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Stimulatory Effect of Certain Plant Sphingolipids on Fruiting of *Schizophyllum commune**

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We reported previously that certain cerebrosides and ceramides from fungi were active upon fruiting of *Schizophyllum commune* (Kawai, G., and Ikeda, Y. (1982) *Biochim. Biophys. Acta* 719, 612–618; Kawai, G., and Ikeda, Y. (1983) (*Biochim. Biophys. Acta* 754, 243–248). This work was undertaken to extend our study to sphingolipids in wheat grain. The cerebrosides from wheat grain were fractionated by high-performance liquid chromatography into at least 40 components with and without the fruiting-inducing activity. Four major active fractions were characterized by thin-layer chromatography, infrared spectroscopy, gas-liquid chromatography, gas-liquid chromatography-mass spectroscopy, and ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The active cerebrosides consist of glucose, 2-hydroxyhexadecanoic acid or 2-hydroxyoctadecanoic acid, and (4*E*,8*Z*)-sphingadienine or (8*Z*)-sphingenine. The cerebroside with (8*Z*)-sphingenine became inactive when the double bond was hydrogenated. Diglycosylceramides were as active as the monoglycosylceramides, but triglycosylceramides were only about 10% as active. The relationship between the structure and the activity is discussed.

Sphingolipids are constituents of the cytoplasmic membrane of eukaryotic cells and are known to function as receptors for certain bioactive compounds and thought to play a role in the cation transport system (1, 2). It has also been reported that sphingolipids have other physiological activities such as, activation of certain enzymes (3–5), antimicrobial and antitumor activity (6), anti-ulcerogenic activity (7), and nerve growth factor-like activity (8).

A distinct and novel activity was found by Kawai and Ikeda (9–11) and Kawai *et al.* (12), who demonstrated that the sphingolipids from *Schizophyllum commune* and other fungi stimulate fruiting body formation of *S. commune*. The major active component from *S. commune* has been identified as (4*E*,8*E*)-*N*-D-2′-hydroxyhexadecanoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine and the functional moiety for the activity is *N*-acylsphingoid. Moreover, the 8*E*-double bond and the methyl branch at C-9 of the sphingoid have been proposed as the key elements determining the activity. Very recently, Mori and Funaki (13) synthesized (4*E*,8*E*)-*N*-D-hydroxyhexadecenoyl-9-methyl-4,8-sphingadienine. This chemically synthesized substance stimulated the fruiting of *S. commune* as much as its natural counterpart.

In this paper we intended to extend our study to the effect of sphingolipids of plant origin on fruiting of *S. commune*, because many plant materials such as grains and beans contain various sphingolipids.

EXPERIMENTAL PROCEDURES

Materials—Diglycosylceramide and triglycosylceramide from wheat grain, and monoglycosylceramides (cerebrosides) from wheat grain (14, 15), rice straw (16), spinach leaf (17), Azuki bean seed (18), and pea seed (19) were prepared as described previously. (4*E*,8*E*)-4,8-Sphingadienine and (4*E*,8*Z*)-4,8-sphingadienine of cerebrosides from rice bran, and fatty acid methyl ester of cerebrosides from wheat grain were also prepared (14, 20) to use them as the standard lipids for chromatography.

Bioassay of Fruiting-inducing Activity—The basic assay method was described previously (9). A dikaryotic strain of *S. commune* (IFO 6502) was spotted on the center of malt-yeast agar medium in a Petri dish and grown at 23–25 °C. Four to five days after the inoculation, paper discs (8 mm in diameter and 0.7 mm in thickness), each charged with 20 μl of solvent-dissolved sample, were dried *in vacuo*, and placed at the margin of the plate together with a control disc. The plate was incubated for another week under white fluorescent light (100–200 lux). Usually, fruiting bodies were observed around the test discs. The minimum amount of active substance giving distinct fruiting-inducing effect on the tester was defined as 1 unit, and the specific activity of each sample was expressed as units/mg. Because of variation in the fruiting response, the unit of activity has a range of accuracy from 0.5 to 2.

