Structure of biologically active and inactive cerebrosides prepared from *Schizophyllum commune*

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Abstract A cerebroside fraction prepared from the mycelia of *Schizophyllum commune* was further fractionated into five components (I–V) by reverse-phase high-performance liquid chromatography. Fruiting-inducing activity was found in I–IV but not in V. By gas–liquid chromatography–mass spectrometry and nuclear magnetic resonance analyses it was shown that these fractions contained: I, a mixture of *N*-2'-hydroxypentadecanoyl-1-*O*-glucosyl-nonadecasphingadienine and *N*-2'-hydroxyhexadecanoyl-1-*O*-glucosyl-sphingadienine; II, (4*E*,8*E*)-*N*-D-2'-hydroxyhexadecanoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine (Kawai and Ikeda. 1983. *Biochim. Biophys. Acta.* **754:** 243–248); III, *N*-2'-hydroxyheptadecanoyl-1-*O*-glucosyl-nonadecasphingadienine; IV, *N*-2'-hydroxyoctadecanoyl-1-*O*-glucosyl-nonadecasphingadienine; V, (4*E*,8*E*)-*N*-2'-hydroxytetracosanoyl-1-*O*-β-glucopyranosyl-9-methyl-4,8-sphingadienine. The only structural difference observed between biologically active and inactive cerebrosides was the chain length of acyl moiety; the cerebroside having an acyl chain of 24 carbon atoms was inactive. —**Kawai, G., and Y. Ikeda.** Structure of biologically active and inactive cerebrosides prepared from *Schizophyllum commune*. *J. Lipid Res.* 1985. **26:** 338–343.

Supplementary key words reverse-phase HPLC • fruiting-inducing activity • gas–liquid chromatography • mass spectrometry • nuclear magnetic resonance spectrometry

Sphongolipids are lipids contained in the cytoplasmic membrane of eukaryotic cells (1, 2), and, in fungi, they are present as free sphingoids, ceramides, cerebrosides, polyglycosylceramides, phosphosphingolipids, and glycophosphosphingolipids (3, 4). We reported previously (5) that a certain type of cerebroside contained in the mycelia of *Schizophyllum commune* stimulated fruiting body formation of the same fungus. The active principles were fractionated into four components (Sch I–IV) by sequential silica gel column chromatography and reverse-phase high-performance liquid chromatography. The principle in the major fraction (Sch II) was identified as (4*E*,8*E*)-*N*-2'-hydroxyhexadecanoyl-1-*O*-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine (6). This finding can be regarded as the first indication that the cerebrosides are not only structural components but are also able to play some biological roles.

This report deals with chemical structures of the active principles in Sch I, III, and IV as well as that in Sch V. Sch V is an additional peak found during the course of HPLC fractionation, but is biologically inactive. This work was undertaken on the thought that a comparative study of these active and inactive principles might provide us with a clue for solving the structure-activity relationship.

METHODS

The methods for assaying the fruiting-inducing activity and for preparing the cerebrosides from mycelia of *S. commune* IFO 6502 were described in previous papers (5, 6) together with the general analytical conditions employed.

HPLC of cerebrosides

A reverse-phase column (7.2 mm ID × 250 mm) was packed with TSK-GEL LS 410 (Toyosodakogyo Co., Ltd., Tokyo) and used for separation of cerebrosides. About 40 μl of the cerebroside-containing solution (2% in EtOH) was loaded on the column, eluted with methanol (1 ml/min) at 30°C, and monitored at 220 nm. The peak-forming fractions were collected and rechromatographed for further purification.

Abbreviations: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; FT, Fourier transform; PRFT, partially relaxed Fourier transform; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; Glc, glucose; d18:2, octadecasphingadienine; d19:2, nonadecasphingadienine; d19:2(4*E*,8*E*,9Me), (4*E*,8*E*)-9-methyl-4,8-sphingadienine; 15h:0, 2-hydroxypentadecanoic acid; 16h:0, 2-hydroxyhexadecanoic acid; 17h:0, 2-hydroxyheptadecanoic acid; 18h:0, 2-hydroxyoctadecanoic acid; 24h:0, 2-hydroxytetracosanoic acid; Sch I–V, substances in the peaks I–V of Fig. 1.

