Phytochemistry, 1980, Vol. 19. pp. 83–87. © Pergamon Press Ltd. Printed in England. 0031-9422/80/0101-0083 $02.00/0

SCHIZONELLIN A AND B, NEW GLYCOLIPIDS FROM *SCHIZONELLA MELANOGRAMMA**

GÜNTHER DEML†, TIMM ANKE†, FRANZ OBERWINKLER†, BRUNO MAX GIANNETTI‡ and WOLFGANG STEGLICH‡

† Institut für Biologie I, Lehrstuhl Spezielle Botanik der Universität, Auf der Morgenstelle 1, D-7400 Tübingen, W. Germany;
‡ Institut für Organische Chemie und Biochemie der Universität, Gerhard-Domagk-Str. 1, D-5300, Bonn, W. Germany

(*Received* 11 *April* 1979)

Key Word Index—*Schizonella melanogramma*; Basidiomycetes; smut fungi; antibiotics; glycolipids; schizonellins.

Abstract—Two new glycolipids, schizonellin A and B, were isolated from cultures of the smut fungus *Schizonella melanogramma*. Their structures were elucidated by chemical degradation and spectroscopic methods. The schizonellins are active against Gram-positive bacteria, as well as against some Gram-negative bacteria and some fungi. DNA-, RNA- and protein-syntheses in Ehrlich carcinoma ascitic cells are inhibited simultaneously after addition of the schizonellins. Strong haemolytic action on bovine erythrocytes was observed.

INTRODUCTION

In the course of an investigation of ultrastructural and chemical characteristics in smut fungi [1], we found two antibiotics in submerged cultures of 4 strains of *Schizonella melanogramma* (DC.) Schroeter (Ustilaginales), a parasite growing on leaves of *Carex* species. The strain GD 325, which we used for all our examinations, was collected in the Fimbertal, Switzerland, in 1975. The compounds, named schizonellin A and B, were extracted from the cells and the medium and purified by repeated column chromatography.

RESULTS AND DISCUSSION

Chemical investigations

The schizonellins are colourless, amorphous, hygroscopic substances which are soluble in methanol and chloroform, and slightly soluble in water. Table 1 shows their chromatographic behaviour on TLC with several solvent systems. The spectroscopic data indicate that schizonellin A and B are closely related glycolipids containing one or two *O*-acetyl residues, respectively. On acetylation both compounds give an identical hexaacetate. Base-catalysed methanolysis leads to a glycoside and a mixture of long chain fatty acid methyl esters, which was analysed by GC–MS (Table 2). On acid hydrolysis the glycoside yields D-mannose and erythritol. Comparison of its mp 155° and $[\alpha]_D$ value of −37.5° with the corresponding data of the known 4-*O*-β-D-mannopyranosyl-D-erythritol (**1**) (160–162°; −38°) [2–4] strongly suggests the identity of both compounds.

Table 1. TLC of **2** and **3** on silica plates Merck 60 F_{254}

Solvent system		R_f **2**	R_f **3**
n-BuOH–EtOH–H_2O	4:1:5 (upper phase)	0.72	0.88
n-BuOH–Me_2CO–H_2O	4:5:1	0.85	0.92
$CHCl_3$–MeOH–H_2O	65:25:4	0.77	0.90

The 270 MHz ^1H NMR data of the schizonellins and their peracetate are in accord with a $^4C_1$-conformation for the D-mannopyranoside unit (Table 3). The value of the C—H coupling constant of the anomeric carbon, $J = 159$ Hz, is characteristic for β-glycosides [5]. The position of the acyl residues can be derived from the appearance of the corresponding CHOAc signals at lower field. Thus in both schizonellins the signals for 2-H and 3-H are found at δ 5.45 and 4.88, respectively. In addition, schizonellin B shows the 6-H signals as the AB part of an ABX system at δ 4.27 and

Table 2. GC–MS analysis of the fatty acid mixture*

Methyl ester	R_t (sec)	%†	M^+(*m/e*)
Myristic acid	737	6.2	242
Δ^x-Hexadecenoic acid	856	19.0	268
Palmitic acid	877	44.1	270
Oleic acid	986	21.0	296
Stearic acid	1004	7.6	298

* For details see Experimental.
† Minor components: 2.1%.

