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Lipids and fatty acids of air breathing fish *Boleophthalmus boddaerti*

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The air breathing fish *Boleophthalmus boddaerti* has been studied for its lipids and fatty acids of body flesh. The nonpolar lipids identified were wax esters, steryl esters, 1-O-alkyl-2,3-diacylglycerol, triacylglycerols and sterols. Phospholipid was predominant (51%), the major component being phosphatidylcholine (67%). Polyenoic fatty acids of the $\omega 3$ series, i.e. EPA and DHA, were at considerably high concentrations in the phosphatidylcholine and phosphatidylethanolamine fractions. Cholesterol was the major component (35%) of the different sterols found. In neutral glyceryl ethers, fatty acids up to 28 carbons were recorded with unsaturated, anteiso, iso and normal chains. In wax esters, up to 32 carbon chains were recorded. © 1997 Elsevier Science Ltd

INTRODUCTION

Boleophthalmus boddaerti, commonly known as 'mud skipper', an air breathing fish, belonging to the family Gobiidae, is abundantly available in the mangrove tidal flats of deltaic Sunderbans. These fishes are detritus consumers and feed only during the ebb tide when the mud flats are completely exposed. Mangroves enrich the ecosystem by contributing large amounts of detritus that support numerous macrobenthic deposit-feeding organisms of which *B. boddaerti* predominates and consumes much of the detritus. Because of its delicious taste and abundant availability under natural wild conditions, the fish is voraciously consumed by the local people. Currently, all over the world, sea foods are encouraged because of their beneficial effects on diseases and disorders and for maintenance of good health (Carroll & Woodward, 1989). Of the polyunsaturated acids (PUFA) of marine lipids, eicosapentaenoic acid ($20:5\omega 3$) and docosahexaenoic acid ($22:6\omega 3$) have received considerable attention because of their various biological activities in health and disease (Wykes, 1993). In recent years the importance of phospholipids has been realised because of their structural and functional roles as essential constituents of biological membranes (Paltauf, 1983; Vance & Vance, 1985).

In the present investigation the lipids and fatty acids of the body flesh of *B. boddaerti* were studied, with special reference to the bioactive lipids, with a view to exploring the possibilities of commercial exploitation of the abundantly available species, particularly for medicinal uses.

MATERIALS AND METHODS

The fish were collected from the south-east coast of Sagar island, situated between latitudes 21°31′ to 22°33′N and longitudes 88°02′ to 88°16′E of the Sunderbans mangrove forest, West Bengal, India. Samples were brought live to the laboratory in muddy sea water. Prior to extraction, the fishes were descaled, the fins, heads and livers were discarded and only body flesh was weighed. Matured fishes of approximately the same lengths and weights were collected during the month of September which is just before the breeding period. Males and females were pooled together for this study.

Extraction of lipid

The total lipid was extracted from the flesh on ice following the method of Bligh & Dyer (1959). BHT (butylated hydroxy toluene) was added at a level of 100 mg/l to the solvent as antioxidant. The isolated lipids were dissolved in redistilled hexane and kept at −20°C for future use.

Column chromatography

A portion of the total lipid was subjected to column chromatography using a silicic acid (Mallinckrodt, 300

mesh) column (Rouser *et al.*, 1967). The neutral-, glyco- and phospholipids were eluted by 10, 40 and 10 column volumes of chloroform, acetone and methanol, respectively. The solvents were evaporated and fractions were kept in redistilled hexane at −20°C. Each class of lipids was estimated by weighing, in a micro balance.

Thin-layer chromatography (TLC)

Thin-layer chromatography was performed on 20×20 chromatoplates covered with silica gel G(0.25-0.50 mm thickness). The neutral lipid was fractionated by preparative TLC using light petroleum ether (40°C–60°C)–diethyl ether–acetic acid (80:20:1, v/v/v) (Mangold, 1969). While sterylesters, 1-O-alkyldiacylglycerol, triacylglycerol and sterols were separated, the overlapping hydrocarbon and wax ester bands could only be resolved by rechromatography using light petroleum ether (40°–60°C)–diethyl ether (98:2, v/v) as solvent system (Misra & Ghosh, 1991). Phospholipids were separated into different classes, i.e. phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI) and sphingomyelin (SPH), using a solvent system of chloroform–methanol–28% ammonia (65:25:5, v/v/v) (Rouser *et al.*, 1976). Phospholipids were estimated according to Bartlett (1959). Spots of separated lipid components were detected by iodine vapour and by specific spray reagents. All the lipids were identified by comparing the Rf values with those of authentic standards. Neutral lipid components were estimated by direct weighing.