Preparation of Fatty Acid Methyl Esters—Cerebrosides were heated under reflux with 1 ml of methanolic 5% HCl for 4 h. The reaction mixture was chilled and extracted with hexane. The extract was washed with water and concentrated to dryness to obtain methyl esters of the component fatty acids. This fraction was then subjected to thin-layer chromatography on Silica Gel G in hexane/ether (4:1, v/v) to separate normal, monohydroxy and dihydroxy fatty acid methyl ester (21).

Preparation of Methylglycosides—The methanolic solution after extraction of the fatty acid methyl esters was made alkaline and extracted with diethyl ether to remove sphingoids, and then deionized by passage through columns of Amberlite IR-120 (H^+) and Amberlite IRA-400 (OH^-) resins. The eluate was evaporated to dryness to yield the constituent sugar as methylglycosides.

Preparation of Sphingoids and Their Derivatives—Cerebrosides were hydrolyzed with 1 N HCl in aqueous methanol under reflux for 18 h at 70 °C (22). The reaction mixture was chilled, and extracted with hexane to remove the fatty acid methyl ester. The sphingoids were extracted with ethyl ether, and purified by silica gel thin-layer chromatography as described below. The purified sphingoids were acetylated by allowing them to stand first for 2 h at 70 °C and then overnight at 20 °C in the mixture of 0.1 ml of dry pyridine and 0.5 ml of acetic anhydride (23).

Thin-layer Chromatography—Cerebrosides were developed on a Silica Gel G plate with chloroform/methanol/water (65:16:2, v/v) and were visualized by spraying with anthrone reagent. Fatty acid methyl esters were developed on a Silica Gel G plate with hexane/ether (7:3, v/v) and were visualized with 50% H_2SO_4. Sphingoids were developed

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on a Silica Gel G plate with chloroform/methanol/2 N ammonia (40:10:1, v/v) and visualized with ninhydrin reagent. *N,O,O*-Triacetylsphingoid was developed on a Silica Gel G containing 10% $AgNO_3$ (24) with chloroform/methanol (98:2, v/v) and visualized with 50% H_2SO_4. Primuline spray (25) was used for non-destructive detection of the lipids which were then removed for further chemical analysis.

Gas-Liquid Chromatography—This procedure was performed on a Hitachi 163 Gas Chromatograph (Hitachi Seisakusho Co. Ltd., Tokyo) equipped with a hydrogen flame ionizing detector. Two glass tubes (3 mm × 2 m) were packed with 1.5% SE-30 on 60–80 mesh Chromosorb WAW-DCMS. Trimethylsilyl ether derivatives of the fatty acid methyl ester fractions and of the methylglycoside fractions were analyzed at 220 and 170 °C, respectively.

Gas-Liquid Chromatography-Mass Spectrometry—This procedure was performed on a Hitachi RMU-6MG instrument. A glass tube (3 mm × 1 m) was packed with 80–100 mesh Diasolid ZT (Nihon Chromato Work Co. Ltd., Tokyo). Trimethylsilyl ether derivatives of the cerebrosides were separated at 320 °C. Mass spectra were obtained under the following conditions: ion source temperature, 210 °C; electron energy, 20 eV; ionizing current; 80 μA; ion accelerator voltage, 3.2 kV.

Spectroscopic Methods—Infrared spectra of KBr pellets of testing substances were taken with a JASCO A-3 infrared spectrophotometer (Japan Spectroscopic Co. Ltd., Tokyo). ^1H and ^{13}C NMR spectra were produced in a JEOL FX-400 (JEOL Ltd., Tokyo) at 400 and 100 MHz, respectively. Insensitive nuclei enhanced by polarization transfer assay (26, 27) was also conducted by the ^{13}C NMR spectrometry. A cerebroside was assayed in $CDCl_3/CD_3OD$ (3:1, v/v). Tetramethylsilane was used as an internal standard and chemical shifts were recorded in parts/million.

