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Mid-chain branched alkanoic acids from "living fossil" demosponges: a link to ancient sedimentary lipids?

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Abstract

The lipid assemblages of the "living fossil" stromatoporoid *Astrosclera willeyana* (Great Barrier Reef) and the demosponge *Agelas oroides* (Mediterranean Sea) were investigated. Large amounts of branched carboxylic acids are present in the sponges studied. These compounds include terminally branched carboxylic acids (*iso-*/*anteiso-*) and abundant mid-chain branched carboxylic acids (MBCA) which are characterized by an intriguing variety of structural isomers present in the C_{15}–C_{25} range. The most prominent MBCA are comprised of isomeric methylhexadecanoic acids and methyloctadecanoic acids. A second cluster of MBCA includes methyldocosanoic acids and methyltetracosanoic acids, but other homologues are also present. Methyl branching points were generally observed between the ω5- and ω9-positions. These complex isomeric mixtures apparently derive from symbiotic bacteria living exclusively in demosponges. Comparison with hydrocarbon compositions of ancient carbonates reveals evidence that the MBCA found are potential lipid precursors of mid-chain branched monomethylalkanes often observed in fossil sediments and oils. As a working hypothesis, we suggest that their bacterial source organisms have been widespread in the geological past, and are found "inherited" in the protective environment of distinctive sponge hosts in recent marine ecosystems. Furthermore, both sponges contain abundant linear, long-chain C_{24}–C_{26} dienoic "demospongic" acids. The demospongic acid distribution and the presence of phytanic acid in *A. willeyana* match the patterns found in *A. oroides* and other members of the Agelasida. These findings confirm the systematic position of *A. willeyana* within this demosponge taxon.

Keywords: Biomarkers; mid-chain branched alkanoic acids; isoprenoic acids; mid-chain branched alkanes; demosponges

1. Introduction

Sponges are ancestral multicellular organisms consisting of only a few specialised cell types and are lacking a central nervous system. They are abundant in nearly all contemporary aquatic environments and have developed an exciting variety of strategies for competing even under unfavourable ecological conditions. Phylogenetically, sponges represent the base of metazoan evolution. Their palaeontological record ranges back to Precambrian times (Steiner et al., 1993; Reitner and Mehl, 1995).

It has been well established that the porifera are one of the richest phyla in toxicogenetic species. This may be due to their sessile, exposed habit and their great potential to be overgrown by competitive organisms. Sponges have therefore attracted the increasing attention of organic chemists and pharmacologists, in

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particular in the anti-cancer, -bacterial and -viral research fields (see Garson, 1994; Munro et al., 1994 for reviews).

In addition to their content of a wide range of natural products, sponges turn out to be rich sources of unusual lipids which play a primary structural and functional role in their plasma membranes. Since the early studies by Litchfield et al. (1976), several investigations have proven the presence of long-chain, unsaturated carboxylic acids in a variety of sponges from different marine environments, all of which are members of the Demospongiae. These compounds display characteristic unsaturation patterns and may, in some cases, exhibit terminal as well as mid-chain branching. They may occur as mono-, di- and trienoic compounds (tetraenoic and pentaenoic exceptionally) and cover a relatively broad carbon-number range, typically between C_{24} and C_{30} (e.g. Walkup et al., 1981; Christie et al., 1992; Carballeira and Emiliano, 1993; Duque et al., 1993; Garson et al., 1994). Other organisms apparently lack these characteristic compounds which have thus been introduced as "demospongic" acids into the literature.

In many sponge species, a significant portion of their total fatty acid content can be attributed to bacterial sources (Gillan et al., 1988). Whereas the intracellular matrix of most terrestrial organisms is sterile, sponges may host vast amounts of prokaryotic organisms located within this matrix (Wilkinson, 1978a,b,c; Gillan et al., 1988). These bacteria account for up to 60% of the total biomass, and exhibit peculiar characteristics which differ strikingly from those observed in the ambient sea water by means of morphology and physiology (Wilkinson, 1978a,b,c; Preston et al., 1996; Schumann-Kindel et al., 1996). In fact, most sponges represent closely associated eukaryotic–prokaryotic biocommunities, and the host–symbiont interaction strongly affects the biology and biochemistry of the organism as a whole.

In this study, we report the occurrence of unique patterns of carboxylic acids from the stromatoporoid coralline sponge *Astrosclera willeyana* and the demosponge *Agelas oroides*. These compounds are most likely derived from specific bacterial symbionts. The molecular characteristics are characterized and compared with those of related organic compounds enclosed in fossil sediments and oils. *A. willeyana* shows close morphological affinities to the much more widespread, reef building stromatoporoids of the Mesozoic and the Palaeozoic (Stearn and Picket, 1994). It represents an ancient line within the phylum porifera and can thus be regarded as a "living fossil". However, its exact systematic position among the porifera has not been fully elucidated by conventional optical techniques. Therefore, a second major goal of the present study was the use of lipid markers as a tool for a chemotaxonomical classification of *A. willeyana*.

2. Materials and analytical methods

2.1. Samples

A. oroides was collected from the Mediterranean Sea at water depth of 20 m near Banyuls sur Mer. The noncalcified demosponge has a heavy spongin skeleton characterized by prominent thick fibres in which the spicules are fixed (cored spicules). It possesses acanthostyle megascleres exclusively; microscleres are absent. *A. oroides* is a typical member of the taxon Agelasida.

A. willeyana was collected by SCUBA-diving at the outer Pearl Reef (Great Barrier Reef, Australia). The sponge (~8 cm diameter) was living in a small reef cave at a water depth of 20 m. The age of the individual could be estimated to be >200 years (Wörheide et al., 1996).