* Part VIII in the series "Antibiotics from Basidiomycetes". For Part VII see Kupka, J., Anke, T., Oberwinkler, F., Schramm, G. and Steglich, W. (1979) *J. Antibiot.* **32**, 130.

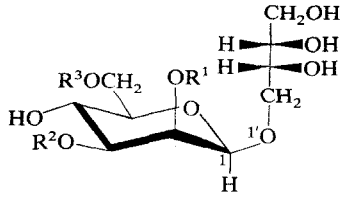
Table 3. ^{1}H NMR spectral data of **2** and **3** (CD_3OD) and the corresponding peracetate ($CDCl_3$) (270 MHz, δ values, TMS as internal standard)

	2	**3**	Peracetate
1-H	4.83 *d*	4.8* *d*	4.64 *d*
2-H	5.45 *dd*	5.46 *dd*	5.47 *dd*
3-H	4.88 *dd*	4.90 *dd*	5.04 *dd*
4-H	3.74 *dd**	3.76 *dd*	5.22 *m*
5-H	3.38 *ddd*	? *ddd*†	3.64 *m*
6-H_A	3.90 *dd*	4.44 *dd*	?
6-H_B	?	4.27 *dd*	?
Further CHOR protons	4.08 *m*, 1 H; 3.47–3.76 *m*, 6 H	4.03 *m*, 1 H; 3.50–3.78 *m*, 5 H	5.22 *m*, 2 H; 4.33–4.12 *m*, 4 *H*; 4.06 *dd* (*J* = 2 and 10 Hz; 1 H); 3.64 *m*, 1 H
OAc	2.00 *s*	2.08 *s*, 2.00 *s*	2.09, 2.06, 2.04, 2.03, 2.02. 1.98 (singlets)
Fatty acid protons	0.90 *t*, 3 H; 1.2–1.4 '*s*' (*br*) 20 H; 1.65 *m*, 2 H; 2.00 *m*; 2.38 *t*, 2 H; 5.34 *m*, 1 H		

J (Hz): 1,2 = 0.75; 2,3 = 3; 3,4 = 9.5; 4,5 = 9.5; 5,6_A = 3 (**2**) or 2 (**3**); 5,6_B = 5 (**2**) or 5.5 (**3**); 6_A,6_B = 12.

* Visible after addition of C_6D_6.

† Visible at δ 3.57 in CD_3OD/C_6D_6 (2 : 1).



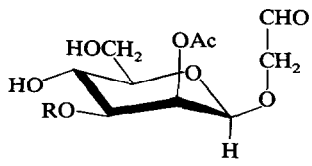
	R^1	R^2	R^3
1	H	H	H
2	Ac	*n*-$C_{15}H_{31}CO$*	H
3	Ac	*n*-$C_{15}H_{31}CO$*	Ac
4	H	*n*-$C_{15}H_{31}CO$*	H

* In admixture with other long chain acid residues (Table 2)

Table 4. Antimicrobial spectrum of **2** and **3**

	MIC (μg/ml) **2**	**3**	
Pseudomonas fluorescens	>200	100–200	Serial dilution test
Aerobacter aerogenes	100–200	100–200	
Escherichia coli K 12	>200	>200	
Proteus vulgaris	100–200	100–200	
Arthrobacter citreus	20–100	20–100	
Bacillus brevis	2–20	2–20	
Bacillus subtilis	>200	2–20	
Corynebacterium insidiosum	100–200	100–200	
Micrococcus roseus	100–200	20–100	
Mycobacterium phlei	20–200	20–200	
Streptomyces sp. ATCC 23836	>200	20–200	
Clostridium pasteurianum	8	8	Plate diffusion test; diameter inhibition zone (mm) with 200 μg per paper disk (6 mm)
Botrytis cinerea	12	12	
Penicillium notatum	10	10	

No inhibition was found with *Aspergillus panamensis*, *Fusarium cubense*, *Paecilomyces variotii*, *Saccharomyces cerevisiae* S 288 C, *Rhizoctonia solani*, *Rhodotorula glutinis* var. *dairenensis*.



