OCCURRENCE OF 1(3),2-DIACYLGLYCERYL-(3)-0-4'-(*N,N,N*-TRIMETHYL)-HOMOSERINE IN *CHLAMYDOMONAS REINHARDI*

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1. Introduction

On investigation of the lipids of the green alga *Chlamydomonas reinhardi*, a major lipid component was detected, which was not identical with any of the usual major membrane lipids of green plants and which was therefore called lipid X [1]. The results presented here demonstrate that the compound is identical with lipid A from *Ochromonas danica* [2,3], which has the structure of a diacylglyceryl-(3)-0-4'-(*N,N,N*-trimethyl)-homoserine [4].

2. Material and methods

2.1. *Cultivation of organisms*

Chlamydomonas reinhardi arg⁻ mt⁺, streptomycin-resistant mutant sr_3 [5], was cultivated mixotrophically [1]. The nutrient was medium I [6] with 0.1 g arginine–HCl and 2 g NaOAc·2 H_2O/litre. Cultures were grown under 10 000 lux fluorescent light for 3 days at 26–28°C.

Ochromonas danica The Culture Centre of Algae and Protozoa, Cambridge, No. 933/2 was cultivated on 30 ml nutrient in 150 ml Erlenmeyer flasks. The nutrient was a modified medium [7] containing (in wt%) 0.2% Trypton (Difco), 0.1% yeast extract (Difco), 0.1% Liebig's meat extract and 0.5% glucose. Cells were cultivated under illumination for 6 days at 26–28°C.

2.2. *Extraction of lipids*

Lyophilized cells were extracted subsequently with hot MeOH and Et_2O, the solvent evaporated and the residue dissolved in Et_2O and centrifuged. The average yield of ether-soluble lipids was 16.5% (per dry wt).

2.3. *Isolation of lipid X*

In a standard experiment, 7.5 g ether-soluble lipid were dissolved in $CHCl_3$ and loaded on a column of 4 cm i.d. containing 75 g silicic acid 100 mesh (Fluka) in $CHCl_3$. The column was successively eluted with 350 ml $CHCl_3$, 350 ml $CHCl_3$–MeOH 2% (v/v), 350 ml $CHCl_3$–MeOH 3% (v/v), 150 ml $CHCl_3$–MeOH 1:1 (v/v) and 150 ml MeOH. Fractions were spotted on a silica gel G plate and developed with $CHCl_3$–MeOH–7 N NH_4OH, 65:30:4 (solvent A) and visualized with 25% $HClO_4$ at 120°C. Pooled fractions yielded a residue of 0.47 g, which was dissolved in 5 ml $CHCl_3$ and rechromatographed on 10 g alumina (alkaline, Merck) activity grade III. Elution was carried out with 60 ml $CHCl_3$, 60 ml $CHCl_3$–MeOH 1:1 (v/v) and 20 ml MeOH. From pooled fractions 0.25 g pure lipid X were obtained.

2.4. *Deacylation and reacylation*

Lipid X, 100 mg, were hydrolyzed with 2 ml KOH–H_2O–EtOH, 1:2:20 (w/v/v) for 30 min at 50°C. The residue was acidified with HCl–H_2O, 1:1 (v/v) and extracted 4 times with 4 ml petroleum ether. The aqueous phase was neutralized with solid $NaHCO_3$ and the mixture diluted with 10 vol. isopropanol and centrifuged. The solvent was evaporated and the residue extracted with isopropanol. The alcohol component was reacted with 0.5 ml propionyl chloride for 30 min at 50°C, the excess acid chloride evaporated and the lipid X propionate chromatographed on thin-layer chromatographic plates with solvent A. The

separation of fatty acids by gas–liquid chromatography has been described [1]. The quantitative determination of lipid X was carried out by a hydroxamate method [8] after 2-dimensional thin-layer chromatography on silica gel G with solvent A (1. dim.) and $CHCl_3$–MeOH–AcOH–H_2O, 170:25:25:6 (v/v/v/v) (solvent B, 2.dim.) [1].