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TLC of cerebrosides

Bovine brain cerebrosides (the 2-hydroxy fatty acid-containing type and the non-hydroxy fatty acid-containing type) and Gaucher spleen cerebrosides were purchased from Sigma Chemical Co. and used as standards. Thin-layer chromatography was conducted either on precoated silica gel 60 plates (E. Merck, Darmstadt, F. R. G.) using chloroform–methanol–water 65:25:1 or chloroform–methanol–99% formic acid 70:18:12 as the developing agent, or on borate-impregnated silica gel plates (7) using chloroform–methanol–water–15 M ammonia 280:70:6:1. The spots were detected by spraying the plates with primuline reagent (8) or α-naphthol-sulfuric acid reagent (9).

NMR spectrometry

^{1}H-NMR spectra were produced in a JEOL GX-400 (JEOL Ltd., Tokyo) in $CDCl_3$–CD_3OD 2:1 (v/v) at 400 MHz. Tetramethylsilane was used as an internal standard and chemical shifts are reported in ppm (δ values). In addition to the normal FT (Fourier transform) method, the PRFT (partially relaxed Fourier transform) method (10) was used to eliminate the large proton signal of methanol-d_4.

GLC and GLC-MS

Gas–liquid chromatography was performed on a Shimadzu GC-6A Gaschromatograph (Schimadzu Seisakusho Co., Kyoto). The glass tubes (3 mm ID × 200 cm) were layered with 2.5% OV-17 on 60–80 mesh Uniport HPS (Gasukurokogyo Co., Ltd., Tokyo) or with 3.8% SE-30 on 80–100 mesh Gaschrom Q. The trimethylsilyl ether derivatives of the ceramide fractions from Sch I, III, and IV were analyzed on the OV-17 column at 320°C. The trimethylsilyl ether derivatives of the sphingoid, the fatty acid methyl ester, and the ceremide from Sch V were

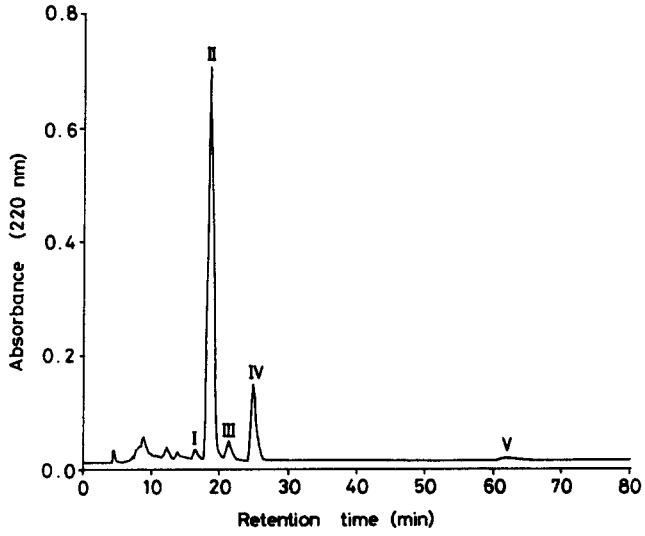

Fig. 1 Reverse-phase high-performance liquid chromatogram of the cerebrosides from *S. commune* IFO 6502. Forty μl of the cerebroside solution prepared from the mycelia of *S. commune* IFO 6502 (2% in EtOH) was loaded on a TSK-GEL LS 410 column (7.2 mm ID × 250 mm). Elution was with methanol (1 ml/min) at 30°C.

TABLE 1. Diagnostic ions in mass spectra of the trimethylsilyl ether derivatives of the ceramides from Sch I, III, IV, and V