Saponification of various lipids

Aliquots of neutral lipids (NL), glycolipids (GL) and phospholipids (PL) and separated components of neutral lipids, i.e., wax esters (WE), steryl esters (SE), 1-O-alkyldiacylglycerol (ADAG), triacylglycerol (TG) and phospholipid components, i.e. PE, PC, PI and SPH, were saponified separately according to Christie (1982). The fatty acids and nonsapoifiables were recovered as usual. In general, the lipid sample (100 mg) was hydrolysed by refluxing with a 1 M solution of potasium hydroxide in 95% ethanol (2 ml) for 1 hr, in an atmosphere of nitrogen gas. The solution was cooled, water (5 ml) was added and the solution was extracted thoroughly with diethyl ether (3×5 ml). Sometimes it was necessary to break the emulsion by centrifugation. The solvent extract was washed several times with water, dried over anhydrous sodium sulphate and the nonsaponifiable materials were recovered on removal of the solvent in a rotary evaporator. The aqueous layer was acidified with 6 N HCl and extracted with peroxide-free diethyl ether (3×5 ml). The free fatty acids were recovered after washing the extracts with water, drying over anhydrous sodium sulphate and removing the solvent in a rotary evaporator.

Methylation of fatty acids

Fatty acids of each fraction obtained by the above process were methylated using an etherial solution of diazomethane (Schlenk & Gallerman, 1960).

Acetylation of alcohols, sterols and 1-O-alkylglycerols

The alcohols, sterols and 1-O-alkylglycerols obtained from alkaline hydrolysis of wax esters, sterylesters and 1-O-alkyl-diacylglycerols respectively, were acelylated from the nonsaponifiables along with the free sterols according to Privette & Nutter (1967).

Esimation of sterols

Sterols obtained by preparative TLC was estimated colorimetrically using ferric chloride reagent, as described by Kates (1972).

Gas liquid chromatography (GLC)

Gas liquid chromatography of the various lipid components was done on a Hewlett Packard instrument, model 5890 series II, equipped with glass column (1.8×2 mm i.d.) and flame ionisation detector (FID). Quantitation was done by an integrator (Hewlett Packard model 3394A) attached to the GLC machine. Columns used were packed columns of 3% OV-17 and 10% DEGS; both the liquid phases were supported on chromosorb-W(HP) 80–100 mesh and packed into the glass column. On-column injections were made for all the samples. The column and standards were purchased from Hewlett Packard Company, Avondate, PA, U.S.A. and Sigma Chemical Company, St. Louis, MO, U.S.A., respectively.

Analysis of alcohol acetates from wax ester

GLC of alcohol acetates (obtained from the nonsaponifiables of wax ester) was done on a 3% OV-17 column. Oven temperature was programmed from 210°C to 330°C at the rate of 6°C/min. The detector and injection port temperatures were 380°C and 350°C, respectively. Nitrogen was used as carrier gas with a flow rate of 30 ml/min. Identification was done by comparing the retention times of the authentic compounds with those of the samples, as well as by semilogarithmic plots at two different isothermal temperatures (Misra & Ghosh, 1991).

Analysis of steryl acetates

GLC of steryl acetates was done on a 3% OV-17 and SE-30 columns. Oven temperatures were kept isothermal at 280°C. Detector and injection port temperatures were 350°C and 330°C, respectively. Nitrogen was used as carrier gas with a flow rate of 30 ml/min. Iden-

tification was done following the methods of Patterson (1971), by comparing the relative retention times (RRT) with those in the literature.

