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GAS CHROMATOGRAPHIC-MASS SPECTROMETRIC IDENTIFICATION OF BETAINE LIPIDS IN *CHROOMONAS SALINA*

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Key Word Index—*Chroomonas salina*; Cryptophyceae; algae; betaine lipids; diacylglyceryl-hydroxymethyltrimethyl-β-alanine; diacylglyceryltrimethylhomoserine; fatty acids; GC-mass spectrometry.

Abstract—The betaine lipids diacylglycerylhydroxymethyltrimethyl-β-alanine (DGTA) and diacylglyceryltrimethylhomoserine (DGTS) can be identified by derivatization of their polar groups. Demethylation, deacylation and silylation of DGTS yields demethyl-deacyl-DGTS-TMSi; dihydro-deamino-deacyl-DGTA-TMSi is produced by deamination, hydrogenation, deacylation and silylation of DGTA. The characterization of the derivatives by GC-MS was used as a diagnostic method for the identification of DGTA in *Chroomonas salina*, where this lipid accounts for 20% of the total polar lipids. Major fatty acids of DGTA (%) are 22:6*n*-3 (22), 20:5*n*-3 (14), 18:1*n*-7 (12), 18:4*n*-3 (11), 16:0 (8) and 14:0 (8), indicating that this lipid contains a broad variety of different molecular species in this alga.

INTRODUCTION

The betaine lipids diacylglyceryltrimethylhomoserine (homoserine lipid, DGTS) and diacylglycerylhydroxymethyltrimethyl-β-alanine (alanine lipid, DGTA) are constituent polar lipids of many algae [1–3]. Labelling experiments strongly suggest that these lipids play an important metabolic role in the desaturation and redistribution of fatty acids among cellular lipids in certain plants. DGTS was strongly labelled in the green alga, *Chlamydomonas reinhardtii* [4], and in the chrysophyte alga, *Ochromonas danica* [16], when fed with [1-^{14}C]oleate. Rapid labelling of DTGA was also observed in the brown algae, *Fucus vesiculosus* and *Ascophyllum nodosum*, fed with [1-^{14}C]acetate [5] and in *Cryptomonas* CR-1 grown in the presence of [^{14}C]$NaHCO_3$ [6]. Recently, in *Chroomonas salina* which, like *Cryptomonas*, is a member of the Cryptophyceae, rapid incorporation of labelled fatty acid precursors into a specific lipid component has been demonstrated [7]. Based on its Dragendorff-positive reaction, this lipid was strongly suggested to be DGTA, but its identity remained to be fully established.

DGTS and DGTA are structural isomers both containing a carboxyl and a trimethylammonium group. The latter is responsible for the Dragendorff-positive reaction, which is typical of both compounds. Although their positions in two-dimensional TLC clearly differ, for final identification, an additional analytical criterion is required. Thus, a GC-MS method was developed and used for the identification of the fast-labelled betaine lipid in *C. salina*. The procedure is applicable to the identification of betaine lipids in other organisms.

RESULTS

The identification procedure is based on the transformation of DGTS (**1**) and DGTA (**3**) into volatile derivatives which can be analysed by GC-mass spectrometry (Fig. 1). The two lipids, although structural isomers containing the same functional groups in their polar moiety, behave differently because of the altered stability of their trimethylammonium groups.

DGTS, as the more chemically stable compound, is easily demethylated by treating with sodium benzenethiolate [8]. After deacylation and silylation, demethyl-deacyl-DGTS-TMSi (**2**) is obtained [8, 9]. DGTS isolated from *C. reinhardtii* was used as a reference. In the GC-mass spectrum, the TMSi derivative gave ions at *m/z* 437 [M$^+$], 422 [M − CH_3]$^+$ and 320 [M − CO_2TMSi]$^+$ Fig. 2.

From the M_r of [M − CH_3]$^+$, a value of 422.2210 was found, which was in accordance with the formula $C_{17}H_{43}O_5NSi_3$ (calculated value 422.2214).

From DGTA (**3**), isolated from *O. danica*, trimethylamine was cleaved off by heating the lipid in chloroform at 65° for a few minutes. In this process, the polar group produced a methacrylic acid-like compound [10], according to the rules of the Hofmann degradation [11].