RESULTS

Fruiting-inducing Activity of Some Plant Cerebrosides—Table I shows the fruiting-inducing activity of some plant cerebrosides. In general, their fruiting-inducing activities were considerably lower than the cerebrosides from fungi. The cerebrosides from spinach leaf showed an activity equivalent to one-sixtieth of that from *S. commune*, and those from rice straw, pea seed, Azuki bean seed, and wheat grain were about one-tenth as active as that of *S. commune*.

High-performance Liquid Chromatography of Wheat Grain Cerebroside—Fig. 1 shows fractionation of cerebrosides from wheat grain by high-performance liquid chromatography. The fractions indicated by arrows displayed high activity. Some of the other fractions also exhibited the activity, but it was quite low. Each of the peaks I–IV was purified by repeated chromatography. Finally, 3.8 mg of I, 12.6 mg of II, 0.9 mg of III, and 1.5 mg of IV were obtained from 100 mg of the cerebroside preparation used. Their specific activities were equal to 6000 units/mg, and this value was equivalent to about half of that of Sch II (the major cerebroside in *S. commune* (10)) and about 8 times of that of the starting cerebroside. The active components in fractions I–IV are referred to as Whe I–IV, respectively, in this paper.

Characterization of Whe I–IV—We have already characterized the cerebrosides in wheat grain (14, 15). The component sugars were either glucose (97.4%) or mannose (2.6%). The component fatty acids were either non-hydroxy fatty acid (3%) or 2-hydroxy fatty acid (97%). The carbon chain length ranged from 14 to 26. The component sphingoids were sphinganine (9%), (*E*)-4-sphingenine (1%), (*E*)-8-sphingenine (24%), (*Z*)-8-sphingenine (47%), (4*E*,8*E*)-4,8-sphingadienine (2%), (4*E*,8*Z*)-4,8-sphingadienine (13%), 4-hydroxysphinganine (1%), (*E*)-4-hydroxy-8-sphingenine (1%), or (*Z*)-4-hydroxy-8-sphingenine (2%). Taking these data into consideration, we attempted to characterize Whe I–IV by use of the conventional method (14, 17, 28) and by ^1H and ^{13}C NMR spectrometry.

TABLE I

Fruiting-inducing activity of some plant cerebrosides

Cerebroside fractions prepared by the method described in the text were assayed fruiting-inducing activity.

Cerebroside	Specific activity
	units/mg
Spinach leaf	200
Rice straw	800
Pea seed	800
Azuki bean seed	1,200
Wheat grain	800
S. commune (Sch II)	12,000

Each of the Whe I–IV yielded a single spot on a silica gel plate developed with $CHCl_3/MeOH/H_2O$ (65:16:2, v/v), and the R_F value (0.67) was the same as that of the lower spot cerebroside of bovine brain origin. Fig. 2 shows infrared spectra of these fractions. The spectra retained the characteristic absorption profile of the original cerebrosides (14), but were not identical to each other because there was a weak absorption at 970 cm^{-1} due to *E*-double bond in the spectra of Whe I and III but not in those of Whe II and IV.

Trimethylsilyl derivatives of Whe I–IV were applied to gas-liquid chromatography-mass spectrometry (28–30). Each of the tested derivatives gave a single peak, and the mass spectrum of this peak was recorded. Table II shows a part of the mass spectra. Suggested components are also indicated. The component sugar was always hexose. The component fatty acid was 2-hydroxyhexadecanoic acid (Whe I and II) or 2-hydroxyoctadecanoic acid (Whe III and IV). The component sphingoid was sphingenine (Whe II and IV) or sphingadienine (Whe I and III).

Methylglycosides obtained by methanolysis were trimethylsilylated and then analyzed by gas-liquid chromatography. Two peaks corresponding to α- and β-methyl-D-glucosides were detected.

Fatty acid methyl esters obtained by methanolysis were trimethylsilylated and then analyzed by gas-liquid chromatography. Peaks were assigned by comparison with the retention times of the standards from wheat grain cerebrosides (14). Each chromatogram had a single peak corresponding to that of 2-hydroxyhexadecanoic acid (Whe I and II) and 2-hydroxyoctadecanoic acid (Whe III and IV), respectively.

