According to this scheme, the peptide bonds in the isolated dipeptide A_2pm-A_2pm and tripeptide A_2pm-A_2pm-A_2pm should extend from an L carbon to a D carbon. This problem is currently under investigation.

Atypical N-ω-(L-R_3)-L-R_3 interpeptide linkages between L-Ala-D-Glu(L-R_3-D-Ala) peptide units—like the A_2pm-A_2pm peptide bonds in Mycobacteria—might occur as minor components of many bacterial peptidoglycans and might have escaped detection during previous studies.

Acknowledgments

We are grateful to Drs. E. Bricas and J. van Heijenoort (Institut de Biochimie, Orsay) for their helpful advice and for samples of A_2pm stereoisomers and dinitrophenyl derivatives. We thank D. Kosloff (Institut Pasteur, Paris) for providing BCG cells, Dr. E. Zissmann (Institut de Chimie des Substances Naturelles, Gif-sur-Yvette) for growing *M. smegmatis* cells, Mr. E. Tzagarakis (Institut de Biochimie, Orsay) for preparing BCG cell walls, and Mr. A. Yapo (Institut de Biochimie, Orsay) for performing amino acid analyses.

References

Adam, A., Petit, J. F., Wietzerbin-Falszpan, J., Sinay, P., Thomas, D. W., and Lederer, E. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 4,* 87.

Azuma, I., Thomas, D. W., Adam, A., Ghuysen, J. M., Bonaly, R., Petit, J. F., and Lederer, E (1970), *Biochim. Biophys. Acta 208,* 444.

Bricas, E., Ghuysen, J. M., and Dezélée, P. (1967), *Biochemistry 6,* 2598.

Coyette, J., Perkins, H. R., Polacheck, I., Shockman, G. D., and Ghuysen, J. M. (1974), *Eur. J. Biochem.* (in press).

Cunto, G., Kanetsuna, F., and Imaeda, T. (1969), *Biochim. Biophys. Acta 192,* 358.

Ghuysen, J. M. (1968), *Bacteriol. Rev. 32,* 425.

Ghuysen, M. M., Tipper, D. J., and Strominger, J. L. (1966), *Methods Enzymol. 8,* 685.

Hash, J. H. (1963), *Arch. Biochem. Biophys. 102,* 379.

Kotani, S., Yanagida, I., Kato, K., and Matsuda, T. (1970), *Biken J. 13,* 249.

Lederer, E. (1971), *Pure Appl. Chem. 25,* 135.

Leyh-Bouille, M., Ghuysen, J. M., Bonaly, R., Nieto, M., Perkins, H. R., Schleifer, K. H., and Kandler, O. (1970), *Biochemistry 9,* 2961.

Schleifer, K. H., and Kandler, O. (1972), *Bacteriol. Rev. 36,* 407.

Wietzerbin-Falszpan, J., Das, B. C., Azuma, I., Adam, A., Petit, J. F., and Lederer, E. (1970), *Biochem. Biophys. Res. Commun. 40,* 57.

Wietzerbin-Falszpan, J., Das, B. C., Gros, C., Petit, J. F., and Lederer, E. (1973), *Eur. J. Biochem. 32,* 525.

Isolation and Characterization of a Novel Lipid, 1(3),2-Diacylglyceryl-(3)-*O*-4′-(*N,N,N*-trimethyl)homoserine, from *Ochromonas danica*

Alfred E. Brown and John Elovson*

ABSTRACT: The phytoflagellate *Ochromonas danica* is unique in containing high concentrations of the detergent-like chlorosulfolipids, about 0.2 μmol/mg of total cell protein; in contrast, the organism contains less than 0.01 μmol of phospholipids/mg of protein. A second novel polar lipid has now been isolated from *O. danica* and characterized as 1(3),2-diacylglycerol-3(1)-*O*-4′-(*N,N,N*-trimethyl)homoserine, by chemical and mass spectrometric analysis. Its concentration in *O. danica* is 0.14 μmol/mg of protein, and it is suggested that this lipid, rather than the detergent-like chlorosulfolipids, largely substitutes for the usual phospholipids in membrane bilayer structures in *O. danica*.

The occurrence of unusual lipids is fairly common in bacteria and protozoa (see reviews by Thompson and Nozawa, 1972; Kates, 1964). Recently, a novel class of lipids has been isolated and characterized in the phytoflagellate, *Ochromonas danica* (Elovson and Vagelos, 1969). These compounds are derivatives of docosane 1,14-disulfate (Mayers and Haines, 1967), where up to six chlorine atoms are substituted for hydrogens on the hydrocarbon backbone (Elovson and Vagelos, 1969, 1970).

The presence of the sulfate groups in these unique lipids make them potent anionic detergents which in fact inhibit a number of enzymes *in vitro* (Elovson and Vagelos, 1969). Since preliminary results suggest that the chlorosulfolipids are enriched in cellular membranes, the present work was undertaken to characterize the other polar lipids of *O. danica*. This paper reports on the isolation and characterization of yet another novel lipid from *O. danica*, which consists of a *N,N,N*-trimethylhomoserine group linked to a 1,2-digylceride moiety through an oxygen-ether bond. This zwitterionic lipid constitutes over 50% of the total "normal" polar lipids in *O. danica*.

Experimental Section

Methods

Culture. Ochromonas danica was obtained from the American Type Culture Collection, ATCC 30004. Growth medium

† From the Department of Biology, University of California—San Diego, La Jolla, California 92037. *Received March 4, 1974.* This investigation was supported in part by research grants from the National Science Foundation (GB-25245-X) and a Public Health Service grant (AM-15836) from the National Institute of Arthritis and Metabolic Diseases.

and conditions for heterotrophic growth have been described previously (Elovson and Vagelos, 1969). Cultures were incubated at room temperature on a rotary shaker operating at 100 rpm. Cells from the stationary phase were harvested by centrifugation, and washed with ½ the culture volume of water. Cell pellets thus obtained were used directly for lipid extractions.

