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CHLOROSULPHOLIPIDS IN ALGAE

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Key Word Index—*Botrydium granulatum*; *Monodus subterraneus*; Chrysophyta; *Nostoc* sp. E; Cyanophyta; *Elakatothrix viridis*; *Zygnema* sp.; Chlorophyta; chlorosulpholipids; docosane-1,14-disulphate.

Abstract—The docosane series of chlorosulpholipids has been detected by GC-MS in two members of the Xanthophyceae, *Botrydium granulatum* and *Monodus subterraneus*, two members of the Chlorophyceae, *Elakatothrix viridis* and *Zygnema* sp. and a member of the Cyanophyceae, *Nostoc* sp. E.

Chlorosulpholipids were originally discovered in the alga *Ochromonas danica* (Chrysophyceae, Chrysophyta [1,2]) where they constitute 15% of the lipids and 3% of the dry weight of heterotrophically-grown, stationary phase cells [2]. Two series of chlorosulpholipids were present, one based on *N*-docosane-1,14-diol disulphate and the other, much less abundant, based on *N*-tetracosane-1,15-diol disulphate. The 13-chloro-, 11,15-dichloro, 2,2,11,13,15-pentachloro- and 2,2,11,13,15,16-hexachloro derivatives of *N*-docosane-1,14-diol disulphate have been identified along with several partly characterized tri- and tetrachloro derivatives [3]. The 14-chloro-, 2,12,14,16,17-pentachloro- and 2,2,12,14,16,17-hexachloro derivatives of *N*-tetracosane-1,15-diol disulphate have also been detected [3]. Chlorosulpholipids have also been shown to occur in *Ochromonas malhamensis* [3,4], but earlier reports [5] suggesting that they were present in a variety of algae, bacteria and higher plants have not been substantiated and have in some cases been negated [3,6]. However, *N*-docosane-1,14-diol disulphate and its mono-, di-, tri-, tetra-, penta- and hexachloro-derivatives have recently been detected in the filamentous alga *Tribonema aequale* (Xanthophyceae, Chrysophyta) [7].

In the present work we report the detection of docosane-1,14-diol and several chlorinated derivatives in two other members of the Xanthophyceae, *Monodus subterraneus* and *Botrydium granulatum*. Chlorosulpholipids have also been found in 2 green algae, *Elakatothrix viridis* and *Zygnema* sp. and a blue–green alga, *Nostoc* sp. E but could not be detected in a preliminary examination of several other Chlorophyceae. Positive identification of these compounds was made, as described previously [7], by GC–MS analysis of the trimethyl-silyl ethers of the mixture of diols produced by solvolysis of the chlorosulpholipid fraction extracted from the alga in question. The chlorosulpholipids of *O. danica* were isolated and analysed in an identical manner for comparison purposes.

GLC analysis of the TMS-ethers of the diols derived from the chlorosulpholipid fractions isolated from *M. subterraneus* (12·2 g wet wt), *B. granulatum* (11·5 g wet wt) and *Nostoc* sp. E. (20 g wet wt) each give seven peaks (A–G) which had identical retention times to those of the TMS-ethers of docosane-1,14-diol and its mono- to hexa-chloro derivatives, isolated from *O. danica*, respectively. The retention times of peaks A–G relative to cholestane and the temperature at which elution occurred were: Peak A, 0·97, 228°; Peak B, 1·24, 241°; Peak C, 1·50, 253°; Peak D, 1·71, 261°; Peak E, 1·93, 263°; Peak F, 2·25, 264° and Peak G, 2·50, 264°.

