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Lipids of the zygomycete *Absidia corymbifera* F-965

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Abstract

The cell lipids of the zygomycete *Absidia corymbifera* F-965 extracted with isopropanol and $CHCl_3$–MeOH mixtures at the exponential growth phase comprise $20 \pm 2\%$ of mycelium dry wt. The lipids consist of: triacylglycerols (51% of the total lipids extracted), diacylglycerols (9%), monoacylglycerols (3%), ergosterol (5%), ergosterol peroxide (5α,8α-epidioxyergosta-6,22-diene-3 β-ol) (3%), fatty-acid esters of ergosterol (less than 0.5%), free fatty acids (4%), glucocerebroside (3%), and glycerophospholipids (22%). The main phospholipids are phosphatidylethanolamine (39% of the total phospholipids), phosphatidyl-*myo*-inositol (17%), diphosphatidylglycerol (12%), phosphatidic acid (7%), phosphatidylcholine (6%), phosphatidylglycerol (3%), and two unusual phospholipids reported earlier, *N*-acetylphosphatidylethanolamine (7%) and *N*-ethoxycarbonyl phosphatidylethanolamine (9%). In addition, two unknown acidic phospholipids are present in traces. Saturated fatty acids of the lipids are dominated by *n*-hexadecanoic acid and unsaturated ones by octadecenoic acid; octadecadienoic and octadecatrienoic acids are present in lesser amounts. Ergosterol peroxide as well as the above glucocerebroside which contains 9-methylsphinga-4(*E*),9(*E*)-dienine have first been revealed in zygomycetes.

Keywords: *Absidia corymbifera*; Acylglycerols; Cerebroside; Ergosterol; Ergosterol peroxide; Fatty acids; Fungus; Glycerophospholipids; Lipids; Zygomycetes

1. Introduction

The genus *Absidia* embraces a wide range of lower filamentous fungi and belongs to the phylum Zygomycota, the class Zygomycetes, the order Mucorales, the family Mucoraceae (Hawksworth et al., 1995). Although the majority of microorganisms of the genus are soil saprophytes, among the strains of *Absidia corymbifera* and *Absidia septata* parasitic ones pathogenic for animals and men have been revealed (Sondhi et al., 1999; Plaza et al., 1998). The cell–lipid content of the *Absidia* fungi varies from 3% to about 30% of dry mycelium weight (Lösel, 1988; Ratledge, 1989), specifically in the strain of *A. corymbifera* studied previously, the total cell lipids accounted for 28% (Lösel, 1988). At present, there is a very scanty information on the lipid composition of the members of this genus. Moreover, as a rule, the identification of lipids was carried out only by TLC, which is obviously inadequate for the reliable inference on the lipid structure to be made. It has been reported (Lösel, 1988) that triacylglycerols are generally the predominant constituents of the total extractable lipids. In the low-polarity lipid fractions, free fatty acids (FAs), sterols, and their FA esters were also found. As in the majority of other zygomycetes (Lösel, 1988; Weete and Gandhi, 1997), the sterol fractions of the *Absidia* species dominate by ergosterol and/or 22,23-dihydroergosterol,

Abbreviations: ECPE, ethoxycarbonylphosphatidylethanolamine; EPO, ergosterol peroxide (5,8-epidioxyergosta-6,22-diene-3-ol); FA, fatty acid; ME, methyl ester; PE, phosphatidylethanolamine.

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phosphatidylethanolamine (PE) and phosphatidylcholine are most commonly prevalent in the phospholipid fractions. Among FAs of the lipids, palmitic acid is generally the main saturated one, while oleic acid prevails in unsaturated FAs (Konova et al., 2002; Lösel, 1988; Ratledge, 1989; Stahl and Klug, 1996). Some species are markedly enriched in octadecadienoic and octadecatrienoic acids, but tetraenoic and more unsaturated FAs, if any, are met with in traces; the sole exception known thus far is *Absidia anomala* wherein the arachidonic acid content of the total FAs comes to 4.3% (Radwan et al., 1996).

