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New insights on the structure of algaenan from *Botryoccocus braunii* race A and its hexane insoluble botryals based on multidimensional NMR spectroscopy and electrospray–mass spectrometry techniques

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Abstract

Through solution state NMR spectroscopy and quadrupole-time-of-flight mass spectrometry (Q-TOF MS) studies of the hexane insoluble botryal extract of the algae *Botryococcus braunii* race A, coupled with high-resolution magic-angle-spinning (HR-MAS) NMR spectroscopy of the algaenan from this alga, it has been possible to advance the structural understanding of this geochemically important biopolymer. It was found that the hexane insoluble botryals in this study constitute a mixture of low molecular weight unsaturated aliphatic aldehydes and unsaturated hydrocarbons with an average chain length of about 40 carbons. Exact assignments were provided for many of the structural units present and describe the average constitution of the mixture as a whole. Reticulation of the low molecular weight chains via acetal and ester links explains the primary make up of algaenan. In this study, it is concluded that the algaenan results from the reticulation of low molecular weight hexane insoluble botryal species rather than the polyaldehyde as previously observed in studies of algae at alternate stages of their growth cycle.
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Keywords: *Botryococcus braunii*; Algaenan; High-resolution magic-angle-spinning nuclear magnetic resonance; Multidimensional spectroscopy; Electrospray ionization mass spectrometry

1. Introduction

Botryococcus braunii race A is a ubiquitous colonial green micro alga that is an important synthesizer of lipids in fresh and brackish water ecosystems (Largeau et al., 1980; Metzger and Largeau, 1994; Metzger et al., 1990; Schouten et al., 1998). A high percentage of hydrocarbons in some petroleums have probably been derived exclusively from this alga (Hillen et al., 1980; Wilson et al., 1988). The majority of these biosynthesized lipids in *B. braunii* are located in the outer cell wall of the algal material (Berkaloff et al., 1983; Bertheas et al., 1998; Gelin et al., 1994a,b; Largeau et al., 1984). Various studies have shown that a type of aliphatic, insoluble, and chemically resistant biopolymeric material termed "algaenan" (Tegelaar et al., 1989) is a major component of the outer cell wall in *B. braunii* (Berkaloff et al., 1983; Blokker et al., 1998, 2000; Derenne et al., 1989; Gatellier et al., 1993; Gelin et al., 1994b; Kadouri et al., 1988).

Algaenan is of great geochemical importance (Gelin et al., 1999; Largeau et al., 1984, 1986) and even economic importance as a precursor to petroleum formation in the aquatic environment (Gelinas et al., 2001). However, the chemical structure of *B. braunii* algaenan is mainly inferred, partly based on the molecular structure of an aliphatic polyaldehyde that can be isolated from the green alga (Metzger et al., 1993). It was suggested that algaenan is derived by a condensation reaction of the soluble aliphatic polyaldehyde material (Bertheas et al., 1998; Gelin et al., 1994b; Metzger et al., 1993). Previous studies have yielded some structural information

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for the algaenan biopolymer and its polyaldehyde extract using NMR (Bertheas et al., 1998), size exclusion chromatography (Bertheas et al., 1998), TMAH thermochemolysis (Allard and Templier, 2000; Zang et al., 2001), and pyrolysis techniques (Gelin et al., 1994a). It has been hypothesized that the soluble polyaldehyde precursor and ultimately the algaenan itself, for the Race L strain of *B. braunii* is formed via an *n*-C_{32} diunsaturated α,ω-dialdehyde by a condensation–polymerization pathway involving an aldolization–dehydration mechanism (Bertheas et al., 1998).

In this study, advanced multidimensional solution state NMR and electrospray ionization mass spectrometry techniques were applied to characterize the "hexane insoluble botryals" from *B. braunii* and use the solution state NMR findings to help identify the signals obtained from the new High-Resolution Magic-Angle-Spinning (HR-MAS) NMR spectroscopy of the insoluble algaenan. The goal was to employ these techniques to evaluate the structural relationships between the soluble and insoluble fractions with the ultimate aim to establish a structure for the insoluble algaenan from B. *braunii* race A.

2. Results and discussion

It has been documented that a polyaldehyde can be isolated from *B. braunii* (Metzger et al., 1993) which upon drying becomes insoluble in organic solvents. However, we find that a fraction of "botryal like" species also co-precipitated with the polyaldehyde during its isolation. These botryals, which are not removed by pre-extraction with hexane (presumably, because they are either not soluble in hexane or entrapped within the polyaldehyde macromolecular structure), can be redissolved in chloroform. For the purpose of this study we will use the term "hexane insoluble botryals" to describe this fraction. It is important to note that, in our cultured algae, the hexane insoluble botryals constitute the dominant fraction of the polyaldehyde/botryal extract because, upon drying and redissolution, most of the material became soluble. These hexane insoluble botryals have very similar structures to those observed in the polyaldehyde and they can be easily studied by solution state NMR and MS, as they are non polymeric in nature and have increased solubility over the polyaldehyde (after drying). Fig. 1 compares the ^{1}H spectra of the polyaldehyde from *B. braunii* race A (kindly provided by Dr. Pierre Metzger, note the polyaldehyde sample was never dried down, isolation was performed directly into deuterated chloroform by the Metzger group) to the hexane insoluble botryals. With the exception of terminal methyl groups (and some terminal unsaturations, not visible in Fig. 1, but discussed later in this paper) in the hexane insoluble botryals both materials contain very similar structural components. Fig. 1 further considers the effect of a "diffusion gate" NMR experiment on the two fractions (Wu et al., 1995). During such an experiment signals from small molecules will be gated, while signals from very large molecules, with very slow diffusion, will be unaffected. Fig. 1 clearly shows that the hexane insoluble botryals are all low molecular weight species (~99% of the signal is destroyed in the diffusion gate experiment), and confirms that the polyaldehyde supplied by Dr. Metzger is indeed a very high molecular weight polymer (only ~3% of the signal intensity is lost). In structural terms the hexane insoluble botryals here are consistent with monomeric units from the polyaldehyde that have been "endcapped" with terminal groups. However, the exact relationship between these hexane insoluble botryals and the polyaldehyde is unclear. Whether these hexane insoluble botryals are in fact precursors to the polyaldehyde, or are specific to a particular growth condition or stage of growth is yet to be determined.

Comparison of the hexane 1-D ^{1}H NMR spectrum for the hexane insoluble botryals to that of the HR-MAS spectrum of the algaenan swollen in DMSO-d_6 (see Fig. 2) also shows strong similarities. Considering this, and in order to learn as much as possible about algaenan, it is beneficial to extract as much information as possible from the hexane insoluble botryal fraction. The resolved NMR spectral information from MS and liquid state NMR from this fraction can be used to aid in the interpretation of less resolved HR-MAS NMR data collected for the insoluble algaenan data.

2.1. Hexane insoluble botryals

Total correlation spectroscopy (TOCSY) identifies through bond couplings between protons in the same spin system. Fig. 3 shows the TOCSY spectra for the hexane insoluble botryals. Connectivities and chemical shifts are consistent with structures 1–5 labeled in Fig. 4. In these structures the CH_2 units adjacent to, and further removed from the functionality, crowd together in the same region of the spectrum which has been labeled as B in Fig. 3. The region labeled A in Fig. 3 is consistent with CH_3 groups and likely results from terminal CH_3 groups ending a number of the chains depicted in Fig. 4.