A. willeyana has a primary spicular skeleton consisting of acanthostyle megascleres and an aragonitic secondary basal skeleton. The soft tissue covers only the ontogenetic younger parts (3–5 mm) of the calcareous basal skeleton (stromatoporoid grade of soft tissue organisation). Microscopic analyses reveal that *A. willeyana* hosts abundant bacteria which are estimated to comprise 30–50% of the soft tissue biomass (up to 70% in some areas, Wörheide, 1997). The exact identity of these organisms and their particular function within the sponge is still under investigation. Despite its outstanding morphology and mode of organisation, *A. willeyana* appears to be taxonomically related to the demosponge taxon *Agelasida* (Reitner, 1992).

2.2. Preparation and analytical methods

The thawed sponges were cleaned mechanically from all visible nonsponge debris adhering to the outer surfaces. *A. oroides* was cut into small pieces. The living tissue of *A. willeyana* was carefully separated from the basal skeleton by scraping off the outer 1–2 mm surface layer. After saponification of the samples in 6% KOH in CH_3OH, the supernatant was decanted and the residue extracted by ultrasonication in CH_2Cl_2/CH_3OH (3:1; v:v) until the solvent became colourless. Subsequently, the combined supernatants were extracted with CH_2Cl_2 vs. water (pH 2). The organic compounds of the CH_2Cl_2 phase were fractionated by column chromatography (Merck silica gel 60, 70 mm; 15 mm i.d.). The acidic fraction was obtained with CH_2Cl_2/CH_3OH (3:1; v:v; 25 mL) as elution agent. The respective carboxylic acids were converted to their methyl esters with diazomethane and analyzed by gas

Table 1
Relative concentration of carboxylic acids in *Agelas oroides* and in *Astrosclera willeyana* (% of total carboxylic acid fractions)

Carboxylic acid		*A. oroides*	*A. willeyana*
Tridecanoic	13	0.1	tr
12-Methyltridecanoic	i-14	0.3	tr
Tetradecanoic	14	3.5	2.8
Methyltetradecanoic (MBCA)	m-14	1.7	
13-Methyltetradecanoic	i-15	2.9	2.1
12-Methyltetradecanoic	ai-15	2.1	0.9
Pentadecanoic	15	1.8	0.3
Methylpentadecanoic (MBCA)	m-15	1.6	tr
14-Methylpentadecanoic	i-16	0.7	1.7
13-Methylpentadecanoic	ai-16	0.3	tr
Hexadecenoic	16:1	0.7	1.9
Hexadecanoic	16	5.1	11.3
Methylhexadecanoic (MBCA)	m-16	9.7	3.4
15-Methylhexadecanoic	i-17	1.3	2.2
14-Methylhexadecanoic	ai-17	1.6	1.1
Heptadecanoic	17	1.0	0.8
Methylheptadecanoic (MBCA)	m-17	0.5	tr
16-Methylheptadecanoic	i-18	tr	tr
3,7,11,15-Tetramethylhexadecanoic (phytanic)	P	7.1	2.0
Octadecadienoic	18:2		0.7
Octadecenoic	18:1	0.5	1.8
Octadecanoic	18	4.3	5.5
Methyloctadecenoic (MBCA)	m-18:1	1.0	tr
Methyloctadecanoic (MBCA)	m-18	26.1	8.8
17-Methyloctadecanoic	i-19	tr	0.2
16-Methyloctadecanoic	ai-19	tr	0.1
Nonadecenoic	19:1	0.4	0.8
Nonadecanoic	19	0.4	0.1
18-Methylnonadecanoic	i-20	0.2	0.4
17-Methylnonadecanoic	ai-20	0.2	0.4
Eicosanoic	20	0.3	0.7
Methyleicosanoic (MBCA)	m-20	1.9	2.2
19-Methyleicosanoic	i-21	tr	2.8
18-Methyleicosanoic	ai-21	0.1	2.2
Heneicosanoic	21	0.1	0.1
Methylheneicosanoic (MBCA)	m-21	1.7	tr
Docosanoic	22	tr	0.1
Methyldocosanoic (MBCA)	m-22	2.4	1.3
21-Methyldocosanoic	i-23	0.3	tr
20-Methyldocosanoic	ai-23	0.1	tr
Methyltricosanoic (MBCA)	m-23	0.8	tr
5,9-Tetracosadienoic	24:2	4.6	11.2
Methyltetracosanoic (MBCA)	m-24	1.5	1.8
5,9-Pentacosadienoic	25:2	5.0	13.6
5,9-Hexacosadienoic	26:2	2.7	14.4
Totals			
Linear carboxylic acids (without demospongic acids)		17.8	26.0
Iso-/anteiso carboxylic acids		10.1	14.1
Mid-chain branched carboxylic acids (MBCA)		48.9	17.5
Demospongic acids		12.3	39.2

chromatography (GC) and combined gas chromatography–mass spectrometry (GC–MS). The identification of the compounds was based on comparison of the mass spectra and of GC retention times with those of published data and of reference compounds.

