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Synthesis of Novel Lysophosphatidylcholine Analogues Using Serine as Chiral Template

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Abstract: Four novel lysophosphatidylcholine (lysoPC) analogues, (*S*)-*N*-stearoyl-*O*-phosphocholineserine methyl ester [**(*S*)-1a**], (*R*)-1-lyso-2-stearoylamino-2-deoxy-*sn*-glycero-3-phosphatidylcholine [**(*R*)-2a**], (*R*)-*N*-stearoyl-*O*-phosphocholineserine methyl ester [**(*R*)-1b**], and (*S*)-1-lyso-2-stearoylamino-2-deoxy-*sn*-glycero-3-phosphatidylcholine [**(*S*)-2b**], were synthesized starting from serine as a chiral template. These synthetic compounds exhibited greatly enhanced hyphal transition inhibitory activity in *Candida* as compared to the natural lysoPC.

Recently, lysophosphatidylcholine (lysoPC) has been of crucial importance in various biochemical processes due to its regulatory activity of signaling enzymes in a range of lysoPC-responsive cells. LysoPC mediates the activation of p38, AP-1, and JNK kinases,[1,2] as well as adenylyl cyclase,[3] and displays the inhibition of platelet aggregation[4,5] while specifically binding to two G-protein-coupled receptors, OGR1 and GPR4, with lower affinity than sphingosylphosphorylcholine.[6] In addition, the presence of lysoPC resulted in the migration of lymphocytes toward the site of inflammatory tissue by inducing the secretion of chemotactic chemicals from macrophages.[7]

In the course of our study in glycerolipid chemistry,[8] we isolated lysoPCs from deer antler extracts, guided by the inhibitory activity of morphogenic transition of yeast to hyphae in *Candida albicans*, and fully characterized their structures.[8e] Despite the growing knowledge of deer antler in immunological function, the molecular basis of a cellular target or mechanism of action is unknown.[8a,9] The effects of antler on immunological function may be the result of stimulation of cellular immunity or the suppression of pathogenic activity. Of the various pathogens in humans, fungi are least vulnerable to medicines. Only a few phospholipids exhibited antifungal activity but none of them targeted on the morphogenic transition suppression of fungi, to the best of our knowledge.[10] We successfully synthesized lysoPC as well as its two regioisomers in racemic form in order to establish a powerful spectroscopic tool for the structural elucidation of lysoPCs in nature.[8f,g] From our previous study, however, the isolated lysoPC was proven to be not as effective as other commercially available drugs such as amphotericin B, clotrimazole, ketoconazole, and fluconazole on the suppression of hyphal transition of *C. albicans* (data not shown). In addition, sphingomyelin and phosphatidylcholine had no effect, suggesting that both phosphocholine moiety and a short chain length at the C1 position of the glycerol backbone may be necessary in the hyphal transition suppressing activity. These results led us to design and synthesize four lysoPC analogues. In this paper, we report the asymmetric synthesis of newly designed lysoPC analogues to search for a novel hyphal transition suppressor in *C. albicans*.

Structures of novel lysoPC analogues are shown in Figure 1. They are (*S*)-*N*-stearoyl-*O*-phosphocholineserine methyl ester [**(*S*)-1a**], (*R*)-1-lyso-2-stearoylamino-2-deoxy-*sn*-glycero-3-phosphatidylcholine [**(*R*)-2a**], (*R*)-*N*-stearoyl-*O*-phosphocholineserine methyl ester [**(*R*)-1b**], and (*S*)-1-lyso-2-stearoylamino-2-deoxy-*sn*-glycero-3-phosphatidylcholine [**(*S*)-2b**].

These four lysoPC analogues are designed in such a way that the OH group at the C1 position of the glycerol backbone is either preserved or transformed into the CO_2-CH_3 group, whereas the OH group at the C2 position is replaced by $NHCO(CH_2)_{16}CH_3$. LysoPC analogues are designed to possess the amide group instead of the ester with an expectation of providing them with resistance to enzymatic hydrolysis.