Fig. 5 shows the HMQC spectrum of the hexane insoluble botryal. Methine units, in structures 1, 2, and 4 are clearly seen and are labeled as 1b, 2a, 4a, respectively. Polarization Enhancement During Attach Nucleus Testing (PENDANT) spectroscopy (which distinguishes carbons on the basis of the number of attached protons) identifies the expected multiplicities for all carbons in structures 1–5 and further confirms their assignments (data not shown). The methine units in structure 3 are not visible in the HMQC as they are

present in such low concentration and are below the detection limit of the experiment. Region B, Fig. 5, is consistent with a range of CH_2 units associated with these structures, while region A contains cross peaks from CH_3 units, which likely terminate chains containing structures 1–5.

In order to confirm all the assignments an HMQC–TOCSY experiment was carried out. HMQC–TOCSY permits the identification of connectivities between protonated carbons that are present in the same spin system. Fig. 6 shows the HMQC–TOCSY of the hexane insoluble botryals. All long range ^{1}H–^{13}C coupling occur as expected further confirming the assignments of structures 1–5 in Fig. 4.

2.2. Quantification of the structural components

Quantification of the structures is best carried out from the 1D spectra. However, overlap makes accurate quantification of the individual resonances very difficult especially in the methylene region. Therefore it is more accurate to consider the main methylene (~25 to 40 ppm carbon) region as a whole. Table 1 shows the integrals obtained from the carbon spectrum (which was

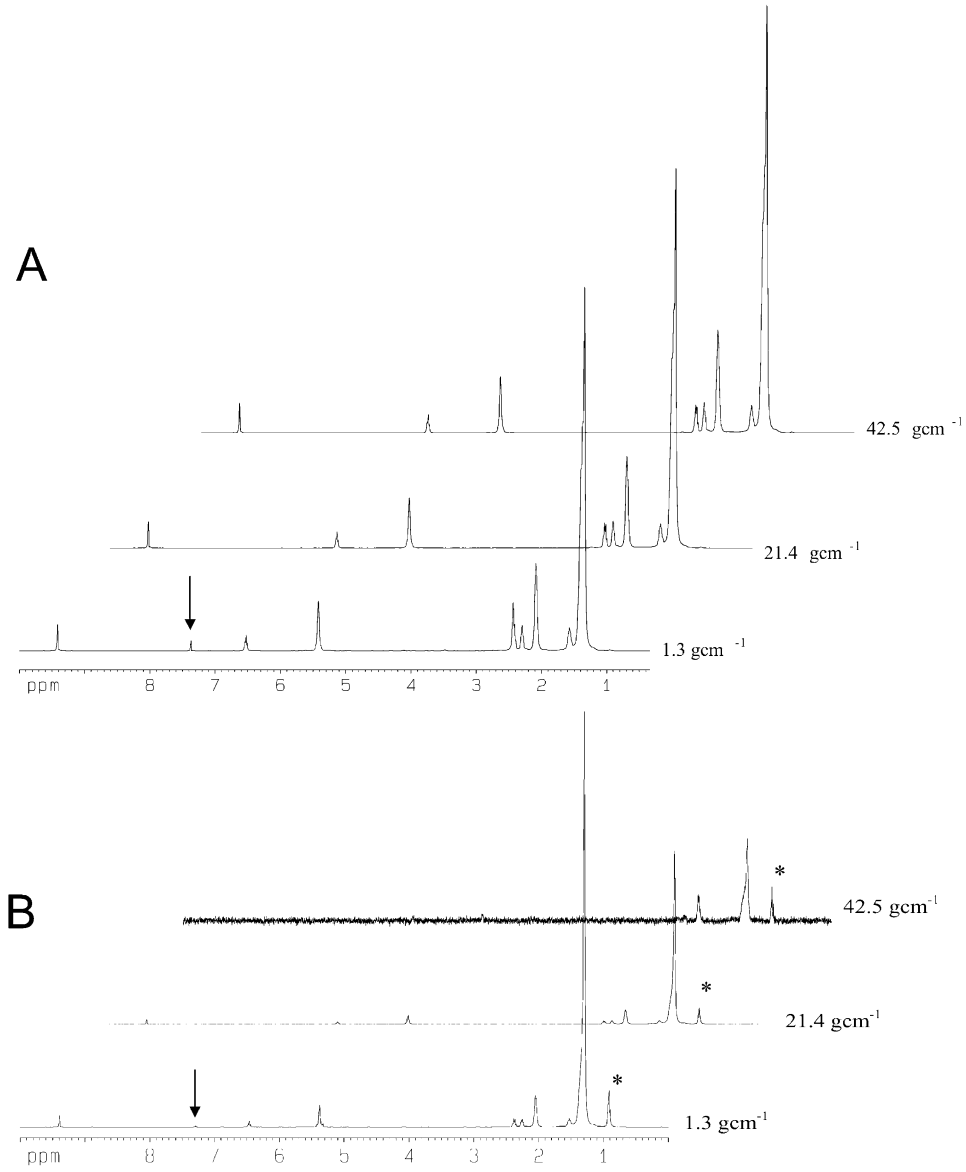


Fig. 1. Comparison of the ^{1}H NMR (conventional spectrum is the foremost slice in each set) of the polyaldehyde extract (A) from *B. braunii* Race A (supplied by the Metzger group) to the hexane insoluble botryals (B). The "diffusion gate" experiment clearly shows the hexane insoluble botryals (B) are small molecular weight while the polyaldehyde (A) is polymeric in nature. The chemical constituents of both species are nearly identical, with the exception of terminal methyl groups (labeled with *) which are abundant in the hexane insoluble botryal fraction. Numbers represent the gradient strength employed in the "diffusion gate" (see Experimental for details). The arrow denotes the position of the chloroform solvent.

carried out under quantitative conditions, see Experimental) and the ratios of various units present. The integrals indicate that structures 3 and 5 signals constitute less than 0.5% of the carbon in the sample, suggesting that they only occur once in every 200 carbons or more, and are therefore relatively minor components in the overall mixture of structures. However terminal units [terminal double bonds (structure 4) and terminal CH_3 units] are in abundance at a total of ~5% of the total carbon intensity. It has been suggested the material has a straight chain type structure (Metzger et al., 1993) which is further supported by the nature of the units identified by NMR spectroscopy. In such a case with ~5% of the carbon intensity resulting from terminal groups, then for every terminal group there will be an average of 20 chain structures. Considering that in order to terminate a chain there will need to be a terminal group at each end of the chain, and the average chain length in the sample will be in the region of about 40 carbons in length.