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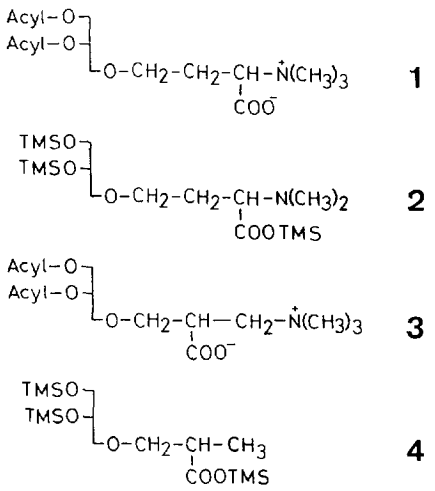

Fig. 1. **1** DGTS; **2** demethyl-deacyl-DGTS-TMSi; **3** DGTA; **4** dihydro-deamino-deacyl-DGTA-TMSi.

After hydrogenation with Pd/H_2, a stable derivative was obtained, which after deacylation and silylation yielded dihydro-deamino-deacyl-DGTA-TMSi (**4**). In the GC-mass spectrum, ions were observed at *m/z* 379 $[M - CH_3]^+$, 321 $[M - TMSi]^+$, 305 $[M - OTMSi]^+$, 291 $[M - CH_2OTMSi]^+$ and 205 $[TMSi - CH_2 - CH - OTMSi]^+$ (Fig. 3).

The $[M]^+$ *m/z* 394 could not be detected under the conditions used. From the M_r of $[M - CH_3]^+$, a value of 379.1795 was measured which corresponded to the formula $C_{15}H_{35}O_5Si_3$ (calculated value 379.1792).

The same procedure was applied to the unidentified Dragendorff-positive compound X isolated from *C. salina* [7]. On a TLC plate, it co-migrated with DGTA from *O. danica* and also gave a IR spectra (data not shown) which was identical to the reference compound. After derivatization as described above, the mass spectra of compound X and of reference DGTA were also identical.

Betaine lipids could not be separated from phosphatidylserine (PS) and phosphatidylinositol (PI) by single dimension HPTLC. When quantitated by HPTLC-scanning densitometry the combined PS, PI and betaine lipid fraction comprised 19.6±1% (mean ±s.d., n = 6 cultures) of total polar lipids in *C. salina*. Analysis by two-dimensional HPTLC showed that the amounts of PS and PI were very much less than that of DGTA. A very faint Dragendorff-positive spot at the site where DGTS normally migrates, indicated the presence of this lipid only in trace amounts. From these data it is concluded that in *C. salina*, DGTA is present as a major lipid component.

Polyunsaturated fatty acids (PUFA) accounted for over 60% of the total fatty acids present in DGTA from *C. salina*, with saturated and monounsaturated fatty acids comprising *ca* equal proportions of the remainder (Table 1).

The major fatty acid was 22:6*n*-3, which accounted for 22.2% of the total fatty acids. The only other fatty