Synthesis of the first target molecule, (*S*)-*N*-stearoyl-*O*-phosphocholineserine methyl ester [**(*S*)-1a**], is illus-

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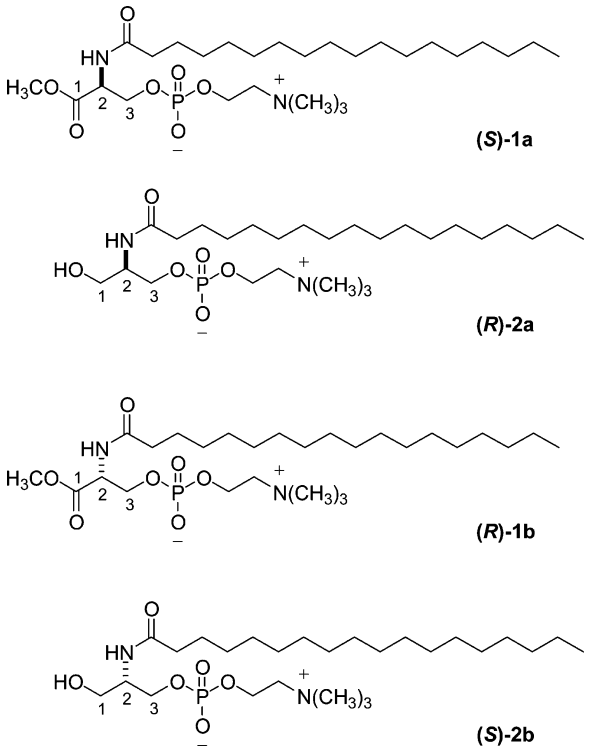
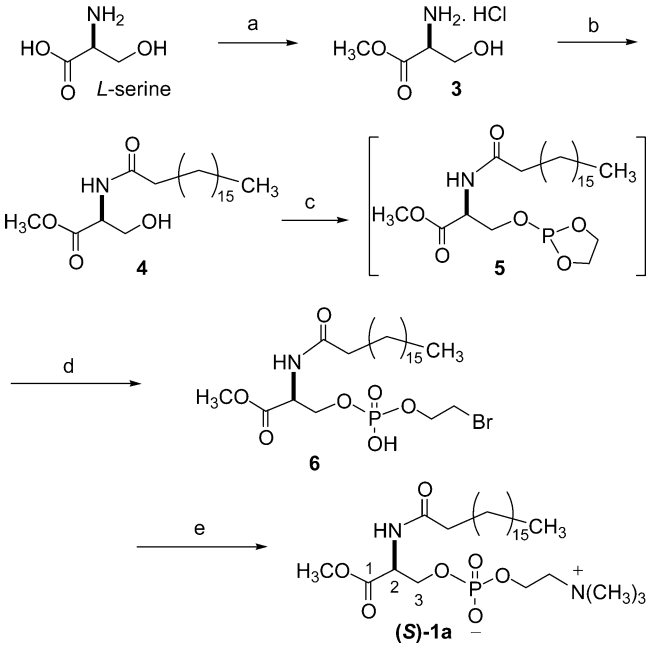


FIGURE 1. Structures of the novel lysophosphatidylcholine (lysoPC) analogues: (*S*)-*N*-stearoyl-*O*-phosphocholineserine methyl ester [**(*S*)-1a**], (*R*)-1-lyso-2-stearoylamino-2-deoxy-*sn*-glycero-3-phosphatidylcholine [**(*R*)-2a**], (*R*)-*N*-stearoyl-*O*-phosphocholineserine methyl ester [**(*R*)-1b**], and (*S*)-1-lyso-2-stearoylamino-2-deoxy-*sn*-glycero-3-phosphatidylcholine [**(*S*)-2b**].

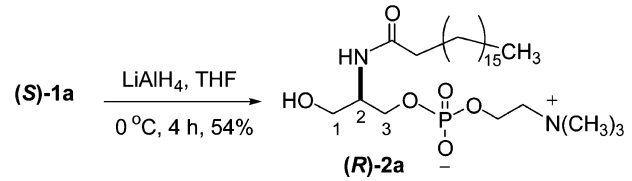
SCHEME 1[a]



[a] Key: (a) dry HCl/MeOH, 2.5 h, 98%; (b) stearic acid, DCC, HOBt, NMM, CH_2Cl_2, 0 °C to rt, 4 h, 90%; (c) ethylene chlorophosphite, DIEA, THF, −15 °C, 1.5 h; (d) (i) Br_2, −15 °C, 15 min, (ii) H_2O, rt, 1 h; (e) aq 40% NMe_3, $CHCl_3$/^{i}PrOH/CH_3CN (3/5/5, v/v), rt, 11 h, 12% (from **4**).