Ion	Sch I		Sch III	Sch IV	Sch V
	I-1	I-2			
M	767 (3)[a]		795 (1)	809 (1)	893 (1)
M − 15	752 (17)		780 (7)	794 (5)	878 (4)
M − 90	677 (15)		705 (6)	719 (6)	803 (9)
M − 103	664 (5)		692 (2)	706 (1)	790 (2)
M − 90 − 15	662 (8)		690 (3)	704 (2)	788 (3)
M − 90 × 2	587 (5)		615 (2)	629 (2)	713 (3)
M − 90 − 103	574 (9)		602 (3)	616 (3)	700 (3)
M − (a − 73)	517 (24),	531 (15)	545 (14)	559 (15)	643 (17)
M − a	444 (100),	458 (91)	472 (100)	486 (100)	570 (100)
M − a − 16	428 (6),	442 (5)	456 (2)	470 (3)	554 (3)
M − (c + 1)	438 (7),	424 (5)	438 (4)	438 (4)	438 (7)
c + 1 + 73	402 (14),	416 (9)	430 (5)	444 (10)	528 (14)
M − (c + 1 + 90)	348 (23),	334 (13)	348 (14)	348 (16)	348 (29)
c + 2	330 (5),	344 (3)	358 (3)	372 (3)	456 (5)
M − (a − 73) − (e − 1)	335 (17)		335 (19)	335 (9)	335 (15)
M − b	323 (32),	309 (24)	323 (19)	323 (23)	323 (37)
d	285 (33),	299 (23)	313 (18)	327 (21)	411 (39)
Suggested formula					
Sphingoid	d19:2	d18:2	d19:2	d19:2	d19:2
Fatty acid	15h:0	16h:0	17h:0	18h:0	24h:0

[a]Numbers in parentheses represent percent relative intensity as compared to base ion which is 100%. For fragment code see Fig. 2.

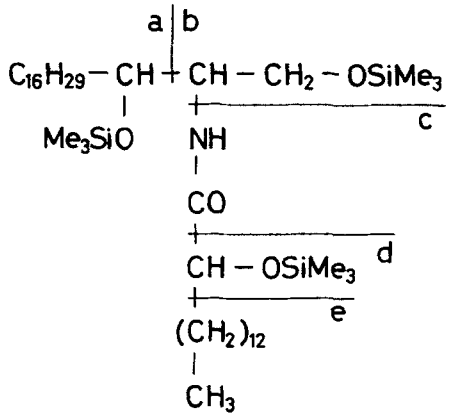

Fig. 2 Designation of mass spectral fragmentations of 1,3,2'-tri-*O*-trimethylsilyl-*N*-2'-hydroxypentadecanoyl-nonadecasphingadienine. Fragmentations are illustrated according to Hammarström et al. (12).

analyzed on the OV-17 column at 230°C, 270°C, and 340°C, respectively.

GLC–MS was performed on a Hitachi RMU-7M instrument (Hitachi Seisakusho Co., Ltd., Tokyo). The column was a stainless steel tube of 3 mm ID × 200 cm layered with 2.5% OV-17 on 60–80 mesh Uniport HPS. The column was operated at the temperatures described above. Mass spectra were produced under the following conditions: ion source temperature, 210°C; electron energy, 70 eV; ion accelerator voltage, 3.2 kV; and ionizing current, 51 μA.

RESULTS

HPLC of the cerebroside fraction of *S. commune*

A cerebroside fraction was prepared from the mycelia of *S. commune* IFO 6502 as reported previously (5). The fraction was purified further by silica gel column rechromatography, followed by reverse-phase high performance liquid chromatography. **Fig. 1** shows a profile of the liquid chromatogram. Fruiting-inducing activity was recovered in Peaks I–IV and each peak was demonstrated to contain cerebroside (5). Peak V showed no activity but the fraction was also collected for chemical analysis. The fractions corresponding to Peaks I–V were named Sch I–V, respectively.

Characterization of Sch I, III, and IV

The active substances in Sch I–IV had been presumed to be glucosylceramides from the results of thin-layer chromatography and infrared spectrometry (5). First, ceramides were prepared from each glucosylceramide according to Carter, Rothfus, and Gigg (11), and then their trimethylsilyl ether derivatives were analyzed by GLC and GLC–MS. Each gave a near single peak; the mass spectra of the peaks are shown in **Table 1.** The components (**Fig. 2**) were characterized by the method of Hammarström, Samuelsson, and Samuelsson (12) and Hammarström (13).

As shown in Table 1, the ceramide from Sch I is a mixture of *N*-2'-hydroxypentadecanoyl-nonadecasphingadienine (major) and *N*-2'-hydroxyhexadecanoyl-octadecasphingadienine (minor). This result explains why the reverse-phase HPLC analysis as well as the GLC analysis failed to distinguish between these two species; they are of equal molecular weight.