Sphingoids obtained by aqueous HCl hydrolysis were analyzed by thin-layer chromatography. Those from Whe II or IV gave a single spot (R_F 0.23) on a silica gel plate with chloroform/methanol/ammonia (40:10:1, v/v), but those from Whe I or III gave three spots (R_F 0.49, 0.33, 0.20) on the plate. The highest spot may be an *O*-methyl derivative of the sphingoid since it became yellow when sprayed with ninhydrin reagent. Aqueous HCl methanolysis of a cerebroside with a double bond at C-4 of the component sphingoid gives various derivatives such as *O*-methyl and *threo* forms (31). The lowest spot may be a *threo* derivative. On these grounds, the spingoids in Whe I and III may be regarded as 4,8-sphingadienine and those in Whe II and IV as 8-sphingenine. The absence of the absorption at 970 cm^{-1} in the infrared spectra of Whe II and IV (Fig. 2) suggests *Z* configuration of the double bond at C-8 of the sphingoid of Whe II and IV. By-product-free sphingoids of Whe I and III were recovered from the spot of R_F 0.33 and were acetylated. These *N,O,O*-triacetyl sphingoids were subjected to analysis to determine configuration of the double bonds by thin-layer chromatography on a silica gel containing 10% $AgNO_3$ (24). Both yielded a single spot corresponding to that of authentic (4*E*,8*Z*)-4,8-sphingadienine (R_F 0.36). The authentic (4*E*,8*E*)-4,8-sphingadienine developed faster (R_F 0.45). Therefore, the sphingoids in Whe I and III were considered to be (4*E*,8*Z*)-4,8-sphingadienine.

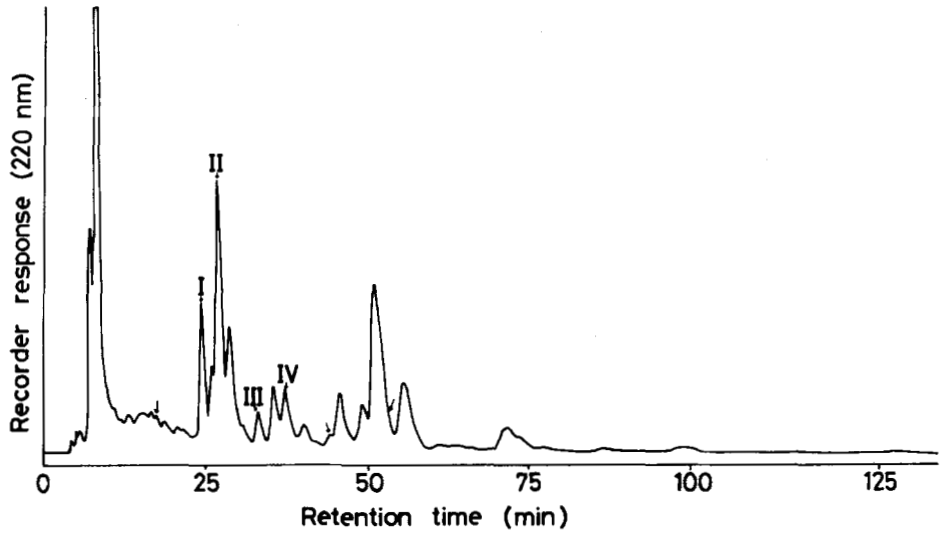


FIG. 1. **High-performance liquid chromatography of cerebrosides from wheat grain.** Forty microliters of the cerebroside solution (10% in $CHCl_3$/MeOH, 1:1, v/v) were loaded on a reverse phase column (size: 7.2 × 250 mm; packing: TSK GEL LS-410). The elution was performed with MeOH/H_2O (25:1, v/v) at 40 °C.