The MS of peaks A–G isolated from *M. subterraneus* exhibited fragmentation patterns characteristic of the TMS-ethers of the docosane series of chlorosulpholipid diols. Peak A had an MS

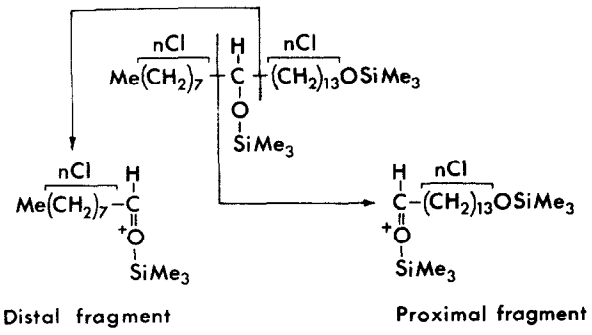

Fig. 1. Principal MS fragmentation of chlorosulpholipid diol TMS-ethers.

identical to that of the TMS-ether of docosane-1,14 diol. The highest *m/e* value in the MS was 471 ($M^+ - Me$) and was accompanied by peaks at *m/e* 373 (proximal fragment) and 215 (distal fragment). The M^+ peak is frequently missing from the TMS-ethers of the chlorosulpholipid diols [2] and the principal fragmentation is shown in Fig. 1, giving the proximal (C-1-OTMS → C-14-OTMS) and distal (C-22 → C-14-OTMS) fragments [2]. The distal fragment (DF) is very abundant and is frequently the base peak. The proximal fragment (PF) is much less abundant and sometimes cannot be seen in the higher chlorinated species; its degree of chlorination may then be elucidated by the characteristic *m/e* values of the "PF minus *n*HCl" fragment ions.

The highest ion cluster of peak B had peaks at *m/e* values of 505 and 507 in the ratio of 2:1 with respect to their intensity. These ions are 34 and 36 mass units greater than the $M^+ - Me$ ion of peak A indicating the presence of one chlorine atom. The presence of fragment ions at *m/e* values of 215 and 407/409 (intensity ratio 2:1) indicates that the chlorine atom is located in the proximal fragment. The precise position of the chlorine cannot be given with any degree of certainty. However the MS has a peak at *m/e* 397 (like *O. danica* [2] but unlike *T. aequale* [7]) which has been interpretted [2] as being due to the loss of TMSCl from the $M^+ - Me$ ion by interaction of Cl and TMS groups, a situation which would be most likely to occur if they were bonded to adjacent carbons. This would place the Cl on C-13.

The highest *m/e* values in the MS of peak C were a Cl_2 cluster at 539 which corresponds to the $M^+ - Me$ fragment ion of the TMS-ether of dichloro-*N*-docosane-1,14-diol. Also present were a non-chlorinated ion at *m/e* 215 and a Cl_1 cluster at *m/e* 249. These correspond to non- and monochlorinated distal fragments suggesting that peak C is a mixture of two dichlorinated diols, one having no Cl atoms distal and two Cl atoms proximal to the secondary alcoholic oxygen and the other having one Cl atom distal and one proximal. This was confirmed by the presence of Cl_1 clusters at *m/e* 407 (monoClPF) and 369 (diClPF-HCl). The presence of a Cl_1 cluster at *m/e* 431 would correspond to $M^+ - [Me + TMSCl]$ and may suggest that one or both the dichloro species has a Cl atom adjacent to a TMS group.

The highest *m/e* values in the MS of peak D were a Cl_3 cluster at 573 corresponding to the $M^+ - Me$ ion. The presence of ions at *m/e* values of 215 (nonClDF), 249 (Cl_1 cluster, monoClDF) 213, monoClDF-HCl), 367 (triClPF-3HCl) and 369 (diClPF-2HCl) indicate that there are two trichloro diol species in the peak, one having all three Cl atoms proximal to the secondary alcoholic oxygen and the other having one Cl atom distal and two proximal to the secondary alcoholic oxygen.

The highest *m/e* values in the MS of peak E was a Cl_4 cluster at *m/e* 607 corresponding to the $M^+ - Me$ ion of a tetrachloro species. Ions at *m/e* values of 283 (Cl_2 cluster, diClDF), 249 (diClDF-HCl), 211 (diClDF-2HCl), 405 (Cl_1 cluster, diClPF-HCl) and 369 (diClPF-2HCl) indicate the presence of a tetrachloro species with two Cl atoms distal and two proximal to the secondary alcoholic oxygen. A Cl_1 cluster at *m/e* 249 (monoClDF) and ions at *m/e* values of 213 (monoClDF-HCl) and 367 (triClPF-3HCl) suggest that a tetrachloro species having one Cl atom distal and three proximal to the secondary alcoholic oxygen is also present in peak E.

