiments, revealed the complex glyceride (Fig. 5). The presence of long aliphatic hydrocarbon chains was indicated by a large band of methylene protons at δ 1.26 in the ^{1}H-NMR spectrum, as well as by a cluster of methylene signals around δ 29.7 in the ^{13}C-NMR spectrum. The latter also indicated the presence of a series of oxygen-bearing carbons, i.e. two oxymethylenes at δ 66.27 (C-1) and 67.78(C-3), and seven oxymethine groups at δ 78.33 (C-1'), 76.25 (C-5'), 74.00 (C-4'), 73.24 (C-6'), 73.06 (C-2'), 72.80 (C-3'), and 69.94 (C-2). The HMQC experiment allowed us to associate these carbons with the attached protons; they were two oxymethylene protons [δ 4.16 and 4.11 (H_2-1), and 3.97 (H_2-3)] and seven oxymethine protons [δ 4.20 (H-2'), 3.97 (H-2), 3.91 (H-1'), 3.76 (H-6'), 3.62 (H-4'), 3.38 (H-3'), and 3.20 (H-5')].

A combination of ^{1}H-COSY, HMQC, and HMBC experiments showed the presence of an inositol and an 1-acyl glycerol moiety confirmed by a 3-bond H-C correlation between H-1 (δ 4.16 and 4.11) and C-1" (δ 175.40) in the HMBC experiment. The inositol part was connected to the 1-acyl glycerol moiety by a phosphate linkage, as determined by the C-P long-range couplings [78.33 (C-1', d, Jcp=6.0 Hz), 73.24 (C-6', d, Jcp=5.5 Hz), 73.06 (C-2', d, Jcp=1.8 Hz), 69.94 (C-2, d, Jcp=7.8 Hz), and 67.78 (C-3, d, Jcp=6.0 Hz)].

To obtain further information on the nature of the acyl chains, the compounds were subjected to methanolysis by refluxing in MeOH/MeONa for 1 h, and the fatty acids were transmethylated with 5% HCl in MeOH at 80°C for 2 h and reextracted with n-hexane. GC analysis of the extract showed the presence of fatty acid methyl esters, identified as methyl palmitate (C16:0, 26%), methyl palmitoleate (C16:1, 18%), and methyl iso-palmitate (i-C16:0, 56%) by comparing GC retention times with those of the authentic samples.

The relative configurations of the inositol ring were determined by proton coupling constants and NOESY experiment. The orientations of the H-1'-H-6' protons by coupling constants were readily assigned as axial, equatorial, axial, axial, axial, and axial, respectively. This interpretation was supported by NOE correlations between H-4' and H-6' and the mutual correlations among H-1', H-3', and H-5'. Thus, the inositol moiety was defined as a myo-inositol. In addition, the ^{1}H-NMR spectrum of this mixture was very similar to that of commercially available lysophosphatidyl inositol (Sigma). Therefore, this fraction was defined to be a mixture of three lysophosphatidyl D-myo-inositols which had palmitic, palmitoleic, and iso-pentadecanoic acid acylated on the sn-1 position, respectively (26% of 1-palmitoyl-sn-glycero-3-phospho-[1-D-myo-inositol], 18% of 1-palmitoleoyl-sn-glycero-3-phospho-[1-D-myo-inositol], and 56% of 1-isopalmitoyl-sn-glycero-3-phospho-[1-D-myo-inositol] as the major component). From the GC analysis and structural point of view, the major component, 1-isopalmitoyl-sn-glycero-3-phospho-[1-D-myo-inositol], seems to be responsible for the antifungal activity, but further separations of this mixture into pure individual compounds were not performed.