5 R = n-$C_{15}H_{31}CO$ in admixture with other long chain acid residues (Table 2)

4.44, due to the influence of the additional acetyl group. To determine the position of the individual acyl groups at C-2 and C-3, a partial methanolysis of schizonellin A was performed. Treatment of the compound for 4 min with a trace of methylate in methanol leads to the deacetyl derivative **4** with retention of the long chain acyl residue. In its ^{1}H NMR the typical signal of 2-H has been shifted into the CHOH multiplets, whereas the 3-H signal remains at δ 4.87. This proves the location of the acetoxy group at C-2 and establishes the structure of schizonellins A and B as **2** and **3**.

Lead tetraacetate oxidation of **2** yields the degraded aldehyde **5** in accord with the position of the acyl residues at the mannose unit. For evaluation of its antibiotic activity, the perpalmitate of **3** was prepared by reaction with palmitic acid, dicyclohexylcarbodiimide and 4-dimethylaminopyridine [6, 7].

A mixture of 4-O-β-D-mannopyranosyl-D-erythritol derivatives, acylated by several long chain fatty acids, has been previously isolated from *Ustilago* sp. PRL-627 [8, 9]. However, the position of the individual acyl residues has not yet been determined [10, 11]. The schizonellins exhibit similar biological activity as ustilagic acids, metabolites from *U. maydis* (DC.) Corda [12, 13], which belong to a different type of glycolipid, however [14, 15].

Biological activity

Table 4 shows the antimicrobial spectrum of the schizonellins, indicating growth inhibition of Gram-negative and Gram-positive aerobic and anaerobic bacteria. In the plate diffusion test only *Botrytis cinerea* and *Penicillium notatum* were inhibited at high antibiotic concentrations. Both schizonellins exhibit almost the same bacteriostatic activity against the tested bacteria. The effect of the schizonellins on macromolecular syntheses in cells of the ascitic form of Ehrlich carcinoma was tested as previously described [16] with the exception that 100 μg/ml glucose was added to the phosphate buffered saline. At 25 μg/ml of schizonellin A or B the incorporation of leucine, uridine and thymidine into 5% TCA insoluble material (protein, RNA and DNA) was completely inhibited (Table 5). Both schizonellins exhibit haemolytic properties when tested according to Kupka *et al.* [17]. At 10 μg/ml of schizonellin A and 30 μg/ml schizonellin B, complete haemolysis of bovine erythrocytes was observed. These findings suggest a detergent-like mode of action of these antibiotics.

EXPERIMENTAL

General. Mps are uncorr. TLC was performed on Si gel plates (Merck 60 F_{254}).

Fermentation. Schizonella melanogramma GD 325 was maintained on agar slants containing a YM-medium of (g/l.): yeast extract 4, malt extract 10, glucose 4. Cells from these cultures were used to inoculate 500 ml conical flasks containing 150 ml of YM-medium. The seed flasks were incubated on a rotary shaker at 22° for 3–4 days. Fermentation was carried out in a New Brunswick FS 314 fermentor containing 5 l. of YM-medium with 5% glucose. After inoculation with 6% of seed cultures and addition of 4 ml Niax polyol antifoam, the fermentation was conducted at 22° for 5 days with aeration (3 l./min) and agitation (170 rev/min). Antibiotic production was followed by the paper disk/agar-diffusion assay using *Bacillus brevis* as test organism.

Isolation. The cells from a 5 l. culture grown for 5 days (wet weight 120 g) were collected by centrifugation, washed several times with H_2O, and extracted twice with 500 ml MeOH. The combined extracts were evapd yielding 24.4 g of brown oil. After addition of $CHCl_3$, a yellow pigment began to crystallize immediately. After filtration, the mother liquor was evapd, and the residual oil (20.7 g) containing the antibiotics was purified by column chromatography on Sephadex LH 20 (2.5 × 180 cm) with MeOH as solvent. The fractions exhibiting antibiotic activity were combined, the solvent evapd, and the residue containing the schizonellins was applied to a column (2.5 × 10 cm) of Al_2O_3 (Merck; activity V), and eluted with BuOH–EtOH–H_2O (4:1:5,

Table 5. Effect of **2** and **3** on DNA, RNA and protein syntheses of Ehrlich carcinoma ascitic cells