Recently we have reported that the strain *A. corymbifera* VKMF-965 produces two unusual glycerophospholipids, *N*-ethoxycarbonylphosphatidylethanolamine (ECPE) and *N*-acetyl-PE (Batrakov et al., 2001). The former had not yet been described in the literature, the latter was revealed in cattle and human brains and a human placenta (Debuch and Wendt, 1967), but in the work quoted its chemical structure was not properly proved. Both the lipids were among the main phospholipids of the strain and comprised 9% and 7%, respectively, of the total phospholipids, or 1.8% and 1.2%, respectively, of the total extractable lipids. The present communication is concerned with the identification of the remaining lipids.

2. Results and discussion

The cell lipids of *A. corymbifera* extracted with isopropanol and $CHCl_3$–MeOH mixtures comprised $20 \pm 2\%$ of dry biomass as found in five identical runs. Analysis of the extract by TLC, including two-dimensional mode, showed that the bulk of the lipids was contributed by low-polarity non-ionic components such as acylglycerols and sterols. Among more polar components, free FAs, glycolipid, and eight phospholipids were revealed. To identify these constituents, each of them was isolated in a chromatographically pure state. This was performed by means of DEAE-cellulose column chromatography which yielded five lipid fractions (A–E; Table 1), and subsequent separation of these into individual lipid classes by silica gel-column chromatography.

The glycerolipids thus obtained were identified using mainly a set of regular procedures (Batrakov and Nikitin, 1996) including: (1) TLC of lipids in various solvent systems along with standards, (2) acidic hydrolysis and alkaline methanolysis followed by TLC of the degradation products, (3) enzymatic hydrolysis of phospholipids with phospholipase D followed by TLC of the hydrolysis products, (4) IR and ^1H NMR spectroscopy (occasionally ^{13}C NMR spectroscopy). The positional distribution of FAs in the neutral glycerides was determined through enzymatic hydrolysis catalysed by porcine pancreatic lipase; in the case of glycerophospholipids, snake venom phospholipase A_2 was applied.

Fraction A eluted with $CHCl_3$ from the DEAE-cellulose column was composed of six constituents, five of them might be considered as typical of the fungal low-polarity lipids. These were triacylglycerols which represented the predominant lipid class of the strain, diacylglycerols, monoacylglycerols, sterol, and FA esters thereof (Table 2; the FA composition of the lipids isolated is given in Table 3). Also, this fraction contained a unusual constituent which had not been found previously in zygomycetes. When analysing by TLC and reversed-phase TLC, it behaved as an individual compound. Its IR spectrum displayed absorption at 860 cm^{-1} suggesting the occurrence of a peroxide group (Bellamy, 1975). In addition, its ^1H and ^{13}C NMR spectra coincided with those of 5α,8α-epidioxyergosta-6,22-diene-3β-ol reported earlier (Gunatilaka et al., 1981; Sgarbi et al., 1997; Takaishi et al., 1992), which argued for the identity of the compounds. This conclusion was supported by the MALDI mass spectrum of the constituent isolated which showed prominent peaks of ions $[M + Na]^+$ at m/z 451, $[M + Na - O_2]^+$ at m/z 419, $[M - O_2 - H_2O]^+$ at m/z 378, and $[M - O_2 - H_2O -$

Table 1
Fractionation of the lipids (1.11 g) extracted from *Absidia corymbifera* F-965 with a DEAE-cellulose colomn (acetate form; 110 ml bed volume)[a]