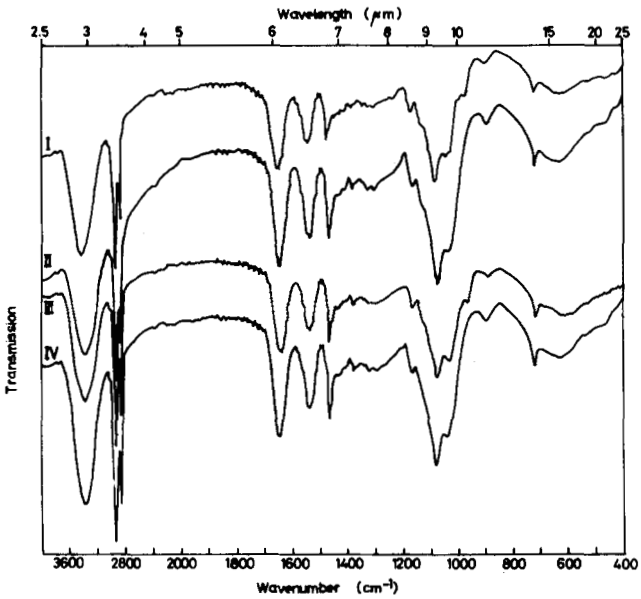


FIG. 2. **Infrared spectra of the fruiting-inducing cerebrosides from wheat grain.** I–IV in Fig. 1 were analyzed. The spectra were obtained with KBr pellets.

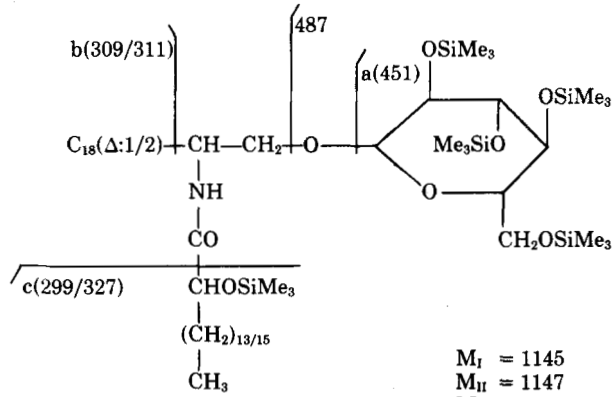
It could be concluded from the results stated above that the Whe I–IV cerebrosides consist of the following components; glucose for sugar, 2-hydroxyhexadecanoic acid or 2-hydroxyoctadecanoic acid for fatty acid, and (4*E*,8*Z*)-sphingadienine or (8*Z*)-sphingenine for sphingoid (Table III).

The assigned structure of the major substance (Whe II) was confirmed by ^1H NMR and ^{13}C NMR spectroscopy. Fig. 3 shows a ^1H NMR spectrum of Whe II. The signals for methyl residues are found at 0.80 ppm which corresponds to the terminal methyl residues of the sphingoid and the acyl moiety. The symmetrical multiplet at 5.29 ppm was assigned to C(8)H and C(9)H. The doublet at 4.21 ppm corresponded to the anomeric proton, and the coupling constant (J = 7.8 Hz) indicated the presence of a β-D-glucopyranoside linkage. Fig. 4 shows a ^{13}C NMR spectrum of Whe II and assignment of carbons. The signal at 26.9 ppm which was assigned to C-7 and C-10 suggested that the double bond between C-8 and C-9 would be of *Z* configuration, since the signal would not be so much shielded if it were of *E* configuration. For instance, the chemical shift values for the carbons next to the double bonds of (*E*)-5-decene and (*Z*)-5-decene are 32.9 and 27.5 ppm, respectively (34).

TABLE II

Partial mass spectra of active cerebrosides from wheat grain

Trimethylsilyl derivatives were prepared from the cerebrosides in I–IV in Fig. 1. They were subjected to gas-liquid chromatography on Diasolid ZT and the mass spectrum of each peak was recorded. The ions were characterized according to the literature (29, 30) and the method reported previously (28). Suggested formula are shown at the bottom of the table.