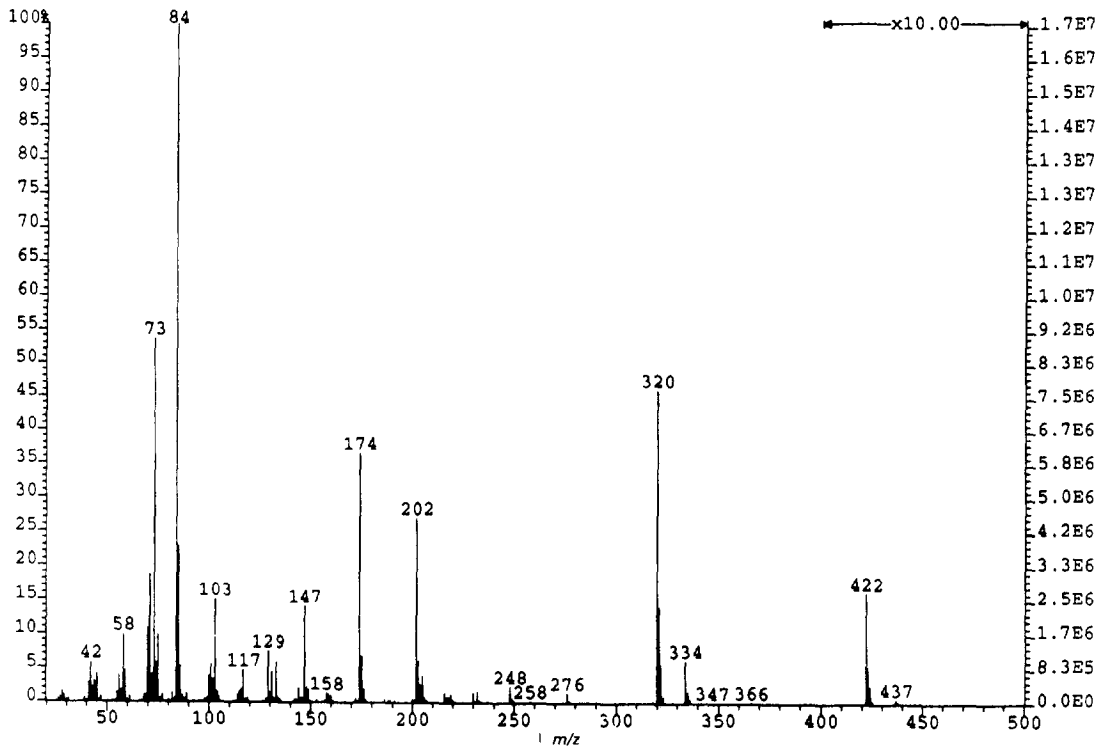

Fig. 2. EI-mass spectrum of demethyl-deacyl-DGTS-TMSi.

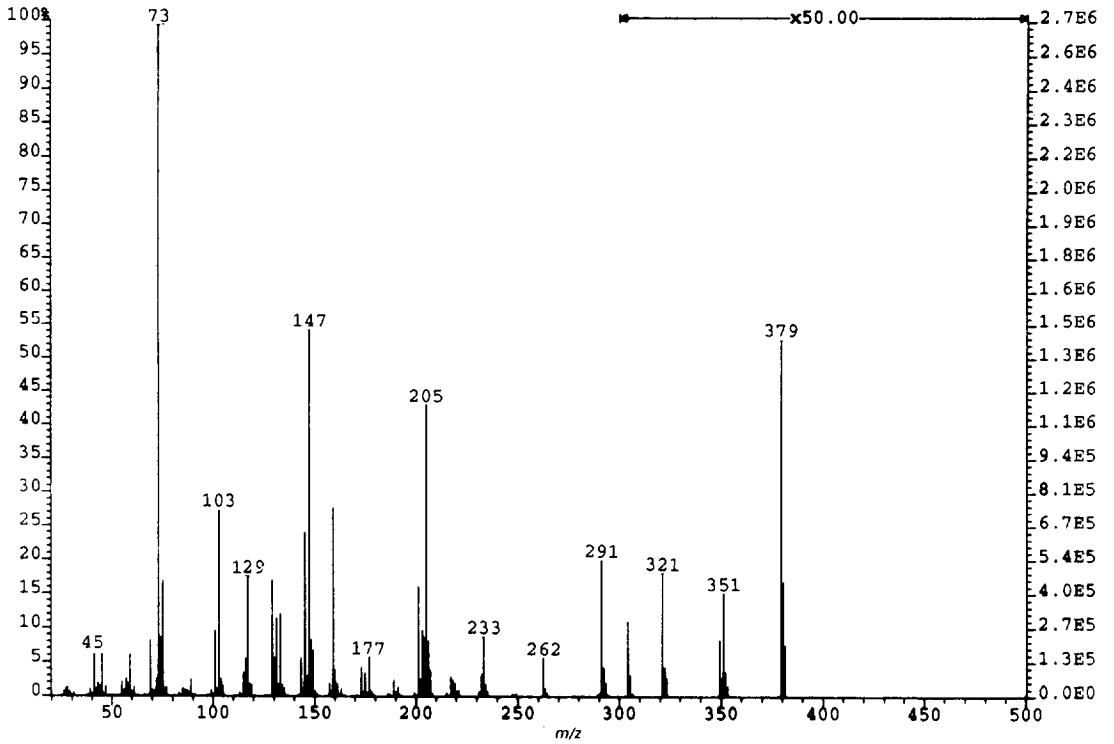

Fig. 3. EI-mass spectrum of dihydro-deamino-deacyl-DGTA-TMSi.

acids present at concentrations greater than 10%, were 20:5*n*-3, 18:1*n*-7 and 18:4*n*-3.