The ceramides from Sch III and IV were suggested to be *N*-2'-hydroxyheptadecanoyl-nonadecasphingadienine and *N*-2'-hydroxyoctadecanoyl-nonadecasphingadienine, respectively (Table 1).

Characterization of Sch V

The infrared spectrum of Sch V was almost identical with those of Sch I–IV, but Sch V migrated slightly faster than Sch II on TLC (**Table 2**). Therefore, Sch V was subjected to aqueous-HCl methanolysis according to Gaver and Sweeley (14) and fractionated into prospective fatty acid methyl ester, sphingoid, and methyl glycoside by the method of Miyazawa, Ito, and Fujino (15). When analyzed by GLC, trimethylsilyl ether derivatives of the methyl glycoside yielded, on the OV-17 column, a single peak having the retention times of authentic preparations of the α and β anomers of methyl-D-glucopyranoside. Trimethylsilyl ether derivatives of the sphingoid yielded seven peaks on the OV-17 column. The pattern was the same as that of the preparation from Sch II (5). Trimethylsilyl ether derivatives of the fatty acid methyl ester gave a single peak on the OV-17 column. The mass spectrum of this peak displayed ions with m/z 470 (M), 455 (M – CH_3), 427 (M – CH_3 – CO), and 411 (M – $COOCH_3$), identifying it as methyl 2-*O*-trimethylsilyltetracosanoate.

The GLC of the trimethylsilyl ether derivatives of the ceramide gave a major peak (96%) and the mass spectrum

TABLE 2. R_f values of some cerebrosides on thin-layer chromatography

	Silica Gel:	Free		With Borax
Cerebroside	Solvent System:	(a)	(b)	(c)
Bovine brain type I		0.51	0.50	0.04
Bovine brain type II		0.59	0.56	0.08
Gaucher spleen		0.60	0.57	0.23
Sch II		0.51	0.50	0.15
Sch V		0.56	0.53	0.18

Cerebrosides were developed on silica gel plates or borate-impregnated silica gel G plates (7) using a), chloroform–methanol–water 65:25:1 (v/v), b), chloroform–methanol–99% formic acid 65:18:12 (v/v), or c), chloroform–methanol–water–ammonia 280:70:6:1 (v/v). Bovine brain cerebrosides type I contains 2-hydroxy fatty acids and the type II contains non-hydroxy fatty acids.

of the peak fraction indicated that the ceramide was *N*-2'-hydroxytetracosanoyl-nonadecasphingadienine (Table 1).

Fig. 3 shows the ^{1}H-NMR spectrum of Sch V. The spectrum indicates that Sch V is identical with Sch II except for the chain length of the acyl moiety (6). The coupling constant of the anomeric proton (4.27, 1", doublet, J = 7.8 Hz) indicates the presence of a β-glucopyranosyl linkage. The large singlet at 1.57 ppm can be assigned to the methyl branch at C-9 of the sphingoid moiety and the identity of the chemical shift value to that of Sch II suggests that the double bond at position 8 is the (*E*) configuration, since the chemical shift of methyl branch of (*Z*)-isoprene units of polyprenols is 0.07 ppm larger than that of the (*E*) isomer (17). The doublet of

