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Unexpected occurrence of hopanoids at gas seeps in the Black Sea

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Abstract

High concentrations of free C₃₂ bis-homohopanoic acids (up to 433 µg/g dry wt) occur in microbial mats at methane seeps in anoxic Black Sea waters. These compounds show a strong preference for the ‘geological’ 17α(H),21β(H)- over the ‘biological’ 17β(H),21β(H)-configuration (αβ/ββ ratios up to 30.7) and indicate the potential formation of αβ-hopanooids in modern environments. Strong ¹³C-depletions (δ¹³C as low as –78.4‰ PDB) indicate an *in situ* generation of these hopanooids by biota involved in the anaerobic cycling of methane carbon. The inferred presence of hopanooids indigenous to a permanently anoxic marine environment is significant because these lipids are not known to occur in strictly anaerobic bacteria.

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1. Introduction

Triterpenoids of the hopane series are classical bacterial lipids. In living bacteria, the so-called biohopanooids comprise simple C₃₀ compounds, such as diploptene or diplopterol, and more functionalised bacteriohopanepolyols (BHP) and composite BHP. The latter compounds have a polyhydroxylated alkyl (sugar-) side chain and additional polar moieties (e.g. amino acids, nucleic acids) which may mediate cell membrane properties and interactions between membranes and polar biomolecules, like proteins and DNA (Cvejic et al., 2000; Neunlist and Rohmer, 1985; Rohmer et al., 1984). A wide distribution of BHP was originally inferred from geochemical studies, as diagenetic derivatives

of these lipids, including intact bacteriohopanetetrol, were found in ancient sediments (Ensminger et al., 1972; Mycke et al., 1987; Ourisson and Albrecht, 1992). These ‘geohopanooids’ are among the most abundant organic molecules in sediments and actually represent the oldest chemofossils to which a discrete biological source can be attributed (Summons et al., 1999). The diagenetic fate of the hopane carbon skeleton is characterized by alteration to its stereochemistry, namely an increase in the ‘geological’ 17α(H),21β(H)- (αβ) and 17β(H),21α(H)- (βα, moretane) vs. the ‘biological’ 17β(H),21β(H)- (ββ) isomers, as well as an increase in the *S*- vs. the *R*- configuration at the asymmetric C-22 atom. Because these alterations progress with increasing burial, they are routinely used as maturity parameters in studies of petroleum and organic matter in ancient rocks (Farrimond et al., 1998; Peters and Moldowan, 1991; Sinninghe Damsté et al., 1995).

An early evolutionary origin of hopanoid biosynthesis has been claimed due to its independence from molecular oxygen and its compatibility with an anoxic, prebiotic

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atmosphere (e.g. Rohmer et al., 1984). However, although consequentially expected, hopanoids have never been found in strictly anaerobic microorganisms, including archaea and sulfate-reducing bacteria (SRB; Ourisson and Rohmer, 1992). Biomarker studies have therefore operated under the assumption that there is no contribution of hopanoids from bacteria within anoxic sedimentary environments (Innes et al., 1997). Nevertheless, ^{13}C depleted hopanoids, namely the C_{30} compounds diplopterol/diploptene have recently been reported from numerous methane-seep deposits (e.g. Elvert et al., 2000; Hinrichs, 2001; Pancost et al., 2000a; Thiel et al., 2001). These settings are controlled by the anaerobic oxidation of methane (AOM), a widespread process which is thought to be performed by syntrophic consortia of archaea and SRB (Boetius et al., 2000; Hoehler et al., 1994; Michaelis et al., 2002; Orphan et al., 2001; Valentine and Reeburgh, 2000). The presence of hopanoids in these sediments has been attributed to contributions from aerobic methanotrophs thriving in the oxic water column (Hinrichs, 2001), but also to anaerobic chemo-organotrophic bacteria associated with AOM (Pancost et al., 2000a). Against this background we extended a previous study (Michaelis et al., 2002; Thiel et al., 2001), and explored the hopanoid distributions in a set of microbial mats associated with methane seeps on the deeper Black Sea shelf. This ecosystem, covered by an oxygen-free water body, offers the unique possibility of studying biomarker patterns controlled by anaerobic methane cycling with minimum interference from aerobic biological processes.

