Monatshefte für Chemie 127, 755–761 (1996)

Monatshefte für Chemie
Chemical Monthly

Cerebrosides from *Fomitopsis pinicola (Sw. Ex Fr.) Karst.*

S. Striegler[1] and **E. Haslinger**[2,*]

[1] Abteilung für Organische Chemie I, Universität Ulm, D-89069 Ulm, Germany
[2] Institut für Pharmazeutische Chemie, Universität Graz, A-8010 Graz, Austria

Summary. A cerebroside fraction was obtained from the fruit bodies of *fomitopsis pinicola* using column chromatography and then separated into six compounds by reversed-phase HPLC. The sugar component of all cerebrosides was *D*-glucose. The major fatty acids were 2-hydroxyfatty acids (C_{14}–C_{18}), the long chain base was identified as 9-methyl-C_{18}-4,8-sphingadienine which is widely distributed in fungi and reported to be essential for the fruit-inducing activity of fungi. Based on degradation studies, fast atom bombardment mass spectrometry, and different ^{1}H and ^{13}C NMR investigations, the structure of the main cerebroside (**1**) was determined to be (4*E*,8*E*,2*S*,3*R*,2′*R*)-N-2′-hydroxypalmityl-1-O-*β*-D-glucopyranosyl-9-methyl-4,8-sphingadienine.

Keywords. Cerebroside; Glycolipid; *Fomitopsis pinicola*; ^{1}H and ^{13}C NMR.

Cerebroside aus *Fomitopsis pinicola (Sw. Ex Fr.) Karst.*

Zusammenfassung. Aus den Fruchtkörpern von *fomitopsis pinicola* wurde ein Cerebrosidgemisch erhalten und durch Säulenchromatographie und HPLC in sechs Verbindungen aufgetrennt. Der Zuckerbaustein aller Cerebroside war *D*-Glucose. Die Fettsäurekomponenten waren 2-Hydroxyfettsäuren mit einer Kettenlänge zwische C_{14} und C_{18}. Der Basenteil konnte als 9-Methyl-C_{18}-4,8-sphingadienin identifiziert werden. Diese Verbindung ist in Pilzen weit verbreitet und für die Fruchtbildung verantwortlich. Aus Abbaustudien, FAB-MS und verschiedenen ^{1}H- und ^{13}C-NMR-Messungen wurde die Struktur des Hauptcerebrosids (**1**) als (4*E*,8*E*,2*S*,3*R*,2′*R*)-N-2′- hydroxypalmityl-1-O-*β*-*D*-glucopyranosyl-9-methyl-4,8-sphingadienin ermittelt.

Introduction

Fomitopsis pinicola, which is often confused with *fomes fomentarius*, belongs to the wood-destroying fungi. Particularly the glycosides of *fomes fomentarius* are well known, but only few components of *fomitopsis pinicola* have been isolated and characterized up to now [1, 2, 3]. The present paper describes the isolation and structural characterization of cerebrosides from *fomitopsis pinicola.* The main cerebroside (**1**) has the same structure as those isolated from *schizophyllum commune* [4], *lentinus edodes* [5], or *penicillium funiculosum* [6]. It has been shown that these cerebrosides have fruit-inducing activity on basidiomycetes [4, 7].

Results and Discussion

Isolation

From 1.3 kg (fresh weight) of the fruit bodies of *fomitopsis pinicola* we obtained a mixture of cerebrosides by extraction with methanol. After several chromatographic purification procedures, 17 mg of the main cerebroside (**1**) were obtained.

Structure determination

The ^{1}H NMR spectrum (Fig. 1) of **1** showed signals at 0.82 and 1.20 ppm which can be attributed to terminal methyls and to methylene groups of aliphatic chains. A singulet centered at 1.54 ppm (3H) indicates a CH_3–C(R) = fragment, and a double triplet and a triplet signal between 1.60 and 2.0 ppm stem from allylic CH_2 groups. The signals between 3.40 and 4.50 ppm were assigned to the protons of the sugar component and some CHOH signals of the aglycone. The signals of three olefinic protons (4.9–6.0 ppm) indicated the presence of two double bonds. A TOCSY experiment demonstrated that both double bonds are situated in the same chain. The coupling constant between the olefinic protons H-4 and H-5 (J_{4-5} = 16.0 Hz) showed (*E*) geometry of the double bond at position 4. The singulet at 1.54 ppm can be assigned to the methyl group at C-9 of the sphingoid moiety. The ^{13}C chemical shift of the methyl carbon at C-19 (16.1 ppm, Table 1) suggests that the configuration of the double bond at position 8 is (*E*), too, the chemical shifts of the C-3 methyl carbons in (*Z*) and (*E*) isomers of 3-methyl-3-hexene being 22.7 and 15.4 ppm, respectively [4, 8–12].