M_I = 1145
M_{II} = 1147
M_{III} = 1173
M_{IV} = 1175

Ion	I		II		III		IV	
	m/z	%	*m/z*	%	*m/z*	%	*m/z*	%
M − b + 73	909	1	909	3	937	0.5	937	3
M − b	836	1	836	5	864	0.3	864	5
M − a + 102	796	15	798	32	824	5	826	30
M − a − 16	678	33	680	100	706	21	708	100
M − a − b + 73	458	52	458	12	486	40	486	12
a	451	5	451	7	451	2	451	6
M − a − b + 31	416	10	416	11	444	6	444	12
a − 90	361	100	361	45	361	100	361	42
b	309	17	311	8	309	11	311	11
c	299	8	299	12	327	5	327	9
a − 90 × 2	271	20	271	8	271	12	271	8
Suggested formula								
Sugar	Hexose		Hexose		Hexose		Hexose	
Fatty acid	16h:0		16h:0		18h:0		18h:0	
Sphingoid	d18:2		d18:1		d18:2		d18:1	

Fruiting-inducing Activity of the Purified Cerebrosides and Oligoglycosylceramides—Table III summarizes the fruiting-inducing activity together with the chemical formula of the active cerebrosides (monoglycosylceramides), the hydrogenated Whe II, and the oligoglycosylceramides. The specific

TABLE III

Structure-activity relationship in the glycosphingolipids from wheat grain

Lipid	Activity	Sugar	Fatty acid	Sphingoid
	units/mg			
Monoglycosylceramide	800	Glc[a]	12–26h:0[a]	d18:0–2[a]
Diglycosylceramide	800	Man-Glc[a]	12–26h:0[a]	d18:0–2[a]
Triglycosylceramide	100	Man-Man-Glc[a]	12–26h:0[a]	d18:0–2[a]
Whe				
I	6,000	Glc	16h:0	d18:2 (4*E*,8*Z*)
II	6,000	Glc	16h:0	d18:1 (8*Z*)
III	6,000	Glc	18h:0	d18:2 (4*E*,8*Z*)
IV	6,000	Glc	18h:0	d18:1 (8*Z*)
H_2-Whe II[b]	< 20	Glc	16h:0	d18:0
Sch II	12,000	Glc	16h:0	d19:2 (4*E*,8*E*,9Me)

[a] In this table, minor components such as mannose, non-hydroxy fatty acids, trihydroxy bases are omitted. Refer to Refs. 14 and 15 for the precise values. (The abbreviations used are: 12–26h:0, 2-hydroxy fatty acids having carbon chain length 12–26; d18:0–2, sphinganine with 0 ~ 2 double bonds; 16h:0, 2-hydroxyhexadecanoic acid; d18:1(8*Z*),(*Z*)-8-sphingenine; d18:2(4*E*,8*Z*), (4*E*,8*Z*)-4,8-sphingadiene; d18:0, sphinganine; 18h:0, 2-hydroxyoctadecanoic acid; d19:2 (4*E*,8*E*,9Me), (4*E*,8*E*)-9-methyl-4,8-sphingadienine.)

[b] The hydrogenated derivative of Whe II.