	Schizonellin added (μg/ml)	Incorporation of radioactivity (cpm) precursor Thymidine	Uridine	Leucine
Control	0	5130	30 800	24 700
2	5	1180	21 400	20 500
	25	108	893	1930
	50	32	59	317
3	5	3480	20 700	18 900
	25	78	291	349
	50	40	98	235

upper phase). The fractions containing the schizonellins were combined, the solvent evapd, and the residual oil loaded on a column (2.5 × 10 cm) of Si gel (Mallinckrodt) in $CHCl_3$. Schizonellin A was eluted with $CHCl_3$–MeOH (19:1); schizonellin B with $CHCl_3$–MeOH (97:3). Yield after evapn of the combined fractions: schizonellin A 244 mg, and schizonellin B 227 mg.

Schizonellin A (**2**). Colourless, amorphous gum; mp 80–85°; $[\alpha]_D$ −44.3° (*c* 1.4, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3660–3480 (m), 2945 (st), 3050 (w), 2870 (m), 1750 (st), 1730 (st), 1465 (w, br), 1370 (w), 1250 (m), 1085 (st, br), 1050 (m, br). ^{13}C NMR (CD_3OD): δ 14.4, 20.8, 23.7, 26.2, 28.1, 30.2, 30.3, 30.4, 30.8, 33.1, 35.5, 46.2, 47.1, 48.1, 50.9, 51.9, 62.5, 64.6, 66.1, 70.7, 72.5, 72.8, 73.6, 75.5, 78.3, 100.3, 130.8, 130.9, 172.0, 175.1. (Found: C, 59.58; H, 9.22. Calc. for $C_{28}H_{52}O_{11}$ (palmitate): C, 59.50; H, 9.20%).

Schizonellin B (**3**). Colourless oil; $[\alpha]_D$ −42.8° (*c* 2, $CHCl_3$). IR similar to that of schizonellin A. ^{13}C NMR: δ 14.4, 20.75, 20.81, 23.7, 26.2, 28.1, 30.1, 30.2, 30.3, 30.5, 30.6, 30.8, 33.1, 35.0, 64.6, 66.1, 70.6, 72.4, 73.0, 73.6, 75.1, 75.6, 100.4, 129.2, 130.8, 130.9, 171.9, 172.7, 174.9. (Found: C, 59.76; H, 8.87. Calc. for $C_{30}H_{54}O_{12}$ (palmitate): C, 59.33; H, 8.89%).

Acetylation. **2** or **3** (10 mg) was reacted overnight in Py (2 ml) and Ac_2O (1 ml). After working up a colourless oil (13 mg) was obtained, which showed only one spot on TLC (R_f 0.76; CCl_4–EtOAc, 3:1). IR ($CHCl_3$) cm^{-1}: 2980 (m), 2950 (st), 2875 (m), 1760 (st), 1750 (st), 1735 (st), 1470 (w, br., 1375 (st), 1250 (st), 1070 (st), 920 (w).

Methanolysis. **2** or **3** (50 mg) was refluxed in MeOH (3 ml) with NaOMe (200 mg) for 2–6 hr. After neutralization with HOAc the soln was extracted ×3 with Et_2O, dried (Na_2CO_3) and analysed by GC–MS (Table 2). The aq. phase was treated with Dowex 50 W (H^+ form) and evapd. **1** was obtained as a colourless solid, mp 155°; $[\alpha]_D$ −37.5° (*c* 0.8, H_2O); R_f 0.18 (PC, BuOH–HOAc–H_2O, 4:1:1). ^1H NMR (D_2O): δ 3.34–4.21 (12 H, *m*), 4.78 (1 H, *d*, *J* = 1 Hz).