No.	Eluent	Eluent volume (ml)	Lipid fraction eluted	Fraction weight (mg)	Fraction composition
1	$CHCl_3$	650	A	791	FA esters of ergosterol, TAG, DAG, ergosterol, and EPO
2	$CHCl_3$–MeOH (9:1)	450	B	46	MAG, cerebroside, PC
3	$CHCl_3$–MeOH (7:3)	500	C	88	PE
4	MeOH	350		5	Peptides
5	$CHCl_3$–HOAc (4:1)	400	D	40	Free FAs
6	MeOH	400		4	
7	$CHCl_3$–MeOH–25% aq. NH_4OH (10:5:1)	600	E	124	*N*-Acetyl-PE, DPG,ECPE, PA, PG, PI, and two unidentified phospholipids

[a] In Tables 1–3, the following abbreviations are used in addition to those given above: DAG, diacylglycerols; DPG, diphospatidylglycerols; MAG, monoacylglycerols; HA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidyl-*myo*-inositol; and TAG, triacylglycerols.

Table 2
Relative percent (by weight) of individual lipid classes in the total lipids extracted from *Absidia corymbifera* F-965 cells (the figures in parentheses stand for the relative percent of phospholipid classes in the total phospholipids as found by the procedure of Vaskovsky et al. (1975))

Lipid class	Relative (%)
Non-phosphorus lipids	
Sterol FA esters	<0.5
TAG[a]	51
DAG	10
MAG	3
Ergosterol	5
EPO	3
Free FAs	4
Cerebroside	3
Phospholipids	
N-Acetyl-PE	1.5 (7)
ECPE	2 (9)
PE	8 (39)
DPG	2.5 (12)
PA	1 (7)
PC	1 (6)
PI	3.5 (17)
PG	>0.5 (3)

[a] For abbreviations used here see footnote under Table 1.

$CH_3]^+$ at m/z 363. It should be stressed that the elimination of O_2 from molecular and other ions is a characteristic feature of the mass spectra of peroxide compounds. Thus, the metabolite under discussion was ergosterol peroxide (EPO) (see Scheme 1).

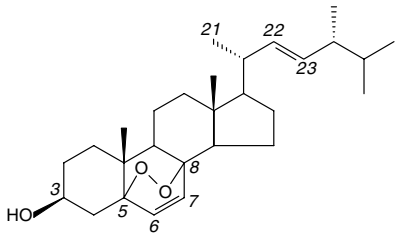
The sterol subfraction was analysed as such and as acetate by the same chromatographic technique. As a result, it was found to consist essentially of only one sterol comigrating with ergosterol on TLC, silver ion TLC, and reversed-phase TLC. Since its ^1H and ^{13}C NMR spectra as well as UV spectrum showed no difference from those of ergosterol (Adler et al., 1977), the sterol was recognised to be ergosterol. The least polar constituent of Fraction A comigrated with ergosterol oleate on TLC, and when subjected to alkaline methanolysis gave ergosterol and FA MEs. Based on these facts, it was characterised as a mixture of FA esters of ergosterol. Because its content of the total lipids extracted was much lesser than 0.5%, the subfraction was not studied in more detail.

Fraction B comprised three components, a glycolipid, monoacylglycerols, and phosphatidylcholine. On TLC, the glycolipid migrated very closely to the cerebrosides isolated previously from the filamentous soil fungus *Mortierella alpina* and containing amide-bound 2-hydroxy FAs (Batrakov et al., 2002). Its IR spectrum displayed the absorption bands of an amide group at 1662 and 1524 cm^{-1} but no absorption of an ester carbonyl. On drastic acidic methanolysis the glycolipid was broken down into methyl D-glucosides, 2-D-hydroxyhexadecanoic acid ME, and a lipophilic amine comigrating with the standard 4-sphingenine on TLC and responding positively to the periodate-Schiff's reagent. The findings described gave grounds to assign the glycolipid to the glucocerebroside type. In its MALDI mass spectrum, only one pseudomolecular ion $[M + Na]^+$, at m/z 750, was seen. Per-*O*-acetate of the glycolipid migrated as an individual compound on both reversed-phase TLC and silver ion TLC indicating that the glycolipid represented the sole molecular species, the