Lipid Extractions. Lipids were extracted essentially as described by Elovson (1974). Cell pellets were suspended in a small volume of 0.01 M EDTA (pH 4.0) and extracted with 3.75 volumes of chloroform–methanol (1:2 v/v) at 0°. Cell material was centrifuged and reextracted twice by the same procedure. Extracts were pooled, and separated into two phases by the addition of 0.01 M EDTA and chloroform to yield a resulting ratio of chloroform–methanol–EDTA of 1:1:1 (v/v). The upper aqueous phase was washed with additional chloroform until colorless; the chloroform fractions were combined, dried over anhydrous magnesium sulfate, and filtered through fritted glass filters. The filtrate was taken to dryness *in vacuo,* dissolved in a small volume of chloroform, and stored at −20° until further use.

Silicic Acid Column Chromatography. Chloroform fractions from whole lipid extracts were fractionated on 200–400 mesh silicic acid columns. Prior to use, the silicic acid was washed and activated for 24 hr at 150°. Approximately 50 mg of lipid extract/g of silicic acid was applied, and the column was washed with 20 volumes each of chloroform, acetone, and methanol. Column fractions were reduced *in vacuo* at 30°. Residues were dissolved in 5–10 ml of chloroform and transferred to screw cap tubes. Fractions were taken to dryness under a stream of nitrogen, and resuspended in chloroform to 5–10 mg of lipid/ml of solvent. Material was stored under an atmosphere of N_2 at −20° in screw cap tubes with Teflon liners.

Thin-Layer Chromatography (tlc). Slurries of silica gel G were applied to glass plates to 0.25- and 0.5-mm thickness. Plates were air dried overnight and activated prior to use in a vacuum oven at 120°. Whole lipid extracts and methanol eluents from silicic acid columns were developed in a solvent system of $CHCl_3$–MeOH–HOAc–H_2O (170:24:12.5:2, v/v). Lipids were visualized by iodine vapor. Amino groups were visualized with ninhydrin, phosphate with the molybdate spray of Dittmer and Lester (1964), and quaternary amino groups with the choline spray of Vaskovsky and Suppes (1971). For preparative tlc extracts were applied as thin bands on 0.50-mm silica gel G plates, and developed in the respective solvent. Appropriate spots were scraped from plates into fritted glass filter funnels, and eluted with a solvent mixture of $CHCl_3$–MeOH–HOAc–H_2O (50:39:1:10, v/v). Lipids were extracted into the organic layer by the addition of about ½ volume of 4.0 M NH_4OH. The organic layer was collected and reduced *in vacuo* at 30°, and the resulting material was suspended in chloroform.

Mild Alkaline Hydrolysis. Lipid (1–5 mg) was dissolved in 50–250 μl of toluene–methanol (1:1 v/v). An equal volume of 0.1 N NaOH in methanol was added and the mixture was incubated at 0° for 2 hr. The reaction was acidified with 2 N HCl and extracted twice with diethyl ether, after the addition of H_2O (50–250 μl). The ether fraction which contained the fatty acids and fatty acid methyl esters was collected and taken to dryness with a stream of nitrogen. Methyl esters were prepared by dissolving samples (100–500 μg) in 0.1 ml of diethyl ether–methanol (9:1) and adding an excess of saturated diazomethane in diethyl ether. Material was taken to dryness in a stream of N_2 and dissolved in an appropriate amount of carbon tetrachloride.

The water-soluble fraction was characterized by thin-layer chromatography in the solvent systems described by Sundler *et al.* (1972). Spots on chromatograms were detected with iodine vapor. Material in this fraction was further purified by ion-exchange chromatography on Dowex 50-X8-400 resin (H^+ form). Columns were prepared by washing with 6 N HCl, followed by water until washes were neutral. After sample application, columns were washed with several volumes of water and the material was eluted with a linear gradient of 0–2 N hydrochloric acid. Samples containing radioactivity were pooled and taken to dryness *in vacuo* at 40°, and the residue was dissolved in methanol.

Liquid Scintillation Counting was performed in a Packard TriCarb spectrometer using Bray's (1960) solution. In some cases, spots on thin-layer plates were scraped directly into vials and counted.

Assay for Choline. Choline assays were performed according to the method described by Bottcher *et al.* (1961).

Determination of Lipid Nitrogen. Lipid nitrogen was determined by the method of Sloane-Stanley (1967), with the following modification. Digestion was carried out for 45 min in 13 × 125 mm screw cap test tubes with Teflon liners in an aluminum heating block (Scientific Products) maintained at approximately 220°. These conditions were found to give the proper refluxing of perchloric acid to effect digestion. Since standard curves for ammonium sulfate and phosphatidylcholine were identical, the latter was used as a standard in all determinations. Nitrogen determinations for intact lipid were in close agreement with values for deacylated product.

Determination of Esterified Fatty Acids. Fatty acid esters were determined as the hydroxamates employing the method of Skidmore and Enteman (1962) with phosphatidylcholine as a standard.

Formaldehyde Determinations. Formaldehyde, formed by periodate oxidation of deacylated lipid A, was determined with chromotropic acid as described by Hantke and Braun (1973). Glycerol was used as a standard in all assays.

Nuclear Magnetic Resonance (nmr) Spectra. Lipid samples were analyzed in 1.0 ml of deuteriochloroform with 1% (v/v) tetramethylsilane. Deacylated lipid was run in deuterium oxide containing 1% (v/v) sodium 3-trimethysilyl-1-propanesulfonate. All samples were run on a Varian HR 220 spectrometer.