Consideration of the integrals for the different chain structures provides some insight into the nature of the structures themselves. For example, the integral in Table 2 indicates that for an average chain length of 40

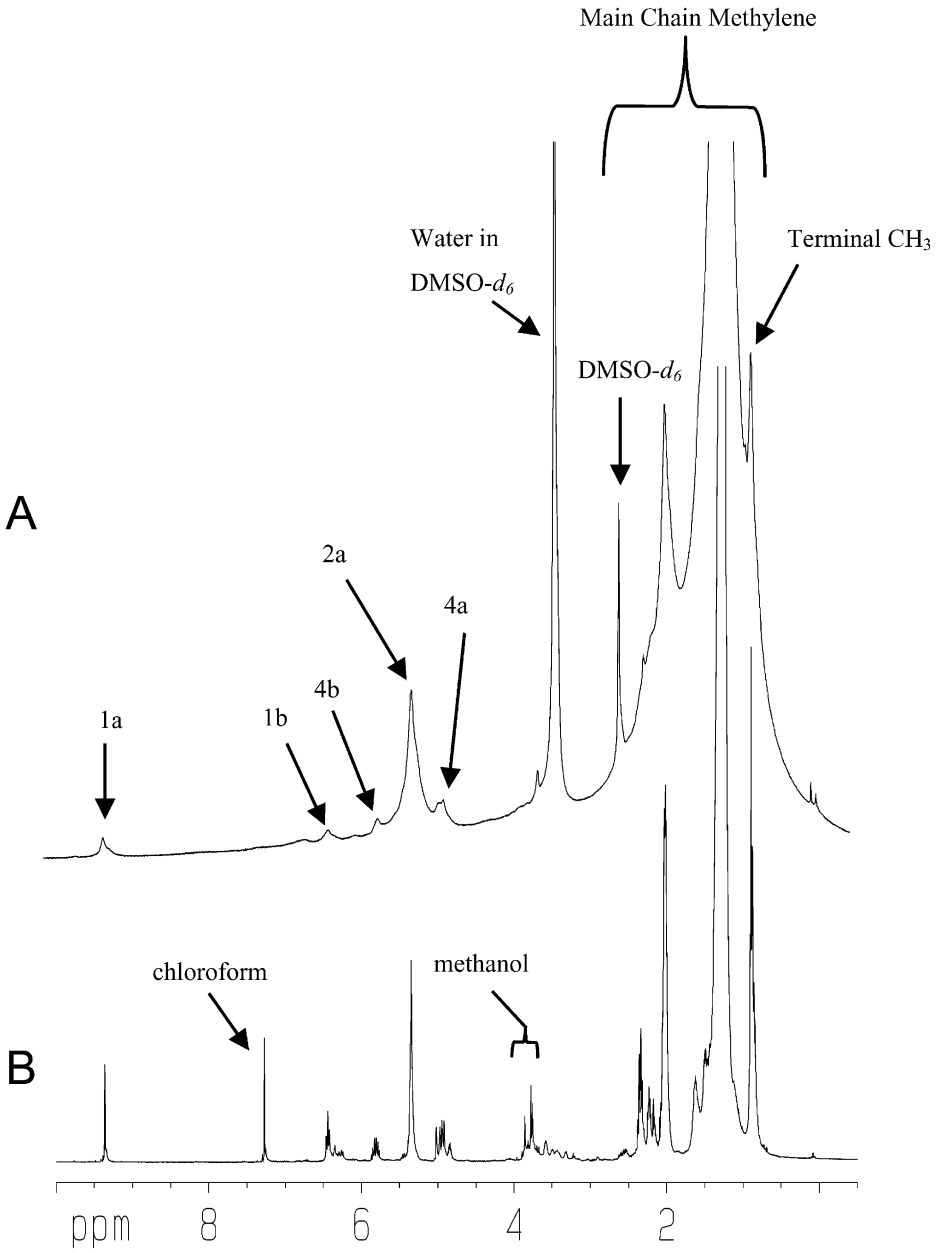


Fig. 2. (A) ^{1}H HR-MAS spectrum of algaenan from *B. braunii* swollen in DMSO-d_6. The labels represent signals from the major components present as shown in Fig. 4 and further discussed in the text. (B) ^{1}H Solution state NMR of the soluble hexane insoluble botryals. Chloroform was employed as the solvent and signals from the methanol used in the extract procedure persist.

carbons there will be 0.33 unsaturated aldehyde groups, i.e. on average, a third of the chains in the sample will contain an unsaturated aldehyde. Similarly we can deduce that, on average, every 40 carbon units will contain a mid-chain unsaturation, half of them will terminate with an unsaturation at one end, all of them will terminate with a methyl group at one end, and another half will terminate with methyl groups at both ends. It is very important to stress that these observations only hold as an average for all the structures in the sample and certainly does not dictate that these individual structures as described are the only possible structures or even that these structures definitely exist as suggested. In reality the sample is likely to contain a mixture of compounds, which may have considerable variation in their individual constitutions, but in summation display the average properties as described above.

Indeed the average functionalities and structures identified here, are in general agreement with structures observed in the polyaldehyde extract (Metzger et al., 1993) in that straight chain units containing unsaturated aldehydes, and a proportion of mid chain unsaturations are in abundance. In organic extracts both small molecular weight botryals and polymerized aldehyde materials have been identified (Metzger and Casadevall, 1989, 1992; Metzger and Largeau, 1994; Metzger et al., 1993). Metzger et al. (1993) suggested the molecular weight of the polymeric material to be in the range 10,000–4,000,000 Daltons. When subjected to NMR spectroscopic studies in our laboratory, the material obtained from the Metzger group (through personal communication) was confirmed to be of high molecular weight polyaldehyde structure (see Fig. 1). In the present study the abundance of terminal functionalities identified by analysis of the NMR spectrum strongly implies that the hexane insoluble botryals are not polymeric in nature. This is confirmed by the ''diffusion gate'' experiment shown in Fig. 1 and the detection of exclusively positive Nuclear Overhauser enhancements (Neuhaus and Williamson, 2000) for all the peaks (data not shown), which suggest all the structures have relatively low molecular weights (less than ~1000 Daltons). Q-TOF MS data show a range of masses from 240 to 840 Daltons, with no higher molecular weight observed in any abundance in the hexane insoluble botryal fraction studied here. From the MS data it is possible to identify the exact structures of some of the aldehyde containing units (see Table 2). The units containing the aldehyde functionality are determined to represent, on average, approximately a third of the structures in the mixture by NMR spectral analysis, and are prominent in the mass spectroscopy data due to the presence of the easily ionized aldehyde functionality. In addition to these aldehydic structures, the NMR spectral data indicate that approximately two thirds of the structures present do not contain an aldehydic unit and are consistent with general structures as shown in Fig. 7 which are known to be common structural units in algal cell

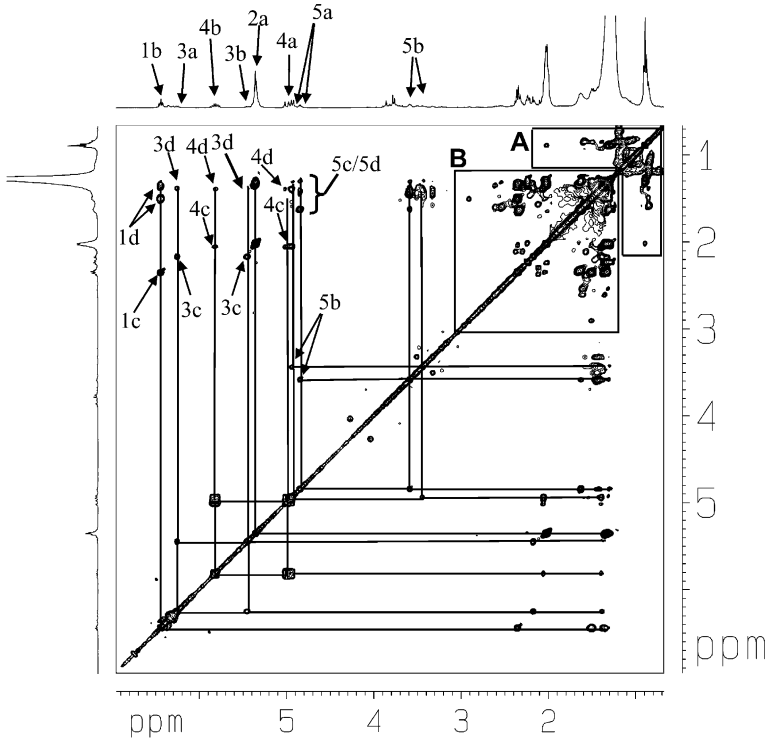


Fig. 3. TOCSY spectrum of the hexane insoluble botryals. The numbered labels represent structures summarized in Fig. 4 and discussed in the text. Region A represents coupling between main chain methylene and terminal methyl. Region B represents CH_2–CH_2 couplings in various environments.