The MS of peak F had very low intensity ion clusters at *m/e* values of 656 (M^+) and 641 ($M^+ - Me$) corresponding to a pentachloro species. These were accompanied by ions at *m/e* values of 283 (Cl_2 cluster, diClDF), 247 (Cl_1 cluster, diClDF-HCl), 211 (diClDF-2HCl), 439 (Cl_2 cluster, triClPF-HCl), 403 (triClPF-2HCl) and 367

(triClPF-3HCl) which show that two Cl atoms are distal and three are proximal to the secondary alcoholic oxygen.

The highest *m/e* value in the MS of peak G was at 675 ($M^+ - Me$) corresponding to a hexachloro species. Also present were ions at *m/e* values of 283 (Cl_2 cluster, diClDF), 247 (Cl_1 cluster, diClDF-HCl), 211 (diClDF-2HCl), 509 (Cl_4 cluster, tetraClPF), 473 (Cl_3 cluster, tetraClPF-HCl), 437 (Cl_2 cluster, tetraClPF-2HCl), 401 (Cl_1 cluster, tetraClPF-3HCl) and 365 (tetraClPF-4HCl), therefore indicating that the hexachloro species has two Cl atoms distal and four proximal to the secondary alcoholic oxygen.

The MS of peaks A–G isolated from *Nostoc* sp. E gave fragmentation patterns almost identical to those of *M. subterraneus* indicating that the same series of compounds is present. GC–MS of the TMS-ethers of the diols isolated from *B. granulatum* gave unequivocal fragmentation patterns for peaks B, C and D; these were virtually identical with the MS of the equivalent peaks isolated from *M. subterraneus* indicating the presence of a monochloro-, two dichloro- and two trichloro derivatives of docosane-1,14-diol disulphate in the alga. The quantity of material present in the other peaks was too small or too contaminated with closely-chromatographing components to give clear-cut MS identification. Nevertheless from GLC retention data and the positive identification of peaks B, C and D it is clear that the range of chlorosulpholipids is present in *B. granulatum*.

GC–MS of the TMS-ethers of *E. viridis* and *Zygnema* sp. gave unequivocal fragmentation patterns for peaks A–C, F and G in the case of the former and peaks A–C in the case of the latter which were almost identical with the corresponding peaks from *M. subterraneus*. Clear cut MS of the remaining GC peaks were not obtained but retention data indicate the presence of the usual range of chlorosulpholipids in both algae.

It therefore appears that within the Chrysophyta, *Ochromonas* spp are not the only algae to produce chlorosulpholipids since three representative members of the Xanthophyceae, *T. aequale* (Heterotrichales), *M. subterraneus* (Heterococcales) and *B. granulatum* (Heterosiphonales) also synthesize them, albeit in much smaller quantities. This ability is not, however, peculiar to the Chrysophyta since the docosane series of chlorosulpholipids has been detected in 2 species of green algae (Chlorophyceae), *Elakatothrix viridis* and *Zygnema* sp. and a blue–green alga (Cyanophyceae), *Nostoc* sp. E. It remains to be seen whether other species within these and other algal classes also produce this group of lipids.