Analysis of 1-O-alkyl-2,3-diacetylglycerols

GLC of alkyldiacetylglycerols was done on a 3% OV-17 column. Oven temperature was programmed from 200°C to 330°C at the rate of 8°C/min. Detector and injection port temperatures were 380°C and 350°C, respectively. Nitrogen flow rate was 30 ml/min. Identifications of the alkoxy compounds were based on: i) TLC, where Rf value corresponded with that of the standards (Mangold, 1969; Mangold & Bauman, 1967); ii) The IR spectrum showed an absorbtion band (Kates, 1986; Snyder, 1972) at 1110 cm^{-1}, characteristic for O-alkylethers, -C-O-C; iii) ^{1}H-NMR (100 MHz) spectra showed peaks at δ3.4 and 3.8, for characteristic protons, $-CH_2-O-$ and [-CH-O-], respectively, according to Snyder (1972) and Pakrashi *et al.* (1989). NMR spectra of 1-O-alkyl-2,3-diacetylglycerols were recorded on a JEOL.FX-100 FT-NMR using $CDCl_3$ as solvent with tetramethylsilane (TMS) as internal, standard. IR spectra were recorded in an instrument model MB-102, FT-IR by Bomem Inc., Quebec, Canada. A reference was first obtained with CsI powder and then with the neat sample (100–200 μg) and transmittance spectrum obtained at 4 cm^{-1} resolution. GLC identification of components was done by the techniques suggested by Misra & Ghosh (1991).

Analysis fatty acid methylesters (FAME)

GLC of FAME was done on a 10% diethylene glycol succinate polyester (DEGS) column. Oven temperature was kept isothermal at 196°C. Detector and injection port temperatures were 255°C and 250°C, respectively. Nitrogen flow rate was 30 ml/min. Identifications of fatty acids were made by i) comparison of retention times of authentic standards, as well as with the fatty acids of cod liver oil, as secondary standards, as suggested by Ackman & Burgher (1965); ii) Semilogarithmic plots (Ackman *et al.*, 1963) of relative retention times (RRT) against carbon chain lengths; iii) comparison of equivalent chain length (ECL) values of the component acids with those reported in the literature (Misra *et al.*, 1983; Ackman, 1969); and iv) comparison of the chromatogram of catalytically reduced fatty acid methyl esters with that of the original sample (Ghosh & Dutta, 1972).

RESULTS AND DISCUSSION

Total lipids, and other various classes and subclasses of lipids of body flesh of *B. boddaerti* are presented in Table 1. The total lipid of the body flesh is low (1.0%) and nonsaponifiable content is high, a characteristic of the lipids of animals of the Sunderbans mangrove estuarine complex, as reported earlier for the king crab (Pakrashi *et al.*, 1989), gastropod molluscs (Dutta *et al.*, 1986; Misra *et al.*, 1986), and the bivalve (Chattopadhyay, 1993). The total lipid of the body flesh of *B. boddaerti* is comparable to that of Atlantic cod, *Gadus morhua* (Addison *et al.*, 1968), which is as low as 0.73%. The seasonal variation of the lipid of *B. boddaerti* has not been studied.

Among the various classes of lipids separated by column chromatography, composition of phospholipid was highest (51.3%) followed by glycolipid (26.4%) and neutral lipid (22.3%). Phospholipid and glycolipid together was as high as 87.4% as was found in the Atlantic cod (Addison *et al.*, 1968), which is comparable to *B. boddaerti*. The high level of phospholipid is similar to other animals of the Sunderbans ecosystem, reported earlier (Dutta *et al.*, 1986; Misra *et al.*, 1986; Chattopadhyay, 1993). In the phospholipid fraction, phosphatidylcholine (PC) is the major component (67.0%). The other three fractions were around 10–11%. In the neutral lipid, triacylglycerol (TG) is the major fraction, comprising 80.7%. Hydrocarbons and sterols were 11.3% and 5.2%, respectively. All other components of NL were within 1%.

Alcohol composition

The level of n-C-18:0 was highest (18.6%) of all the components reported in Table 2. Table 2 shows that, among the four classes of alcohols, the highest proportion was of n-chains (53.3%), whereas the proportions

Table 1. Composition of various classes of lipids obtained from body flesh of *Boleophthalmus boddaerti*

Lipids	% w/w
Total lipid (TL)[a]	1.0
Neutral lipid (NL)[b]	22.3
Glycolipid (GL)[b]	26.4
Phospholipid (PL)[b]	51.3
Neutral lipids	
Hydrocarbons (HC)[c]	11.3
Wax esters (WE)[c]	1.0
Sterylesters (SE)[c]	0.8
1-O-alkyl-2,3-diacylglycerols (ADAG)[c]	1.0
Triacylglycerols (TG)[c]	80.7
Sterols (ST)[c]	5.2
Phospholipids	
Phosphatidylethanolamine (PE)[d]	10.0
Phosphatidylcholine (PC)[d]	67.0
Phosphatidylinositol (PI)[d]	11.3
Sphingomyelin (SPH)[d]	11.7