biomass (Allard et al., 2002; Youngblood et al., 1971). Such structures without polar functionalities are less likely to undergo electrospray ionization so will be underestimated in the mass spectrometry data, yet evidence can still be seen for the presence of these structures from the Q-TOF data (Table 2).

2.3. Algaenan

It has been suggested that algaenan is derived by a condensation reaction from the aliphatic polyaldehyde material in *B. braunii* Race A (Bertheas et al., 1998; Gelin et al., 1994b; Metzger et al., 1993). In the present study the hexane soluble botryals display greater similarities to the algaenan than the polyaldehyde from the same race (see Figs. 1 and 2). The signals in the ^{1}H HR-MAS data are broader than those observed in the hexane insoluble botryals (Fig. 2). This results from the anisotropy that cannot be totally removed by spinning and solvent swelling, and the related fast T_2 relaxation

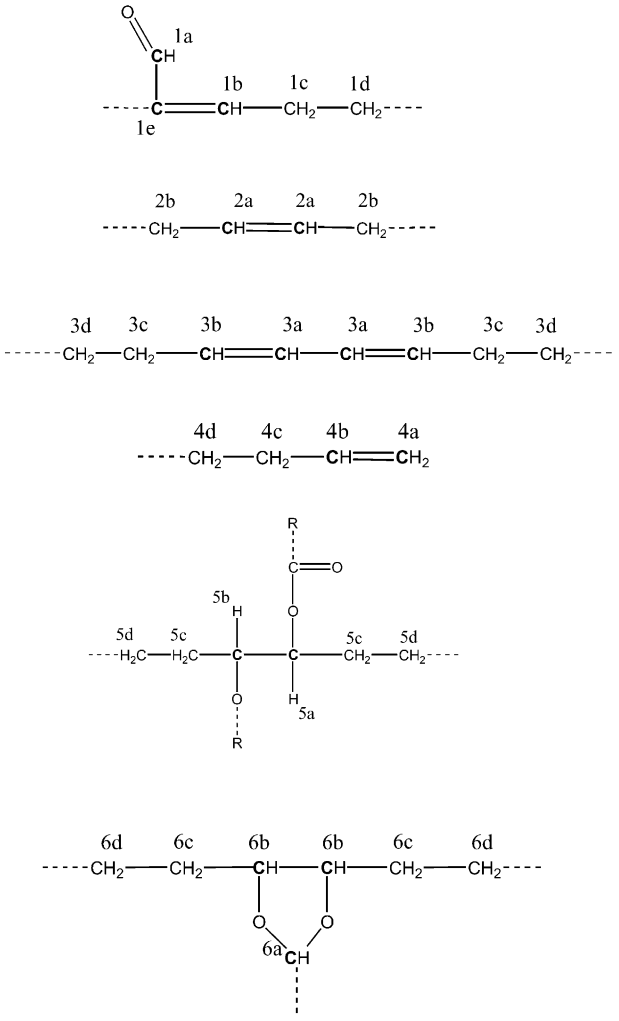


Fig. 4. Structures 1–4 identified in the hexane insoluble botryals from *B. braunii*. The presence of structures 5 and 6 are uncertain. For full discussion see text.

which is characteristic of large macromolecular networks. In both the hexane insoluble botryals and the insoluble algaenan an abundance of terminal unsaturations and methyl groups can be seen that are not apparent and have not been previously reported present in the polyaldehyde. The structural components of the algaenan can be further confirmed by considering 2-D HR-MAS data.

The two dimensional NMR spectral data support the concept that the hexane insoluble botryals and the insoluble algaenan are similar in composition. Both the TOCSY (Fig. 8), and HSQC (Fig. 9) data for the algaenan show cross peaks characteristic of the most abundant units in the hexane insoluble botryals. It is interesting to note that contribution from terminal units (both terminal methyl and terminal double bonds) are in roughly the same abundance (determined from the integral of signals in the ^{13}C HR-MAS data, spectrum shown in Fig. 10) in the algaenan and the soluble extract. This strongly suggests the chain lengths in the two materials are very similar, and thus the polymeric nature of the algaenan is likely explained by the mid-chain reticulation of these chains. Comparison of the ^{13}C spectra for the two materials (Fig. 10) reveals the intensities of the signals present in both sample are similar with the exception of (1) the mid-chain unsaturations, which appear approximately twice as intense in the algaenan, and (2) the absence of signals from structure 5 (Fig. 4) in the algaenan.

In addition to signals observed in the extract, the ^{13}C HR-MAS data for the algaenan contains additional signals that are consistent with ester structures, and acetals (various NMR prediction packages predict acetal structures as proposed (Fig. 4, structure 6) to be 101 ppm and 89 ppm for units 6a and 6b, respectively) in roughly equal amounts (Fig. 11). These units suggest the algaenan to be an esterified/acetalated network of the hexane insoluble botryrals. Note that with both these structures methine units adjacent to the oxygen groups would be expected to be present in the HSQC spectrum. Lowering the contour threshold of the 2-D HR-MAS spectrum does indicate peaks that may be consistent with the peaks expected (data not shown), but the crosspeaks are so close to the baseline noise that assignments in the HSQC cannot be made with confidence. However this said, the inherently weak nature of the signals are expected given the nature of HR-MAS NMR. HR-MAS is carried out on swollen solids. Thus, T_2 relaxation will occur very fast, and relaxation during the evolution period of the 2-D experiment will be rapid. However, T_2 relaxation for the methine protons adjacent to the acetal or esters linkers, will be especially enhanced for three reasons. Firstly, the oxygen atom in proximity will provide an excellent T_2 relaxation pathway. Secondly, the rigidity of the algaenan polymer will not be uniform. Terminal functionalities (such as the

