Fruiting-Inducing Activity and Antifungal Properties of Lipid Components in Members of Annelida

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Fruiting-inducing activity and antifungal properties of lipid components in the phylum Annelida were examined. Some amphoteric cerebrosides carrying a phosphocholine group showed fruiting-inducing activity on *Schizophyllum commune*, and one of them possessed activity comparable to that of *Sch* II, the most potent substance known. Furthermore, alkyl lysophosphatidylcholines were found to have an inhibitory effect on the growth of phytopathogenic fungi, *Alternaria kikuchiana* and *Phomopsis mali*. The relationship between structure and biological activities is discussed.

Key words amphoteric cerebroside; fruiting-inducing activity; *Schizophyllum commune*; alkyl lysophosphatidylcholine; antifungal activity; phytopathogenic fungi

Our recent studies on the lipid components of Annelida, such as *Pheretima asiatica*,[1] *Hirudo nipponica*,[2] *Marphysa sanguinea*,[3] and *Neanthes diversicolor*[4] have revealed the presence of unique zwitterionic galactosylcerebrosides, as well as large amounts of alkyl ether phospholipids. In 1982, Kawai and Ikeda noted that fruiting bodies were formed around a colony of *Penicilium funiculosum* A-1 which appeared as a contaminant in a culture of the Basidiomycete *Shizophyllum commune*.[5] They isolated several fruiting-inducing substances from *S. commune* itself and identified the major active principle as [(4*E*,8*E*,2*S*, 3*R*,2′*R*)-*N*-2′-hydroxyhexadecanoyl-1-*O*-(β-D-glucopyranosyl)-9-methyl-4,8-sphingadienine (*Sch* II), together with related compounds.[6] Later, Kawai and his collaborators examined the structure-bioactivity relationship among various cerebrosides.[7]

Since the glycosylceramides isolated in our studies are, unlike those analyzed by Kawai *et al.*, amphoteric cerebrosides carrying the phosphocholine unit in the sugar moiety, we were interested in their fruiting-inducing effect, and we therefore examined the activity of twenty homogeneous cerebrosides on *S. commune* N-1.

Potent antimicrobial activities of alkyl lysophospholipids have been found by Tsushima and co-workers,[8] so we also examined the antifungal activity of naturally occurring phospholipids from Annelids against two species (*Alternaria kikuchiana* IFO 6444 and *Phomopsis mali* IFO 31010) of phytopathogenic fungi.

Experimental

Materials The samples used for the bioassay were obtained from *Pheretima asiatica* (**5**—**8**, **17**, **18**, **21**—**28**, fr. a, fr. b, fr. d), *Hirudo nipponica* (**1**—**4**, **15**, **19**, **20**), *Marphysa sanguinea* (**9**, **13**, **14**, **16**, fr. c) and *Neanthes diversicolor* (**10**—**12**). The 2-acylated phosphatidylcholines (**23a**, **23b**, **23c**) were prepared by acylation of **23** with the corresponding acyl anhydrides in pyridine at room temperature for 2 d followed by purification by column chromatography or recrystallization. Authentic *Sch* II and a dikaryotic strain of *S. commune* N-1 were donated by Dr. G. Kawai. A standard sample, Fosetyl® (ethyl phosphonate) was purchased from Shionogi Corporation.

Bioassay of Fruiting-Inducing Activity The assay was conducted according to the method described by Kawai *et al.*[6c] An appropriate amount of the test sample was dissolved in a solvent ($CHCl_3$–MeOH, 1 : 1 v/v). The solution (20 μl) was applied to a paper disc (8 mm diameter,

