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LETTER

Novel, resistant microalgal polyethers: An important sink of organic carbon in the marine environment?

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Abstract—Five out of seven marine microalgal species investigated were found to biosynthesize nonhydrolysable, mainly aliphatic, biomacromolecules (algaenans). The molecular structure of the algaenan isolated from the microalga *Nannochloropsis salina* of the class Eustigmatophyceae was determined by solid state ^{13}C NMR spectroscopy, Curie point pyrolysis–gas chromatography–mass spectrometry, and chemical degradations with HI and RuO_4. The structure is predominantly composed of C_{28}–C_{34} linear chains linked by ether bridges. The algaenan isolated from a second eustigmatophyte (*Nannochloropsis* sp.) was structurally similar. Algaenans isolated from two chlorophytes also possess a strongly aliphatic nature, as revealed by the dominance of alkenes/alkanes in their pyrolysates. Accordingly, we propose that the aliphatic character of numerous Recent and ancient marine kerogens reflects selectively preserved algaenans and that these algaenans may act as a source of *n*-alkanes in marine crude oils.

1. INTRODUCTION

A wide variety of novel, insoluble, nonhydrolysable aliphatic biomacromolecules has recently been isolated from several lacustrine and terrestrial primary producers. These macromolecules have been recognised as algaenans in common, ubiquitous fresh-water green microalgae (Philp and Calvin, 1976; Berkaloff et al., 1983; de Leeuw et al., 1991; Derenne et al., 1992b; de Leeuw and Largeau, 1993), as cutans and suberans in higher plant cuticles (Nip et al., 1986; Tegelaar et al., 1989a) and periderm tissue (Collinson et al., 1994), respectively, as tegmens in inner seed coats of fresh-water plants (van Bergen et al., 1994), as sporopollenins in spores and pollen grains (Guilford et al., 1988; Hemsley et al., 1993), and as polycadinenes in resins (van Aarssen et al., 1990). Because of their chemical stability and their resistance against bacterial decay, these macromolecules are selectively preserved (Zelibor et al., 1988; Nip et al., 1989) and make up a significant part of organic carbon in nonmarine sediments ranging in age from Recent to over 300 million years. They eventually can act as the source of high-wax crude oils upon burial and thermal cracking (Tegelaar et al., 1989b). Similarly, it has been proposed that the bulk of marine sedimentary organic matter (OM) derives from marine algaenans (Tegelaar et al., 1989c), but evidence for that has been rather circumstantial (Hatcher et al., 1983; Derenne et al., 1992c) and the existence of a marine algaenan was only shown for an uncommon monospecific chlorophyte (Derenne et al., 1992a). Accordingly, the possible occurrence of algaenans in five common marine green microalgae (Chlorophyceae) and two marine microalgae of the class Eustigmatophyceae has now been investigated.

2. Experimental

2.1. Culture Conditions

All strains were obtained from the Culture Collection of the Natural Environment Research Council (U.K.). Algae were grown in batch cultures [in a medium described in Veldhuis and Admiraal (1987)] in a light/dark cycle of 16/8 h at 15°C. Cultures were kept axenic. Algaenans were isolated from the dry algal biomass by ultrasonic extraction with different organic solvents and by successive hydrolyses with KOH, HCl, and H_2SO_4. Extracts, saponified extracts, and organic fractions isolated after the KOH and HCl hydrolyses of the extracted algal biomass were analysed by GC and GC-MS.

2.2. Solid State ^{13}C NMR

The NMR spectrum was obtained at a ^{13}C frequency of 25.2 MHz using cross polarization with magic-angle spinning. Pertinent experimental parameters are the following: contact time = 1 ms, pulse delay = 1 s, acquisition length = 0.5 K and line broadening = 30 Hz. The chemical shifts scale is referred to tetramethylsilane at 0 ppm.

2.3. Curie Point Pyrolysis–Gas Chromatography–Mass Spectrometry

Samples were pyrolysed using a FOM-4LX Curie point pyrolysis unit directly coupled to a Hewlett Packard 5890 series II gas chromatograph fitted with a 25 m × 0.32 mm CP-Sil 5 (film thickness 0.12 μm) fused silica capillary column, temperature programmed from 0 to 320°C (hold time 10 min) at a rate of 3°C/min. Structural assignments were made based on MS and retention time data. MS conditions: VG Autospec Ultima instrument, ionization energy 70 eV, mass range 40–800, cycle time 1.8 s.