Complementary to the sponge samples, several massive carbonates were analyzed for the presence of related biomarker compounds in fossil materials. These rocks included (1) a Jurassic reef carbonate from the Swabian Alb, Germany, (2) a limestone from the alpine Triassic Cassian beds and (3) a microbialite from the Pleistocene Searles Lake, U.S.A. (see Geological and Paleobiological Implications (Section 3.6) for details). From each sample, ~100 g were taken and broken to small pieces. In order to release the organic matter as completely as possible from the microcrystalline rock matrix, 200 mL of doubly-distilled water were added and the carbonate was slowly dissolved by dropwise adding 2 N HCl. To avoid artifacts caused by excessive acidification, the reaction was stopped when ca. 80% of the carbonate had been dissolved. The remaining carbonate pieces were removed and the residue was separated from the solution by centrifugation. After washing with double-distilled water > pH 5, the samples were dried and extracted ultrasonically in CH_2Cl_2/CH_3OH (3:1; v/v) until the extract became colourless. The hydrocarbon fraction was separated from the combined organic extract by column chromatography (Merck silica gel 60; 50 mm × 15 mm i.d.) with 10 mL *n*-hexane and was analyzed by GC/GC–MS.

GC analyses were performed on a Carlo Erba Fractovap 4160 gas chromatograph equipped with a 30 m fused silica capillary column (DB5, J&W Scientific, 0.3 mm i.d., 0.25 μm film thickness). Injector: "on column". Detector: FID. Carrier gas: H_2. Temperature program: 80°C isothermal for 3 min; from 80 to 300°C at 4°C min^{-1}; isothermal for 30 min. The GC–MS system was a Finnigan MAT CH7A mass spectrometer interfaced to a Carlo Erba 4160 gas chromatograph equipped with a 50 m fused silica capillary column (DB5-HT, J&W Scientific, 0.3 mm i.d., 0.25 μm film thickness). Carrier gas: He. Temperature program: 80°C isothermal for 5 min; 80–300°C at 4°C min^{-1}; 300°C isothermal for 20 min. Quantification was carried out by GC peak-area integration relative to an internal standard of known concentration (5α(H)-cholestane).

2.3. Synthesis of 12-methyloctadecanoic acid methyl ester

1 g of 12-hydroxy-octadecanoic acid was esterified using two equivalents of 1 M TMCS (trimethylchlorosilane; Pierce) in methanol. After evaporation of the reagents the obtained products were purified using a silica gel column (Merck silica gel 60; 2.5% ether in dichloromethane). About 900 mg of 12-hydroxyoctadecanoic acid methyl ester was oxidised using excess pyridiniumdichromate (Aldrich) in 20 mL dry dichloromethane. After 6 h of stirring in a nitrogen atmosphere at room temperature the mixture was filtered over silica gel (Merck, silica gel 60). Subsequently, the resulting product was converted to a 12-methylene derivative using Wittig reagent. 15 equivalents of butyllithium (1.6 M in hexane; Aldrich) were slowly added to a mixture of 20 equivalents of methyltriphenylphosphonium bromide (Aldrich) in 10 mL dry tetrahydrofuran. After 15 min, 1 equivalent of the 12-oxooctadecanoic acid methyl ester was added to the orange coloured reaction mixture which was subsequently boiled under reflux (stirring). After 3 h, the reaction was quenched with water and the products were extracted with ether. The 12-methylenecarboxylic acid methyl ester was purified by silica gel chromatography (Merck, silica gel 60). For reduction of the double bond, palladium on charcoal (Merck) was added to the 12-methyleneoctadecanoic acid methyl ester dissolved in ethyl acetate. The mixture was vigorously stirred under a hydrogen atmosphere for 4 h, filtered over silica gel and the 12-methyloctadeanoic acid methyl ester was purified using thin layer chromatography and dichloromethane as developer (0.5 mm, Merck, silica gel 60).

Coelution experiments were performed on fused silica capillary columns: HP-1 (Hewlett Packard; 50 m, 0.32 mm i.d., 0.17 μm film thickness) and DB5-HT (J&W Scientific; 60 m, 0.32 mm i.d., 0.1 μm film thickness); GC-temperature program: 60°C (1 min), 60–150°C (10°C/min), 150–250°C (1°C/min), 250–320°C (10°C/min). Coelution experiments by GC–MS: temperature program: 80°C (5 min), 80–150°C (10°C/min), 150–250°C (1°C/min), 250–320°C (10°C/min).

3. Results and discussion

3.1. Linear short-chain carboxylic acids

Linear, short-chain carboxylic acids with 12–22 carbon atoms comprise 26.0 and 17.8% of the carboxylic acid fractions in *A. willeyana* and *A. oroides*, respectively (Table 1). They are characterized by large amounts of straight chain, even carbon-numbered alkanoic acids, with $C_{16:0}$ (palmitic acid) and $C_{18:0}$ (stearic acid) predominating. Linear unsaturated acids are less abundant and mainly consist of monounsaturated $C_{16:1}$ and $C_{18:1}$ homologues. These compounds may either derive from the sponge cells themselves or from associated prokaryotic symbionts. Cell fractionation experiments have shown that linear short chain acids are present in both symbiont and sponge cells,

where as enhanced levels were observed in the bacterially derived matter (Zimmerman et al., 1989, 1990). Therefore, the origin of these compounds in the sponges studied cannot definitely be assigned.

3.2. Long-chain unsaturated (demospongic) acids

Large amounts of demospongic acids are present in the carboxylic acid fractions of *A. willeyana* (39.2%) and *A. oroides* (11.2%). The main demospongic acids are the 5,9-tetracosadienoic ($C_{24:2}$), 5,9-pentacosadienoic ($C_{25:2}$) and 5,9-hexacosadienoic ($C_{26:2}$) acids which appear in very similar distributions in both sponges (Table 1). Only trace amounts of other demospongic acids were found.

Demospongic acids are constituents of the sponge cell membranes and their occurrence is not influenced by the presence of bacterial symbionts (Lawson et al., 1988). It has been shown that sponges synthesise demospongic acids via elongation of external carboxylic acid precursors followed by subsequent desaturation commonly starting at the Δ^5 or the Δ^9 position (e.g. Walkup et al., 1981).