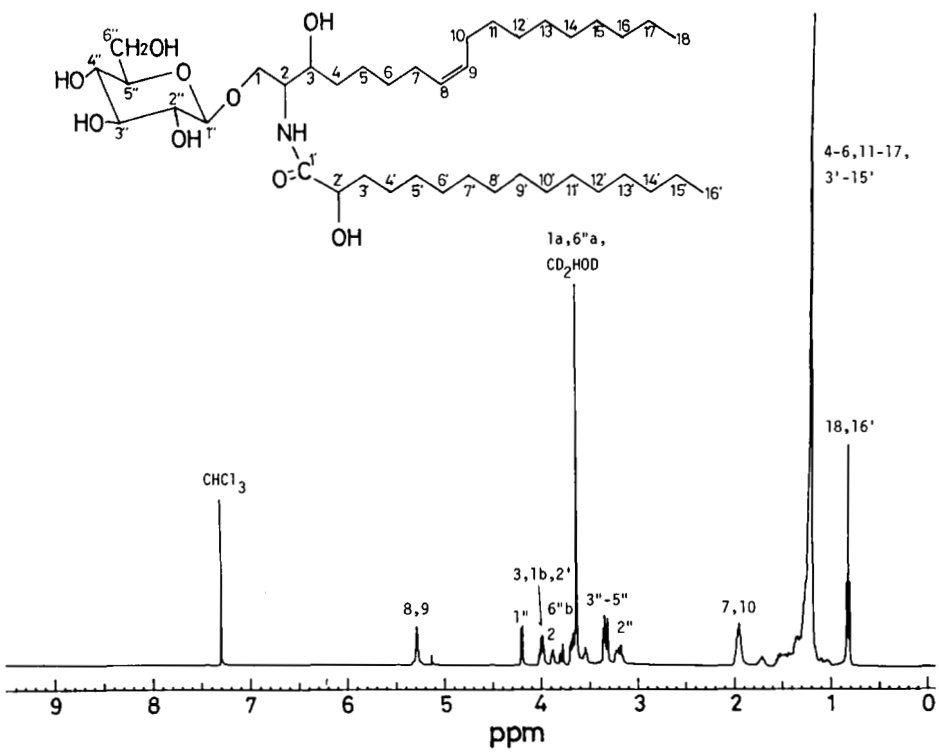


FIG. 3. **^{1}H NMR spectrum of Whe II in $CDCl_3/CD_3OD$ (3:1, v/v) at 400 MHz.** The *letters above* the peaks indicate proton assignments as described previously (10–12).

activity of the purified cerebrosides from wheat grain was about half of the major cerebroside (Sch II) from *S. commune*. They have (4*E*,8*Z*)-4,8-sphingadienine or (*Z*)-8-sphingenine as sphingoids. Hydrogenation of 8*Z*-double bond completely abolished the activity. The ceramide moieties in the di- and triglycosylceramides are identical to those of monoglycosylceramides (14, 15). Nevertheless, the diglycosylceramides have activity equal to that of monoglycosylceramides, but the triglycosylceramides have only one-eighth the activity.

DISCUSSION

According to high-performance liquid chromatographic analysis, wheat grain appears to contain at least 40 molecular species of cerebrosides (Fig. 1). The number of cerebrosides studied in this paper is limited, but the selected four active cerebrosides were demonstated to have the following common characters: glucose as the sugar component, either 2-hydroxyhexadecanoic acid or 2-hydroxyoctadecanoic acid as the fatty acid component, either (4*E*,8*Z*)-4,8-sphingadienine or (*Z*)-8-sphingenine as the sphingoid component. Some of the peak fractions appearing earlier than I or later than IV in Fig. 1 also exhibited the fruiting-inducing activity, but their activities were significantly lower than those of Whe I–IV. It is possible that the active cerebrosides in the earlier and the later fractions are structurally similar to the Whe I–IV, but have either fewer than 16 or more than 18 carbon atoms in the acyl moiety. Thus, about 20% of the cerebrosides in wheat grain are biologically active on the basis of fruiting-inducing activity.

The selected cerebrosides from wheat grains are not as active as those from *S. commune* or other fungi. Their structural similarity and difference are as follows. The cerebrosides from fungi have the (4*E*,8*E*)-9-methyl-4,8-sphingadienine structure in common (10–12). The cerebrosides from wheat grain also have a similar structure, but they have an 8*Z*-double bond instead of an 8*E*-double bond and a 9-methyl group. This structural difference may be related in some way to the observed difference in biological activity. The diglyco-