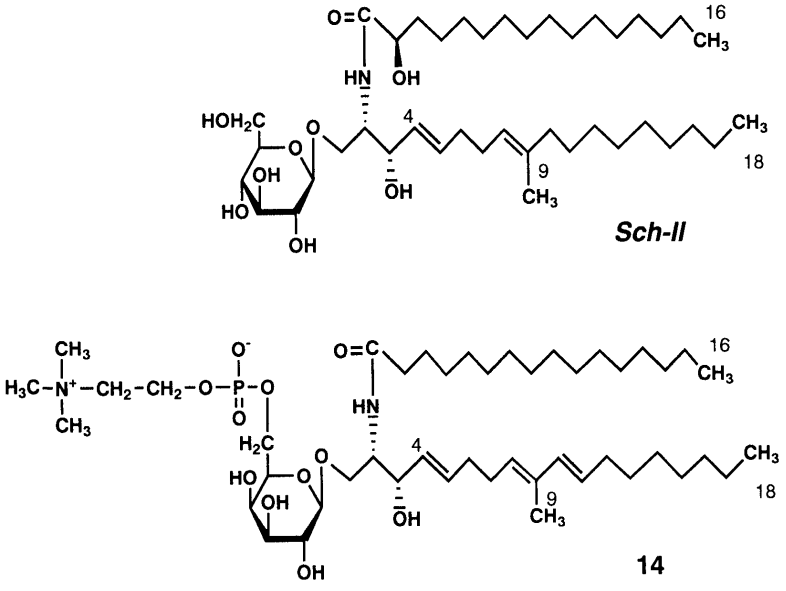

Fig. 1

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0.7 mm thickness: Advantec Toyo) and the solvent was removed under reduced pressure. A control disc was prepared in the same way by applying the solvent instead of the test solution. A dikaryotic strain of *S. commune* N-1 was inoculated at the center of the malt-yeast agar medium[9] in a 9 cm Petri dish, and the plate was incubated at 24 °C under white fluorescent light (100—200 lux). When the colony reached about 4 cm in diameter. (3—5 d), the control and test discs were placed on the margin (about 7 mm from the colony) of the plate. The plates were placed upside down and incubated for another two weeks under the same conditions as described above. Fruiting response around the discs was checked on day 14. The cultures without discs were incubated similarly, and natural fruiting was examined on day 30. Usually, more abundant fruiting bodies were observed around the test discs. All procedures were carried out aseptically.

When the minimum effective concentration was to be determined ($\times 10^{-9}$ mol/disc), the original solution was diluted two-fold serially with $CHCl_3$–MeOH (1 : 1 v/v), and the least quantity which apparently gave a larger number of fruiting bodies than the control was defined. In this system, the true minimum effective concentration is within the range of 0.5—2.0 times the given value (reflecting the two-fold dilution). The assay was carried out in duplicate and experiments were repeated three times.

Antifungal Activity Phytopathogenic fungi, *Alternaria kikuchiana* (IFO 6444) and *Phomopsis mali* (IFO 31010), were used as test organisms. The bioassay was conducted with paper discs of 6 mm diameter (Advantec Toyo).[10] An appropriate amount of the test sample was dissolved in $CHCl_3$–MeOH (1 : 1 v/v). A test sample (10 μl) was placed on a paper disc and the solvent was removed under reduced pressure. A control disk was prepared in the same way by the solvent applying instead of the test solution. Each fungus was spotted on the center of the corn-meal agar medium (Difco) in a 9 cm petri dish, and grown at 24 °C. Two to three days after the inoculation, the control and test discs were placed in four opposing positions (about 20 mm from the colony margin). After incubation for another 3 d under the same conditions as described above, the zone of inhibition produced around the disc was measured. No inhibition zone appeared with any control disc.

The inhibitory effect was compared with that of Fosecyl®, and expressed in terms of the equivalent amount of Fosecyl® (0—4.84×10^{-6} g/disc inhibitory concentration. IFO 6444; $r = 0.9939$, IFO 31010; $r = 0.9991$). The assay was carried out in triplicate and experiments were repeated four times.

Results and Discussion

Fruiting-Inducing Activity Table 1 summarizes the results of the fruiting-inducing assay together with the chemical formulae of the tested samples. All active cerebrosides have two or three double bonds in the sphingosine unit, and, regardless of the structural difference of their sugar residue, those containing (4*E*)-sphingenine or sphinganine are inactive. Among the active cerebrosides, compound **14** showed low active concentration values (1.3×10^{-9} mol/disk) for inducing fruiting body formation, and had an activity almost equal to that of *Sch* II (1.4×10^{-9} mol/disk). The other active compounds showed about a quarter to half the activity of *Sch* II.