Previous studies of demospongic acids revealed considerable variation of their molecular characteristics with respect to the species studied. Although care must be taken when applying single biochemical features for chemotaxonomical considerations, the presence of the three consecutive C_{24}, C_{25} and C_{26} homologues with $\Delta^{5,9}$ unsaturation as the principal demospongic acids appears to be a typical feature of the Agelasida (Carballeira et al., 1987; Carballeira and Emiliano, 1993; Duque et al., 1993) but is rarely observed in other sponges (Garson et al., 1994). Combined with additional biomarker evidence like the presence of specific sterols and brominated pyrrole derivatives (Thiel, 1997) this pattern may be taken as a clear hint for a taxonomic position of *A. willeyana* within the Agelasida.

3.3. Isoprenoic acids

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is present in *A. willeyana* (2.0%) and *A. oroides* (7.1%) in significant amounts (Table 1).

Isoprenoic acids have been reported from several demosponges (e.g. Ayanoglu et al., 1982; Barnathan et al., 1992; Christie et al., 1992; Carballeira and Emiliano, 1993; Garson et al., 1994). However, they are not ubiquitously observed within the porifera. If isoprenoic acids are present in a sponge, their occurrence is commonly confined to either phytanic acid or 4,8,12-trimethyltridecanoic acid. With one known exception (Christie et al., 1992), both compounds do not cooccur in the same sponge and have thus been regarded as species-selective (Carballeira et al., 1987). As the presence of phytanic acid appears to be another common feature of agelasid demosponges (*Agelas dispar*, Carballeira et al., 1987; *Agelas* sp., Carballeira and Emiliano, 1993; *Agelas axifera*, Löwenberg, unpublished results), our observation further supports a taxonomic relationship of *A. willeyana* with the *Agelasida*.

It should be noted that the prominent occurrence of isoprenoic acids in certain sponges implies the potential of "primitive" metazoans as a biological source for functionalized isoprenoids. This finding may affect the interpretation of fossil isoprenoic acids and their corresponding hydrocarbons which are ubiquitously found in ancient sediments and oils.

However, a direct biosynthesis of isoprenoic acids as functional lipid constituents has been proven neither for sponges nor for any other living organism. Although the location of isoprenoic acids within the sponge cell membranes has been clearly demonstrated (Lawson et al., 1988; Garson et al., 1994), it is not yet known whether these compounds are produced *de novo* or are metabolites from the modification of dietary precursors, i.e. chlorophyll derived phytyl units. An origin of sponge isoprenoids from the metabolism of chlorophyll a derived from cyanobacterial symbionts has been discussed (Gillan et al., 1988). For *A. willeyana*, such an origin can be excluded due to the observed absence of phototrophic organisms in the specimen studied.

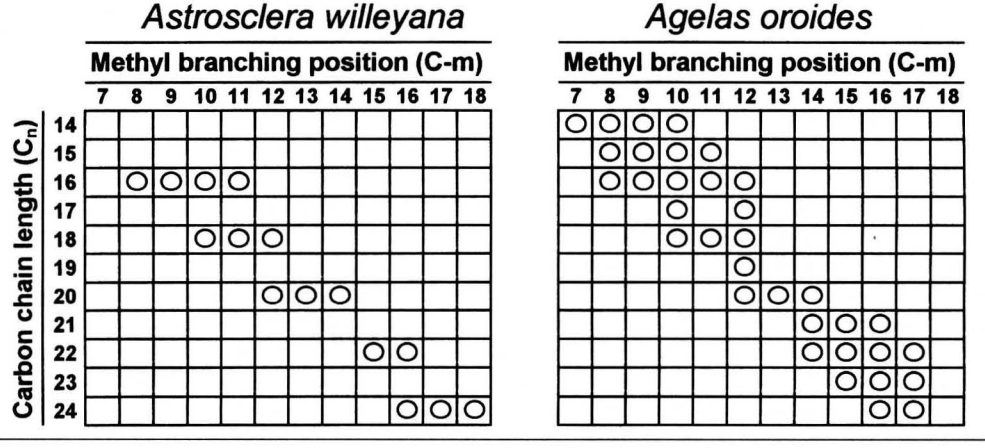
3.4. Iso- and anteiso-carboxylic acids

Carboxylic acids showing methyl branching at the ω2- and ω3-positions are referred to as *iso*- and *anteiso*-acids (*i*-/*ai*-). They comprise 14.1 and 10.1% of the total carboxylic acid fractions of *A. willeyana* and *A. oroides*, respectively (Table 1).

In both sponges, these acids are present over a broad range of carbon chain lengths (Table 1). Particularly large amounts are observed for the *i*-/*ai*-C_{15} and *i*-/*ai*-C_{17} homologues which clearly exceed those of their neighbouring linear homologues. A remarkable feature is the prominent occurrence of the unusual *i*-/*ai*-C_{21} acids in *A. willeyana* (5%).

I- and *ai*-acids are widespread lipid constituents of anoxygenic bacteria (for a review see Kaneda, 1991) but have not been reported from cyanobacteria (e.g. Cohen and Vonshak, 1991). They have also been found in a variety of other organisms including molluscs, fungi and marine phytoplankton (see Perry et al., 1979, and references cited therein). Nevertheless, because they are generally observed in much lower concentrations in other organisms than in bacteria, they are regarded as molecular markers for bacterially derived organic matter (Leo and Parker, 1966; Cooper

Table 2
MBCA isomers/homologues identified in *Astrosclera willeyana* and in *Agelas oroides*. Circles mark the presence of individual MBCA as unambigously determined by GC–MS. Trace compounds revealing faint mass spectra are not included



and Blumer, 1968; Cranwell, 1973, 1974; Grimalt and Albaigés, 1990).