EXPERIMENTAL

Monodus subterraneus Peterson 848/1 Lewin U.S.A. and *Botrydium granulatum* L. Greville 805/3a Vischer were obtained from the Culture Collection of Algae and Protozoa, The Botany School, Cambridge whilst *Zygnema* sp., *Elakatothrix viridis*, *Ulothrix subtilissima*, *Stigeoclonium* sp., *Cosmarium botrytis* and *Scenedesmus quandricauda* were a gift from Dr. A. K. Jones, Dept. of Botany, U.C.W., Aberystwyth. All were grown for a period of 28–42 days on Bold's Basal Medium [8] contained in Roux bottles standing upright 15 cm from a double bank of 'warm white' fluorescent tubes (4750 lx) and continuously aerated; these conditions provided a temp. of 20–21°. The algae were harvested by centrifugation at 15000 *g* for 20 min. The sediment was then washed by resuspending in H_2O and recentrifuging 2×. Resulting algal pellets were then extracted 3× $CHCl_3$–MeOH (2:1,) after first disrupting the cells by cycles of freezing and thawing followed by grinding with acid-washed silver sand. The resulting extracts were then put through the chlorosulpholipid isolation procedure described for *O. danica* [9]. The putative chlorosulpholipid fraction was then solvolysed [10] to yield diols which were silylated and analysed by GLC and GC–MS. GLC was carried out on a 150 × 0·4 cm column packed with 1% SE-30 on 80–100 mesh GasChrom Q programmed to 5 min isothermal at 210° followed by a linear increase of 5°/min up to a nominal 265° and then held. The carrier gas flow through the column was O_2-free N_2 (GLC) and He (GC–MS) at 50 ml/min. GC–MS was carried out on a Pye 104 gas chromatograph linked via a single-stage silicone rubber membrane separator [11] to an AEI MS-30 mass spectrometer. Low resolution MS were obtained with an electron energy of 24 eV, an emission current of 300 μA and a source temp. of 250°.

Nostoc sp. E was a gift from Dr. A. J. Smith, Dept. of Biochemistry and Agricultural Biochemistry, U.C.W., Aberystwyth. This organism was originally isolated [12] from the coralloid roots of the cycad, *Macrozamia lucida*, L. Johnson. It was grown photoautotrophically at about 35° in 10 l. flasks illuminated by "warm white" fluorescent tubes (5000 lx) in the Cg10 medium of Van Baalen [13] modified by doubling the KNO_3 content and adding $KHCO_3$ (0·9 g/l). The medium was continuously gassed with a mixture of 95% air/5% CO_2 giving a final pH of 8·2. The cells were harvested after 14 days by continuous-flow centrifugation. The resulting cell paste was resuspended in 5 mM Tris buffer, pH 7·6 and then recentrifuged at 10000 *g* for 30 min. The cells were disrupted by freezing and thawing and then extracted with Me_2CO. The Me_2CO extract was reduced to dryness by rotary evaporation and then extracted with $CHCl_3$–MeOH –2:1). The same isolation and analytical procedure as was used for the other algae was then followed.

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REFERENCES

1. Haines, T. H., Pousada, M., Stern, B. and Mayers, G. L. (1969) *Biochem. J.* **113,** 565.
2. Elovson, J. and Vagelos, P. R. (1969) *Proc. Nat. Acad. Sci. U.S.* **62,** 957.
3. Haines, T. H. (1973) *Ann. Rev. Microbiol.* **27,** 403.
4. Haines, T. H. (1973) *Lipids and Biomembranes of Eukaryotic Microorganisms* (Erwin, J. A., ed.) pp 197–232, Academic Press, New York and London.
5. Haines, T. H. (1965) *J. Protozool.* **12,** 655.
6. Emanuel, D., Stern, A. and Haines, T. H. (1972) quoted in Ref. 4.
7. Mercer, E. I. and Davies, C. L. (1974) *Phytochemistry* **13,** 1607.
8. Deason, T. R. and Bold, H. C. (1960) *University of Texas Publication*, No. 6022, 1.
9. Thomas, G. and Mercer, E. I. (1974) *Phytochemistry* **13,** 797.
10. Mayers, G. L., Pousada, M. and Haines, T. H. (1969) *Biochemistry* **8,** 2981.
11. Hawes, J. E., Mallaby, R. and Williams, V. P. (1969) *J. Chromatog. Sci.* **7,** 690.
12. Bowyer, J. W. and Skerman, V. D. B. (1968) *J. Gen. Microbiol.* **54,** 299.
13. Van Baalen, C. (1968) *Plant Physiol.* **43,** 1689.