trated in Scheme 1. L-Serine was chosen as the chiral template and protected by treatment with saturated HCl in methanol to provide methyl ester **3**. Coupling reaction between compound **3** and stearic acid was achieved by using standard DCC/HOBt methodology[11] to provide the desired amide **4** in good yield. It is noteworthy to mention that protection of the C3 hydroxyl group of compound **3** was not necessary prior to executing the amide bond formation at the C2 amino group. Final installation of the phosphocholine moiety into the C3 hydroxyl group of compound **4** was the key step in our strategy. Attempts to synthesize the phosphocholine moiety by using triethylammonium phosphocholine,[12] 2-bromoethyl dichlorophosphate,[13] and 2-chloro-2-oxo-1,3,2-dioxaphospholane[14] were unsuccessful. Interestingly, we found that the phosphocholine moiety was successfully introduced by using the ethylene chlorophosphite−trimethylamine protocol, according to the procedure reported by Bittman.[8g,15] Thus, compound **4** was treated with ethylene chlorophosphite in the presence of *N*,*N*-diisopropylethylamine in THF at −15 °C to produce cyclic phosphite **5**. Compound **5** was not purified due to its decomposing nature on silica. After ring-opening reaction of compound **5** with bromine and subsequent hydrolysis, the resulting compound **6** was quaternized with 40% aqueous trimethylamine in $CHCl_3$/^{i}PrOH/CH_3CN. However, we could not trace the desired product by thin-layer chromatography, in contrast with our previous result in the synthesis of lysoPC.[8g] At this point, we decided to purify the intermediate **6** by means of recrystallization with acetone/methylene chloride before proceeding to the next step. The first target, **(*S*)-1a**, was successfully synthesized from purified **6** by treatment with aqueous trimethylamine (12% yield from **4**). As a result, compound **(*S*)-1a** was obtained from L-serine in six steps and 11% overall yield.

SCHEME 2



Synthesis of the second target, **(*R*)-2a**, was accomplished by reduction of the methyl ester group of **(*S*)-1a** to the corresponding alcohol with lithium aluminum hydride in THF,[16] in 54% yield (Scheme 2).

Synthesis of the third and fourth target molecules, **(*R*)-1b** and **(*S*)-2b**, followed the same synthetic protocol as for **(*S*)-1a** and **(*R*)-2a**. Synthesis began with D-serine as

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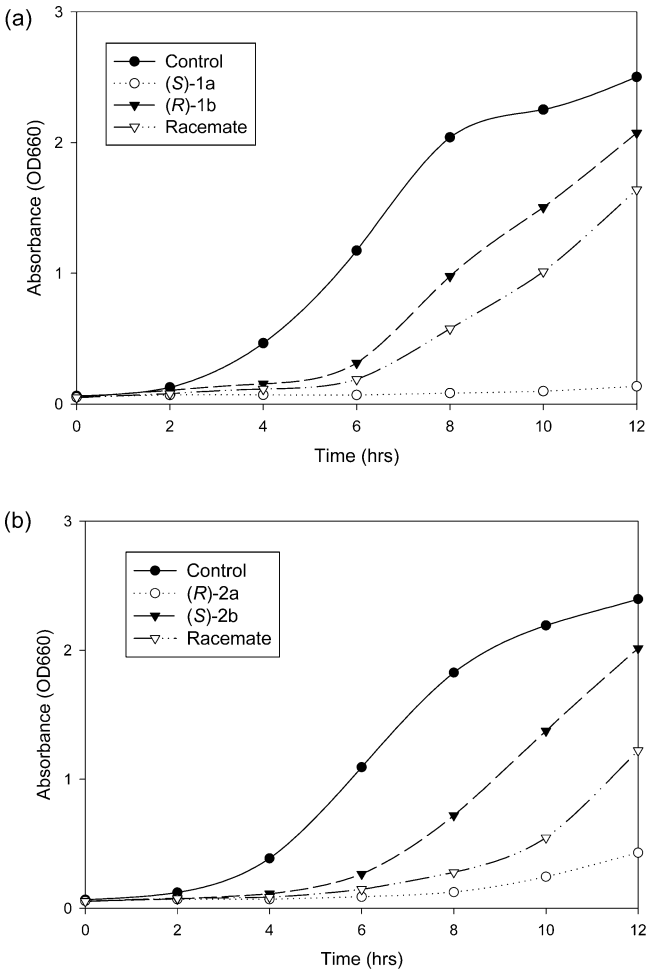

FIGURE 2. Effects of (a) **(*S*)-1a** and **(*R*)-1b** and (b) **(*R*)-2a** and **(*S*)-2b** on the growth of SC5314. SC5314 was cultured in Sabouraud's dextrose broth supplemented with each isomer at 30 °C, and growth was determined by measuring the absorbance at 660 nm (OD660).