Partial methanolysis. **2** (15 mg) was treated at room temp. for 4 min with a trace of methylate in MeOH. The reaction was stopped by adding dil HCl, and the soln evapd. After TLC (CCl_4–EtOAc, 3:1, R_f 0.67) **4** was isolated as an oily substance. $[\alpha]_D$ −8.3 (*c* 0.4, MeOH). IR ($CHCl_3$) cm^{-1}: 3680 (m), 3500–3200 (st, br), 3020 (st), 2985 (st), 2870 (m), 1740 (m), 1460 (w), 1130 (m), 1080–1020 (st, br), 980 (m). ^1H NMR (90 MHz, $CDCl_3$): δ 0.89 (3 H, *t*, *J* = 6 Hz), 1.31 (21 H, *br s*), 1.59 (2 H, *m*), 2.02 (1 H, *m*), 2.31 (2 *H*, *t*), 3.49–4.11 (11 H, *m*), 4.56 (H_1, *J* = 0.75 Hz), 4.87 (H_3, *dd*, J_1 = 3, J_2 = 9.5 Hz), 5.33 (1 H, *m*).

Acid-catalysed hydrolysis. The aq. phase from methanolysis was refluxed for 2 days at 110° with 2N H_2SO_4. After neutralization with $Ba(OH)_2$ and centrifugation of the ppt., the aq. soln was evapd. PC yielded two oily substances, which crystallized after several days. Their physical and chemical properties (^1H NMR R_f, colour reactions, $[\alpha]_D$, mp) were identical with those of D-mannose and erythritol.

Lead tetraacetate oxidation. **2** (5 mg) was stirred for 4 hr with $Pb(OAc)_4$ (20 mg) in HOAc (2 ml). After treating with a soln of oxalic acid in HOAc and working up (centrifugation, evapn, TLC ($CHCl_3$–MeOH, 9:1, R_f 0.34)), **5** (3.6 mg) was obtained. Colourless oil, which crystallized after a few days. Mp 69–73°, $[\alpha]_D$ −41.5 (*c* 0.45, MeOH). IR ($CHCl_3$) cm^{-1}: 3560–3400 (m, br), 2950 (st), 2870 (m), 1750 (st), 1735 (m), 1680 (w, br), 1600 (w), 1465 (w), 1375 (w), 1260–1250 (m, br), 1085 (st). ^1H NMR ($CDCl_3$): δ 0.91 (3*H*, *t*, *J* = 6 Hz), 1.29 (21 H, *br s*), 1.67 (2 H, *m*), 2.08 (3 H, *s*), 2.11 (2 H, *m*), 2.42 (2 H, *m*), 2.67–3.31 (2 H, OH), 3.31–4.18 (4 H, *m*), 4.27 (2 H, *d*, *J* = 1 Hz), 4.8 (1 H, *d*, *J* = 0.75 Hz), 4.89 (1 H, *dd*, J_1 = 4.5 Hz, J_2 = 9 Hz), 5.38 (1 H, *m*), 5.62 (1 H, *dd*, J_1 = 0.75, J_2 = 4.5 Hz), 9.71 (1 H, *d*, *J* = 1 Hz). MS: *m/e* (rel. intensity %) M^+ [stearate] 530 (0.1), 513 (0.5), 499 (0.5), 485 (0.8), 471 (2), 443 (2), 411 (3), 383 (2), 337 (2), 333 (2), 311 (2), 288 (7), 273 (9), 265 (10), 256 (10), 246 (15), 239 (100), 211 (21), 187 (17).

Schizonellin B perpalmitate. **3** (10 mg) was stirred at room temp. with a mixture of palmitic acid (16.4 mg), 4-dimethylaminopyridine (2 mg) and dicyclohexylcarbodiimide (14 mg) in CH_2Cl_2 (5 ml) for 2 days. After filtration of the ppt. and column chromatography (Si gel, 2.5 × 130 cm, eluent CH_2Cl_2) a colourless gum (18.9 mg) was isolated (71%); R_f 0.58 (TLC, CCl_4–EtOAc, 3:1), $[\alpha]_D \pm 0$ (*c* 3, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 2960 (st), 2870 (m), 1710 (st), 1450 (w), 1360 (w). ^1H NMR ($CDCl_3$): δ 0.89 (*ca* 15 H, *t*, *J* = 6 Hz), 1.29–2.11 (*ca* 120–130 H, *br m*), 2.4 (*ca* 10 H, *m*), 3.24–4.4 (*ca* 13 H and OH, *br m*). MS *m/e* (rel. intensity %) M^+ 1558 (0.02), 1525 (0.1), 1499 (0.1), 1356 (0.5), 1355 (0.5), 1329 (1), 1303 (1), 945 (2), 919 (3), 847 (3), 819 (70), 749 (7), 723 (9), 635 (4), 623 (3), 609 (20), 581 (7), 565 (6), 309 (6), 267 (11), 265 (8), 239 (100), 237 (8), 169 (34).