Regarding porifera, *i*- and *ai*-carboxylic acids are abundant in the extracts of virtually all symbiont bearing species and can be taken as a good measure for the portion of bacterially derived compounds among the lipids of the total sponge (Gillan et al., 1988).

3.5. Mid-chain branched carboxylic acids (MBCA)

The most striking feature of the carboxylic acid inventories of the two sponges is the abundance of mid-chain monomethylated carboxylic acids (MBCA). In total, these compounds account for 17.5 and 48.9% of the carboxylic acid fractions in *A. willeyana* and *A. oroides*, respectively (Table 1). Based on their mass spectrometric properties, an intriguing variety of structural isomers over a wide range of carbon chain lengths was observed in *A. willeyana* and *A. oroides* (Table 2).

The separation of single MBCA is only in part achieved by routine GC-analyses. Partial resolution was obtained for the homologues of low molecular-weight, namely for the methyl-$C_{14:0}$ and the methyl-$C_{15:0}$ series present in *A. oroides* (see enlarged section in Fig. 1). MBCA of increased chain length were typically observed as a single, slightly broadened peak eluting at >0.5 equivalent chain length (ECL) units less than their corresponding linear counterparts. Nevertheless, the presence of each MBCA isomer can be deduced from the occurrence of characteristic ion fragments resulting from chain cleavage next to the respective branching point. Mass spectra showing the fragmentation behaviour of individual isomers are given in Fig. 2 for some selected MBCA. Isomer identifications were further confirmed by coinjection with an authentic reference compound (Fig. 3).

In both sponges, MBCA cover a carbon-number range from C_{15} to C_{25}. The site of methyl branching is, in each case, located between the ω5- and ω9-positions. The most prominent MBCA in the short chain range are the methylhexadecanoic acids (3.4 and 9.7%) and methyloctadecanoic acids (8.8 and 26.1% for *A. willeyana* and *A. oroides*, respectively; Table 1). An exciting feature of *A. oroides* is the occurrence of a particularly broad spectrum of branching sites and the presence of significant concentrations of MBCA over the entire carbon-number range from C_{15} to C_{25}. In this sponge, up to five isomers of a given homologue were observed, together accounting for a total of 33 different MBCA (Table 2). In *A. willeyana*, 15 MBCA isomers were characterized. In this sponge, only minor amounts of additional homologues accompany the prominent methylhexadecanoic and methyloctadecanoic acids.

These complex patterns are in contrast to those so far reported from natural samples, since previous reports on the occurrence of MBCA in marine organisms and sediments refer to the presence of single isomers only.

Individual MBCA have been observed in several pure cultured prokaryotes, for example 10-methylhexadecanoic acid in *Desulfobacter* sp. (Dowling et al., 1986). 11-methyloctadec-11-enoic acid was found in the soil bacterium *Rhizobium leguminosarum* (Orgambide et al., 1993). A prominent occurrence of MBCA in prokaryotes is observed within the *Actinomycetales* which commonly show abundant 10-methylhexadecanoic acid and/or 10-methyloctadecanoic acid (Campbell and Naworal, 1969; Ballio and

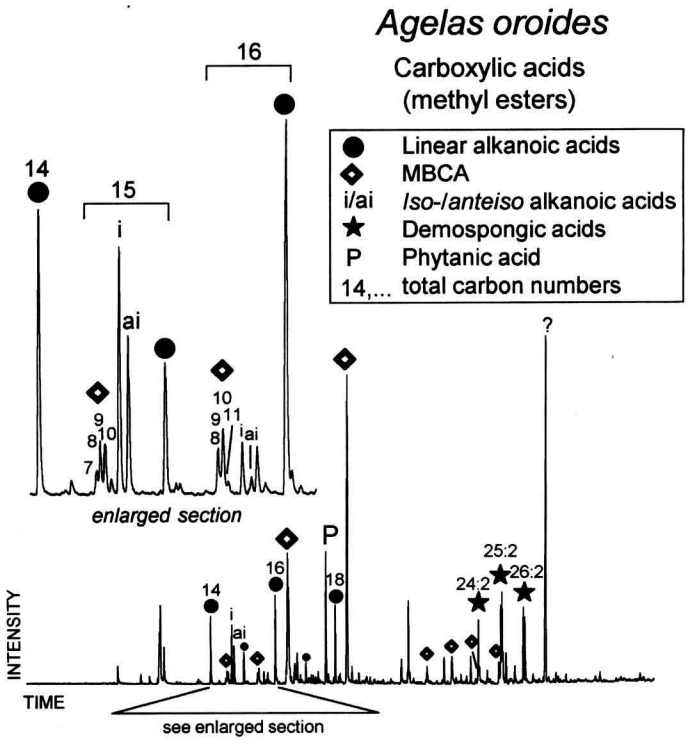


Fig. 1. Gas chromatogram displaying the total carboxylic acid fraction (methyl esters) obtained from *Agelas oroides*. The presence of partially resolved, complex isomer mixtures of methyl-$C_{14:0}$ and methyl-$C_{15:0}$ MBCA is shown in the enlarged section. Small numbers indicate the site of methyl branching (e.g. "10" = 10-methyltetradecanoic and 10-methylpentadecanoic acid, respectively).

Barcellona, 1971; Larsson et al., 1980; Brown et al., 1985). Furthermore, 10-methyloctadecanoic acid (tuberculostearic acid) is a typical constituent of mycobacteria, probably reflecting their close taxonomic relationship with the order *Actinomycetales* (Julák et al., 1980; Larsson et al., 1980; Schlegel, 1985; Fourche et al., 1990).