2. Materials and methods

During a German–Russian–Ukrainian joint expedition in July 2001 we studied methane seeps on the deeper

Black Sea shelf south-west of the Crimean Peninsula (180–235 m water depth, upper anoxic zone). Using the manned submersible ‘Jago’ launched from the R.V. ‘Professor Logachev’, we discovered and sampled up to 4 m high carbonate buildups associated with bubbling gas (Michaelis et al., 2002). Up to 10 cm thick microbial mats grow on these carbonate structures which consist of high-Mg-calcite and aragonite; $\delta^{13}\text{C}$ values of around -30% PDB reveal that the carbonate carbon predominantly derives from the oxidation of methane which comprises $>90\%$ of the seep gases and is biogenic in origin ($\delta^{13}\text{C} = -62.4$ to -68.3% PDB; Michaelis et al., 2002). Laboratory experiments previously showed the capacity of the seep-associated microbial mats for (i) AOM, (ii) methanogenesis from H_2/CO_2 and acetate, and (iii) sulfate reduction (Pimenov et al., 1997). Recent microbiological work revealed archaea of the methane-metabolizing cluster ANME-1 and sulfate reducing bacteria (SRB) of the *Desulfococcus/Desulfosarcinales* group as prominent members of the microbial population (Michaelis et al., 2002; Tourova et al., 2002).

We collected three mat samples using TV-grab, multicorer and submersible (Table 1). The samples were kept at $-20\text{ }^\circ\text{C}$ until they were analysed. Surface sediment (0–4 cm) from a nearby non-seep area was used as a control sample. The sediment recovered from that site included a 30 cm thick, finely laminated Black Sea Unit I succession showing neither indications of bioturbation nor metazoan fossils. This signifies that no major oxic events occurred during the last 2500 years. The mat samples were saponified in 6% KOH in CH_3OH (MeOH) and neutral lipids were removed by extraction with *n*-hexane. After acidification to pH 1, carboxylic acids were extracted with CH_2Cl_2 (DCM) *vs.* water and methylated with a trimethylchlorosilane (TMCS):MeOH mixture (1:8, v:v, 1 h, $70\text{ }^\circ\text{C}$). The reaction mixture was dried (N_2) and the resulting methyl esters were eluted

Table 1
Locations and sample characteristics

Sample No. and device	Position	Water depth (m)	Sample	Composition
86-1 Multicorer	44.78 N 31.96 E	184	Control site (non-seep), surface sediment 0–4 cm	Siltstone, thin calcite layers (coccoliths)
10-10 TV-grab	44.78 N 31.99 E	235	Pink microbial mat, ≈ 10 cm thick, filaments up to $\approx 100\text{ }\mu\text{m}$ in length	Massive biomass with aragonite globules
118-1 ^a Submersible	44.78 N 31.98 E	180	Pink microbial mat, thriving at 10 cm sediment depth	Biomass, few aragonite globules
55-4B ^b Submersible	44.77 N 31.98 E	226	Pink microbial mat, similar to 10-10	Biomass, few aragonite globules

^a No massive carbonate formation observed.

^b Detailed description in Michaelis et al. (2002).

over a silica gel column (Merck silica gel 60, 15 mm i.d. \times 25 mm length) with 4 column volumes (cv) of DCM, and analysed by gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), and GC–combustion–isotope–ratio–MS (GC–C–IRMS) as described elsewhere (Thiel et al., 2001). Isotope ratios are given as δ -values ($\delta^{13}\text{C}$ [‰] vs. PDB) for 3 analytical replicates. *Bis*-homohopanoic acid epimer identifications are based on their retention behaviour (t_R : $\alpha\beta < \beta\alpha < \beta\beta$; $22S < 22R$), mass spectra ($\alpha\beta$, m/z 191 $>$ m/z 263; $\beta\alpha$, m/z 191 \approx m/z 263; $\beta\beta$, m/z 191 $<$ m/z 263; e.g.

Jaffé et al., 1988) and coinjection with a carboxylic acid fraction from the Late Eocene Pysht formation (USA) which contains the typical $22S/22R$ diastereoisomers of the $\alpha\beta$ - and $\beta\alpha$ -epimers, and one $\beta\beta$ -($22R?$)-epimer (Fig. 2). The presence of BHP was checked in a DCM:MeOH (1:1, v:v) extract of the microbial mat sample 10-10. To examine the occurrence of GC-amenable compounds, the extract was acetylated using an acetic acid anhydride:pyridine mixture (1:1, v:v, 14 h, RT) and analysed by GC and GC–MS using a 15 m DB5-HT capillary column (0.32 mm i.d., 0.1 μm film