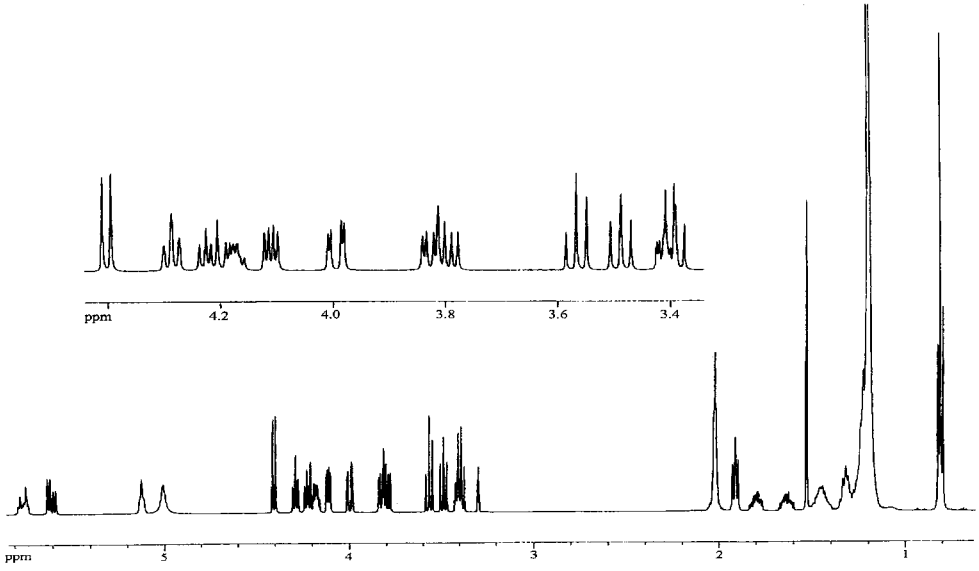


Fig. 1. ^{1}H NMR spectrum of **1** (methanol-d_4/pyridine-d_5 = 2:3)

Table 1. ^{13}C chemical shifts of **1** (CD_3OD:pyridine-d_5 = 2:3)

Carbon	δ(ppm)	Carbon	δ(ppm)	Carbon	δ(ppm)
1	69.9	1′	177.0	1″	105.0
2	54.8	2′	73.0	2″	75.2
3	72.8	3′	35.9	3″	78.3
4	131.4	4′	28.7	4″	71.8
5	134.0	5′	23.6	5″	78.3
6	32.9	6′	23.6	6″	62.8
7	28.7	7′	23.6		
8	124.8	8′	23.6		
9	136.6	9′	23.6		
10	40.7	10′	23.6		
11	29.0	11′	23.6		
12	29.0	12′	23.6		
13	23.6	13′	23.6		
14	23.6	14′	23.6		
15	23.6	15′	23.6		
16	23.6	16′	14.5		
17	23.6				
18	14.5				
19	16.1				

Table 2. ^{1}H chemical shifts of **1** (CD_3OD:pyridine-d_5 = 2:3)

Proton	δ(ppm)	Proton	δ(ppm)	Proton	δ(ppm)
1	3.82/4.21 dd/dd (10.3; 5.9; 3.7)	1′	–	1″	4.40 d (7.8)
2	4.17 m (6.1; 5.9; 3.7)	2′	4.10 t (7.9; 7.9)	2″	3.40 dd (7.8; 9.0)
3	4.27 dd (6.9; 6.1)	3′	1.79/1.63 d/d (10.1; 7.9)	3″	3.57 dd (8.9; 9.0)
4	5.59 dd (15.4; 6.9)	4′	1.45 m	4″	3.48 dd (8.9; 9.0)
5	5.73 br dt (15.4)	5′	1.20 br s	5″	3.40 m (2.4; 9.0)
6	2.03 br s	6′	1.20 br s	6″	3.99/3.77 dd/dd (2.1; 2.4; 11.8)
7	2.03 br s	7′	1.20 br s		
8	5.12 br s	8′	1.20 br s		
9	–	9′	1.20 br s		
10	1.92 t	10′	1.20 br s		
11	1.31 m	11′	1.20 br s		
12	1.20 br s	12′	1.20 br s		
13	1.20 br s	13′	1.20 br s		
14	1.20 br s	14′	1.20 br s		
15	1.20 br s	15′	1.20 br s		
16	1.20 br s	16′	0.82 t		
17	1.20 br s				
18	0.82 t				
19	1.54 s				