The major component of fraction B isolated under the same chromatographic condition was also identified by combined spectroscopic methods and GC analysis. The mass spectrum of fraction B showed a prominent molecular ion peak at *m/z* 581 that was significantly different from those of the major constituents of the fraction A. However, the ^{1}H-NMR data for this fraction were almost identical to those obtained for the fraction A. A combination of 2D NMR experiments also revealed that the major component of this fraction possessed the same sn-glycero-3-phospho-[1-D-myo-inositol] moiety as the major components of A. Therefore, the structural differences between these compounds are the length and/or nature of the fatty acid side chain. The acidic methanolysis of the fraction B

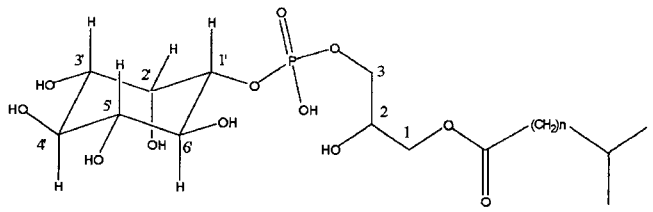

Fig. 6. Structure of antifungal lysophosphatidyl inositol; n=12 for compound A, 11 for compound B.

followed by GC analysis indicated the isopentadecanoic acid (i-C15:0) as the fatty acid. Thus, the major component of fraction B was most likely 1-isopentadecanoyl-sn-glycero-3-phospho-[1-D-myo-inositol]. The structures of compounds A and B were shown in Fig. 6.

An interesting phenomenon has been observed for antifungal activity of these compounds. Neither did the fatty acid moiety of these antifungal lysophosphatidyl inositol, such as iso-myristic acid and iso-pentadecanoic acid nor the lysophosphatidyl inositol with unbranched fatty acid such as sn-1 palmitic acid show any activity (data not showed), suggesting that the combination of these moieties is essential for the antifungal activities. The result also suggested that the antifungal compound in the fraction A, a mixture of 3 different lysophosphatidyl inositols, is the one having iso-pentadecanoic acid which is also the major component in the mixture (56%). These compounds displayed highly selective antifungal activity against *C. albicans* with MIC values of 5 μg/ml (compound A) and 2.5 μg/ml (compound B), while almost negligible antibiotic activity against *E. coli* and *P. aerogenosa* with a MIC value higher than 50 μg/ml, and no cytotoxic activities against human myeloma leukemia K562 (LD_{50}>100 μg/ml). Therefore, structurally, these compounds can be regarded as good candidates for the large-scale production by either a fermentation or phospholipid modification process through enzymatic or chemical processes.

Lysophospholipids are usually produced from corresponding phospholipids by the action of phospholipase A_2, and there are many reports on the physiological function of this class of lipid, such as being a precussor of platelet activation factor (PAF). Many branched fatty acids (iso- or anteiso-) are known to be involved in eubacteria and actinomycetes, and used for the taxonomical studies [1]. The exact function of branched fatty acids is not well known, however, there are reports about the branched fatty acid (14,16-dimethyl stearic acid) derivatives possessing very selective antibiotic activity against *Legionella* species [7, 13]. Five membered cyclitol glycolipids with branched fatty acids have been reported from marine sponges [3]. There have also been several reports about the involvement of branched or unsaturated fatty acyl moieties in some antifungal compounds such as the zaragozidine and echinocabdin series [12]. It is quite interesting that the branched fatty acids acylated to the 1-phosphoinositol group show selective antifungal activity.

Table 3. Assignment for key protons and carbons of compounds A and B.

Moiety	Position No*	^{13}C-NMR (ppm)	^{1}H-NMR (ppm)
Glycerol	1	66.27	4.16, 4.11
	2	69.94	3.97
	3	67.78	3.97
Inositol	1'	78.33	3.91
	2'	73.06	4.20
	3'	72.80	3.38
	4'	74.00	3.62
	5'	76.25	3.20
	6'	73.24	3.76
Fatty acid	1"	175.40	-
	2"	34.92	2.35
	3"	26.00	1.62
	4"-13"	29.71	1.26 (cluster)
	14"	29.15	1.51
	15", 16"	23.05	0.87

*Compare with the structure in Fig. 6.

Acknowledgments

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Table 4. The MIC value of antifungal lysophosphoinositol A and B against *E. coli, P. aerogenosa, C. albicans,* and human leukemia K-562.

	E. coli	*P. aerogenosa*, MIC (μg/ml)	*C. albicans*	human leukemia K-562 LD_{50} (μg/ml)
Compound A*	>50	>50	2.5	>100
Compound B	>50	>50	5	>100

*Because compound A is a mixture, the MIC values were calculated on the assumption that 56% of the mixture is the iso-palmitoyl lysophosphatidyl inositol.

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