2.5. *Demethylation of lipid X propionate*

Sodium benzenethiolate was prepared as in [9]. The demethylation was carried out as in [4]; 0.23 g lipid X propionate were mixed with 1.2 g Na-benzenethiolate and 6 ml dioxane (distilled over $LiAlH_4$) and the mixture refluxed for 1 h. The solvent was evaporated and the residue extracted with isopropanol. The residue of the isopropanol solution was acidified with conc. HCl–H_2O, 1:1 (v/v) and extracted 4 times with Et_2O, neutralized with solid $NaHCO_3$ and the mixture diluted with 20 vol. isopropanol and then centrifuged. Evaporation of the solvent gave the demethylated alcohol component which was reacylated with propionyl chloride. On a thin-layer chromatographic plate (solvent A), the R_F value of the demethylated lipid X propionate was slightly greater than the one of lipid X propionate. For mass spectrometric analysis the demethylated alcohol component was silylated with pyridine–bis(trimethylsilyl)acetamide–trimethylsilyl chloride, 2:2:1 (v/v/v) for 30 min [4].

2.6. *Mass spectrometry*

A Varian MAT CH 5/DF was used. Infrared spectra were carried out with a Perkin-Elmer IR 457 with a liquid film on a KBr support. For NMR spectra a Varian XL-100 was used at 100 MHz for protons and 25 MHz for ^{13}C.

3. Results and discussion

The R_F values of lipid X from *Chlamydomonas* in 2-dimensional thin-layer chromatography are 0.76 (solvent A) and 0.64 (solvent B). Therefore, lipid X is slightly more polar than monogalactosyl diglyceride (R_F 0.83/0.85). Since molybdenum blue [11] and anthrone reagent [12] gave no reaction, both phosphorus and sugar are absent from lipid X unlike in known major lipids of green plants. However, its

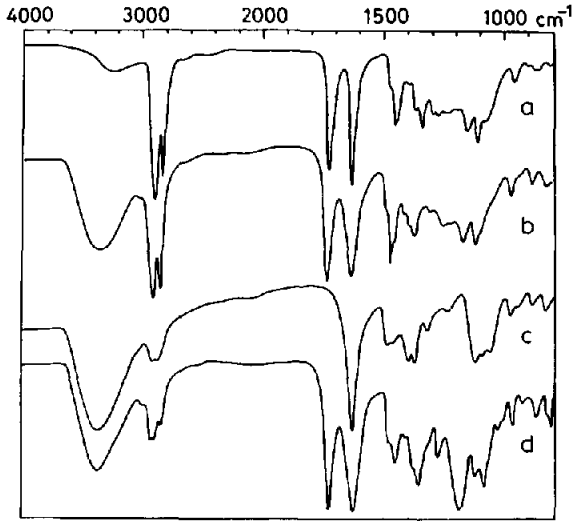

Fig.1. Infrared absorption spectra (in KBr) of (a) lipid X from *Chlamydomonas*, (b) lipid A from *Ochromonas*, (c) lipid X deacylated, (d) lipid X propionate.

properties are very similar to lipid A detected in *Ochromonas danica* [3] and later shown to be 1(3),2-diacylglyceryl-(3)-*O*-4′-(*N*,*N*,*N*-trimethyl) homoserine (DGTS) [4]. The aim of the work presented here was to demonstrate the identity of lipid A from *Ochromonas* and lipid X from *Chlamydomonas*.

By co-chromatography of both components on thin-layer chromatographic plates (2-dimensional, solvents A + B) a single spot was obtained. The infrared spectra are identical, as shown in fig.1a,b, containing the characteristic band of ester carbonyl at 1735 cm^{-1}, which disappears on deacylation (fig.1c), but reappears on reacylation (fig.1d).

Mass spectra were obtained from demethylated, deacylated and silylated lipid X. As demonstrated in fig.2a, characteristic peaks appear at *m/e* 437 (M^+), 422, 350, 334, 320, 262, 248, 232, 205, 202, 174, 117, 103 and 84. Except for the peaks at *m/e* 350 and 262, the same fragments were also observed with or postulated for demethylated, deacylated and silylated lipid A from *Ochromonas* in [4]. In accordance with [4] our results are consistent with the structure shown in fig.3.

Identical spectra are also observed, when lipid X propionate (fig.2b) and lipid A propionate (fig.2c)

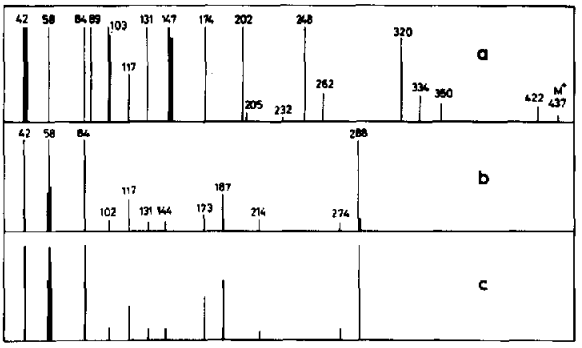

Fig.2. Mass spectra of (a) lipid X (demethylated, deacylated, silylated) from *Chlamydomonas*, (b) lipid X propionate, (c) lipid A propionate from *Ochromonas*.