Me_3Si[1] *Derivatives.* Samples (10–100 μg) were dried under a stream of N_2; the residue was treated with 0.025 ml of pyridine–bis(trimethylsilyl)acetamide–trimethysilyl chloride (2:2:1 v/v) at room temperature for 30 min prior to analysis.

Gas–Liquid Chromatography: Glass columns (6 ft × ⅛ in. i.d.) packed with 1% SE-30 on 100–120 mesh Gas-Chrom Q, or 15% HI-EFF 2BP on 80–100 Chromosorb W were used in a Varian aerograph 2100 instrument, equipped with a hydrogen flame detector. Hydrogen flow was 22 ml/min, rotameter readings were at 14. Peak areas were determined with a series 200 Disc integrator.

Gas Chromatography–Mass Spectrometry. Analysis was performed on an LKB 9000 instrument. Direct, on-column injections (1–2 μl) were made, utilizing a 6-ft column of 1% SE-30 on 100–120 mesh Gas-Chrom Q, at a helium flow rate of 30 ml/min. Temperature programs of 10°/min were run from 60 to 200°, with the flash heater at 220°. Spectra were recorded at ionizing energies of 70 eV, with the ion source at 290° and separator at 300°.

To verify peak assignment of ions containing Me_3Si groups

[1] Abbreviation used is: Me_3Si, trimethylsilyl.

TABLE I: Lipids of *O. danica*.

Lipid P, total	0.24 μmol/10^9 cells
Lipid N, total	3.77 μmol/10^9 cells
"Lipid A"	2.78 μmol/10^9 cells
Chlorosulfolipids	5.88 μmol/10^9 cells
Total cell protein	27.0 mg/10^9 cells

their spectra were compared to those of the respective perdeuterated Me_3Si ether derivatives (Elovson and Vagelos, 1970).

Demethylation of Lipid A. Sodium benzenethiolate was prepared according to the method of Jenden *et al.* (1968). In a large-scale experiment 0.3 g of lipid was dissolved in dioxane (5 ml) freshly distilled from lithium aluminum hydride. Sodium benzenethiolate (0.17 g) was added, and the reaction mixture was refluxed for 1 hr under an atmosphere of nitrogen gas. The reaction was stopped by the addition of cold 1 ml of 2 N HCl, and the monodemethylated lipid was extracted into chloroform (Stoffel *et al.*, 1971). The course of the reaction was followed by analytical tlc on silica gel in a solvent system of chloroform–methanol–water (65:25:4, v/v); the same system was used preparatively for purification of the product.

Periodate Oxidation. Deacylated, demethylated lipid A (38 μmol of N) was dissolved in 1 ml of 1 N HCl; 2.3 ml of 0.05 M $NaIO_4$ was added and the mixture left overnight at room temperature. The reaction mixture was taken to dryness *in vacuo* at 30°; the residue was triturated with methanol, filtered through Whatman 1 filter paper, and taken to dryness under a stream of nitrogen. The residue was dissolved in 1 N HCl (0.2 ml), heated for 1 hr at 100°, dried *in vacuo*, and dissolved in methanol. The resulting *N,N*-dimethylhomoserine lactone was subjected to the analytical procedures described in the appropriate sections. In some cases the lactone was converted to the sodium salt by heating with an excess amount of sodium hydroxide. Product was recovered by trituration with pyridine, followed by filtration over Whatman 1 filter paper.

Synthesis of N,N-Dimethylhomoserine. The synthesis of *N,N*-dimethylhomoserine is based upon the method of Reinhold *et al.* (1968). Homoserine (1 mmol) was dissolved in about 2 ml of H_2O; catalyst (50 mg of 5% Pd/C) and 37% formaldehyde solution (0.17 ml) were added, and the volume was adjusted to 3.0 ml. The reaction was run overnight at ambient temperature on a Parr low-pressure apparatus at a pressure of 3 atm of hydrogen. The reaction mixture was filtered over Whatman 1, the catalyst was washed with several milliliters of water, and the filtrate and washings were combined. The product was taken to dryness *in vacuo* at 30°, and repeatedly triturated with methanol. The solution was taken to dryness under a stream of N_2, and the residue was dissolved in methanol. This product was compared to periodate-treated, deacylated, demethylated lipid A as described in the proper section.

High-voltage electrophoresis was employed to analyze *N,N*-dimethylhomoserine and the periodate treated product of demethylated, deacylated lipid A. Samples were run for 2 hr on Whatman 3M filter paper at 3 kV in a buffer of 0.5% pyridine acetate (pH 3.5). Methylated amines were detected with iodine; amino groups were visualized with ninhydrin.

Materials

O. danica was from the American Type Culture Collection. Silylation reagents, hexamethyldisilazane, trimethylsilyl chloride, and bis(trimethylsilyl)acetamide, were obtained from Pierce Chemical Co.; perdeuterated bis(trimethylsilyl)acetamide was from Merck, Sharp and Dohme of Canada. Methyl esters, phospholipid standards, SE-30 silicone gum, and HI-EFF polyester resin were from Applied Science. Silica gel G was from Brinkmann; ready-made plates were from Brinkmann or Supelco, Inc. Benzenethiol was from Matheson Coleman and Bell. Homoserine was purchased from Sigma. L-[*methyl*-^{14}C]Methionine and [2-^{3}H]glycerol were obtained from Amersham/Searle. Tetramethylsilane and deuterium oxide were from Mallinckrodt.

TABLE II: Fatty Acid Methyl Esters Obtained by Transmethylation of Lipid A.