Determination of the structure of the sugar unit

The coupling constant of the anomeric proton (4.40 ppm, doublet, 1-H′, $J = 7.8$ Hz) in the ^{1}H NMR spectrum of **1** indicated β configuration, and from the coupling constants of the carbohydrate resonances it was clear that a glucopyranosyl residue was part of the molecule. Differentiation between *D*- or *L*-configuration was performed as described by *Reznicek et al.* [13]: after hydrolysis, the sugar was isolated and glycosidated with *S*-(+)-2-butanol; GC analysis of the silylated glycoside [14, 15] showed that the glucose has D-configuration.

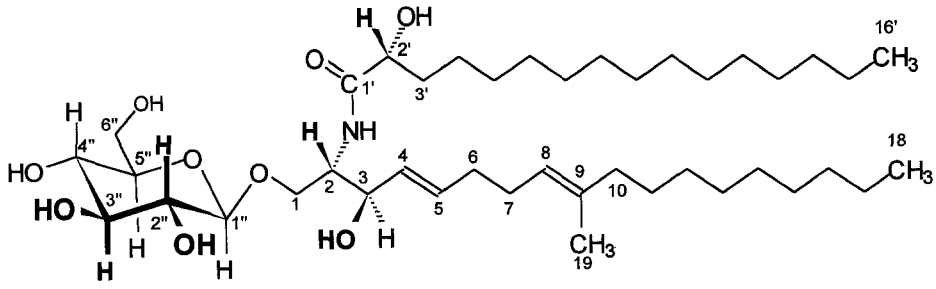
Determination of the configuration of C-2′

After acid hydrolysis of **1** the product mixture was extracted with *n*-hexane and treated with diazomethane. The fatty acid ester was isolated, purified by HPLC, and identified as α-hydroxypalmitinic acid methylester by GC analysis and MS. An optical rotation of $[\alpha]_D^{20} = -1.2°$ ($c = 0.05$, EtOH); (literature values for (*R*)-α-hydroxypalmitinic acid methylester: $[\alpha]_D^{20} = -1.3°$ ($c = 2.8$, EtOH); $[\alpha]_D^{20} = -1.5°$ ($c = 10.0$, EtOH)) [16] suggests that the configuration of C-2′ is *R*. The configuration of the α-hydroxy fatty acid ester has also been determined by derivatization with *S*-(+)-α-methoxy-α-trifluormethylphenylacetic acid (*MTPA*). The ^{1}H NMR shifts of the methoxy group can be used to determine the configuration of the α-carbon of the fatty acid. The chemical shift of the *MTPA* ester of the hydroxy fatty acid obtained from **1** in CCl_4 was $\delta = 3.64$ ppm. According to literature values for *R*-(3.63–3.67 ppm) and *S*-configuration (3.54–3.56 ppm) [17], we assume *R*-configuration for C-2′.

Determination of the configuration of C-2 and C-3

The ceramide moiety was obtained by hydrolysis of **1** [18]. Optical rotation of the cermide was $[\alpha]_D^{20} = +7.1°$ (see Experimental). Values from synthetic products 2*R*,3*S*-*erythro*-ceramide: ($[\alpha]_D^{20} = +10.6°$ ($c = 0.56$, $CHCl_3$); 2*S*,3*R*-*erythro*-ceramide: $[\alpha]_D^{20} = +6.4°$ ($c = 0.76$, $CHCl_3$); [19]) suggest 2*S*,3*R*-configuraton.