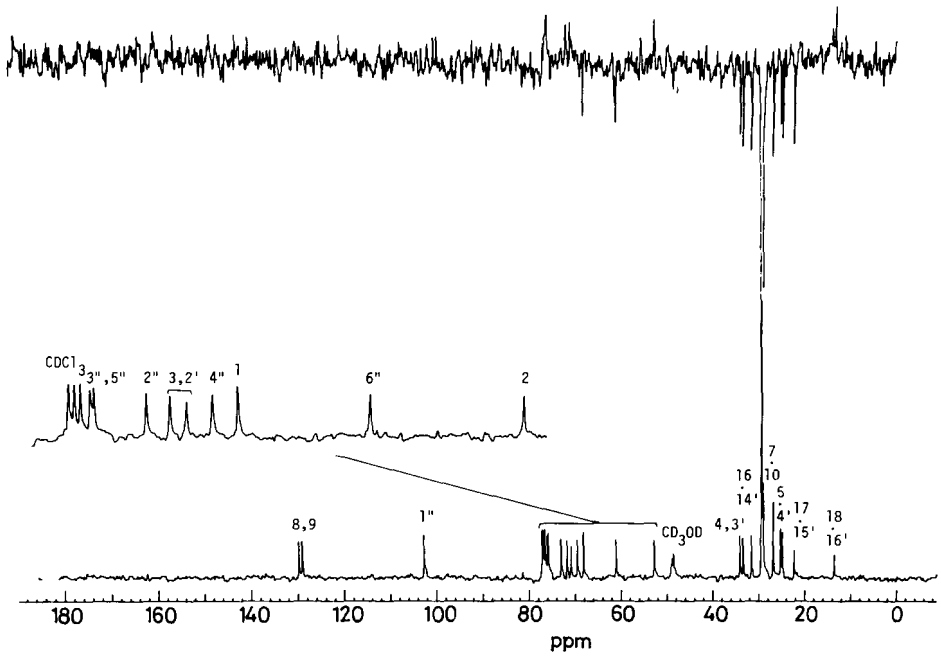


FIG. 4. **^{13}C NMR spectrum of Whe II in $CDCl_3/CD_3OD$ (3:1, v/v) at 100 MHz.** The *upper line* represents insensitive nuclei enhanced by polarization transfer spectrum. The *letters above* the peaks indicate the carbon assignments shown in the structural formula in Fig. 3. The assignments were done according to the previous report (10), Dorman and Roberts (32), and Dabrowski *et al.* (33).

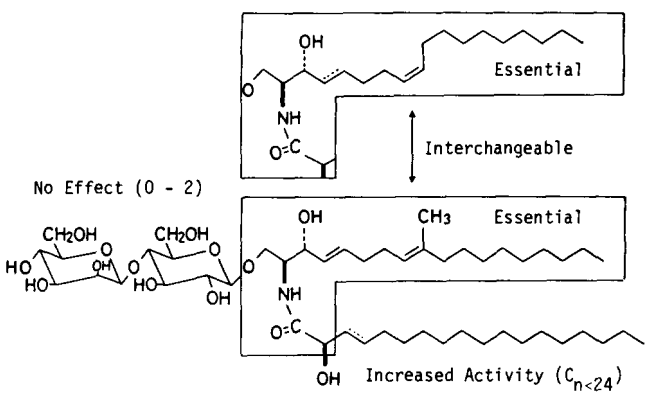


FIG. 5. **Functional moiety in the fruiting-inducing substances.** The *N*-acylsphingoid constitutes the essential part of the active substance. The 8*E*-double bond and the methyl group at position 9 of the sphingoid moiety can be substituted by an 8*Z*-double bond. The sugar is unnecessary, but a sugar moiety with more than 2 hexose resiudes sometimes interfere with the activity. The fatty acid having a chain length of less than 24 and/or a 2-hydroxy group seems to be related in some way to the activity.

sylceramide from wheat grain is as active as the monoglycosylceramide from the same source, but the triglycosylceramide is not. We have reported previously (10) that the glucose moiety in the active ceramides has nothing to do with the activity. However, as far as the sugar-containing ceramides from wheat grain are concerned, the number of hexose residue seems to have some correlation with the activity.

A tentative scheme of the relationship between structure and activity is given in Fig. 5. 1) The acid amide linkage, and the 8*E*-double bond and methyl group at C-9, are essential for achievement of high activity although the latter can be replaced with an 8*Z*-double bond. 2) An acyl moiety with a carbon chain of fewer than 24 units and/or a 2-hydroxy group increases the activity (11). 3) The sugar moiety has nothing to do with the activity, but in certain cases the presence of more than 2 sugar resiudes is inhibitory. To get further insight, synthesis of various ceramides is in progress.

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