Fatty Acid	%
C14:0	36.82
C16:0	5.14
C18:0	2.27
C18:1	13.58
C18:2	22.61
C18:3	5.73
C20:4[a]	8.18
C20:5[a]	5.67

[a] Tentative identification.

Results

Composition of Polar Lipid Fraction. About 40% of the chloroform-soluble lipids extracted from dark-grown *Ochromonas* cells in late-logarithmic–early stationary phase appear in the methanol eluate from a silicic acid column; the balance consists mostly of glycerides, sterols, and other neutral lipids which elute in the first chloroform fraction, with only very small amounts of material recovered in the intermediary acetone fraction. When analyzed by thin-layer chromatography the methanol fraction separated into at least five components which stained with iodine vapors. Two of the minor components chromatographed with standard phosphatidylethanolamine and phosphatidylcholine in several tlc systems, and showed the appropriate staining reactions for amino groups, choline, and phosphate expected for these lipids. However, the major component was an unidentified lipid with a chromatographic mobility intermediate between that of cardiolipin and phosphatidylethanolamine in the system used. This component, called lipid A, was ninhydrin-negative but gave a strong positive reaction with the spray reagent for quaternary amino groups; however, it gave no reaction with the spray reagent for phosphate. Quantitative analysis of material eluted from the tlc plate verified the complete absence of phosphate and showed the presence of about 1.1 μmol of quaternary amino groups/mg of lipid. Table I shows quantitative analysis of those polar lipids in *Ochromonas danica* which are recovered in the chloroform phase after partitioning against methanol water in the extraction procedure. It is seen that phospholipids constitute less than 10% of these. Although not completely identified the data summarized above suggest the presence of phosphatidylethanolamine and phosphatidylcholine in this fraction. Lipid A is the major component, and accounts for most of the lipid nitrogen, the balance of the latter is found in the phospholipids, and in two other unidentified components with lower chromatographic mobilities than lipid A. One of these components yields the same deacylation product as does lipid A (see below), and may be a lyso compound, most likely an artifact of preparation; the

TABLE III: Chemical Analysis of Lipid A.

Analysis	μmol/μmol of Lipid A[a]
Fatty acids	1.8[b]
Ester groups	2.0[c]
Formaldehyde equivalents	2.0[d]

[a] Standardized to the nitrogen content of lipid A (Sloane–Stanley, 1967). [b] Determined by gas chromatography with heptadecanoic acid as an internal standard. [c] Determined on the intact lipid after the method of Skidmore and Enteman (1962). [d] Determined on deacylated lipid after the method of Hantke and Braun (1973).

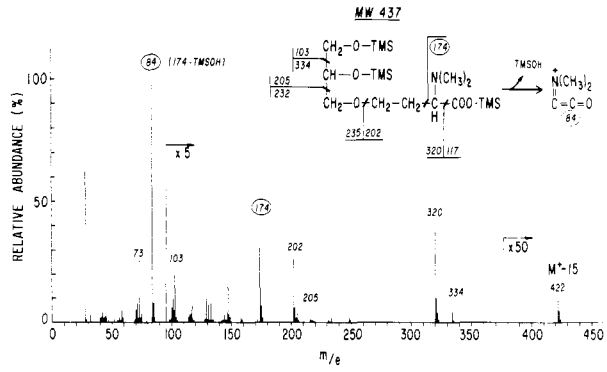

FIGURE 1: Mass spectrum and fragmentation pattern of the pertrimethysilyl derivative of deacylated, demethylated lipid A.

other unknown also contains quaternary amino nitrogen and no phosphate, but has not been further identified.

Structure Determination of Unknown Lipid A. Fatty Acid Content and Composition. Results of gas chromatographic analysis of fatty acids from lipid A are shown in Table II. Identical results were obtained when methyl esters were prepared by acid-catalyzed methanolysis of the intact lipid or by mild alkaline hydrolysis followed by treatment with diazomethane. The predominant species are the unsaturated C-18 acids, identified by cochromatography with authentic standards of polar and nonpolar stationary phases; by the same criteria smaller amounts of arachidonic and an eicosopentaenoic acid were also present. The lipid is highly enriched in myristic acid, which makes up over one-third of the total fatty acids; palmitic and stearic acids are present in much lower concentrations. Quantitative determination of total fatty acids with heptadecanoic acid as internal standard in the gc analysis showed a ratio of 1.8 fatty acids/nitrogen. Independent analysis with the hydroxamate method gave a value of 2.0 ester groups/nitrogen (Table III).

Analysis of Water-Soluble Products of Deacylated Lipid A. The water-soluble material released on mild alkaline hydrolysis of lipid A was recovered and analyzed by thin-layer chromatography as described in the Experimental Section. A single spot (R_F 0.56) appeared on exposure to iodine vapors, indicating the presence of a quaternary ammonium group, as expected; this spot also gave a positive reaction for 1,2-diol groups with a periodate–benzidine spray agent. These findings, together with the 2:1 ratio of ester to nitrogen suggested that lipid A was a glycerolipid, carrying two fatty acyl groups and some unknown quaternary amine derivatives. However, when larger amounts of the purified water-soluble material were prepared, attempts to release glycerol from this product by vigorous treatment with acid (6 N HCl, 100° overnight) or alkali (2 N NaOH, 100° overnight) were unsuccessful: the material was recovered unchanged, although these procedures completely hydrolyzed phosphatidylcholine into glycerol, phosphate, and free choline. On the other hand, when lipid A was isolated from cells grown in the presence of [^{3}H]glycerol it accounted for 25% of the total lipid radioactivity, and 65% of that in the polar lipid fraction; over 90% of the label in lipid A was recovered in the purified water-soluble deacylated product. Similarly, lipid A accounted for 40% of the total lipid radioactivity and 65% of that of the polar lipids when cells were grown on [^{14}C]methionine; about 80% of this radioactivity was recovered in the purified water-soluble deacylated product. Furthermore, when the deacylated material was subjected to periodate oxidation under acid conditions, 2 equiv of formaldehyde/nitrogen were in fact obtained (Table III).