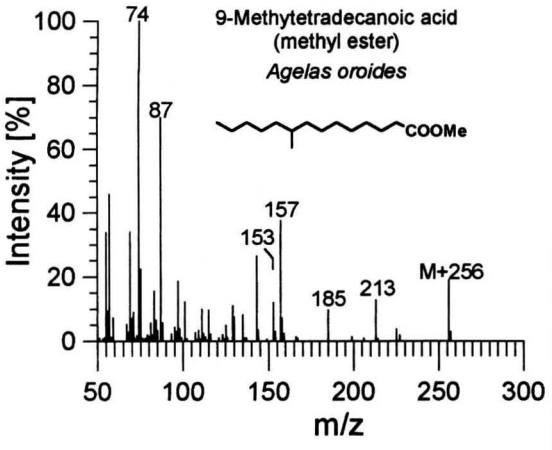
A bacterial origin is also proposed for mid-chain branched carboxylic acids which have previously been observed among the lipids of demosponges (Walkup et al., 1981; Carballeira and Reyes, 1990; Duque et al., 1993; Barnathan et al., 1994). According to the known presence of MBCA in bacteria, these compounds are generally attributed to nonphototrophic symbiont lipids rather than to direct synthesis by the respective sponge (e.g. Gillan et al., 1988, Garson et al., 1994).

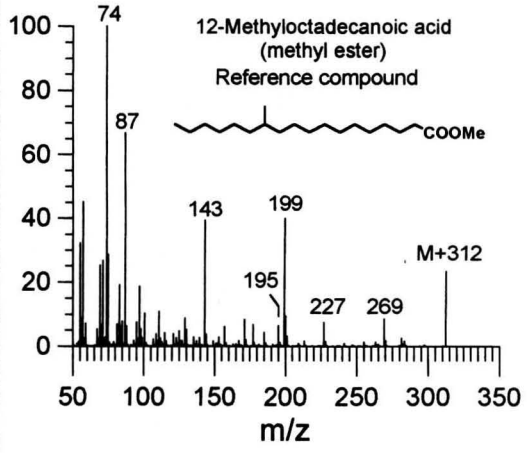
In general, previous studies report the presence of single MBCA isomers, rather than of complex mixtures as observed in the present study. The only cooccurrence of MBCA isomers, of 9- and 10-methylhexadecanoic acids and of 11- and 12-methyloctadecanoic acids, has been reported from four species of reef dwelling demosponges (Gillan et al., 1988). Nevertheless, our results obtained from *A. willeyana* and *A. oroides*, as well as from several other demosponges (Thiel, 1997; Löwenberg, unpublished results), strongly suggest that complex MBCA mixtures are a specific feature of demosponge symbionts. Their poor resolution on conventional capillary columns may have hampered an accurate assignment of isomeric MBCA during previous investigations. MBCA occur in demosponges regardless of the host species and their geographical positions. They appear to be absent in other sponge taxa and have not been found in marine sediments and sea water. We therefore conclude that demosponges have hosted their specific bacterial symbionts over geological timescales.

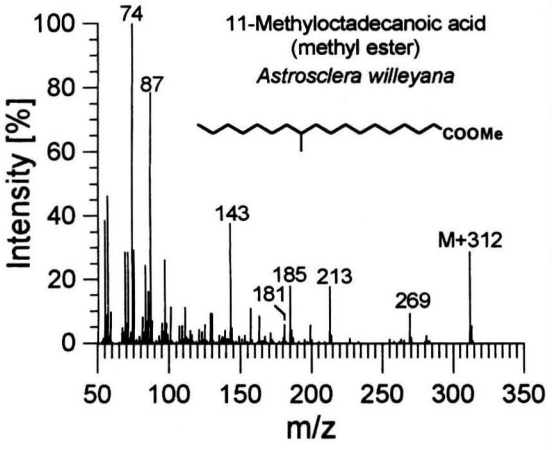
3.6. Geological and palaeobiological implications

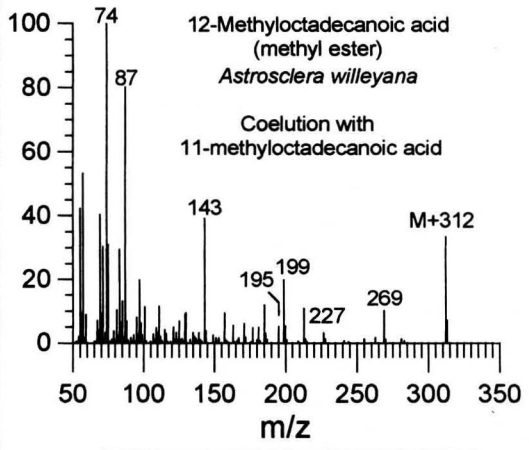
Our observation of complex MBCA patterns derived from recent marine bacteria consequently raises the attention to fossil marker molecules which can reasonably be attributed to such functionalized precursors. It has been well documented that linear carboxylic acids represent suitable substrates for the generation of sedimentary linear alkanes under geological conditions, namely by α-decarboxylation (Kissin, 1987) and, catalyzed by the presence of $CaCO_3$, by β-cleavage (Shimoyama and Johns, 1972).