terminal unsaturations; note these appear clearly in the HSQC) will have increased molecular motion (as they are at the end of flexible hydrocarbon chains) relative, to the methine groups associated with crosslinking units. The crosslinking units themselves will make the biopolymer locally rigid, and thus the T_2 relaxation will be increased in these locations. Thirdly, solvation will likely be reduced around the reticulations as it will be harder for solvent molecules to penetrate the biopolymer structure, this will further favor T_2 relaxation at these specific sites. Indeed these T_2 effects can be seen in the lineshape of the ^{13}C signals (Fig. 10). The width of the peaks for the ester (and associated methine, see Fig. 10) and acetal signals are greater than those observed for the terminal carbon of main chain structures (note the spectral line width, i.e. peak width at half height, is determined by the reciprocal of T_2 (Cowan, 1997). As argued above this supports the fact that the units are centered in rigid domains and in proximity to oxygen atoms. Both ester and acetal functionalities are relatively unstable in strong acidic conditions, yet they survive the 6 M HCl reflux that is employed in the isolation of the algaenan. We believe that due to the hydrophobic nature of the algaenan the 6 M HCl simply cannot penetrate the algaenan structure to break these bonds. This is supported by studies by Largeau et al. (1986) who also observed "protected esters" in algaenan. Furthermore, acetalated species have also been identified in both the L (Bertheas et al. 1998) and B strains (Okada et al., 1996) of *B. braunii,* but to the authors' knowledge, this is the first time acetalated species have been found in algaenan from the A strain. Gelin et al. (1994b) hypothesize that the algaenan is simply a more condensed or reticulated form of the polyaldehyde that is made insoluble due to oxygen cross-linking. While our data agree with the notion of cross-linking, we find in this study it is not the cross-linking of a linear polyaldehyde that results in algaenan but the reticulation of low molecular weight botryals. Note that if the algaenan in this study were simply a result of the cross-linking of the polyaldehyde, then terminal methyl groups and terminal unsaturations would not be present in the structure, and these are clearly seen in abundance in the HR-MAS spectra for the algaenan studied here. From the NMR spectroscopic data, it is possible to hypothesize an average structure for the algaenan in this study (see Fig. 11). It is important to remember such a structure describes only the average case. From the NMR spectral data it is not possible to predict the exact number of main chain methylene units separating each functionality, which may or may not vary throughout the network, and it is

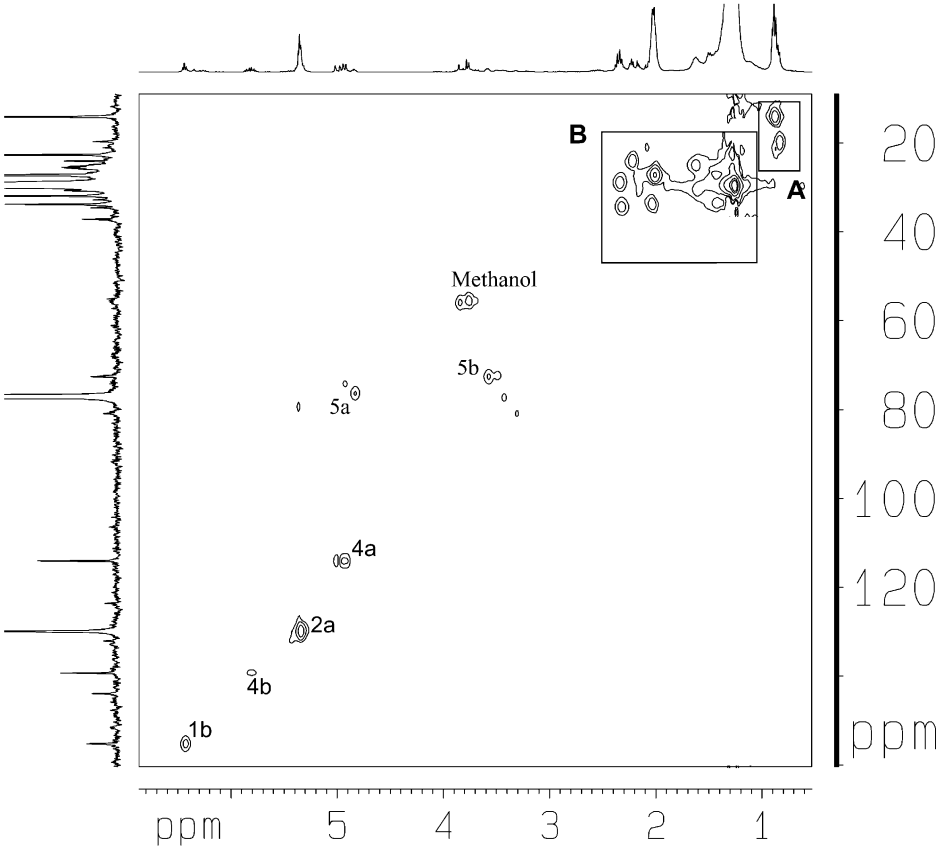


Fig. 5. HMQC spectrum of the hexane insoluble botryals. The numbered labels represent structures summarized in Fig. 4. Region A represents terminal methyl units, and region B methylene (CH_2).

important to note the chain lengths only represent the average, which is likely to vary considerably.

3. Concluding remarks

Through solution state NMR spectroscopy and electrospray MS studies of the soluble hexane insoluble botryals from *B braunii*, and with HR-MAS NMR spectroscopy of the isolated algaenan, it has been possible to advance the structural understanding of the geochemically important biopolymer. We find that the hexane insoluble botryals are consistent with a mixture of unsaturated polyaldehydes, and unsaturated hydrocarbons with an average chain length of about 40 carbons. We find in this study that the algaenan results from the reticulation of low molecular weight chains, similar to those found in the extract. In the algaenan studied here we find that mid-chain cross-linking occurs via ester and acetals to produce the biopolymer algaenan from the low molecular weight botryals. Previous studies (Gelin et al., 1994b) suggest that the algaenan can originate from the reticulation of polyaldehyde chains. While this is a definite possibility in some cases, the similarity of the algaenan structure to the low molecular hexane insoluble botryals in this study suggests it is not the pathway of formation of algaenan in this study. While previous work had focused on *B. braunii* in various stages of growth (Metzger et al., 1993) our studies were based exclusively on this algae grown to a late stationary phase and this may explain why our conclusions differ from previous ones. In addition to the polyaldehyde extract, Dr. Metzger provided samples of the algaenan recovered from the same batch of algae from which the polyaldehyde extract was obtained. While it will be the subject of another manuscript and is somewhat beyond the scope of this paper, the solid-state ^{13}C NMR spectral data suggest that this algaenan sample is different in composition than the one examined in this study. The primary difference is the presence of terminal methyl groups that are, in comparison, substantially reduced in the algaenan supplied by Metzger. This serves to further corroborate the fact that there may be product/precursor relationships for algaenan that are dependent on the growth conditions of *B. braunii*. Further HR-MAS NMR spectroscopic studies of a diverse range of algaenans grown under a variety of conditions will be needed in order determine the range