GC–MS. Quadrupole instrument with data system; column: 10 m capillary column OV 101; carrier gas: He, pressure 9 psi; analysed quantity: 1 μl of a soln in *n*-hexane; temp. programming: start 60°, 45 sec isothermal, then linear programme 8°/min, final temp. 210°. MS: EI, 48 eV, 0.43 mA emission, scan time: 1.2 sec/10 mass units. Results see Table 2.

Test organism and antimicrobial spectrum. *Corynebacterium insidiosum* and *Proteus vulgaris* were grown on YM-medium, *Clostridium pasteurianum* on RCM medium 5411 (Merck) and all other bacteria on nutrient broth (Difco). The incubation temp. was 27° for *Arthrobacter citreus*, *C. pasteurianum*, *C. insidiosum*, *Pseudomonas fluorescens* and *Streptomyces* sp.; the other bacteria were incubated at 37°. *Aspergillus panamensis* and *Penicillium notatum* were grown on malt extract (20 g/l.), *Saccharomyces cerevisiae and Rhodotorula glutinis* on yeast nitrogen base (Difco), and the other fungi on YM-medium. Incubation temp. for all fungi was 27°.

Macromolecular syntheses in cells of the ascitic form of Ehrlich carcinoma. The effect of **2** and **3** on the macromolecular syntheses in cells of the ascitic form of Ehrlich carcinoma was tested as described previously [16].

Test for haemolytic activity. The effect of **2** and **3** on bovine erythrocytes was tested by a modified hemiglobinecyanide method [17].

Acknowledgements—Financial support by the Deutsche Forschungsgemeinschaft is thankfully acknowledged. We thank Prof. A. Prox, Biberach, for the GC–MS spectra, and Dr. G. Höfle, Stöckheim, for the 270 MHz ^1H NMR spectra.

REFERENCES

1. Deml, G. (1977) Dissertation, Universität Tübingen.
2. Gorin, P. A. J. and Perlin, A. S. (1961) *Can. J. Chem.* **39,** 2474.
3. Charlson, A. J. and Perlin, A. S. (1956) *Can. J. Chem.* **34,** 1804.
4. Gorin, P. A. J., Haskins, R. H. and Spencer, J. F. T. (1960) *Can. J. Biochem. Physiol.* **38,** 165.
5. Bock, K. and Pedersen, C. (1975) *Acta Chem. Scand.* **B29,** 258.
6. Neises, B. and Steglich, W. (1978) *Angew. Chem.* **90,** 556.

7. Neises, B. and Steglich, W. (1978) *Angew. Chem. Int. Ed. Engl.* **17,** 522.
8. Haskins, R. H., Thorn, J. A. and Boothroyd, B. (1955) *Can. J. Microbiol.* **1,** 749.
9. Boothroyd, B., Thorn, J. A. and Haskins, R. H., (1956) *Can. J. Biochem.* **34,** 10.
10. Fluharty, A. L. and O'Brien, J. S. (1969) *Biochemistry* **8,** 2627.
11. Bhattacharjee, S. S., Haskins, R. H. and Gorin, P. A. J. (1970) *Carbohydr. Res.* **13,** 235.
12. Haskins, R. H. and Thorn, J. A. (1951) *Can. J. Botany* **29,** 585.
13. Reed, R. W. and Holder, M. A. (1953) *Can. J. Med. Sci.* **31,** 505.
14. Lemieux, R. U. (1951) *Can. J. Chem.* **29,** 415.
15. Lemieux, R. U., Thorn, J. A., Brice, C. and Haskins, R. H. (1951) *Can. J. Chem.* **29,** 409.
16. Anke, T., Oberwinkler, F., Steglich, W. and Höfle, G. (1977) *J. Antibiot.* **30,** 806.
17. Kupka, J., Anke, T., Oberwinkler, F., Schramm, G. and Steglich, W. (1979) *J. Antibiot.* **32,** 130.