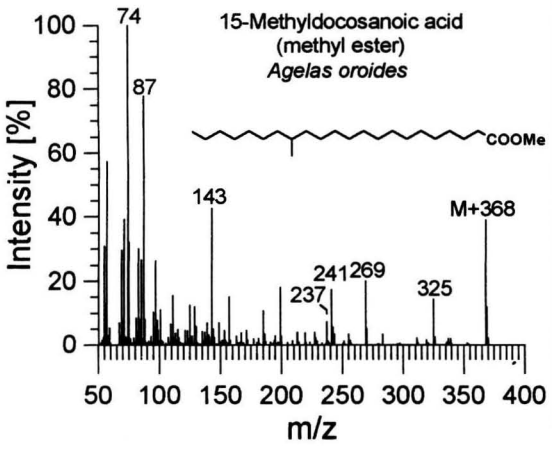
An origin of fossil monomethylalkanes from monomethyl-branched carboxylic acid precursors is particu-

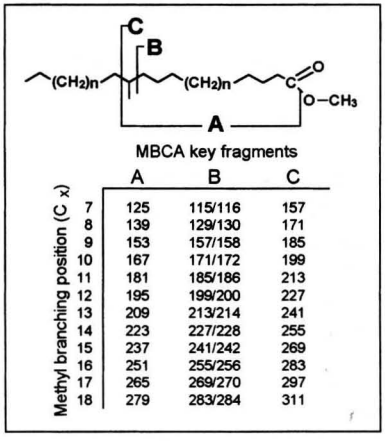












Methyl branching position (C x)	A	B	C
7	125	115/116	157
8	139	129/130	171
9	153	157/158	185
10	167	171/172	199
11	181	185/186	213
12	195	199/200	227
13	209	213/214	241
14	223	227/228	255
15	237	241/242	269
16	251	255/256	283
17	265	269/270	297
18	279	283/284	311

Fig. 2. Electron impact mass spectra of selected MBCA. Ionization energy 70 eV; ion source temperature 250°C. MBCA key fragments are given in the bottom table. See text for further explanation.

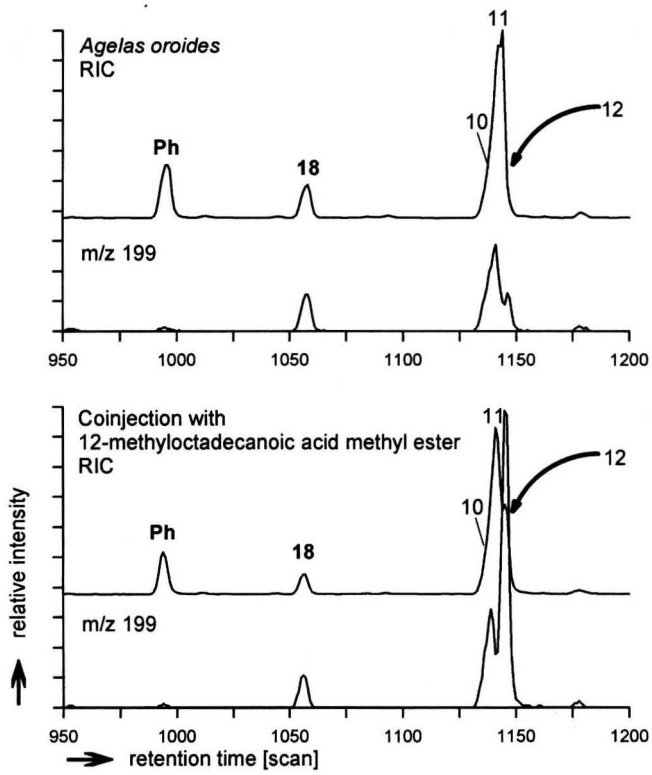


Fig. 3. GC–MS coinjection of synthetic 12-methyloctadecanoic acid (methyl ester) with the carboxylic acid fraction (methyl esters) obtained from *Agelas oroides*. 18 is *n*-octadecanoic acid and Ph phytanic acid. 10, 11, 12 indicate the site of methyl branching for individual MBCA isomers with a C_{18} carbon chain. The presence of 12-methyloctadecanoic acid within the tailing edge of the natural MBCA mixture can be inferred from the *m/z* 199 ion chromatogram (fragment "B" in Fig. 2).

larly evident for ancient sediments in which preferentially 2- and 3-methylalkanes are observed. These compounds are most likely derived from *iso*- and *anteiso*-branched carboxylic acids which may have been contributed by anoxygenic bacteria. A fossil example showing a prominent occurrence of 2- and 3-methylalkanes are the deep-water microbialithic limestones from the Triassic Cassian beds (Fig. 4; Dolomites, northern Italy; for palaeontological background see Müller-Wille and Reitner, 1993). It is noteworthy that a distinct preponderance of the 2- over the 3-methyl homologues which is commonly found in modern prokaryotes is retained in the branched-alkane pattern of fossil rock extracts. This observation indicates that neither a methyl-group rearrangement nor a radical or ionic transformation of linear into branched carbon chains (Klomp, 1986; Kissin, 1987) had a significant impact on the methylalkane pattern observed. Under mild thermal conditions, the molecular integrity of *iso*- and *anteiso*-compounds may thus be retained over geological timescales.