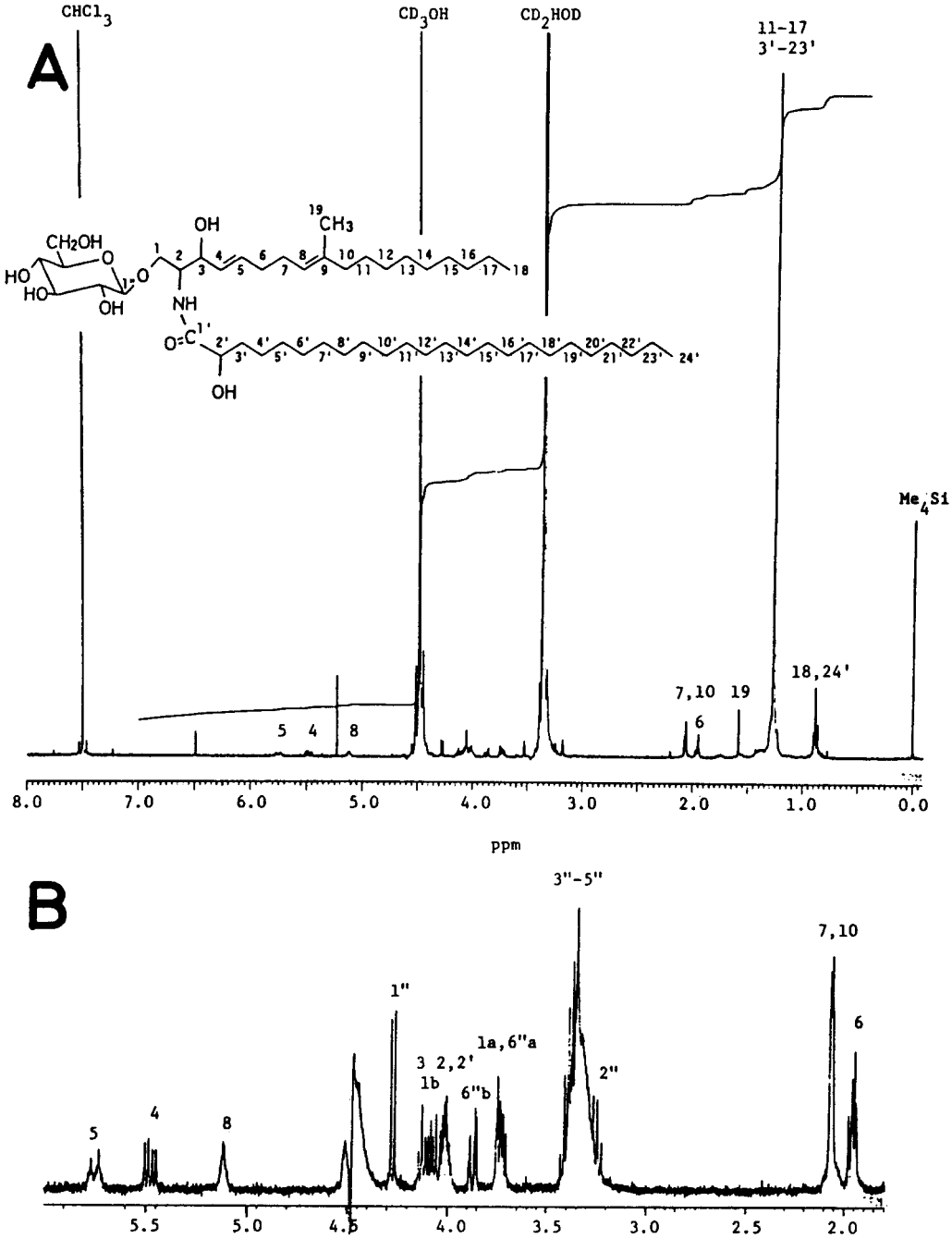

Fig. 3 ^{1}H-NMR spectrum of Sch V in $CDCl_3$–CD_3OD 2:1 (v/v) at 400 MHz. A, Ordinary FT spectrum by 16 scans; B, partial PRFT spectrum by 64 scans. Letters above the peaks indicate the proton assignments shown in the structural formula. The assignments were done according to the previous report (6) and Koch and Perlin (16).

TABLE 3. Summary of the chemical analysis of the cerebrosides from *S. commune*

Cerebroside	Relative Amount	Activity	Glycoside	Fatty Acid	Sphingoid
	%				
Sch I	2.5	+	Glc	[15h:0 16h:0]	[d19:2 d18:2]
Sch II	77.5	+	Glc	16h:0	d19:2(4*E*,8*E*,9Me)
Sch III	4.0	+	Glc	17h:0	d19:2
Sch IV	14.5	+	Glc	18h:0	d19:2
Sch V	1.5	-	Glc	24h:0	d19:2(4*E*,8*E*,9Me)

Relative amount was calculated from the dry weights. Fruiting-inducing activity was assayed as described previously (5), and is indicated with the symbols + and −; + represents 9,000–12,000 units/mg and − represents <20 units/mg.

doublets at 5.47 ppm and the broad doublet at 5.73 ppm can be assigned to the C-4 and C-5 olefinic protons of the sphingoid moiety and the coupling constant (J = 15.3 Hz) establishes the (*E*) geometry of the double bond at the position 4.