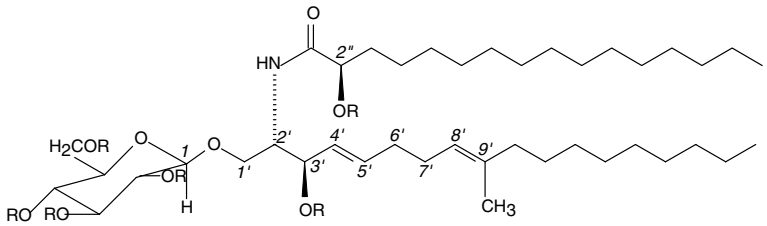
Table 3
Fatty acid composition (mol%) of the *Absidia corymbifera* F-965 lipids as found by GLC[a]

Lipid class	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	20:0	20:4	22:1
TAG[b] total	1	Tr	18	2	17	33	7	15	2	1	4
TAG *sn*-1(3)	2	ND	27	3	25	15	5	17	3	1	2
TAG *sn*-2	1	ND	5	1	2	67	10	12	1	Tr	1
DAG *sn*-1(3)	1	ND	17	3	8	36	12	18	Tr	ND	5
DAG *sn*-2	Tr	4	7	2	2	50	13	22	Tr	Tr	Tr
MAG	Tr	Tr	15	1	5	45	12	18	ND	4	ND
Free FAs	2	Tr	22	3	16	27	9	12	2	2	5
ECPE *sn*-1	2	Tr	25	3	5	27	9	27	ND	Tr	2
ECPE *sn*-2	Tr	ND	2	1	ND	59	20	18	ND	ND	ND
N-Acetyl-PE *sn*-1	3	Tr	55	5	4	27	2	4	ND	ND	ND
N-Acetyl-PE *sn*-2	Tr	Tr	15	3	2	62	13	5	ND	ND	ND
DPG total	1	Tr	15	2	Tr	30	13	39	ND	ND	ND
PA total	Tr	ND	8	1	2	37	17	29	Tr	1	5
PE *sn*-1	2	Tr	60	3	4	23	2	6	ND	ND	ND
PE *sn*-2	Tr	Tr	2	Tr	Tr	63	17	18	ND	ND	ND
PI *sn*-1	2	Tr	72	3	6	9	3	5	ND	ND	ND
PI *sn*-2	Tr	Tr	10	1	Tr	49	24	16	Tr	ND	ND
PC *sn*-1	Tr	2	42	6	4	28	9	8	1	ND	ND
PC *sn*-2	ND	1	10	7	1	52	19	10	ND	ND	ND
PG total	2	1	51	Tr	11	26	6	2	1	ND	Tr

[a] ND, not detected; Tr, trace.
[b] For abbreviations used here see footnote under Table 1.



Ergosterol peroxide (5α ,8α -epidioxyergosta-6,22-diene-3β -ol)



I R = H; II R = $COCH_3$

Scheme 1. Ergosterol peroxide (5α,8α-epidioxyergosta-6,22-diene-3β-ol) and glucocerebroside (**I**; **II**, per-*O*-acetate) from *Absidia corymbifera* F-965.