[a]Expressed as % w/w of wet tissue.
[b]Expressed as % w/w of total lipid.
[c]Expressed as % w/w of neutral lipids.
[d]Expressed as % w/w of total phospholipids.

of the other three classes were comparable and were approximately 15–16%.

The alcohol composition in the present study shows qualitative similarities (with a minor variation in quantitative aspects) to those reported earlier in the lipids of detritivorous benthic animals (Pakrashi *et al.*, 1989; Chattopadhyay, 1993) as well as in plant leaves (Misra *et al.*, 1987) of this ecosystem. *B. boddaerti* is a detritivorous animal; it is probable that the alcohols from the plant waxes of the ecosystem might be transmitted through the food chain to this animal.

Sterol composition

Sterol compositions of free and esterified compounds are presented in Table 3. Cholesterol is the major component present in both the samples, in comparable amounts. An appreciable number of components of steroidal nature could not be identified following the method of Patterson (1971). The stereochemistry of the sterols identified in the present study was not determined. *B. boddaerti*, basically a detritus feeder, consumes various types of sterols present in detritus. These sterols are stored and modified according to need by the organism. Hence there is a good agreement between sterol compositions obtained from *B. boddaerti* and various mangrove plants (Misra *et al.*, 1984; Ghosh *et al.*, 1985).

Alkyl chain composition of 1-O-alkyl-2,3-diacylglycerol

Inspection of Table 4 indicates that C-20:0 was the major (26.0%) component in monoalkylated ether lipids of *B. boddaerti*. Of the other components, C-15:0, C-16:0, C-17:U, C-17:0; C-18:0 and C-22:0 were present in appreciable quantities. Among the four classes, the n-alkyl chain was the major component (65.6%), whereas, the proportions of the other three classes were approximately 8–14%. In this investigation, alkyl chain lengths from 10 to 28 were determined with odd and even chain lengths and containing saturated, iso-, anteiso- and unsaturated moieties which were very similar to the

Table 2. Composition of alcohols obtained from wax esters of body flesh of *Boleophthalmus boddaerti*

Carbon number	Normal chain[a]	Unsaturated chain[a]	Anteiso chain[a]	Iso chain[a]
16	0.3	7.9	—	—
17	1.4	2.2	—	1.3
18	18.6	0.9	2.3	1.1
19	0.7	—	0.5	0.5
20	2.0	—	1.0	0.9
21	—	—	1.0	0.5
22	1.8	3.9	0.8	—
23	3.7	—	1.2	0.8
24	6.7	—	—	1.4
25	—	—	0.5	—
26	0.9	—	—	1.1
27	2.0	—	0.4	—
28	—	—	2.9	4.6
29	9.2	—	1.1	0.6
30	3.5	—	1.4	0.8
31	1.5	—	1.7	1.5
32	1.0	—	1.4	0.5
Total	53.3	14.9	16.2	15.6

[a]Expressed as % w/w of total alcohols obtained by hydrolysis of wax esters.

Table 3. Composition of free and esterified sterols from the body flesh of *Boleophthalmus boddaerti*, analysed as acetates by GLC

Sterols (free)	Percentage[a]	RRT
Unidentified (6 Components)	29.6	< 0.92
22-Dehydrocholesterol	15.9	0.92
Cholesterol	37.3	1.00
Zymosterol	6.6	1.14
24-Dihydrolanosterol	8.3	1.41
Sitosterol	0.6	1.62
24-Methylenecycloartanol	1.3	2.00
Saringosterol	0.4	2.49
Sterols (esterified)		
Unidentified (12 Components)	50.4	< 1.00
Cholesterol	34.9	1.00
Zymosterol	0.3	1.14
$\Delta^{7,22}$ Ergostadienol	2.4	1.25
Campestanol	0.5	1.34
Unidentified	8.2	1.49
$\Delta^{7,25}$ Stigmastadienol	0.4	1.73
Citrostadienol	1.7	2.15
Unidentified	0.4	2.34
Unidentified	0.8	2.61

[a]Expressed as % w/w of total sterols obtained from free and esterified sterols of the sample.