the chiral template. The carboxylic group of D-serine was protected as the methyl ester, which was then reacted with stearic acid to afford *N*-stearoyl-D-serine methyl ester in 87% yield for two steps. Sequential reactions of the resulting amide with ethylene chlorophosphite, bromine, and water followed by aqueous trimethylamine produced the third target, **(*R*)-1b** (10% overall yield from D-serine). For the installation of the hydroxyl function at the C1 position, the ester group of **(*R*)-1b** was reduced with lithium aluminum hydride to afford the fourth target, **(*S*)-2b** (53% yield), with a rotation value of −5.3 (*c* 2.04, CH_2Cl_2/MeOH). Comparison with **(*R*)-2a**, which has a positive rotation value of +5.3 (*c* 1.92, CH_2Cl_2/MeOH), revealed that the target lysoPC analogues were synthesized in enantiomerically pure forms. All structures of lysoPC analogues were characterized by spectroscopic analysis and compounds **(*S*)-1a** and **(*R*)-2a** were further confirmed by fast atom bombardment mass (FAB-MS) spectrometry.

For the antifungal activity data, *Candida* strains were used as the test microorganism and grown in the Sabouraud-dextrose broth at 30 °C for 16 h. Then *C. krusei*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, and *C. albicans* were transferred to cornmeal agar plates supplemented with lysoPC analogues: at 1.9 μg/mL for **(*S*)-1a** and **(*R*)-1b** and at 7.8 μg/mL for **(*R*)-2a** and **(*S*)-2b**. After 5 days of incubation, each strain was observed with a photometric microscope. All of the lysoPC analogues showed selective inhibitory activity against the hyphal transition on various species of *Candida*, such as *C. albicans*, *C. krusei*, *C. guilliermondii*, and *C. parapsilosis*, but not *C. tropicalis*. The minimum inhibitory concentration (MIC) and IC_{50} were determined as reported previously.[8e] The log-phase yeast culture of *C. albicans* was transferred to RPMI liquid media and grown at 37 °C for 7 h with or without each of the lysoPC analogues at various concentrations. The percentage of hyphae was calculated by counting hyphae and the total number of

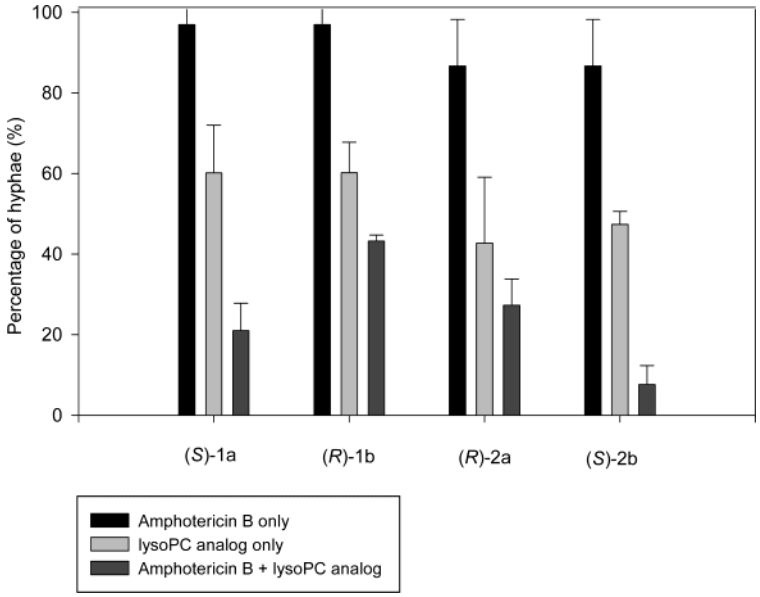

FIGURE 3. Additive effects of four lysoPC analogues with amphotericin B on the suppression of hyphal transition in *Candida albicans*. Lower and less-toxic doses of amphotericin B (0.02 μg/mL) and IC_{50} values of four lysoPC analogues were added to the culture for **(*S*)-1a** and **(*R*)-1b** (at 1 μg/mL), for **(*S*)-2b** (at 4 μg/mL), and for **(*R*)-2a** (at 2 μg/mL). Hyphae were counted after 7.5 h of growth.