Thus, the inactive principle in Sch V is (4*E*,8*E*)-*N*-2'-hydroxytetracosanoyl-1-*O*-β-glucopyranosyl-9-methyl-4,8-sphingadienine.

DISCUSSION

The results presented in this paper are summarized in **Table 3.** The hexose moiety of the cerebrosides (Sch I–V) is glucose. Every fatty acid moiety contains a 2-hydroxy group. All sphingoids except Sch I are nonadecasphingadienine. The sphingoid of Sch I is a mixture of nonadecasphingadienine (major) and octadecasphingadienine (minor). Since the cerebrosides in Sch II, III, and IV equally exhibit the fruiting-inducing activity, the cerebroside having the nonadecasphingadienine structure may be potent. We do not know, however, whether the cerebroside having the octadecasphingadienine moiety is biologically active or not. Noteworthy is the observation that the fruiting-inducing cerebrosides, excepting the minor component in Sch I, are structurally alike and that difference is found only in the chain length of the fatty acid moieties.

The biologically inactive cerebroside in Sch V differs from that in Sch II only in the chain length (24 carbon atoms) of the fatty acid moiety. This suggests that the threshold dividing the active from the inactive cerebrosides may lie between 18 and 24 with regard to the chain length of the fatty acid moiety.

A comparative study reported previously of the fruiting-inducing activity of the derivatives from Sch II has established that: *1*) the sphingoid moiety with a 9-methyl branch constitutes an essential part of the activity; *2*) the fatty acid having a certain chain length and/or a 2-hydroxy group may play an enhancing role; and *3*) the glycoside moiety is not essential (6). To these conclusions, this study adds a new view that the chain length stated in *2*) must be less than 24 carbons. Our view with regard to the functional moieties at the present time is illustrated in **Fig. 4.**

With regard to the question of what is the lowest limit of the chain length of the fatty acid moiety which sustains expression of biological activity, our knowledge is limited. At least, a cerebroside having a chain length of 15 carbon atoms is active enough. The peak fractions which appeared earlier than Sch I in Fig. 1 were colored yellow and were quantitatively too small for chemical analysis. This subject will be challenged anew.

Sch I, II, III, and IV exhibited no extinction at wave length above 240 nm and their absorption maxima were around 205 nm. Nevertheless, we monitored them at 220 nm. The reason for this is that the absorption at 205 nm is not proportional to the amount of testing sample when the amount exceeded 0.02 mg, probably because of the disturbance by methanol used as the eluent. The relationship was linear, however, in the range from 0 to 0.2 mg when monitored at 220 nm. This study established that reverse-phase HPLC can be used as a powerful method to

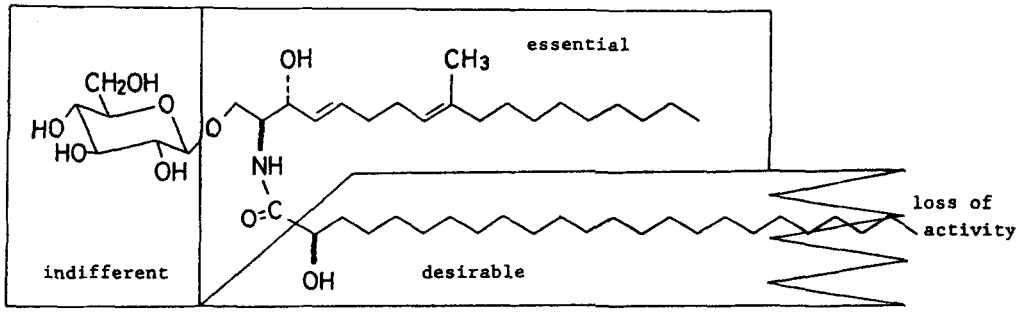

Fig. 4 Functional moiety of fruiting-inducing substance. The *N*-acyl-sphingoid constitutes the essential part of the active substance. The glycoside is not essential. The fatty acid having a chain length of less than 24 and/or a 2-hydroxy group seems to be related to the activity in some way.

fractionate minor molecular species of cerebrosides. Recently Kushi and Handa (18) prepared samples of glycosphingolipids by reverse-phase HPLC and subjected them to field desorption mass spectrometric analysis. The wave length they used was 206 nm.

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