structure of which involved the residues of 2-D-hydroxyhexadecanoic acid, C_{19} long-chain base with two double bonds, and D-glucose. The ^1H and ^{13}C NMR spectra of the glycolipid and its per-*O*-acetate closely agreed with those of glucocerebrosides and the corresponding acetyl derivatives containing 2-hydroxy FAs and a methyl-branched diene base, 9-methyl-4(*E*),8(*E*)-sphingadienine (Batrakov et al., 2002; Boas et al., 1994; Gao et al., 2001a; Toledo et al., 1999). The evidence presented established unambiguously that the glycolipid isolated had the structure of 1-*O*-β-D-glucopyranosyl-2-*N*-(2′-D-hydroxyhexadecanoyl)-9-methylsphinga-4(*E*),8(*E*)-dienine (**I**; **II** per-*O*-acetate; Scheme 1). The anomeric β-configuration of the glucopyranose moiety was derived from the following spectral data. In the ^1H NMR spectrum of native lipid (in 1:1 $CDCl_3/CD_3OD$) the anomeric proton manifested itself as a doublet at δ 4.25 ppm with the coupling constant of 8.0 Hz and in the ^{13}C NMR spectrum, the anomeric carbon resonated at δ 102.7 ppm; as for ^1H NMR spectrum of per-*O*-acetate (in $CDCl_3$) the anomeric proton manifested itself as a doublet at δ 4.47 ppm with the coupling constant of 7.8 Hz and in the ^{13}C NMR spectrum, the anomeric carbon resonated at δ 101.0 ppm (cf. Boas et al., 1994; Bock and Thoegersen, 1982; Gao et al., 2001a; Toledo et al., 1999).

Fractions C and D consisted of chromatographically pure PE and free FAs, respectively. Fraction E comprised eight phospholipids, two of which were present as traces and therefore were not further analysed. The fact that all the phospholipids could be eluted from the DEAE-cellulose column only by ammonia-containing solvent system, testified to their strongly acidic character (Rouser et al., 1963). Among the low-polarity components of the fraction, there were *N*-acetyl-PE and ECPE whose structures had already been reported (Batrakov et al., 2001). The remaining phospholipids were identified as diphosphatidylglycerol, phosphatidylglycero1, phosphatidic acid, and phosphatidyl-*myo*-inositol.

As to phospholipid composition, *A. corymbifera* F-965 is primarily distinguished from other zygomycetes sufficiently studied in this respect in its containing *N*-acetyl-PE and ECPE which rank among the main phospholipids of the strain. An additional point to emphasise is a very low level of phosphatidylcholine, which is not typical of the fungal cell lipids at all. The bulk of phospholipids is contributed by strongly acidic ones such as *N*-acetyl-PE, ECPE, diphosphatidylglycerol, phosphatidylglycerol, phosphatidic acid, and phosphatidyl-*myo*-inositol which comprise together more than 60% of the total lipids extracted, with weakly acidic PE being the major phospholipid. The most unsaturated phospholipids are phosphatidic acid (more than 90% unsaturation), diphosphatidylglycerol (84%), and ECPE (83%), with diphosphatidylglycerol standing out because of the highest level of dienoic and trienoic FAs (Table 3). It is of interest that in ECPE, octadecatrienoic acid is predominantly located at the *sn*-1 position of the glycerol residue. Also, this phospholipid is remarkable for a rather high unsaturation level of the FA residues occurring at this position. These features together with unusual structure of the "polar head" of ECPE raise the question of its biochemical origin and possible biological role. Tetraenoic FAs are virtually absent from the phospholipids, with exception of phosphatidic acid containing about 1% of eicosatetraenoic acid. The percentage of this FA is somewhat greater in non-phosphorus lipids, monoacylglycerols (4%), and free FAs (2%). As quoted above, such a FA pattern is characteristic of the fungi of the *Absidia* ge-

nus. Of very long-chain FAs containing more than 20 carbons in the chain, only docosenoic acid was detected. It is present in free FAs, triacylglycerols, diacylglycerols, ECPE, and phosphatidic acid in very little amounts and is linked to the *sn*-1 position of the glycerol moiety.