When subjected to argentation chromatography the DGTA of *C. salina* could be separated into 12 distinct fractions. The most abundant fraction ($R_f = 0.68$) accounted for almost 33% of the total DGTA and contained 56.2% PUFA (mainly in the form of 20:5*n*-3, 20:6*n*-3, 22:5*n*-3 and 18:4*n*-3), with the remainder being made up of equal proportions of saturated and monounsaturated fatty acids (Table 2).

Table 1. Fatty acid composition (wt %) of DGTA from *Chroomonas salina*

Fatty acid	%
14:0	8.0±0.3
15:0	1.6±0.4
16:0	7.6±3.2
16:1*n*-7	1.9±0.9
18:0	2.5±0.4
18:1*n*-9	4.3±0.3
18:1*n*-7	11.6±0.8
18:2*n*-6	4.2±0.7
18:3*n*-6	1.9±1.3
18:3*n*-3	3.5±1.1
18:4*n*-3	10.8±0.2
20:1*n*-9	0.5±0.2
20:4*n*-6	0.3±0.2
20:4*n*-3	1.6±0.2
20:5*n*-3	13.8±2.2
22:5*n*-3	2.9±0.7
22:6*n*-3	22.2±1.6

Values are means ± s.d. of four cultures.

Highly unsaturated fractions with low R_f value comprised 11% of DGTA and had the highest content of PUFA (87.6%), mainly in the form of 20:5*n*-3 and 22:6*n*-3. A fraction in which 22:6*n*-3 accounted for 35% of the total fatty acids constituted 14.4% of the total DGTA and another fraction containing 18:4*n*-3 as the major PUFA was also present at a significant level (8.5%).

DISCUSSION

The different chemical reactivity of the polar group of DGTS and DGTA is a useful diagnostic tool for the identification of the two betaine lipids. Demethylation, deacylation and silylation of DGTS leads to a stable compound of M_r 437 which was already described earlier [8] and of which the $[M-15]^+$ fragment (M_r 422) is diagnostic. For DGTA, the trimethylammonium group of which is more labile, deamination leads to a methacrylic acid-like product [10], which can be stabilized by hydrogenation of the double bond. Subsequent deacylation and silylation yields a product of M_r 394; however, the $[M-15]^+$ fragment (M_r 379) was only observed under the GC-mass spectrometry conditions used. This saturated compound is considered to be more chemically stable than the unsaturated derivative (M_r 334) obtained from DGTA by methanolysis and silylation [6]. Another lipid of the betaine-type has recently been described as 1,2-diacylglyceryl-3-*O*-

Table 2. Fatty acid composition (wt %) of DGTA fractions from *Chroomonas salina* separated by argentation chromatography

R_f	0.03	0.16	0.23	0.32	0.42	0.51	0.59	0.68	0.72	0.90	0.95	0.99
% total DGTA	11.1	14.4	8.5	6.5	2.5	3.3	3.0	32.7	1.8	3.4	5.3	7.5
Fatty acid												
14:0	0.7	13.1	16.0	11.3	13.6	10.0	7.2	1.0	4.6	5.5	6.4	16.7
15:0	0.7	0.9	1.5	1.7	1.8	0.9	0.9	0.3	0.8	1.6	1.1	2.1
16:0	1.7	14.4	9.1	14.3	9.6	1.6	9.4	14.1	11.6	17.1	24.7	23.3
16:1*n*-7	3.1	—	1.7	1.9	4.4	7.5	3.9	5.4	0.8	3.7	6.4	3.9
18:0	1.9	2.0	1.5	2.4	2.7	2.5	3.4	7.2	7.0	7.6	10.5	5.7
18:1*n*-9	2.2	1.1	3.2	10.8	4.4	5.8	10.3	6.3	17.0	28.0	5.4	23.1
18:1*n*-7	0.9	17.7	8.9	8.0	15.9	28.8	7.7	3.2	0.8	—	17.2	12.6
18:2*n*-6	0.4	1.1	1.3	1.1	19.4	—	2.9	1.0	2.4	5.5	2.6	3.2
18:3*n*-6	0.2	—	0.2	2.8	—	—	0.9	0.2	1.5	0.9	0.7	0.6
18:3*n*-3	0.9	0.8	0.8	16.2	1.7	3.7	1.7	0.9	13.1	6.3	1.7	0.6
18:4*n*-3	5.1	1.3	37.4	2.1	2.7	—	15.4	4.4	11.6	6.7	1.5	0.6
20:1*n*-9	0.9	0.5	0.6	0.9	1.3	—	3.4	6.3	3.9	8.3	6.8	5.4
20:4*n*-6	2.6	0.9	1.9	0.5	—	—	—	2.2	—	2.1	1.1	0.2
20:4*n*-3	1.1	0.3	4.9	0.9	1.3	—	3.9	1.0	4.6	3.0	0.7	0.2
20:5*n*-3	41.4	9.3	5.9	1.9	7.9	22.9	12.8	23.0	10.9	3.7	7.1	0.6
22:5*n*-3	1.1	1.6	1.5	0.5	1.3	—	1.7	8.8	—	—	2.6	—
22:6*n*-3	34.8	35.0	3.6	22.6	11.9	16.2	14.5	14.7	9.2	—	3.6	0.9
Total saturated	5.0	30.4	28.1	29.7	27.7	15.0	20.9	22.6	24.0	31.8	42.7	47.8
Total monounsaturated	7.1	19.3	14.4	21.6	26.0	42.1	25.3	21.2	22.5	40.0	35.8	45.0
Total PUFA	87.6	50.3	57.5	48.6	46.2	42.8	53.8	56.2	53.3	28.2	21.5	6.9