The ^{13}C NMR spectrum of **1** showed the shift values for C-1, C-2 and C-3 given in Table 3. Comparison of these values with shifts from synthetic ceramides [9] indicates *erythro*-configuration for **1**. **1** is therefore (4*E*,8*E*,2*S*,3*R*,2′*R*)-*N*-2′-hydroxyhexadecanoyl-1-O-β-*D*-glucopyranosyl-9-methyl-4,8-sphingadienine.



1

Table 3. ^{13}C chemical shifts of C-1, C-2, and C-3 in **1** compared with shifts from synthetic ceramides in $CDCl_3$

C	**1** δ ^{13}C (ppm)	*erythro*-Ceramide [9] δ ^{13}C (ppm)	*threo*-Ceramide [9] δ ^{13}C (ppm)
1	61.2	62.1	63.7
2	53.6	54.5	54.9
3	73.4	74.2	72.5

Table 4. Molecular masses of cerebrosides **1**–**5** (M_1) and their corresponding 2-hydroxy fatty acid methyl esters (M_2)

cerebroside	yield (mg)	M_1 (g/mol)	M_2 (g/mol)	α-hydroxy fatty acid (chain length)
1	~30	727	286	16
2	~10	713	272	15
3	~5	741	300	17
4	~4	755	314	18
5	~4	699	258	14

Isolation and identification of other cerebrosides

Four additional cerebrosides (**2**, **3**, **4**, and **5**) were isolated using HPLC on RP-8. Their molecular masses and their corresponding fatty acid methyl esters were obtained and identified in the same way as described for **1**. The results are shown in Table 4.

Experimental

Thin layer chromatography of cerebrosides

Thin layer chromatography was performed on 0.25 mm precoated silica gel Alugram Sil G/UV_{254} (Machery-Nagel) plates using $CHCl_3$:MeOH (11:1; v/v). The spots were detected by spraying with methanol-sulfuric acid (9:1; v/v).

Analytical methods

NMR spectra were measured with a Bruker AMX 500 NMR spectrometer at 500 MHz for ^1H and 125 MHz for ^{13}C in CD_3OD or CD_3OD/Pyridine-d_5 (2:1) at 310 K. The methanol signal was used as internal reference. UV spectra: Perkin-Elmer Lambda 17; IR spectra: KBr, Bruker IFS 133V (Sektion Schwingungsspektroskopie, University of Ulm); MS: Finningan MAT 8500 (EI, CI) and Finnigan TSQ7000 (FAB) (Sektion Massenspektrometrie, University of Ulm; GC and GC-MS: GC 8000 Fison instruments.

Isolation of the cerebroside

The fruit bodies of *f. pinicola* were gathered in the Austrian province Styria in August 1992. The mushrooms (fresh weight 1.3 kg) were frozen with liquid nitrogen, pulverized, and freeze-dried (dry weight 1020 g). The powder was defatted with 23 l cyclohexan and extracted with 30 l methanol at room temperature. The solvent was evaporated, and the resulting residue (80 g) was distributed between 750 ml water and 1500 ml *n*-butanol. The oily crude residue of the butanolic phase (72 g) was subjected to column chromatography (CC) on silica gel (60 Å) with chloroform/methanol (11:1; v/v) followed by CC on Sephadex LH-20-100 (Fluka) with methanol yielding a mixture of different cerebrosides (60 mg). That mixture was further separated into six fractions by HPLC (Beckman) with methanol/water on a silica gel RP-18 (5 m) column (ID 10 mm, length 250 mm, Ultrasphere ODS, Beckman) using the following gradient program: start with 60% methanol, isocratic for 10 min, increase the methanol portion to 100% at a rate of 4% min^{-1}, and rinse the column for 20 min with 100% methanol (UV detection at 210 nm and 230 nm; flow rate: 1 ml/min). The main components of each fraction were purified by rechromatography with methanol (100%, isocratic) on a silica gel RP-8 (5m) column (ID 10 mm, length 250 mm, Ultrasphere OCTYL, Beckman; flow rate: 0.8 ml/min; detection at 210 nm), yielding 17 mg of the main cerebroside (**1**). Progress of the purification was monitored by thin-layer chromatography. *(4E,8E,2S,3R,2'R)-N-2'-hydroxypalmitic-1-O-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine* (**1**).