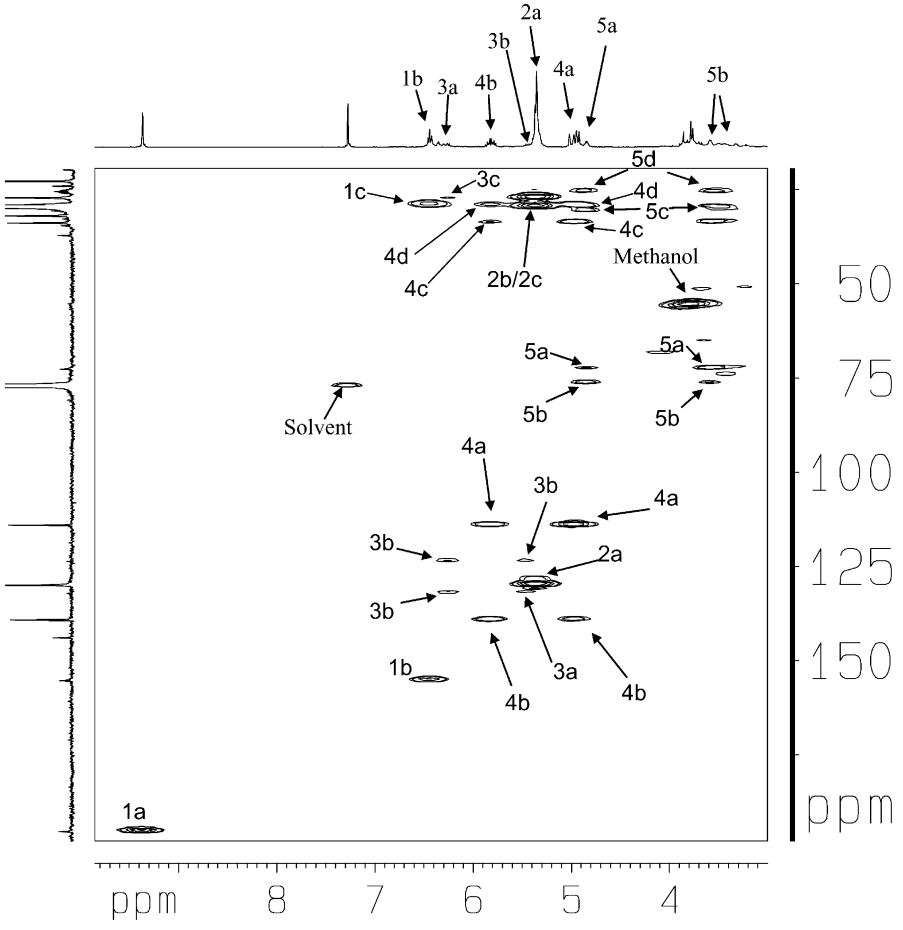


Fig. 6. HMQC–TOCSY spectrum of the hexane insoluble botryals. Labels represent structures summarized in Fig. 4 and discussed in the text.

and details of the pathways of formation for algaenan in the environment.

4. Experimental

4.1. Materials

The alga was grown based on the procedure described previously (Zang et al., 2001). Briefly, the cultures of *B. braunii* race A (obtained from Dr. C. Largeau and originally grown from the UTEX 572 strain that has been under study in the Largeau and Metzger groups, Metzger et al., 1993) were grown in modified CHU medium (Largeau et al., 1980) in 20 l carboys. The culture medium was supplemented with $NaHCO_3$ at a final concentration of 1 mM, representing a typical concentration of bicarbonate in river water. The cultures were grown (110–170 μE m^{-2} s^{-1} light intensity; 12:12 light: dark cycle; 25 °C; > 6 l min^{-1} aeration with 0.2 μm filtered air) to late-stationary phase of growth, with dry weights used to assess growth.

4.2. Algaenan isolation

The *B. braunii* cell suspensions were centrifuged and the aqueous supernatants were removed. The freeze-dried biomass (200 mg) was extracted ultrasonically with methanol (5 × 25 ml) followed by dichloromethane (3 × 25 ml) to remove free lipids. These residues were

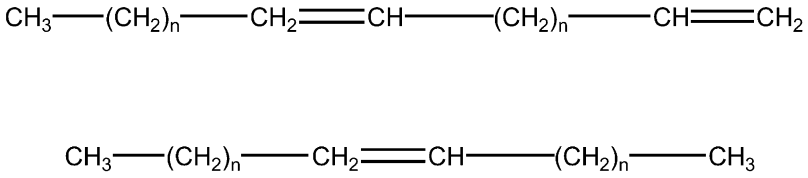


Fig. 7. Example structures for the non-aldehydic portion of the hexane insoluble botryals.

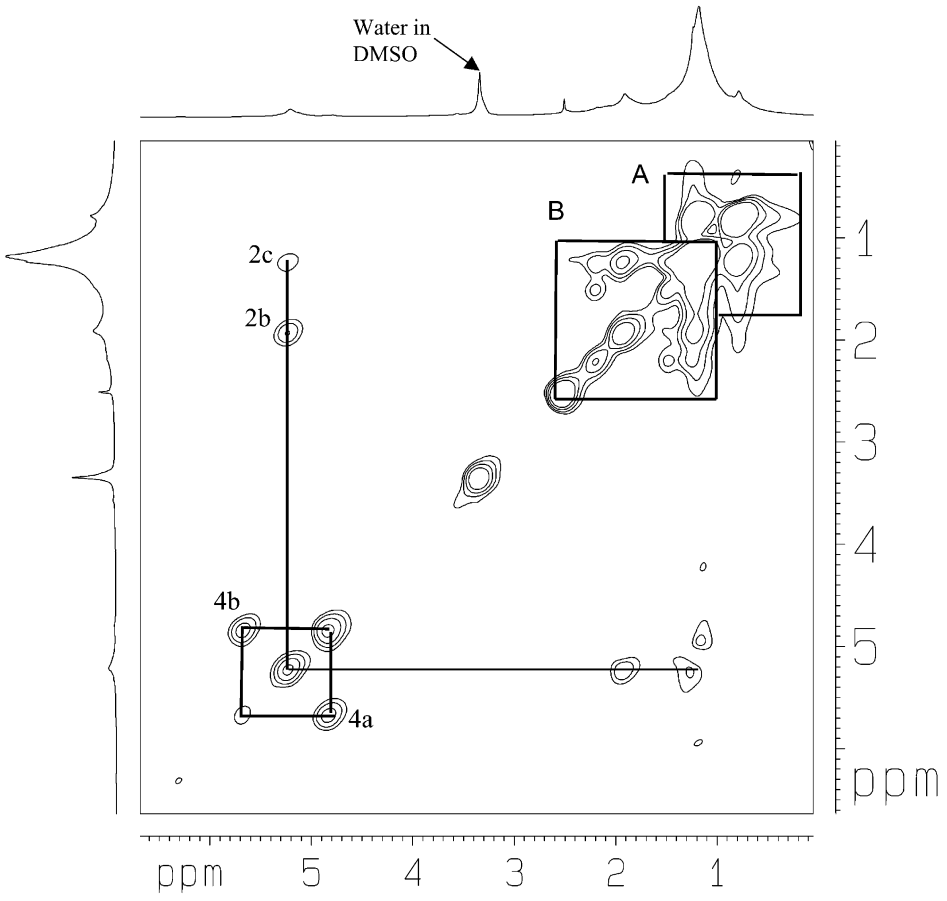


Fig. 8. TOCSY spectrum of the algaenan obtained with the HR-MAS probe. The numbered labels represent structures summarized in Fig. 4 and discussed in the text. Region A represents coupling between main chain methylene and terminal methyl. Region B represents CH_2–CH_2 couplings in various environments.

subsequently extracted ultrasonically with a 1 M KOH solution in methanol (3 × 100 ml) in order to extract esterified organic matter and some of the peptides. Following ultrasonication in water and acidification to pH 3 using 2 M hydrochloric acid, the residues were further extracted ultrasonically with water (2 × 25 ml), methanol (2 × 25 ml) and dichloromethane (25 ml). Subsequently, 4.5% sodium paraperiodate in an acetic acid solution (pH 4.1) was added to the residues to remove carbohydrates (Zang et al., 2001; Zelibor et al., 1998). After a 6 M HCl hydrolysis under reflux for 6 h, the residues were washed with water, freeze-dried and stored at room temperature before analysis.