carboxyhydroxy-methylcholine (DGCC) in the haptophyte alga, *Pavlova lutheri* [12]. It was, however, characterized only in the underived form by FAB-mass spectrometry.

Our results clearly establish the presence of DGTA in *C. salina*. In addition, they allow a comparison of the lipid composition of this alga to the closely related *Cryptomonas* CR-1 which has been analysed by Sato [13]. In *C. salina*, the amount of DGTA comprises *ca* 20% of the polar lipids, while DGTS is present in trace amounts. In *Cryptomonas* CR-1, in contrast, the corresponding values are 9.7% and 1.0% for DGTA and DGTS, respectively [13]. This suggests that in the Cryptophyceae, both DGTS and DGTA are present, with a clear predominance of the latter. The ratio of the two lipids, however, may vary from species to species.

The fatty acid composition of DGTA found here for DGTA from *Chroomonas* is markedly different from that reported previously for the same lipid in *Cryptomonas* CR-1 [13]. Whereas 22:4*n*-3 was found to be a major fatty acid in the DGTA of the *Cryptomonas* species [13], this PUFA was not detected in the DGTA of *C. salina* which contains instead 22:6*n*-3 as its major fatty acid. In addition, 18:4*n*-3 is a major PUFA in the DGTA of *C. salina* but not in those of *Cryptomonas* CR-1.

We have previously shown that in *C. salina* the major PUFA of monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG) are C_{18} components, whereas C_{20} and C_{22} PUFA predominate in PC. By virtue of its high content of 20:5*n*-3 and 22:6*n*-3, DGTA is more similar to PC than MGDG or DGDG. This is similar to the situation which exists in *Cryptomonas* CR-1 [13]. On the basis of the fatty acid composition of the fractions separated by argentation chromatography, no conclusion can be drawn as to the principal molecular species of DGTA present in *C. salina*. However, it is notable that a fraction containing 41.4% and 34.8% of 20:5*n*-3 and 22:6*n*-3, respectively, accounted for over 10% of the total DGTA. This suggests that the molecular species of DGTA containing the two long-chain PUFA occur in this species of *Chroomonas*; it is further evidence that DGTA has an extrachloroplastic site of synthesis.

The identification of DGTA in *Chroomonas* is of some significance, because this lipid seems to play an important role in the lipid metabolism of this alga. Henderson and Mackinlay [7] found that after feeding [2-^{14}C]acetate for 60 min, 56% of the label appeared in the polar lipids. Of this, 10% was found in DGTA, which was the most strongly labelled non-glycolipid polar lipid of the cells. Exceptional labelling of DGTA also occurred when [1-^{14}C]-18:0, -18:1, -18:2 and -18:3($n-3$) acids were supplied as precursors. In chase-experiments, up to 70% of the fatty acid label disappeared from DGTA and reappeared mainly in triacylglycerols [7].