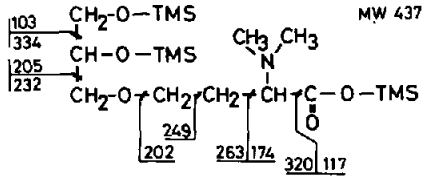

Fig.3. Structure and fragmentation of demethylated, deacylated and silylated lipid X, as demonstrated [4].

are fragmented, but they have not yet been fully interpreted.

These results demonstrate that lipid X from *Chlamydomonas* is identical to lipid A from *Ochromonas*. In addition, analytical data strongly indicate that lipid X has the same structure as DGTS (fig.4). Based on a molecular weight of 347 for the zwitterionic form of DGTS propionate, 1.95 mol fatty acids and 1.09 mol alcohol moiety were obtained gravimetrically after alkaline hydrolysis of native lipid X. In addition, the determination of ester [8] gave 1.98 mol ester group and the elementary analysis gave 0.85 mol N.

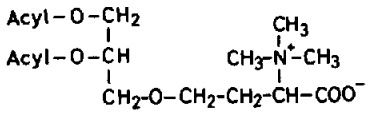

Fig.4. Structure of lipid X from *Chlamydomonas* (DGTS).

The presence of a trimethylammonium group is demonstrated by the formation of trimethylamine from the native lipid at 160°C, and by NMR spectra showing peaks at δ3.26 ppm (^{1}H) and δ52.52 ppm (^{13}C, quartet in the off-resonance spectrum). Evidence for a free carboxylate group is obtained by an infrared absorption band at 1625 cm^{-1} (fig.1) and by an NMR singlet peak at δ171.85 ppm (^{13}C). In the ^{13}C NMR spectra of deacylated lipid X doublet bands at 77.89 and 72.19 ppm, and 4 triplet bands at 73.48, 68.41, 64.21 and 28.91 ppm appear, which can be attributed to the 2 methine groups and the 4 methylene groups, respectively, of the alcohol component of lipid X. The simplification of the multiplets at 4.3 and 3.7 ppm corresponding to 2 methylene groups by decoupling of a methine proton at 5.3 ppm in the ^{1}H spectrum proves the presence of the glycerine-carbon skeleton. The formation of an AB-system for a methylene group at 2.1 ppm by decoupling both a methine- and a methylene-group at 3.5–3.8 ppm points to the structure of the ionized side chain (fig.4).

From these results we conclude that *Chlamydomonas* contains DGTS as a major lipid component accounting for about 10% total amount of ether-soluble lipids. The occurrence of this phosphorus-free zwitterionic lipid in a green alga shows its presence in algae other than *Ochromonas* (Chrysophyceae), where it accounts for more than 50% total lipids and has been suggested to substitute for phospholipids [4]. *Chlamydomonas*, instead, contains phosphoglycerides and DGTS in about the same proportions. The general distribution of DGTS among lower plants remains to be cleared up. Its significance as a membrane lipid component and its intracellular localization are not known at present. In *Chlamydomonas* DGTS was detected in different cell fractions. Since it is present also in the lipids of photosystem I and II particles [10], it might be a constituent of the thylakoid membranes of this alga. As to the acyl pattern of DGTS of *Chlamydomonas*, it should be noted that 18:4, 18:3ω6 and 16:0 acids are present in much higher proportions than in total lipid, as shown in table 1. This could indicate a particular role for DGTS in the biosynthesis of γ-linolenic acid. A similar role in the biosynthesis of unsaturated fatty acids has been suggested for phosphatidyl choline and monogalactosyl diglyceride by different authors [13].

Table 1
Fatty acid composition of lipid X and total lipid from *Chlamydomonas*

	Weight% total fatty acids											
	14:0	16:0	16:1	16:2	16:3	16:4	18:0	18:1	18:2	α-18:3	γ-18:3	18:4
Lipid X	tr	31.9	2.3	0.6	0.8	2.9	tr	3.0	8.5	8.6	33.1	8.1
Total lipid	tr	19.7	4.2	1.0	3.1	20.7	tr	8.4	8.6	24.7	10.0	tr

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