2.4. Chemical Degradations

Oxidation with RuO_4 followed the procedure described by Boucher et al. (1990). The acid products were derivatized with diazomethane to form methylester derivatives.

Table 1. Summary of the presence and nature of algaenans in the microalgae investigated.

Name	Algaenan[a]	Main series of pyrolysis products of algaenan[b]				Chemical nature
		n-alk-1-enes/ *n*-alkanes	*n*-alkan-2-ones, mid-chain ketones[e]	alkyl-benzenes	alkyl-phenols	
Brachiomonas submarina	--[c]		--			--
Chlorella spaerckii	1.2	C_7-C_{41} (C_{14})	C_7-C_{22} (C_{19})	C_6-C_{10} (C_7)	C_6-C_9 (C_7)	aliphatic/aromatic
Chlorococcum sp.	1.0	C_7-C_{34} (C_{26})	C_7-C_{25} (C_{19})	C_6-C_{10} (C_7)	C_6-C_9 (C_7)	aliphatic/aromatic
Nannochloris sp.	~1	--	--	C_6-C_{10} (C_7)	C_6-C_9 (C_7)	aromatic
Stichococcus bacillaris	--[c]		--			--
Nannochloropsis salina	1-2[d]	C_7-C_{36} (C_{16}, C_{28})	C_7-C_{22} (C_{18},C_{19}) C_{20}-C_{34} (C_{29})	C_6-C_{22} (C_7)	--	aliphatic
Nannochloropsis sp.	1.5	C_7-C_{33} (C_{15}, C_{26})	C_7-C_{25} (C_{19}) C_{22}-C_{32} (C_{26})	C_6-C_{22} (C_7)	--	aliphatic

[a] In % of the dry algal biomass. [b] Carbon number range of the indicated series of the algaenans pyrolysis products. The maxima are indicated in brackets. [c] The entire biomass was dissolved after HCl hydrolysis. [d] The range indicated reflect 3 different batch cultures of this alga. [e] Series of mid-chain *n*-alkanones (only present in the pyrolysates of the *Nannochloropsis* algaenans) are indicated below the series of *n*-alkan-2-ones.

Treatment with hydroiodic acid (HI) followed the procedure described by Panganamala et al. (1971). Iodide was displaced with sodium methyl thiolate in methanol for 18 h at room temperature to obtain thiomethyl ethers. The thiomethylether derivatives show a highly characteristic fragmentation pattern upon GC–MS analyses (Carlson et al., 1989).

3. RESULTS AND DISCUSSION

Freeze-dried algal cells, obtained from axenically grown batch cultures, were extracted and the residues were treated with base (KOH) and acids (HCl and H_2SO_4) to remove free lipids, ester-bound lipids, proteins, and carbohydrates, respectively. These treatments afforded 1–2% of an insoluble residue, i.e., algaenan, in the case of three chlorophytes, *Chlorella spaerckii, Chlorococcum* sp., and *Nannochloris* sp., and the eustigmatophytes *Nannochloropsis* sp. and *Nannochloropsis* salina (Table 1). The algaenan of the latter alga was analysed by solid state ^{13}C NMR (Fig. 1). The chemical shift maximizing at 33 ppm indicates the highly aliphatic nature of this algaenan since at least 95% of the NMR response is represented by this chemical shift of CH_2 moieties.

The results of pyrolysis–gas chromatography–mass spectrometry (Py–GC–MS) of this algaenan are in full agreement with the NMR data. The pyrolysate of *N. salina* algaenan (Fig. 2a) is strongly dominated by homologous series of C_7 to C_{36} *n*-alkanes and *n*-alk-1-enes maximizing at C_{16} and C_{28}, revealing the presence of long polymethylenic moieties in its algaenan. Series of ketones identified as co-eluting *n*-alkan-17-ones* and *n*-alkane-18-ones with up to 34 carbon atoms (Fig. 2b) indicate the presence of oxygen functionalities mainly at the C-17 and C-18 positions and the maximum carbon chain length, i.e., 34 carbon atoms, of the building blocks of the macromolecules. The presence of such oxygen functionalities was confirmed by the highly specific distribution patterns of *n*-alkan-2-ones which maximize at C_{18} and C_{19} (Fig. 2b). The additional occurrence of mono-unsaturated mid-chain ketones with the unsaturation located in the shortest alkyl chain suggests the presence of another functionality, probably located at the first carbon atom. Based on earlier pyrolysis studies with model compounds and algaenans derived from freshwater microalgae, it