Similar observations were made with the closely related species, *Cryptomonas* CR-1 [6]. After feeding [^{14}C]$NaHCO_3$ to this alga for 60 min, 5.6% of the acyl label was found in DGTA compared to 4.4% in PC.

Similar results were also obtained with macroalgae of the order Fucales [5], which are taxonomically distant from Cryptophytes. *A. nodosum* and *F. vesiculosus* fed during 8 hr with [2-^{14}C]acetate, incorporated 30% of the label into DGTA which was the most strongly labelled polar lipid. Experiments with long-chain fatty acids as precursors have, however, not been carried out with these species of macroalgae.

Despite the differences in substrates and conditions,

all of the above experiments have demonstrated that radioactivity from fatty acid precursors is predominantly incorporated into the betaine lipid DGTA, suggesting an exceptional metabolic role for this lipid in organisms of different taxonomic positions.

It is interesting to note that on labelling *Chroomonas* and *Cryptomonas* with [^{14}C] acetate, a very high portion of radioactivity rapidly appeared in the plastidial lipid MGDG, namely 17% in *Chroomonas* [7] and 68% in *Cryptomonas* [13]. This is surprising, because the MGDG of these algae mainly consists of molecular species of the 'eukaryotic' type (84% in *Cryptomonas* [13]). This means that the *sn*-2 position of this lipid is occupied mainly by C_{18} or longer-chain fatty acids. According to the concept of Roughan and Slack [14], this type of MGDG is synthesized through the cytoplasmic phospholipid PC, for which a transient high acyl label is expected. Since in these algae, the intermediate label is lower in PC than in DGTA [6, 13], the operation of this biosynthetic pathway seems questionable. As already expressed clearly by Sato [6], the rapid synthesis of 'eukaryotic' MGDG in these chromophytic algae remains unclear. The betaine lipid DGTA could be a potential candidate for a key position in these biosynthetic pathways.

EXPERIMENTAL

Algae. Chroomonas Salina (Wislouch) was obtained from the CCAP, Dunstaffnage Laboratory, Oban, U.K. and cultivated according to ref. [7]. *C. reinhardtii* 137c arg-2$^-$ mt$^+$ was cultivated as described in ref. [4]. *O. danica* 933/2b (CCAP, Cambridge) was cultivated in a medium according to ref. [15].

Lipid isolation and analysis. Chroomonas was extracted with $CHCl_3$–MeOH (2:1) [7]. *Chlamydomonas* and *Ochromonas* were extracted with MeOH containing 0.05% butyl hydroxytoluene and then with Et_2O. Total lipids were sepd on silica gel plates (Merck 5715) using $CHCl_3$–MeOH–H_2O (65:25:4) (solvent 1). Spots were detected with 2′,7′-dichlorofluorescein under UV 366 nm. After elution with MeOH, lipids were rechromatographed with $CHCl_3$–MeOH–*iso*-$PrNH_2$–conc NH_3 (65:35:0.5:5) (solvent 2).

For the estimation of polar lipid classes, total lipids from *C. salina* were sepd into component classes by HPTLC on silica gel G using a double development system followed by scanning densitometry as described previously [17]. To further identify component lipid classes, total lipid was subjected to two-dimensional HPTLC using MeOAc–*iso*PrOH–$CHCl_3$–MeOH–0.25% KCl (25:25:25:10:9) for development in the first dimension followed by development in the second dimension with $CHCl_3$–MeOH–7N NH_4OH (65:30:4). Developed chromatograms were stained with Cu $(OAc)_2$ and charred to visualize sepd components [17] or subjected to stains for specific functional groups [18].

For analysis of fatty acid composition, DGTA was purified by two-dimensional TLC on silica gel G using the solvent systems described above. Sepd components were visualized by spraying the developed chromatograms lightly with 2′,7′-dichlorofluorescein in 98% MeOH and viewing under UV light. The area of adsorbent containing DGTA was scraped into a test tube and the lipid eluted with $CHCl_3$–MeOH–H_2O (5:5:1). DGTA obtained from several chromatograms was pooled. A portion was subjected to acid-catalysed transesterification to produce the Me esters of the fatty acid components (FAME) [19]. FAME were purified by HPTLC using hexane–Et_2O–HOAc (85:15:1) and recovered from the adsorbent by elution with Et_2O. Purified FAME were then analysed by GC using 50 m × 0.32 mm i.d. fused silica capillary column coated with FFAP (SGE). H_2 was used as carrier gas and sample injection was on-column. During each analysis the oven temp. was programmed to increase from 50° to 225°. Sepd components were identified by reference to known standards and by comparison with a well-characterized fish-oil.