White solid; mp.: 182–190 °C; $[\alpha]_D^{20} = +5.3° \pm 0.3$ ($c = 0.11$ in MeOH); $[\alpha]_D^{20} = -6.9° \pm 0.3$ ($c = 0.1$ in $CHCl_3$); $R_f = 0.14$ ($CHCl_3$:MeOH = 11:1); $R_f = 0.53$ (MeOH:H_2O = 8:1); IR: ν(KBr) = 3425 (H-bonded OH and NH), 2956, 2850, 1468, 1384, 1079, 1038, 970 cm^{-1}; FAB-MS (pos.): $m/z = 727$.

Hydrolysis of cerebroside **1**

3 mg of **1** were treated with 5 ml 9% dry methanolic HCl for 3 h at 60 °C; then, 5 ml H_2O were added. Extraction with 3 times 10 ml *n*-hexane and subsequent treatment of the combined hexane solutions with diazomethane yielded the α-hydroxypalmitinic acid methylester. The aqueous phase was evaporated; from the residue, the sugar component was obtained.

MTPA Ester

300 µl dry pyridine, 0.1 mg α-hydroxy palmitinic acid methyl ester (from **1**), 300 µl CCl_4, and 26 µl *R*-(+)-*MTPA*-Cl were mixed at 25 °C. After 6 h, the mixture was diluted with 24 ml, dimethylamino-1-propylamine and 50 ml Et_2O, washed with cold dil. HCl, Na_2CO_3 soln., dried over $NaSO_4$, and evaporated. The residue was dissolved in CCl_4 and used for NMR measurements.

Determination of the configuraion of the glucose unit

The residue of the aqueous phase obtained by hydrolysis of **1** was mixed with 45 µl *S*-(+)-2-butanol and 5 µl HCl_{conc}, heated to 90 °C for 6 h, and evaporated. The residue was dissolved in 50 µl pyridine, treated with 50 µl N, O-*bis*-trimethylsilyl-trifluoracetamid, heated to 75 °C for 20 min, and evaporated. The product was used for GC-analysis.

Isolation of the ceramide moiety

1 (3 mg) was hydrolyzed with 0.1 *M* HCl in $CHCl_3$ for 72 h at 25 °C [18]. The ceramide was extracted with chloroform and purified by HPLC on a silica gel RP-8 column (MeOH, isocratic 22 °C, UV det. at 210 and 230 nm). Yield: 0.5 mg; $[\alpha]_D^{20} = +7.1°$ ($c = 0.21$, $CHCl_3$).

References

[1] Mizuno T, Hayashi K (1981) Shizuoka Daigaku Nogakubu Kenkyu Hokoko **31**: 65
[2] Mizuno T, Usui T (1980) Shizuoka Daigaku Nogakubu Kenkyu Hokoko **30**: 41
[3] Asawa K, Yoshimoto T (1980) Mokuzai Gakkaishi **26**: (12) 819
[4] Kawai G, Ikeda Y (1983) Biochim Biophys Acta **754**: 243
[5] Kawai G (1989) Biochim Biophys Acta **1001**: 185
[6] Kawai G, Ikeda Y (1985) Agric Biol Chem **49**: (7) 2137
[7] Kawai G, Ikeda Y (1982) Biochim Biophys Acta **719**: 612
[8] Mori K, Funaki Y (1985) Tetrahedron Lett **41**: (12) 2369
[9] Dill D, Eckau H, Budzikiewicz H (1985) Z Naturforsch **40b**: 1738
[10] De Haan JW, van de Ven LJM (1973) Org Magn Resonance **5**: 147
[11] Sitrin RD, Chan G (1987) J Antibiot **41**: (4) 469
[12] Karlsson K-A, Leffler H, Samuelson BE (1979) Biochim Biophys Acta **574**: 79
[13] Reznicek G, Susman O, Böhm K (1993) Sci Pharm **61**: 35
[14] Chapman Jr GW, Horvat RJ (1989) J Agric Food Chem **37**: 947
[15] Rothenburger J, Haslinger E (1994) Liebigs Ann Chem 1113
[16] Kuwata T (1973) J Am Chem Soc **95**: 5112
[17] Yasukara F, Yamaguchi S (1980) Tetrahedron Lett **21**: 2827
[18] Kawashima K, Shibuya H (1990) Chem Pharm Bull **38**: (11) 2933
[19] Weete R (1980) Fungal Lipid Biochem. Distrib. and Metabolism. Plenum Press, New York

Received January 19, 1996. Accepted (revised) February 6, 1996