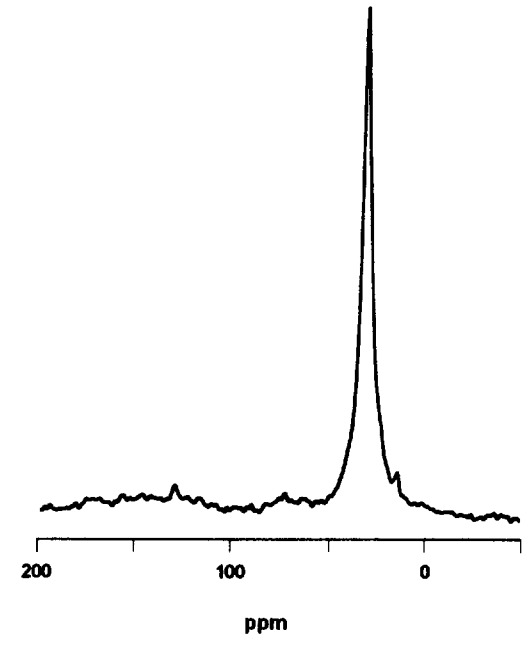

FIG. 1. Solid state ^{13}C NMR spectrum of *N. salina* algaenan. The large peak at 33 ppm is assigned to long-chain polymethylenic structures, the peak at 15 ppm to methyl groups, the broad region between 50 and 90 ppm is due to O-bound alkyl carbons, the region between 100 and 160 ppm corresponds to sp^2 carbons, and the broad resonance between 160 and 180 ppm is assigned to carbonyl or amide carbons.

* The nomenclature used for the mid-chain ketones is purposely not correct and should be, for example, C_{30} alkan-13- and alkan-14-ones. The reason for using such numbering is due to the common, specific positions of the keto group for all the mid-chain ketones.