The fact that the latter reaction occurred at all suggested that deacylated lipid A contained a quaternary ammonium base bonded to the primary hydroxyl group of a glycerol moiety; the greatly enhanced acid lability of the oxidation product suggested that the bond was an O-ether linkage. To pursue this question further the deacylated lipid A product was converted to a volatile form suitable for gas chromatographic and mass spectrometric analysis.

Demethylation of the Lipid A and Analysis of Its Deacylated Product. In order to obtain a volatile derivative intact lipid A, obtained in doubly labeled form from cells grown on [^{3}H]glycerol and [^{14}C]methionine, was treated with sodium benzenethiolate to yield a demethylated dimethylammonium derivative as described in the Experimental Section. Preparative thin-layer chromatography of the reaction mixture gave a major product which chromatographed with an R_F of 0.6, well ahead of unreacted lipid A, R_F 0.48; several minor components with lower R_F values were also recovered, presumably lyso compounds, since they gave the same water-soluble deacylation product as did the major component at R_F 0.6. After purification on Dowex 50 the yield of tritium label in this combined demethylated–deacylated product was about 80% of that in the starting material. Its Me_3Si derivative was prepared and analyzed by gas chromatography and mass spectrometry as described in the Experimental Section.

The Me_3Si derivative gave a single peak on gas chromatography with a retention time of 4.61 min at 150° on a 1% SE-30 column. Mass spectrometric analysis of this peak gave the spectrum shown in Figure 1. The molecular weight of 437, indicated by the weak $M^+ - 15$ ion at highest mass *m/e* 422, and the presence of three Me_3Si groups in the molecule, are consistent with a structure such as that shown in the insert of Figure 1. Ions at *m/e* 103 and 205, with one and two Me_3Si groups, respectively, would derive from the glyceryl moiety as indicated. The ready loss of the carboxytrimethylsilyl group, to give the most intense high-mass ion at *m/e* 320, is consistent with an α heteroatom substituted carboxylic acid derivative. The intense Me_3Si containing ion at *m/e* 174, which loses the elements of trimethylsilyl hydroxide to form the base peak at *m/e* 84, is most simply accounted for by cleavage between the α and β carbons of an α-*N,N*-dimethylamino acid derivative. In other experiments (data not shown) where the demethylated, deacylated lipid A was first treated with diazomethane before forming the Me_3Si derivative, the appropriate shifts were observed for all ion fragments which now contained the carboxymethyl rather than carboxytrimethylsilyl group.

Periodate Oxidation of Demethylated, Deacylated Lipid A; Identification of the Product N,N-Dimethylhomoserine Lactone. As shown in Table III, and Figure 4, upper diagram, per-

TABLE IV: Important Ions in the Mass Spectrum of *N,N*-Dimethylhomoserine Derived from Lipid A.[a]

m/e	Actual Composition	Calculated Mass	Measured Mass[a]
85	$C_5H_{11}N$	85.089145	85.089037
84	$C_5H_{10}N$	84.081320	84.080809
71	C_4H_9N	71.073496	71.073351

[a] Samples were run on a single focusing Varian Mat CH-5 mass spectrometer, at an apparent resolution of 5000. Spectra were recorded at ionizing energies of 70 eV with an accelerating voltage of 3 kV, and filament current of 1 mA. Perfluoroalkanes were used as internal standards for peak matching.

iodate oxidation of deacylated lipid A yielded 2 formaldehydes/nitrogen; the same stoichiometry was obtained for the deacylated, demethylated lipid A. In a large-scale experiment 38 μmol of demethylated, deacylated lipid A was oxidized with periodate as described in the Experimental Section, and the methyl-^{14}C-labeled cationic product was purified by absorption to and elution from Dowex 50 as described in the Experimental Section; 33 μmol of labeled product was obtained. When a portion of this material was taken up in pyridine and analyzed by glc a single peak was observed with a retention time of 2.66 min at 60° on a 1% SE-30 column; a linear temperature program up to 200° failed to elute any other components. When the material was taken through the Me_3Si derivatization procedure before glc, the same major peak was obtained, but a second peak which accounted for about 15% of the material, now eluted at 108° during the temperature program.

The mass spectrum of the major component is shown in Figure 2. The intense ion at highest mass *m/e* 129 is consistent with the molecular ion for *N,N*-dimethylhomoserine lactone. High-resolution mass spectrometry showed that the major fragments at *m/e* 84 and 85 and the base peak at *m/e* 71 all retained the nitrogen atom (Table IV), and clearly arise by transannular elimination of carbon dioxide for the ion of *m/e* 85, coupled with loss of an extra hydrogen and an extra methylene group for fragments at *m/e* 84 and 71, respectively.

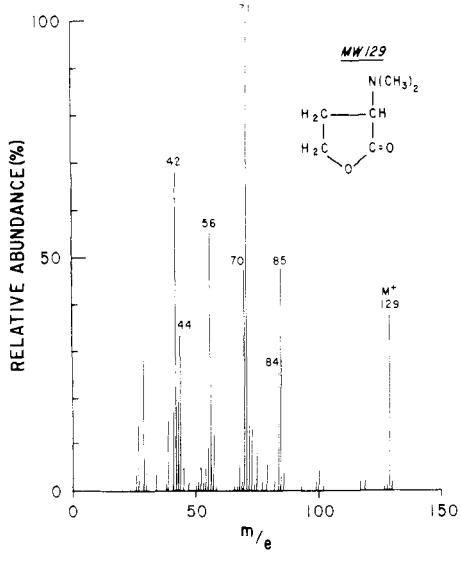

FIGURE 2: Mass spectrum of *N,N*-dimethylhomoserine lactone obtained from the periodate treatment of deacylated, demethylated lipid A.