Table 4. Composition of alkylchains obtained from 1-O-alkyl-2,3-diacylglycerol of body flesh of *B. boddaerti*

Carbon number	Unsaturated chain[a]	Anteiso chain[a]	Iso-chain[a]	Normal chain[a]
10	—	—	—	1.1
11	—	0.2	0.7	0.4
12	—	—	0.3	0.6
13	—	—	—	0.3
14	—	0.5	—	—
15	—	0.5	0.2	5.8
16	—	—	0.7	4.9
17	4.2	—	—	5.2
18	—	1.4	—	3.1
19	2.9	—	—	0.5
20	—	1.4	0.3	26.0
21	—	—	—	0.8
22	—	0.8	0.5	8.4
23	—	—	1.7	0.4
24	2.1	—	1.2	1.4
25	2.1	—	2.3	1.6
26	3.2	—	1.9	2.9
27	—	1.6	2.2	2.2
28	—	1.5	—	—

[a]Expressed as % w/w of total alkylchains obtained from 1-O-alkyl-2,3-diacylglycerols.

Table 5. Fatty acid composition of neutral lipid and its four different subclasses and glycolipid of body flesh of *B. boddaerti*

Fatty acids[ac]	NL[b]	WE[b]	SE[b]	ADAG[b]	TG[b]	GL[b]
14:0	6.7	1.2	1.0	1.1	5.8	5.4
16:0	28.4	30.2	41.0	33.7	13.0	24.7
16:1ω9	16.2	8.7	2.0	1.2	15.2	14.7
16:2ω6	12.3	0.7	0.3	—	7.9	4.3
18:0	2.5	10.9	20.3	25.5	12.6	10.0
18:1ω9	9.0	15.7	16.0	9.4	9.4	9.9
18:2ω9	2.1	—	—	—	2.1	0.7
18:2ω6	0.9	3.1	1.7	2.7	1.8	1.1
20:0	0.7	0.3	0.7	0.5	1.1	0.7
18:3ω3	0.8	4.7	2.5	2.7	1.1	1.0
18:4ω6	1.1	0.2	0.1	0.8	1.4	0.9
20:3ω9	0.5	0.3	—	—	1.3	1.3
22:0	0.4	1.1	1.4	1.1	0.6	0.8
20:4ω6	2.4	6.1	2.6	2.6	3.5	4.7
22:2ω6	0.7	1.2	0.9	—	1.1	0.6
20:5ω3	9.3	6.1	0.2	3.0	13.1	10.2
24:0	0.2	1.5	2.4	2.6	0.4	0.7
22:4ω6	0.8	1.7	0.3	0.5	1.4	1.2
22:4ω3	0.3	1.7	4.7	3.6	0.6	0.3
22:5ω6	2.9	1.5	—	0.8	4.1	4.2
22:6ω3	1.8	2.6	0.7	2.3	2.5	2.6
24:5ω6	—	0.2	0.9	3.6	—	—
24:5ω3	—	0.3	0.3	2.3	—	—
Saturates/unsaturates	0.6	0.8	2.0	1.8	0.5	0.7
ω3 series fatty acids	12.2	15.4	8.4	13.9	17.3	14.1
ω6 series fatty acids	21.1	14.7	6.8	11.0	21.2	17.0

[a]First and second figures represent, carbon chain length: number of double bonds. The W-values represents the methyl end chain from the centre of double bond furthest removed from the carboxyl end.
[b]Expressed as % w/w of total fatty acids present in each component.
[c]Components below 0.1% w/w of total fatty acids present are not reported in the table.

alkyl chains isolated from the cod fish muscle (Ratnayake *et al.*, 1986). Some naturally occurring ether lipids with long alkyl chains exhibit a variety of physiological activities (Mangold & Paltauf, 1983). Several of these substances are used in clinical diagnosis, medical research, and in cancer therapy (Berdel *et al.*, 1985).