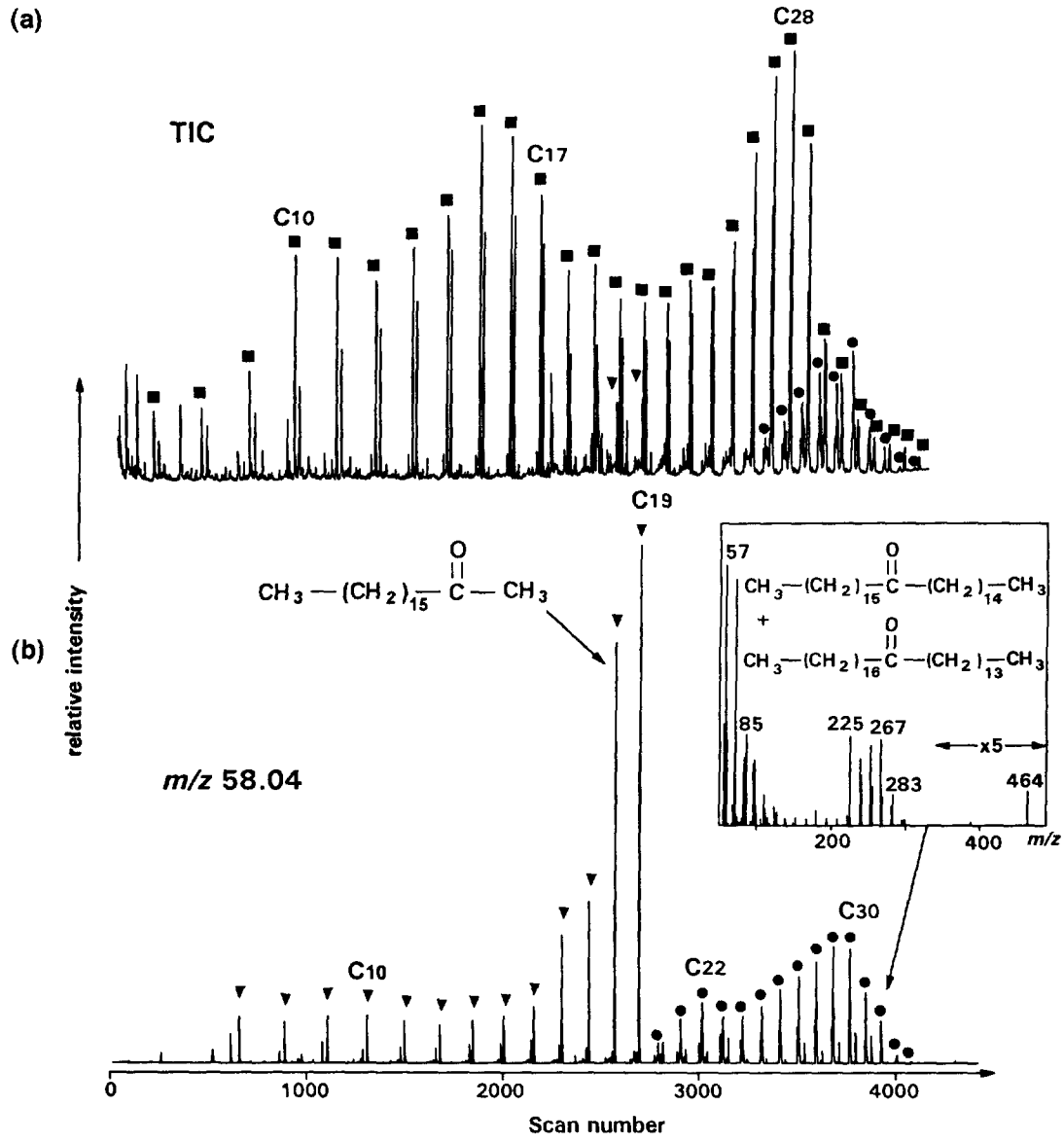

FIG. 2. Analysis of *N. salina* algaenan by Curie-point pyrolysis–gas chromatography–mass spectrometry; (a) total ion current trace (TIC) and (b) mass chromatograms of *m/z* 58.04 revealing the distribution of ketones. Filled squares indicate the *n*-alk-1-ene/*n*-alkane doublets, filled triangles indicate the *n*-alkan-2-ones and filled circles indicate the mid-chain ketones. Numbers indicate the total numbers of carbon atoms. The insert in the bottom figure shows the mass spectrum of the coeluting C_{32} alkan-15- and -16-ones, identified based on MS data from previous studies (de Leeuw et al., 1981; Gatellier et al., 1993).

is concluded that both functionalities represent ether linkages (Gelin et al., 1993, 1994). This is confirmed by the presence of a small but recognizable peak for alkyl-O carbons in the ^{13}C NMR spectrum at approximately 70 ppm (Fig. 1).

Chemical degradation of the *N. salina* algaenan with ruthenium tetroxide (RuO_4) further supports the presence of ether bonds at the ω-17 or ω-18 positions and likely indicates ether bonds at the C-1 position as indicated by the generation of C_{24}–C_{34} ω-17- and ω-18-keto-alkanoic acids (Fig. 3a). A series of C_{24}–C_{34} ω-17,18-diketo-alkanoic acids was also produced by RuO_4 oxidation of this algaenan and reveals the presence of three ether linkages located at the C-1, ω-17 and ω-18 positions of the polymethylenic carbon chains (Fig. 3b). The third functionality is probably involved in cross-linking so that a three-dimensional network is built up, helping to explain the insolubility of the algaenan. The C_8–C_{18} *n*-alkanedioic and C_{12}–C_{18} *n*-alkanoic acids which represent the major products of the RuO_4 degradation result from mid-chain oxidations at the vicinal ether positions (Fig. 3c,d). The long-chain fatty acids, maximizing at C_{29}, probably result from the oxidation of ether-linked alkyl moieties. The prevalence of ether-linked long-chain alkyl units in the *N. salina* algaenan structure was further demonstrated by the release of predominantly 1,ω-17- and 1,ω-18-C_{28}–C_{36}

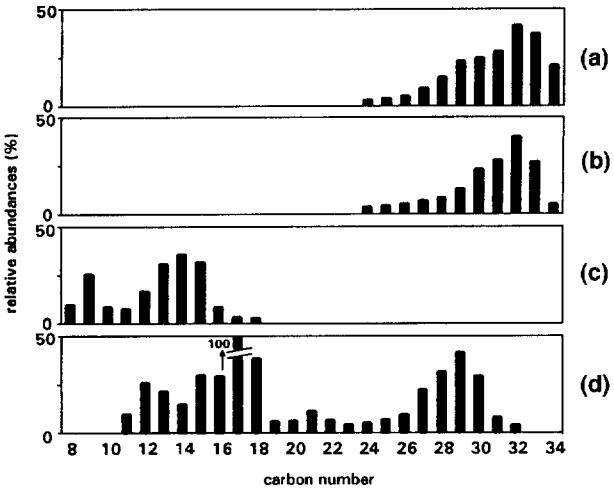

FIG. 3. Histograms displaying the major series of oxidation products derived from RuO_4 degradation of *N. salina* algaenan; (a) co-eluting n-ω^{17}- and n-ω^{18}-keto-alkanoic acids, (b) n-$\omega^{17,18}$-diketo-alkanoic acids, (c) n-alkanedioic acids, and (d) n-alkanoic acids.

diiodo-alkanes after hydroiodic acid treatment, known to selectively cleave ether bonds (Panganamala et al., 1971).