Ergosterol is in realty the only sterol of the strain studied. Such composition of the sterol fraction is not a rarity in the Zygomycota (Weete and Gandhi, 1997). However, to our knowledge, EPO has not been found thus far in these organisms. By now this metabolite has been identified in a number of higher fungi, mainly in basidiomycetes, and also in yeasts, lichens, microalga, and marine invertebrates (see, e.g., Gao et al., 2001; Gunatilaka et al., 1981; Keller et al., 1997; Lobo et al., 1988; Lourenco et al., 1996; Miyamoto et al., 1988; Sgarbi et al., 1997; Sheffer et al., 1986; Sakaki et al., 2001 and relevant communications quoted there). It is worthy of note that EPO is the most abundant lipophilic constituent of the cytoplasmic membrane in the halotolerant microalga *Dunaliella salina* (Sheffer et al., 1986). In fungi, EPO is synthesised from cell ergosterol through H_2O_2-dependent enzymatic oxidation (Bates et al., 1976). It is believed that in some fungi, specifically in the yeast form of the pathogenic fungus *Sporothrix schenckii*, ergosterol may well function as a highly active antioxidant which scavenges reactive oxygen species with the resulting formation of EPO (Sgarbi et al., 1997). Alternatively, *S. schenckii* is capable of converting EPO into ergosterol. EPO was shown to be toxic to hepatoma and fibroblast cells in vitro and to have an antitumor activity against Walker carcinosarcoma and human mammary adenocarcinoma cell lines (Jong and Donovick, 1989). The cytotoxicity of EPO is assumed to be due to its conversion to ergosterol accompanied by formation of highly toxic peroxide products.

Some fungi produce similar peroxides of other sterols, e.g., 5α,8α-epidioxyergosta-6,9(11)-diene-3β-ol has been isolated from a marine fungus, *Cladosporium sphaerospermum* (Kuznetsova et al., 1998).

Cerebrosides are the most widely spread class of the fungal glycolipids (Lösel, 1988; Lösel and Sancholle, 1996). In the *A. corymbifera* strain F-965, only one cerebroside species has been found. Its structure (I, Scheme 1) involves along with 2-D-hydroxyhexadecanoic acid, a long-chain methyl-branched base, 9-methylsphinga-4(*E*),8(*E*)-dienine, which has been revealed in a number of higher fungi (see, e.g., Boas et al., 1994; Gao et al., 2001a; Toledo et al., 1999, and relevant communications there cited) but not in zygomycetes. In some of fungal cerebrosides, this base is N-acylated with unusual 2-hydroxyoctadec-3(*E*)-enoic acid which has so far been found only in fungi. In the cerebroside isolated from *A. corymbifera* F-965, this FA has not been detected. Earlier, it has been shown that cerebrosides similar to that described here are capable of stimulating the formation of fruit bodies in some fungi (Boas et al., 1994; Kawai, 1989; Toledo et al., 1999). A necessary condition for this kind activity to be expressed is the occurrence of the above branched long-chain base and 2-hydroxy FA 16:0 or 18:0 (but not 24:0) in the molecule while the nature of monosaccharide is not essential. The related cerebrosides isolated from a basidiomycete, *Ganoderma lucidum*, in addition to the same activity, are able to inhibit selectively the activity of α-type replicative DNA polymerase of a wide range of eukaryotes but only slightly affect, if ever do, the activity of DNA polymerases of other types, except for δ-type replicative DNA polymerase which is inhibited to a moderate degree (Mizushina et al., 1998). It is of interest that cerebrosides containing the above methyl-branched chain have first been revealed in the sea anemone *Metridium senile* (Karlsson et al., 1979).

3. Experimental

3.1. General methods

Analytical TLC was carried out with silica gel 60-precoated plates (Merck, Germany) using the following solvent systems for development: $CHCl_3$–MeOH–H_2O, 65:25:4 [1] (**1**) and 80:20:2 (**2**); $CHCl_3$–MeOH–25% aq. NH_4OH, 65:25:4 (**3**) and 60:30:5 (**4**); $CHCl_3$–MeOH–HOAc–H_2O, 65:25:5:4 (**5**) and 80:20:5:2 (**6**); CH_2Cl_2–EtOAc, 5:1 (**7**); *n*-hexane–Et_2O–HOAc, 80:20:1 (**8**), for lipophilic substances, and MeCN–HOAc–H_2O, 25:7:6 (**9**) or MeCN–EtOAc–HOAc–H_2O, 25:5:3:5 (**10**), for water-soluble compounds. Two-dimensional TLC was performed with the same plates using solvent system **1** in the first direction and systems **4** or **5** in the second direction.