Kawai *et al.* reported that the 8*E*-double bond and the methyl group at the C-9 position of the sphingosine unit are essential for high activity, but can be substituted by an 8*Z*-double bond, and that a 2′*R*-hydroxy group of the fatty acid residue increases the activity.[7a,c] In comparison with the structure of *Sch* II, as pointed out by Kawai *et al.*, **14** consists of a sphingosine unit having the 8*E*-double bond and 9-methyl group, but **14** has a 10*E*-double bond in the long-chain base instead of a 2′*R*-hydroxy group in the fatty acid unit, which enhances the activity (Fig. 1). Furthermore, in spite of the lack of the 9-methyl group in the sphingosine unit, compound **13** having 4*E*,8*E*,10*E*-double bonds still retained considerable activity. These observations suggested that the 10*E*-double bond in the sphingosine unit may increase the activity.

As each of compounds **19** and **20** contains an 8*Z*-double bond in the sphingosine unit,[7a,c] these compounds were expected to have high activity. However, both of them possessed only about one-fifth of the activity of *Sch* II. These results suggested that the component fatty acid or the sphingosine unit having a carbon chain length of more than C_{18} decreased the activity.

Table 1. Fruiting-Inducing Effect of Amphoteric Galactosylcerebrosides on *S. commune*

Sample	No.	Sphingoid	Fatty acid	Activity	Concentration ($\times 10^{-9}$ mol/disc)
Galα 1-6 Galα 1-6 Galβ 1-Cer	**1**	19 : 1 iso (4*E*)	24 : 0	−	
	2	18 : 1 (4*E*)	24 : 0	−	
	3	19 : 1 (4*E*)	23 : 0	−	
Galα 1-6 Galβ 1-6 Galβ 1-Cer	**4**	18 : 1 (4*E*)	16 : 0	−	
Glcα 1-4 Galβ 1-6 Galβ 1-Cer	**5**	18 : 1 (4*E*)	22 : 0	−	
Galβ 1-6 Galβ 1-Cer	**6**	19 : 1 iso (4*E*)	24 : 0	−	
Cho-P-6 Galβ 1-6 Galβ 1-Cer	**7**	18 : 1 (4*E*)	22 : 0	−	
Galβ 1-Cer	**8**	19 : 1 iso (4*E*)	24 : 0	−	
	9	18 : 1 (4*E*)	16 : 0	−	
Cho-P-6 Galβ 1-Cer	**10**	18 : 0	16 : 0	−	
	11	18 : 1 (4*E*)	16 : 0	−	
	12	18 : 2 (4*E*, 8*E*)	16 : 0	+	6.4
	13	18 : 3 (4*E*, 8*E*, 10*E*)	16 : 0	+	3.2
	14	18 : 3 (4*E*, 8*E*, 10*E*, 9-Me)	16 : 0	+	1.3
	15	18 : 1 (4*E*)	18 : 0	−	
	16	18 : 1 (4*E*)	20 : 0	−	
	17	18 : 1 (4*E*)	22 : 0	−	
	18	18 : 1 (4*E*)	24 : 0	−	
	19	22 : 3 (4*E*, 8*Z*, 11*Z*)	22 : 0	+	6.8
	20	22 : 3 (4*E*, 8*Z*, 11*Z*)	24 : 0	+	6.6

Cho-P, phosphocholine unit; Cer, ceramide unit; −, no activity at the dose tested (128×10^{-9} mol/disc).

Antifungal Activity The antifungal activities were estimated by comparison with those of a widely used commercial fungicide, ethyl phosphonate (Fosetyl®). Some glycerophospholipid fractions obtained in the course of our studies were also subjected to preliminary screening for inhibitory activity against *Alternaria kikuchiana* and *Phomopsis mali*. The alkyl-lysophosphatidylcholine fraction (fr. a) inhibited the growth of both fungi, while the other glycerophospholipid fractions having an ester linkage showed no appreciable inhibition (Table 2).