Pyrolysis of the algaenan of *Nannochloropsis* sp. showed very similar distributions of alkenes, alkanes, and alkanones in its pyrolysate, indicating that both eustigmatophytes biosynthesize structurally related algaenans (Table 1).

Analyses of the extracts of the two eustigmatophytes revealed the presence of free and ester-bound C_{30}–C_{32} alken-1-ols and C_{30}–C_{36} n-alkadiols with alcohol groups at ω-17 or ω-18 and C-1. These findings are in agreement with those of a previous study of the lipids of *N. salina* and other *Nannochloropsis* species (Volkman et al., 1992). The structural similarity of these lipids with units in the algaenan strongly indicates that the specific C_{30}–C_{34} diols are the main building blocks of the *Nannochloropsis* algaenan. Since their identification in Black Sea sediments (de Leeuw et al., 1981), C_{30}–C_{32} diols have shown to be ubiquitous in marine sediments in relatively high abundances (e.g., Smith et al., 1983; ten Haven et al., 1987a–c, 1992; Morris and Brassell, 1988; McCaffrey et al., 1991; Hoefs et al., 1995a), implying that their macromolecular counterparts can be important contributors to the OM of marine sediments.

The abundant alkene/alkane patterns noticed in the pyrolysates of the eustigmatophyte algaenans were also observed, though with different distributions, in the pyrolysates of algaenans of the marine chlorophytes, *C. spaerckii* and *Chlorococcum* sp. (Table 1). Because homologous series of alkenes and alkanes often dominate pyrolysates of marine kerogens isolated from both Recent (Klok et al., 1984; Eglinton et al., 1994) and ancient (Derenne et al., 1990; Douglas et al., 1991) sediments, it is concluded that a common source, which could be algaenans, although not necessarily derived from the four microalgae investigated here, are probably important contributors to marine sedimentary OM. Further support of this comes from stable carbon isotope studies of alkenes and alkanes isolated from pyrolysates of marine kerogens. Their $\delta^{13}C$ values are rather uniform and thus seem to indicate a common source, i.e., algaenan (Eglinton, 1994; Collister et al., 1994; Hoefs et al., 1995b). However, very specific alkene/alkane distribution patterns, such as those found in the pyrolysates of the *Nannochloropsis* algaenans, have not been found so far in the pyrolysates of marine kerogens. This is readily explained by assuming that a suite of structurally diverse algaenans derived from different algae make up these kerogens. Although these different algaenans may be isotopically distinct, they all produce a full range of n-alkanes upon pyrolysis. This will lead to averaged carbon isotopic compositions for all n-alkanes which will not differ much from each other. These considerations indicate that the contribution of algaenan-producing microalgae to marine OM should be traced via specific biomarkers, i.e., diols in eustigmatophytes, rather than through specific pyrolysis patterns of their algaenans.

Aromatic and phenolic compounds were significantly present in the pyrolysates of the two chlorophyte algaenans already mentioned and were predominant in that of *Nannochloris* sp. (Table 1). The origin of the aromatic moieties observed in algaenan pyrolysates is still unclear, whereas the phenols are likely generated by the thermal cleavage of polyphenolic macromolecules such as phlorotannin (van Heemst et al., 1995).

Our results strongly indicate that specific marine eustigmatophyte and chlorophyte microalgae biosynthesize algaenans. These biomacromolecules, due to their highly resistant nature, are thought to escape from mineralization and become selectively preserved in marine sediments, as it has been observed previously for algaenans of freshwater microalgae in lacustrine environments (Goth et al., 1988; Tegelaar et al., 1989c; Derenne et al., 1991; Sinninghe Damsté et al., 1993). Therefore, we regard marine algaenans as a major sink of organic carbon in marine sediments. Upon burial in the Earth crust these materials may act as an important source of n-alkanes in marine petroleums.

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