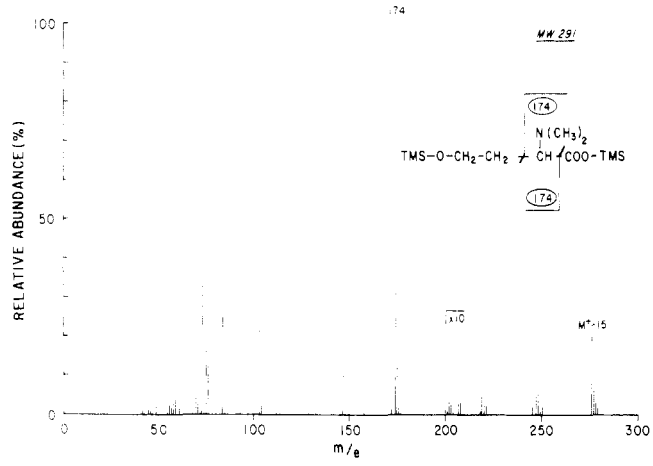

FIGURE 3: Mass spectrum and fragmentation pattern of the Me_3Si ether-Me_3Si ester of *N,N*-dimethylhomoserine.

The spectrum of the minor component which appeared only after subjecting the sample to the Me_3Si derivatization procedure is shown in Figure 3. The molecular weight of 291, defined by the intense $M^+ - 15$ ion at *m/e* 261 and the presence of two Me_3Si groups, clearly establishes this as the Me_3Si ether-Me_3Si ester derivative of *N,N*-dimethylhomoserine. The position of the *N,N*-dimethylamino group on the α carbon is clearly shown by the intense base peak at *m/e* 174, which happens to be the mass of the nitrogen and charge-retaining ion obtained by simple α cleavage on either side of the α carbon.

The relationship between these two compounds was confirmed by treating the periodate oxidation product with base before subjecting it to the Me_3Si derivatization procedure. Under those conditions the major early peak due to the lactone form was completely eliminated, with a concomitant increase in the amount of material in the Me_3Si-ether-Me_3Si ester peak.

Identity of N,N-Dimethylhomoserine Lactone and Periodate Oxidized, Demethylated, Deacylated Lipid A. The structure of periodate oxidized, demethylated, deacylated lipid A was unambiguously established by comparison to the compound prepared by chemical synthesis. Homoserine was reductively methylated with formaldehyde as described in the Experimental Section, treated with 2 N HCl at 60° for 2 hr to ensure its conversion to the lactone, and purified by ion-exchange chromatography. After Me_3Si derivatization, this material showed the same gas chromatographic pattern as described above for the degradation products from lipid A. The mass spectra of these authentic samples of *N,N*-dimethylhomoserine lactone and the open-chain *N,N*-dimethylhomoserine Me_3Si ether-Me_3Si ester were indistinguishable from those of the products which had been so assigned in Figures 2 and 3.

To ascertain that the mixture of the lactone and open-chain form of *N,N*-dimethylhomoserine observed by glc mass spectrometry in fact represented the only major products of the periodate oxidation of demethylated, deacylated lipid A, the products of the latter procedure were also analyzed by high-voltage electrophoresis, as described in the Experimental Section; 1.8 μmol of periodate oxidation product, 1853 dpm, was analyzed in parallel with a sample of the chemically synthesized *N,N*-dimethylhomoserine lactone. After exposure to iodine vapors each of the samples gave one single spot with identical mobilities; there was complete coincidence between staining and radioactivity in the sample of periodate oxidized, demethylated, deacylated lipid A.

Nmr Analysis of Intact Lipid A and Its Demethylated, Dea-

cylated Product. The 220-MHz spectrum of lipid A in chloroform shows a major singlet peak for its trimethylammonium protons at δ 3.29, in about the same position as that reported for phosphatidylcholine in the literature (Chapman and Morrison, 1966; Birdsall *et al.*, 1972; Hague *et al.*, 1972); the spectrum also shows the expected dominant peaks contributed by protons of the fatty acyl groups. The spectrum for demethylated, deacylated lipid A in deuterium oxide shows that the singlet for the dimethylamino group protons is shifted upfield to δ 3.94; comparison with the spectrum of glycerolphospherylcholine reported in the literature (Chapman and Morrison, 1967) suggests that a collection of peaks at δ 3.3–4.0 is contributed by the glycerol moiety, and the α and γ protons of the *N,N*-dimethylhomoserine moiety; a rather poorly resolved 2-proton quartet at δ 2.26 has no counterpart in the spectrum of glycerylphosphorylcholine, and could be contributed by the extra β-carbon protons in the *N,N*-dimethylhomoserine moiety.

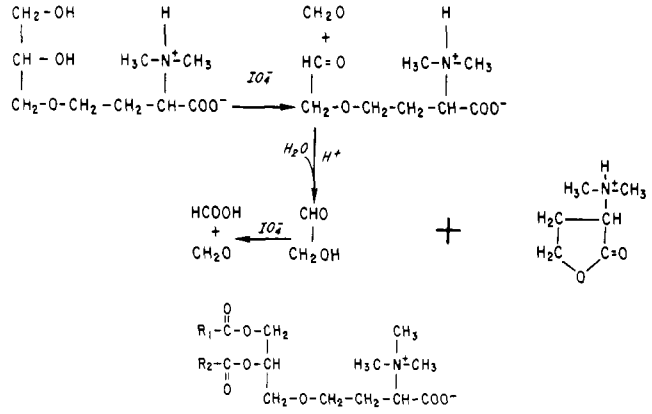

FIGURE 4: (upper) Stepwise degradation of deacylated, demethylated lipid A. (lower) Structure of lipid A.