Substances were visualised by spraying chromatograms both with non-specific reagents (such as 10% ethanolic phosphomolybdic acid followed by heating at 105–110 °C, and 10% methanolic H_2SO_4 followed by charring at 170–180 °C) (Kates, 1986) and with reagents specific to phospholipids (Vaskovsky et al., 1975), phosphorus-containing compounds (Vaskovsky and Latyshev, 1975), glycosides and amino, amido, ester and 1,2-diol groups (Kates, 1986). Phospholipids were quantitated on chromatograms by the procedure of Vaskovsky et al. (1975).

Reversed-phase TLC was done using plates precoated with modified silica gel, RF-18F_{254}S (Merck), and $CHCl_3$–MeOH, 1:1, MeOH–EtOAc, 1:1, and $CHCl_3$–MeOH–MeCN, 1:1:1, as developing solvent systems. Substances were detected in the ultraviolet light and by treating chromatograms with iodine vapours.

[1] In this paper, volume ratios are given for all solvent mixtures used.

The plates for silver ion TLC were prepared according to Nikolova-Damyanova (1992).

Non-hydroxylated FA MEs were analysed by GC on a model 3700 chromatograph (''Chromatograph'', Russia) equipped with a flame-ionisation detection system and a 2500×3 mm i.d. column packed with 17% diethyleneglycolsuccinate on Chromosorb WAW-DMCS-HP, 80–100 mesh; the column temperature was maintained at 175 °C. 2-Hydroxy FA MEs were chromatographed with a similar-size column packed with 5% SE-30 on Chromosorb WAW, 80–100 mesh; the column temperature was programmed from 160 to 260 °C (10 °C min^{-1}). Helium (50 ml min^{-1}) served as a carrier gas.

Alkaline methanolysis, acidic hydrolysis, and phospholipase A_2 (EC 3.1.1.4)-catalysed hydrolysis of lipids were conducted as described in an earlier communication (Batrakov et al., 2001). Acetylation was performed with 1:1 Ac_2O–pyridine at 20–25 °C for 16–20 h.

Enzymatic hydrolysis of triacylglycerols and diacylglycerols with porcine pancreatic lipase (EC 3.1.1.3) (Sigma, USA) was carried out in 0.1 M sodium-borate buffer (pH 7.2) at 37 °C for 1 h. The hydrolysis products were extracted with CH_2C1_2 and methylated with CH_2N_2. The FA MEs were purified by preparative TLC using CH_2C1_2 as a developing solvent.

The absolute configuration of the α-hydroxy-FA settled as follows: a ME of α-hydroxy-FA was analysed with 2,4-dinitrophinyl-L-or D-proline and the adduct formed was analysed by TCL using 1:1 *n*-hexane/diethyl ether as solvent system (3- or 5-fold development) as described earlier (Batrakov et al., 1998).

IR spectra were recorded on a Specord model 71 IR spectrograph (Carl Zeiss, Germany) with solvent-free films. 200 MHz ^1H NMR and 50 MHz ^{13}C NMR spectra were taken on a Gemini 200 instrument in $CDCl_3$ or 1:1 $CDCl_3$–CD_3OD; TMS served as an internal chemical shift reference. Assignment of the signals observed in the ^1H NMR spectra was mainly done by using ^1H–^1H COSY experiment; in the case of ^{13}C NMR spectroscopy, the attached proton test was applied. Mass spectra were measured using the martix-assisted laser desorption ionisation (MALDI) technique in the positive-ion mode with a time-of-flight mass spectrometer Vision 2000 (Thermo Bioanalysis, UK) equipped with a nitrogen laser (337 mm; pulse, 3 ns; the maximum pulse energy, 250 μJ). 2,5-Dihydroxybenzoic acid served as a matrice (delayed extraction).