In some cases, mid-chain branched alkanes of sediments and oils may directly derive from biosynthetic hydrocarbons. In modern organisms, mid-chain branched alkanes are characteristic constituents of cyanobacterial lipids (e.g. Han and Calvin, 1969; Paoletti et al., 1976). They occur in about two thirds of the species so far examined, whereas other groups of organisms apparently lack these compounds (for review see Shiea et al., 1990). In pure cultured cyanobacteria, the biosynthesis of mid-chain branched alkanes is typically restricted to two or three isomers of a single homologue. 7- and 8-methyl-heptadecanes are very common, but other isomers/homologues may also occur, depending on the species and, presumably, the culture conditions. Their suitability as specific biomarkers for cyanobacterial biomass in recent environments has been substantiated by their pronounced occurrence in naturally grown cyanobacterial mats (e.g. Dobson et al., 1988; Shiea et al., 1990, 1991; Kenig et al., 1995; Thiel et al., 1997). According to the patterns found in pure cultured cyanobacteria, only limited ranges of methylated isomers are observed in modern cyanobacterially controlled mat systems. Exceptions with a broader range of structural isomers have been reported from only a few settings, e.g. the

Fig. 4. Partial gas chromatograms of the total hydrocarbon fractions of fossil microbialithic carbonates showing typical distributions of mid-chain branched alkanes and their proposed precursor molecules. Dots mark *n*-alkanes ("17" is *n*-heptadecane). Small numbers indicate the site of methyl substitution for the respective branched alkane homologues (e.g. "5" = methyl branching at C-5).

hypersaline Gavish Sabkha (de Leeuw et al., 1985) and hot springs from Iceland (Robinson and Eglinton, 1990).

With respect to fossil samples, the assignment of mid-chain branched alkanes as cyanobacterially derived biomarkers (Robinson and Eglinton, 1990; Hefter et al., 1993) faces a certain discrepancy. There are only few reports of geological samples matching the confined patterns observed in recent cyanobacteria or cyanobacterial mats. These materials are of Holocene to Tertiary age (Michaelis et al., 1988; Schaeffer, 1993; Kenig et al., 1995). Nevertheless, the presence of only a few mid-chain branched alkanes in these samples illustrates that a cyanobacterial hydrocarbon fingerprint may be incorporated into sediments and may thus be retained over geological timespans. As a reference for a direct preservation of mid-chain branched alkanes, hydrocarbons extracted from a >30 kyr old carbonate microbialite (thrombolite) from Searles Lake (NV, U.S.A.) are given in Fig. 4 (top). In this material, the significant occurrence of only one particular isomer, 7-methylnonadecane, is consistent with an origin from cyanobacterial organic matter.

In addition to these confined monomethylalkane distributions, many ancient sediments and oils (typically Mesozoic and older) are characterized by the presence of complex suites of mid-chain branched alkanes (Kissin, 1987). They may cover a wide range of carbon chain lengths, sometimes resulting in the presence of ten or more homologous series, each showing the presence of many, if not all, possible structural isomers. High abundances were observed in samples of late Precambrian and early Cambrian ages in which mid-chain branched alkanes may be particularly prevalent with carbon numbers $>C_{20}$ (Klomp, 1986; Fowler and Douglas, 1987; Summons, 1987; Summons et al., 1988).

We have observed such complex features in a sponge–microbially derived, micritic carbonate crust cored from the Upper Jurassic sponge bioherms of the Swabian Alb (southern Germany). In addition to pronounced 2- and 3-methylalkanes, this carbonate sample is characterized by abundant mid-chain branched alkanes (in particular methyl-C_{16} and -C_{17}) which cover the entire range of possible methyl branching positions (Fig. 4, bottom). Such patterns clearly differ from the marked distributions found in cyanobacteria and support the suggestion of an origin from functionalized lipid precursors, e.g. MBCA or branched alcohols. However, no suitable lipid precursors from living organisms have yet been specified. Also, a direct contribution of complex mid-chain branched alkane series from now extinct microorganisms has been discussed (Fowler and Douglas, 1987). This idea was followed up by Summons et al. (1988), who suggested a primitive biogenic source, in particular bacteria, which are now "considerably less abundant or reside in environments where they can no longer contribute in a quantitatively significant way to sedimentary lipids".

The results from our present study verify that marine bacteria are capable to produce complex suites of structural isomers of mid-chain branched alkanoic acids which can be considered as potential precursors for complex branched alkane patterns found in ancient sediments. It is of particular interest to note that these MBCA distributions occur in bacterial symbionts of recent sponges which can be regarded "living fossils", since they mark a very early evolutionary stage of multicellular organisms. This is particularly obvious for *A. willeyana* which is restricted to pristine ecological niches in recent marine ecosystems, but shows a close affinity to fossil reef building sponges with a much more extant distribution. Despite the general importance of sponges as members of recent as well as fossil biocommunities, their potential for contributing sedimentary organic matter has not yet been evaluated. Likewise, little is known about the nature and abundance of bacterial symbionts in ancient sponge populations. However, it is evident that a prominent occurrence of mid-chain branched alkanes is not confined to fossil sediments for which the former presence of sponges has been reported. We therefore suggest that these bacteria have been widespread in the geological past and are found "inherited" only in the protective environment of some sponge hosts in recent marine ecosystems. In this respect, we regard the study of contemporary sponge–microbial communities as a promising tool for the interpretation of ancient sedimentary lipid assemblages, but clearly more work is needed to unravel the nature and the biochemical properties of these particular microorganisms.

4. Conclusions

1. Complex isomeric mixtures of mid-chain branched alkanoic acids were found in the demosponge *A. oroides* and in the stromatoporoid *A. willeyana*.
2. The similarity in demospongic acid patterns and the presence of phytanic acid clearly demonstrate a taxonomic relationship of *A. willeyana* with the taxon *Agelasida*.
3. The observed *iso*-, *anteiso*- and mid-chain branched carboxylic acids (methyl-C_{14} to methyl-C_{24}) are attributed to specific, heterotrophic bacteria living in symbiosis with demosponges.
4. The found mid-chain branched carboxylic acids represent potential biological precursors for series of mid-chain branched alkanes present in ancient sediments and oils.

5. As a working hypothesis, it is suggested that the sponge-dwelling bacteria comprise relict populations with a much more widespread distribution in ancient marine ecosystems.

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