Discussion

Although lipid A actually contains fewer components than the normal glycerophospholipids its unusual structure made conventional analysis of its water-soluble deacylated product difficult. The O-ether bond between the glycerol and nitrogenous base is stable to hydrolysis in acid or alkali and the nature of *N,N,N*-trimethylhomoserine moiety was not immediately apparent after removal of the glycerol moiety by acid periodate treatment. However, as soon as a volatile derivative of the deacylated product became available a first, essentially correct, structure suggested itself after gas chromatographic–mass spectrometric analysis. Since the nonspecific tests for choline actually measure quaternary ammonium ions the obvious choice to obtain a volatile derivative was demethylation to a dimethylamino compound. Reaction of deacylated lipid A with benzenethiolate in dioxane gave poor yields, probably due to solubility problems, but demethylation of the intact lipid gave a good yield in a procedure modeled on that used for demethylation of phosphatidylcholine by Stoffel *et al.* (1971). After deacylation the water-soluble product, which accounted for 80% of the original material, readily gave a tritrimethylsilyl derivative suitable for gc–mass spectrometric analysis. The information obtained in that analysis then guided the simple sequence of reactions which established the 1(3),2-diacylglyceryl-3(1)-*O*-4′-(*N,N,N*-trimethyl)homoserine structure for the intact lipid A. The analysis does not distinguish between a glycerol C-1 and C-3 position for the ether; the latter position is of course the expected one, but at least one instance of the glycerol lipid with the polar head group bonded to carbon-1 of glycerol has been reported (Kates *et al.*, 1965).

The structure in Figure 4, lower diagram, is the only one consistent with the data presented above. An O-ether bond to the secondary hydroxyl on the glycerol moiety was excluded by the results of the periodate oxidation since this structure would have rendered the deacylated product completely refractile to this treatment. Another alternative, based on threonine rather than homoserine, was ruled out by formation of the stable lactone after removal of the glycerol moiety, and the simple demonstration that this product behaved identically to chemically synthesized *N,N*-dimethylhomoserine lactone; the homoserine structure is also consistent with the nmr spectrum of the demethylated, deacylated material.

The structure of lipid A is thus unique in several respects. It is the first instance of a glycerol lipid which has an oxygen–ether bond joining the glycerol moiety to polar head group of the molecule; in all other ether lipids described this bond joins the glycerol moiety to a nonpolar long chain fatty alcohol residue, which substitutes for the nonpolar fatty acyl group found in normal glycerol phospholipids (Kates *et al.*, 1965). Very recently an interesting analog has been described in the *Escherichia coli* cell wall lipo protein, where a diglyceride has been shown to be joined to the polypeptide chain through a thioether bond to a N-terminal cysteine (Hantke and Braun, 1973). The polar head group of lipid A is in itself also unique, and is the first lipid betaine of an α-amino acid described. Naturally occurring betaines of α-amino acids are themselves relatively rare; they appear to be exclusively fungal metabolites of unknown function, such as ergothioneine (Melville *et al.*, 1956; Reinhold *et al.*, 1970). With the notable exception of phosphatidylserine, lipids in eucaryotes do not appear to contain amino acids in any form. On the other hand, amino acid esters of phosphatidylglycerol were identified some time ago in several gram-positive bacteria (Macfarlane, 1962) and more recently several ornithine-containing lipids have been identified in different bacilli (Kates, 1964; Brooks and Benson, 1972; Thiele and Schwinn, 1973). Although these two classes of lipids are quite different in structure, their amino acid residues are joined to glycerol or diol moieties by an ester linkage, blocking the amino acid carboxyl groups and making these lipids the only cationic lipids known. In lipid A, on the contrary, where the trimethylaminohomoserine moiety is linked to the diglyceride moiety through its γ-hydroxyl group, the carboxyl group remains free, making lipid A a zwitterionic polar lipid. In fact, although unique in chemical detail, the overall structure of lipid A is quite in line with that of other amphipathic glycerolipids found in biological membranes, notably the phospholipids. Thus, lipid A resembled phosphotidylcholine in having a quaternary amino group, and phosphatidylserine in having a free carboxyl group; however, since it lacks the phosphate anionic group it is a zwitterion and its physical-chemical properties should be more similar to those of phosphatidylcholine rather than to those of the acidic phosphatidylserine.

Lipid A is also somewhat unusual in its high ratio of myristic to palmitic acid; however, this is also true for the overall fatty acid composition of this organism.

The most striking component of *Ochromonas danica* lipids are the chlorosulfolipids (Elovson and Vagelos, 1969) (Table I); if freely soluble their concentration would amount to 15 mM in the cell. They are potent anionic detergents, with a critical micellar concentration six orders of magnitude higher than that reported for phosphatidylcholine (Smith and Tanford, 1972), and only about one order of magnitude less than that for sodium dodecyl sulfate (J. Elovson, manuscript in preparation). When the extremely low concentrations of phospholipids

in these cells (Table I) were first noted, the possibility had to be entertained that the abundant chlorosulfolipids somehow substituted for phospholipids in the membranes, although their physical-chemical properties are so strikingly different. The findings of high concentrations of lipid A in this organism now shows that it, rather than the awkward chlorosulfolipids, can substitute for the phospholipids in a conventional bilayer membrane, even though its chemical details are unusual. Since the darkgrown cells used in this study have rudimentary chloroplasts the large amount of lipid A in these cells must be present mainly in other cellular membranes. The occurrence of two such unique lipids in one organism may perhaps suggest that lipid A and the chlorosulfolipids coevolved in *Ochromonas danica*, at the expense of the usual phospholipids; what possible advantage this might have conferred remains completely unknown.