To examine the relationship between structure and the activity, we assayed eight pure 1-alkyl-lysophosphatidylcholines. Figure 2 shows that all these compounds inhibited the growth of *P. mali* and compound **23** having the pentadecyl group was 11 and 14 times more active than Fosetyl® against *A. kikuchiana* and *P. mali*, respectively. The inhibitory effects of compounds with a shorter or a longer alkyl chain were found to be less potent than that of the C_{15} alkyl chain compound. Compound **21** with a C_{14} alkyl chain showed no detectable inhibition of *A. kikuchiana*.

We next examined the activity of 2-acetyl- (**23a**), 2-propionyl- (**23b**) and 2-butyryl-1-pentadecylphosphatidylcholines (**23c**), but none of them showed significant inhibition against either of the fungi. Our results, together with those of Tsushima *et al.*,[8] indicate that the chain length of the alkyl group has a profound effect on the activity, and the inhibitory effect was lost when an acyl group was introduced on the glycerol moiety.

The results of our study on the minimum concentration needed for inhibition will be reported separately.

Table 2. Growth-Inhibitory Activeties of Lipid Components from Annelids against *A. kikuchiana* and *P. mali*

Fraction		Activity
Fr. a	1-Alkyl-lyso PC	+
Fr. b	1-Acyl-lyso PC	−
Fr. c	1-Alkyl-2-acyl PC	−
Fr. e	Amphoteric galactosylceramides	−

PC, phosphatidylcholines; −, no activity at a concentration of 100 μg/disc.

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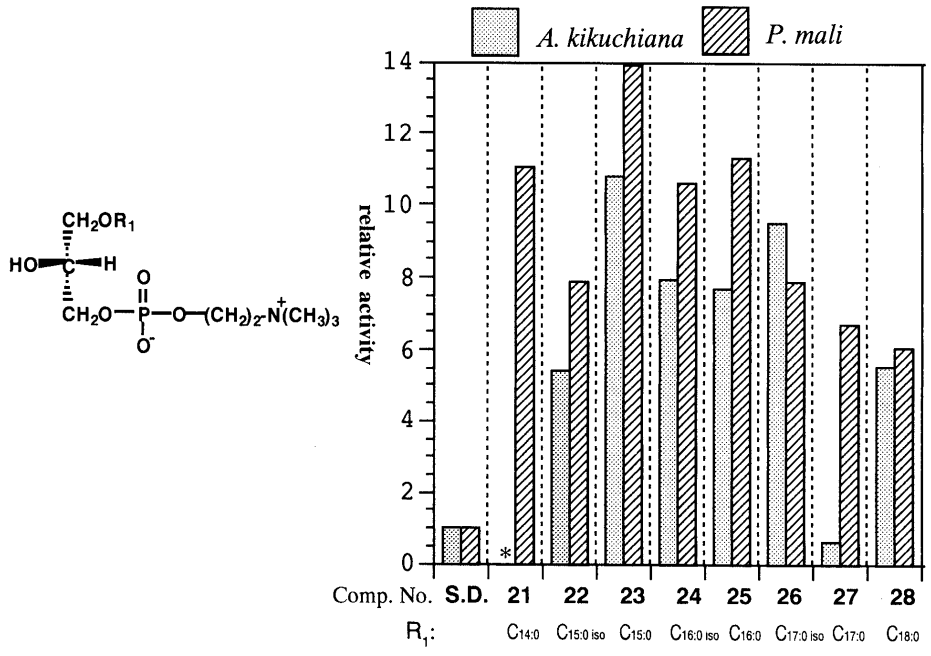

Fig. 2. Growth-Inhibitory Effect of 1-Alkyl-lysophosphatidylcholines

S.D., ethyl phosphonate. *, No activity at a concentration of 100 μg/disc.