4.3. Isolation of the hexane insoluble botryals

An extraction based on the procedure described by (Metzger et al., 1993) was employed. Briefly, after the dried biomass of *B. braunii* alga was pre-extracted with hexane (35 ml), a crude fraction was obtained with a subsequent chloroform extraction (35 ml) of the residue. An equivalent volume of methanol added to the chloroform extract yielded a pale green precipitate that was further purified by repeated solution in chloroform and subsequent addition of methanol. After three repetitions of the process, a light yellowish material was obtained. This material was then air-dried. After drying, the fraction that could be redissolved in chloroform we term the ''hexane insoluble botryal fraction''. The portion that remained insoluble constituted the polyaldehyde polymer.

4.4. NMR spectroscopy

HR-MAS NMR experiments were carried out on a Brüker Avance 600 MHz DRX, fitted with a ^{1}H, ^{13}C, ^{15}N HR-MAS probe. Algaenan (~30 mg) was swollen in DMSO-d_6 and packed into a 4 mm Zirconium Rotor with a Kel-F cap. Proton spectra were collected using 256 scans, using a CPMG sequence with a delay of 40 μs to help reduce baseline roll. 1-D ^{13}C NMR spectral data (32,000 scans) were acquired using inverse gating, and a 12 s recycle delay. The resulting spectrum was processed with an exponential multiplication with 20 Hz line broadening. TOCSY (128 scans, TD (F1) 1024, TD (F2) 512) data were acquired using time proportional phase incrementation (TPPI), and a mixing time of 40 ms. The spectrum was processed using a gaussian function (line

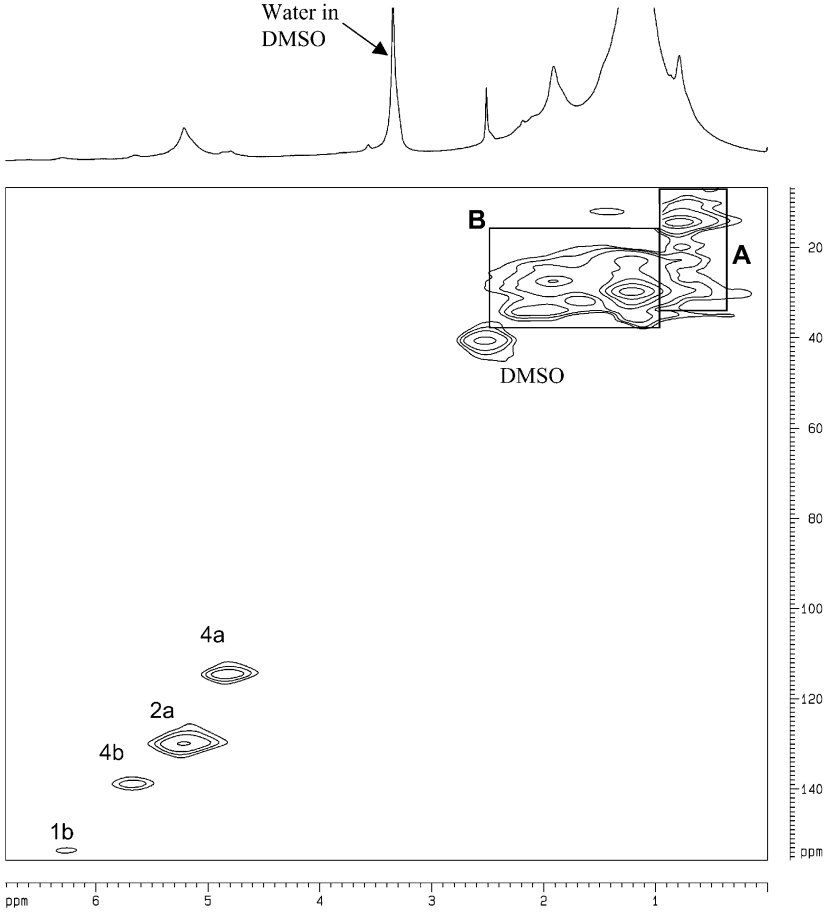


Fig. 9. HSQC spectrum of algaenan obtained with the HR-MAS probe. The numbered labels represent structures summarized in Fig. 4 and discussed in the text. Region A represents terminal methyl units, and region B methylene (CH_2) units.

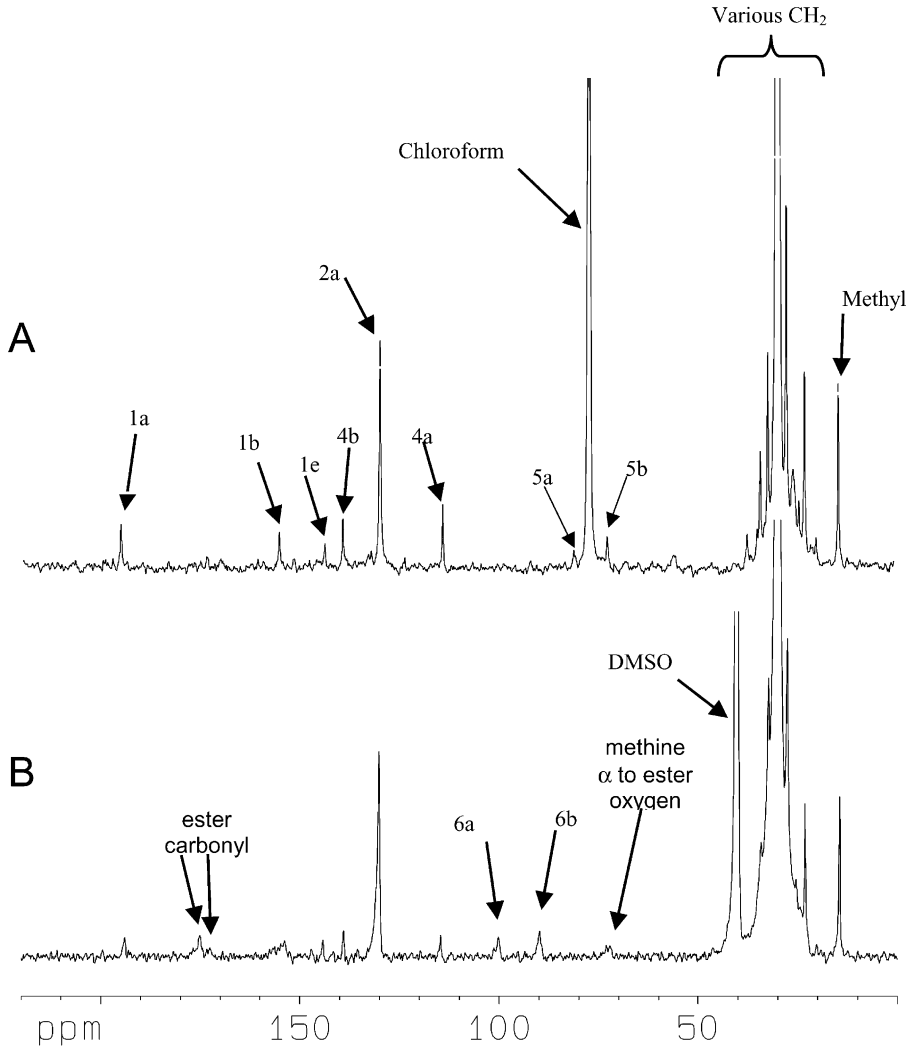


Fig. 10. (A) ^{13}C solution NMR spectrum of the hexane insoluble botryals; (B) ^{13}C HR-MAS NMR spectrum of the algaenan. For assignments see Fig. 4.