The structure of lipid A suggests some possible pathways for its biosynthesis. The O-ether bond between the glycerol and the homoserine betaine moieties may be formed in two ways. One route could be initial activation by esterification of the γ-hydroxyl group on the homoserine followed by displacement of the acyl group by the primary glycerol hydroxyl group; such a mechanism would be analogous to the formation of the cystathionine thioether bond (Nagai and Flavin, 1967) and the recently reported synthesis of O-alkylhomoserine ethers in some bacteria (Murooka *et al.*, 1970); it would also be somewhat analogous to the formation of the usual long-chain alkylglycerol ether lipids (Snyder, 1969). Perhaps a more attractive alternative would be the opposite one, where the O-ether bond could arise by attack of homoserine on an activated glycerol derivative, either CDP-diglyceride, a normal intermediate in complex lipid biosynthesis, or a glycerophospholipid such as PC. The stage at which the homoserine moiety is methylated is also unknown, although by analogy to the biosynthesis of phosphatidylcholine one might perhaps expect this event to occur as the last steps in the biosynthesis of lipid A (Wilson *et al.*, 1960). The origin of the methyl groups from *S*-adenosylmethionine appears established by our labeling data with [*methyl*-^{14}C]methionine *in vivo*. It is interesting that Lust and Daniel established this reaction for the biosynthesis of phosphatidylcholine in *Ochromonas malhamensis* (Lust and Daniel, 1964); it is not clear if their procedure would have picked up any labeled derivative of lipid A, if this lipid indeed is present in this close relative of *Ochromonas danica*. We are presently analyzing the lipids of *Ochromonas danica* to determine if it contains minor amounts of unmethylated or partially methylated precursor forms of lipid A.

Acknowledgments

We thank Dr. Nicholas Ling of the Salk Institute for obtaining the high-resolution mass spectrometry; we also thank Mr. Thomas Warner for assistance with the nuclear magnetic resonance spectra.

References

Birdsall, N. J. M., Feeney, J., Lee, A. G., Levine, Y. K., and Metcalf, J. (1972), *J. Chem. Soc., Perkin Trans. 2*, 1441–1445.

Bottcher, C. J. F., Pries, C., and van Gent, C. M. (1961), *Recl. Trav. Chim Pay-Bas 80*, 1169–1173.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Brooks, J. L., and Benson, A. A. (1972), *Arch. Biochem. Biophys. 152*, 347–355.

Chapman, D., and Morrison, A. (1966), *J. Biol. Chem. 241*, 5044–5052.

Dittmer, J. C., and Lester, R. L. (1964), *J. Lipid Res. 5*, 126.

Elovson, J. (1974), *Biochemistry 13*, 3483.

Elovson, J., and Vagelos, P. R. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 957–963.

Elovson, J., and Vagelos, P. R. (1970), *Biochemistry 9*, 3110–3126.

Hantke, K., and Braun, V. (1973), *Eur. J. Biochem. 34*, 284–296.

Haque, R., Tinsley, I. J., and Schmedding, D. (1972), *J. Biol. Chem. 247*, 157–161.

Jenden, D., Hanin, I., and Lamb, S. I. (1968), *Anal. Chem. 40*, 125–128.

Kates, M. (1964), *Advan. Lipid Res. 2*, 17–90.

Kates, M., Yengoyen, L. S., and Sastry, P. S. (1965), *Biochim. Biophys. Acta 98*, 252–268.

Lust, G., and Daniel, L. J. (1964), *Arch. Biochem. Biophys. 108*, 414–419.

Macfarlane, M. G. (1962), *Biochem. J. 82*, 40p.

Mayers, G. L., and Haines, T. H. (1967), *Biochemistry 6*, 1665.

Melville, D. B., Genghof, D. S., Inamine, E., and Kovalenko, V. (1956), *J. Biol. Chem. 223*, 9–17.

Murooka, Y., Seto, K., and Harada, T. (1970), *Biochem. Biophys. Res. Commun. 41*, 407.

Nagai, S., and Flavin, M. (1967), *J. Biol. Chem. 242*, 3884.

Reinhold, V., Ishikawa, Y., and Melville, D. B. (1968), *J. Med. Chem. 11*, 258–260.

Reinhold, V. N., Ishikawa, Y., and Melville, D. (1970), *J. Bacteriol. 101*, 881–884.

Skidmore, W. D., and Enteman, C. (1962), *J. Lipid Res. 3*, 356–363.

Sloane-Stanley, G. H. (1967), *Biochem. J. 104*, 293–295.

Smith, R., and Tanford, C. (1972), *J. Mol. Biol. 67*, 75–85.

Snyder, F. (1969), *Prog. Chem. Fats Other Lipids 10*, 121–156.

Stoffel, W., LeKim, D., and Tschung, T. S. (1971), *Hoppe-Seyler's Z. Physiol. Chem. 352*, 1058–1064.

Sundler, R., Arvidson, G., and Åkesson, B. (1972), *Biochim. Biophys. Acta 280*, 559–568.

Thiele, D. W., and Schwinn, G. (1973), *Eur. J. Biochem. 34*, 333.

Thompson, G. A., and Nozawa, Y. (1972), *Annu. Rev. Microbiol. 26*, 249–278.

Vaskovsky, V. E., and Suppes, Z. S. (1971), *J. Chromatogr. 63*, 455–456.

Wilson, J. D., Gibson, K. D., and Undenfriend, S. (1960), *J. Biol. Chem. 235*, 3212.