For the separation of frs on the basis of degree of unsaturation of component fatty acids, DGTA was applied as a streak to a TLC plate precoated with silica gel G which had been sprayed with 2 g $AgNO_3$ in MeCN and activated at 110° for 30 min. The plate was developed twice in $CHCl_3$–MeOH–H_2O (65:25:4) and sepd components visualized by spraying with 2′,7′-dichlorofluorescein. Bands of separated frs were scraped off individually into test tubes and, after the addition of a known amount of 17:0 as int. standard, subjected to acid-catalysed transesterification as described above. During extraction of FAME, the organic phase was washed with 2 ml 20% NaCl. FAME were purified and analysed by GC as described above.

Derivatization of DGTS. Demethylation was carried out according to ref. [9] using Na benzenethiolate (Fluka) in dioxane dist. over $LiAlH_4$ before. The product was isolated by TLC (Merck 5715) using solvent 1. Deacylation was done with 0.2 M KOH in MeOH at 40° for 30 min and the product extracted with *iso*PrOH. For silylation, the dry residue was treated with *N* - methyl - *N* - trimethylsilyltrifluoroacetamide (Macherey-Nagel) for a few min and the reaction mixt. used for GC-MS.

Derivatization of DGTA. DGTA was deaminated by dissolving in $CHCl_3$ and heating to 65° for 15 min. In order to eliminate the double bond of the methacrylic acid-type product, the solvent was evapd and replaced by Me_2CO. Pd black was added and the mixt. shaken in a H_2 atmosphere for 3 hr at room temp. Deacylation and silylation was carried out as described above for DGTS.

Spectrometry. IR spectra were recorded with a KBr cell without solvent. For EI-MS, a 70 eV ionization energy at 8 kV acceleration voltage was applied.

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REFERENCES

1. Sato, N. (1992) *Bot. Mag. Tokyo* **105**, 185.
2. Eichenberger, W. (1993) *Plant Physiol. Biochem.* **31**, 213.
3. Eichenberger, W., Araki, S. and Müller, D. G. (1993) *Phytochemistry* **34**, 1323.
4. Giroud, C. and Eichenberger, W. (1989) *Plant Cell Physiol.* **30**, 121.
5. Jones, A. L. and Harwood, J. L. (1993) *J. Exp. Botany* **44**, 1203.
6. Sato, N. (1991) *Plant Cell Physiol.* **32**, 845.
7. Henderson, R. J. and Mackinlay, E. E. (1992) *Plant Cell Physiol.* **33**, 395.
8. Brown A. E. and Elovson, J. (1974) *Biochemistry* **13**, 3476.
9. Eichenberger, W. and Boschetti, A. (1978) *Febs Letters* **88**, 201.
10. Vogel, G., Woznica, M., Gfeller, H., Müller, C., Stämpfli, A. A., Jenny, T. A. and Eichenberger, W. (1990) *Chem. Phys. Lipids* **52**, 99.
11. Saunders, W. H., Jr. and Cockerill, A. F. (1973) *Mechanisms of Elimination Reactions*, John Wiley & Sons, New York.
12. Kato, M., Adachi, K., Hajiro-Nakanishi, K., Ishigaki, E., Sano, H. and Miyachi, S. (1994) *Phytochemistry* **37**, 279.
13. Sato, N. (1991) *Plant Cell Physiol.* **32**, 819.
14. Roughan, P. G. and Slack, C. R. (1982) *Annu. Rev. Plant Physiol.* **33**, 97.
15. Aaronson, S. and Baker, H. (1959) *J. Protozool.* **6**, 282.
16. Vogel, G. and Eichenberger, W. (1992) *Plant Cell Physiol.* **33**, 427.
17. Henderson, R. J. and Mackinlay, E. E. (1989) *Phytochemistry* **28**, 2943.
18. Henderson, R. J. and Tocher, D. R. (1992) in *Lipid Analysis: a Practical Approach* (Hamilton, R. J. and Hamilton, S., eds), pp. 65–111. IRL Press, Oxford.
19. Christie, W. W. (1982) *Lipid Analysis*. Pergamon Press, Oxford.