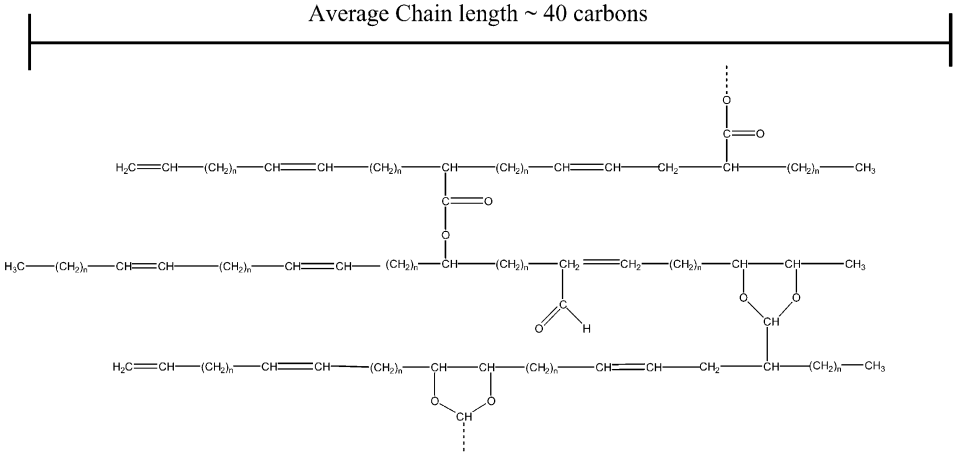
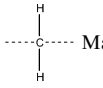
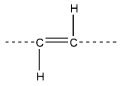
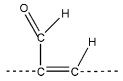
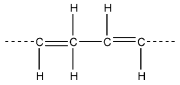


Fig. 11. An average structural representation of the algaenan in this study. It is not possible to determine the number of connecting methylene units in each case (n), however the total chain length can be shown on average to be ~40 units (see text).

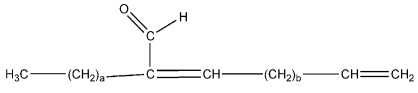
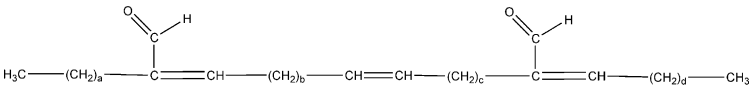
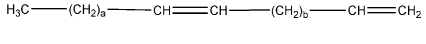
broadening −1, gaussian broadening 0.001) and a zero filling factor of 2 in both dimensions. HSQC [128 scans, TD (F1) 1024, TD (F2) 512, ^{1}J (^{1}H–^{13}C) 145 Hz] data were acquired using echo/antiecho-TPPI gradient selection with decoupling during acquisition. F1 was processed with a sine-squared function with phase shift of 90° while F2 was processed with a gaussian broadening of 0.005 and line broadening of −1.

Table 1
Integral values from the ^{13}C NMR spectrum of the hexane insoluble botryals

Structural fragment	% of total carbon signal	Estimated average ratio of major units present[a]
 Main chain CH_2 groups	88	35
 Terminal CH_3 groups	3.2	1.5
	4.4	1
	2.1	0.5
	2.0	0.33
Structures 5 and 6 (see Fig. 4) and other trace structures	<0.5%	Not considered
	<0.5%	Not considered

[a] Normalized on a basis of the number of carbons in each structure shown and presented as the ratio of units that would be present in chain of 40 carbons (see text for full details).

Table 2
Example structural fragments identified by Q-TOF mass spectrometry

Structure	No. of methylenes (a + b + c + d)	M + 1/z value	Actual mass M + 1
	24	433.41	433.43
	26	461.43	461.47
	27	475.43	475.49
	29	503.44	503.52
	30	517.47	517.53
	31	531.50	531.54
	32	545.51	545.56
	33	559.51	559.57
	34	573.52	573.59
	30	571.49	571.54
	31	585.51	585.55
	32	599.50	599.57
	33	613.51	613.59
	39	697.64	697.68
	41	725.68	725.71
	26	433.48	433.47
	30	489.50	489.53
	32	517.51	517.56

Solution NMR spectroscopic data were acquired using a Brüker Avance 400 MHz NMR fitted with a QNP ^{1}H, ^{13}C, ^{15}N, ^{31}P probe. Sample (~30 mg) was dissolved in $CDCl_3$ (0.75 ml). To ensure quantitative conditions 1-D ^{13}C NMR spectra (1024 scans) were acquired using inverse gating, and a 12 s recycle delay, the resulting spectrum was processed with an exponential multiplication with 20 Hz line broadening. PENDANT (1024 scans) data were acquired using a 2 s recycle delay and a ^{1}J (^{1}H–^{13}C) of 145 Hz, and processed with 5 Hz line broadening. 1-D Proton NMR (128 scans) were carried out with 2 s recycle delay and processed with 0.3 Hz line broadening. TOCSY spectra [64 scans, TD (F1) 1024, TD (F2) 512] were acquired using a 80 ms mixing time, with TPPI. Processing was carried out using a sine-squared function with phase shift of 90° in both dimensions. HMQC [128 scans, TD (F1) 1024, TD (F2) 512, ^{1}J (^{1}H–^{13}C) 145 Hz] were acquired using a BIlinear Rotation Decoupling (BIRD) pulse train, and TPPI. F1 was processed with a sine-squared function with phase shift of 90° while F2 was processed with a gaussian broadening of 0.01 and line broadening of −1. NOESY spectra (256 scans) were collected with a 200 ms mixing time, TPPI, TD (F1) 1024, TD (F2) 256 and processed with sine-squared functions with phase shift of 90° in both dimensions. 2-D HMQC–TOCSY spectra [128 scans, 1024, TD (F2) 512, ^{1}J (^{1}H–^{13}C) 145 Hz] were acquired with 80 ms mixing time, and processed with sine-squared functions with phase shift of 90° in both dimensions. ''Diffusion gate'' experiments were collected using a BPPLED pulse sequence. Scans (16) were collected using 2.5 ms sine shaped gradient pulses (5 ms bipolar pulse pair) and a diffusion delay of 100 ms. Gradient strength ranged from 1.3 to 42.5 gauss cm^{-1} in three linear increments.

4.5. Electrospray ionization mass spectrometry

All experiments were performed on a Micromass Q-Tof™ II (Micromass, Wythenshawe, UK) mass spectrometer equipped with an orthogonal electrospray source (Z-spray) operating in positive ion mode. Polyalanine and alanine were used for mass calibration for a calibration range of m/z 100–2000. The hexane insoluble botryals were prepared in a solution containing 75% $CHCl_3$ and 25% MeOH infused into the electrospray source at a rate of 5–10 μl/min. Optimal ESI conditions were: capillary voltage 3000 V, source temperature 110 °C and a cone voltage of 60 V. The ESI gas was nitrogen. Q1 was set to optimally pass ions from m/z 100 to 2000 and all ions transmitted into the pusher region of the TOF analyzer were scanned over m/z 200–2000 with a 1 s integration time. Data were acquired in the continuum mode until acceptable averaged data was obtained.

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