TABLE 1. Minimum Inhibitory Concentration (MIC) and IC_{50} Values for Inhibition of Hyphal Transition of the Four LysoPC Analogues

compd	MIC (μM)	IC_{50} (μM)
(*R*)-**2a**	9.5	3.8
(*S*)-**2b**	15.3	7.6
(*S*)-**1a**	3.5	1.8
(*R*)-**1b**	7.0	1.8

cells (Table 1). The four lysoPC analogues exhibited substantial antifungal activity—as measured by the hyphal transition suppressing activity of the yeast.

They also inhibited the growth of yeast (Figure 2). Compound **(*S*)-1a** is strongly effective in the suppression of hyphal transition as well as in inhibition of yeast growth. Their additive effects with amphotericin B are shown in Figure 3. Combining the lysoPC analog with amphotericin B suppressed hyphal transition more strongly than each product alone. Compound **(*S*)-2b** represented the strongest additive effect on the suppression of hyphal transition.

We also investigated the effect of lysoPC and the synthetic compounds, **(*S*)-1a** and **(*R*)-1b**, on the mobilization of intracellular calcium concentration in spleen lymphocytes (Figure 4). The intracellular calcium concentration strongly increased when spleen lymphocytes were stimulated with 25 g/mL **(*S*)-1a** (Figure 4E-b) and **(*R*)-1b** (Figure 4D-b) as well as lysoPC (Figure 4C-b). Because the intracellular calcium mobilization occurs early in the lymphocyte activation, investigation of the subsequent effect on signal transduction would confirm whether lysoPC and synthesized lysoPC analogues affect the immune system by the same mechanism.

In conclusion, (*R*)- and (*S*)-enantiomers of novel lysoPC analogues were synthesized from commercially available L- and D-serine as starting materials by a short and efficient method. These newly designed and synthesized lysoPC analogues exhibited much enhanced hyphal transition inhibitory activity against *Candida* species as compared to the natural lysoPC. Compounds are obtained as crystalline powder and are very hydrophilic and lipophilic. Therefore, they could be safely administered

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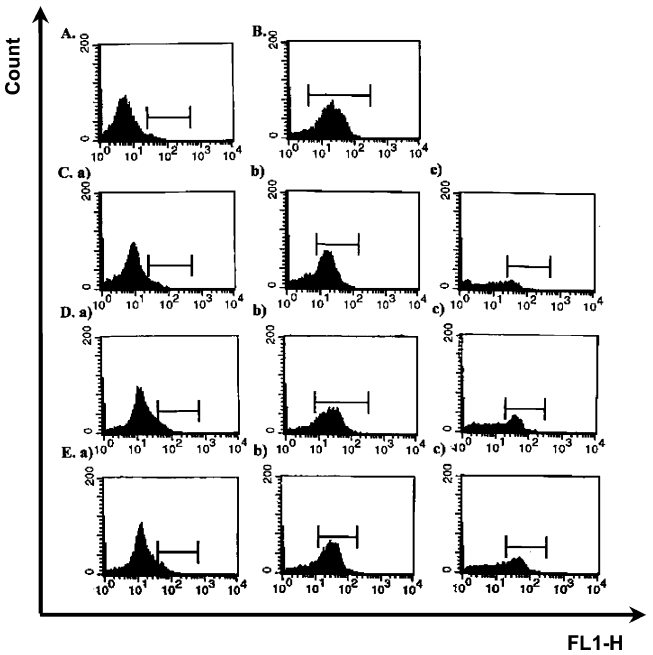

FIGURE 4. Calcium mobilization in response to **(*S*)-1a** and **(*R*)-1b** stimulation. (A) Spleen lymphocytes (10^6 cells/mL) were loaded with Fluo-3/AM (2 μg/mL). These cells were stimulated with (B) ionomycin (2 μg/mL), (C-a) lysoPC (12.5 μg/mL), (C-b) lysoPC (25 μg/mL), (C-c) lysoPC (50 μg/mL), (D-a) **(*R*)-1b** (12.5 μg/mL), (D-b) **(*R*)-1b** (25 μg/mL), (D-c) **(*R*)-1b** (50 μg/mL), (E-a) **(*S*)-1a** (12.5 μg/mL), (E-b) **(*S*)-1a** (25 μg/mL), and (E-c) **(*S*)-1a** (50 μg/mL). Changes in $[Ca^{2+}]_i$ were determined as the percentage of positive cells that mobilized intracellular calcium, as assessed by flow cytometry.

either parenterally or orally as pharmaceutical compositions of various dosages.

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Supporting Information Available: Experimental section and ^1H and ^{13}C NMR spectra as well as FAB-MS spectra of compounds **(*S*)-1a** and **(*R*)-2a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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