3.2. Organism and cultivation conditions

The strain *A. corymbifera* F-965 was obtained from the Russian Collection of Microorganisms and supported on a wort agar medium. The biomass was grown as a submerged culture at 26–28 °C with shaking in 250-ml Erlenmeyer flasks, each contained 50 ml of a liquid medium of the following composition: glucose (Sigma), 4.0%; bactopeptone (Merck), 2.0%; yeast extract (Sigma), 0.1%; KH_2P0_4, 0.14%; and $MgSO_4 \cdot 7\ H_2O$, 0.025% (pH 6.2–6.4). At the end of exponential growth phase (48 h growth), the cells were separated and washed with distilled water.

3.3. Extraction of cell lipids

The cells separated were stirred twice with 30-fold volumes of isopropanol at 22–24 °C for 1 h. The cells debris were treated twice in the same fashion with 2:1 $CHCl_3$–MeOH and then with 1:1 $CHCl_3$–MeOH. The combined extract was evaporated to dryness, the residue was mixed with 20-fold volume of 2:1 $CHCl_3$–MeOH and kept for three days at −5 to 6 °C. The precipitate was filtered off, the filtrate was dried, and the residue was analysed by TCL in solvent systems **1**–**8** and by two-dimensional TLC.

3.4. Initial fractionation of the lipids extracted

The total lipids extracted were chromatographed on a column of DEAE-cellulose (acetate form; Reanal, Hungary) pre-treated according to the procedure described by Rouser et al. (1963). Lipid fractions A–E were eluted as indicated in Table 1.

3.5. Isolation of individual lipids

Fractions A, B, and E were chromatographed with columns of silica gel L 100/160 (Lachema, Czech Republic). While chromatographing Fraction A, elution was carried out syccessively with 1:1 *n*-hexane–C_6H_6, C_6H_6, C_6H_6–$CHCl_3$ mixtures with the concentration of $CHCl_3$ gradually increased from 5% to 75%, and finally with $CHCl_3$; the process was monitored by TLC in solvent systems **6**–**8**. This resulted in successive elution of ergosterol esters, triacylglycerols, ergosterol, diacylglycerols, and EPO. Fraction B was chromatographed eluting with $CHCl_3$ containing gradually increased concentrations of MeOH (from 2% to 50%) the process was monitored by TLC in solvent systems **2**, **5**, and **7**. Monoacylglycerols, cerebroside and phosphatidylcholine were washed out in success. In the case of Fraction E, elution was made with $CHCl_3$–MeOH–25% aq. NH_4OH mixtures, 80:10:1, 70:10:1, 50:10:1, and 40:10:1; the process was monitored by TLC in solvent systems **1**–**5**. This gave pure ECPE, *N*-acetyl-PE, diphosphatidylglycerol, and phosphatidylglycerol. Then, a mixture of phosphatidic acid and phosphatidyl-*myo*-inositol was washed out with 95% ethanol. The mixture was chromatographed once again; phosphatidic acid and phosphatidyl-

myo-inositol were eluted with 7:1 $CHCl_3$–MeOH and 50:10:1 $CHCl_3$–MeOH–H_2O, respectively.

3.6. Acidic methanolysis of cerebroside

An aliquot of the cerebroside fraction was heated with 5:1 MeOH–11 M HCl at 75 °C for 18 h, after which the mixture was diluted with water and 2-hydroxy FA MEs were extracted with *n*-hexane. The aq.-methanolic phase was adjusted to pH >8 with 10% aq. Na_2CO_3, and a long-chain base was extracted with $CHCl_3$. The aq. phase was deionised with Dowex 50 W × 8 (H^+) and Dowex 2 × 8 (HCO_3^-) (Serva, Germany) and dried. The resultant methyl glycosides were analysed by TLC in solvent systems **9** and **10** (triple development) and then by GLC as described earlier (Batrakov et al., 2002).

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