Fatty acid compositions of neutral and glycolipid

Fatty acid composition of glycolipid, neutral lipid and its different fractions of body flesh of *B. boddaerti* are presented in Table 5. In the neutral lipid fraction, NL, WE, TG and glycolipid contained the larger proportions of unsaturates, as was evident from the ratio of saturates to unsaturates, which were below 1.0, whereas, SE and ADAG were mainly saturates.

Fatty acid compositions of individual classes of phospholipids

Table 6 shows that unsaturated fatty acids predominated over the saturates except in SPH, where considerably high levels of palmitic (16:0) and stearic (18:0) acids were found to occur (ca 53%). In SPH and PI, ω6 fatty acids predominated over ω3 fatty acids. In all other samples high levels of ω3 fatty acids were present, which is characteristic of marine animals, but because of the low content of lipids in this particular animal, their actual amount is low. Of the ω3 polyunsaturated fatty acid, major contributions were made by 20:5ω3, 22:6ω3 and also appreciably in most of the samples by 22:5ω3. The biological and pharmacological role of ω3 polyunsaturated fatty acids is of interest since it was found that, eicosapentaenoic acid, 20:5 ω3 (EPA), is very effective in the treatment of some cardiovascular diseases (Dyerberg *et al.*, 1978). EPA has a protective effect against thrombosis, atherosclerosis and some inflammatory diseases (Dyerberg *et al.*, 1978; Lee *et al.*, 1985). EPA reduces the concentration of cholesterol and triglycerides in the plasma by lowering the rate of synthesis of LDL (low density lipoprotein) and VLDL (very low density lipoprotein) by the liver and vascular tissues (Illingworth *et al.*, 1984). Similar studies with docosahexaenoic acid, 22:6 ω3 (DHA), indicate that it is effective in skin disorders, aids brain development and also forms a good part of the retina of the eye (Lee *et al.*, 1985). The lipid composition of air-breathing fish, *Boleophthalmus boddaerti* is almost in accordance with that of other detritivore benthic animals of the Sunderbans mangrove area.

Table 6. Fatty acid composition of different classes of phospholipids of body flesh of *B. boddaerti*, analysed by GLC as methyl esters

Components[ac]	PL[b]	PE[b] (10.0% of PL)	PC[b] (67% of PL)	PI[b] (11.3% of PL)	SPH[b] (11.7% of PL)
14:0	5.1	1.3	2.8	0.6	2.3
16:0	11.2	11.9	22.7	22.6	25.4
16:1ω9	7.5	10.8	13.2	18.0	0.8
16:2ω6	3.8	—	2.2	3.6	2.5
18:0	13.3	16.4	11.7	10.7	28.3
18:1ω9	8.7	8.7	6.7	12.2	3.8
18:2ω9	—	—	—	0.4	—
18:2ω6	1.0	0.7	1.5	0.6	0.4
20:0	0.7	0.4	1.3	0.9	0.6
18:3ω3	0.2	0.5	0.9	0.2	0.2
18:4ω6	0.3	0.3	0.5	—	0.4
20:3ω9	1.6	1.0	2.3	1.1	0.7
22:0	0.9	0.7	1.2	0.5	3.9
20:4ω6	8.4	7.9	4.4	11.7	5.6
22:2ω6	0.3	0.3	0.8	0.3	1.7
20:5ω3	12.4	8.1	14.1	4.4	5.3
24:0	1.5	0.6	4.9	0.9	3.7
22:4ω6	2.2	2.4	1.3	1.2	2.8
22:5ω6	1.4	2.7	0.9	1.6	2.1
22:5ω3	7.4	7.5	1.2	3.9	3.3
22:6ω3	12.1	16.9	4.5	3.5	5.6
24:5ω6	—	0.9	—	0.4	0.6
24:5ω3	—	—	—	0.7	—
Saturates/unsaturates	0.5	0.5	0.8	0.6	1.8
ω3 fatty acids	32.1	33.0	21.6	12.7	14.4
ω6 fatty acids	17.4	15.2	11.6	19.4	16.1

[a]Given elaborately in Table 5.
[b]Expressed as % w/w of total fatty acids present in total phospholipid and in each individual class of phospholipid.
[c]Components below 0.1% w/w of total fatty acids present are not reported in the table.
PL—Total phospholipid; **PC**—Phosphatidylcholine; **PE**—Phosphatidylethanolamine; **PI**—Phosphatidylinositol; **SPH**—Sphingomyelin.

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