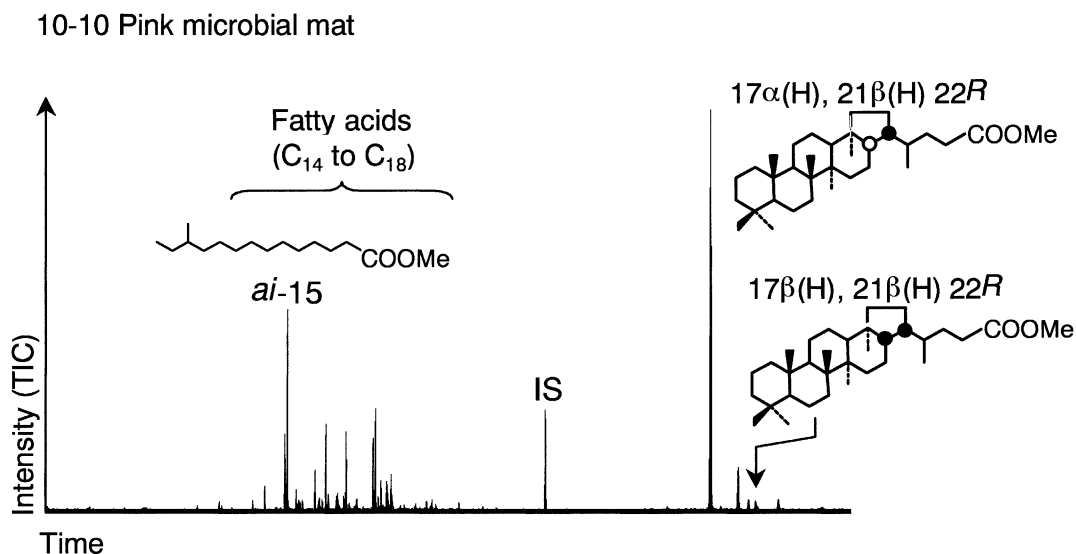


Fig. 1. Total ion current (TIC) of the carboxylic acid fraction from the microbial mat 10-10, illustrating the quantitative distributions of hopanoic acids vs. fatty acids, and the predominance of $\alpha\beta$ - over $\beta\beta$ -*bis*-homohopanoic acid ($22R$). IS = internal standard $5\alpha(\text{H})$ -cholestane.

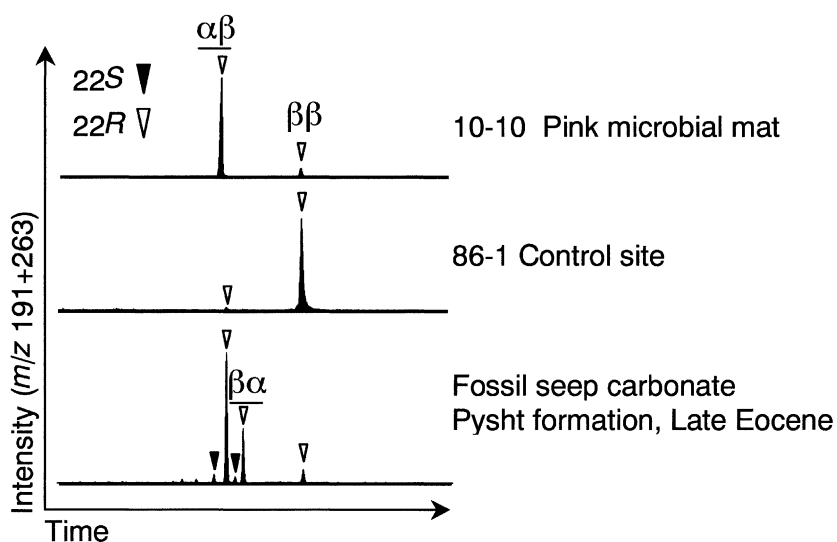


Fig. 2. Partial ion chromatograms (summed m/z 191 + m/z 263) visualizing the epimer abundances of *bis*-homohopanoic acid in selected samples.

thickness; GC temperature program: 3 min at 80 °C, from 80 °C to 330 °C at 10 °C/min, 30 min isothermal at 330 °C). For GC–MS analyses, the interface and ion source temperatures were raised to 320 and 230 °C, respectively. Authentic $\beta\beta$ -bacteriohopanetetrol tetraacetate was used as a reference compound. The presence of composite BHP (not amenable to conventional GC) was examined by *cis*-diol cleavage induced by H_5IO_6 oxidation, and subsequent $NaBH_4$ reduction after the method described by Rohmer et al. (1984). The efficiency of the reaction was controlled and affirmed by parallel treatment of an authentic $\beta\beta$ -bacteriohopanetetrol standard. Alcohols formed during this procedure were analyzed as their acetates. The relative abundances of free *vs.* bound (OH-labile) *bis*-homohopanoic acid were checked by parallel analysis of a free DCM:MeOH (1:1, v:v) extract of the microbial mat 10-10. Free acids in the raw extract were methylated using diazomethane, and the resulting methyl esters were recovered by column chromatography (DCM, 4 cv). Concentrations were determined by GC using $5\alpha(H)$ -cholestane as an internal standard.

3. Results and discussion

C_{32} *bis*-homohopanoic acids occur in high concentrations (up to 433 $\mu\text{g/g}$) in two of the mats studied (Fig. 1, Table 2). These values are much higher than in the control sample (2.2 $\mu\text{g/g}$) and those previously reported for some lacustrine sediments (0.8–3.0 $\mu\text{g/g}$, Buchholz et al., 1993; Rohmer et al., 1980). They are similar to those observed in surface sediments of Priest Pot ($\beta\beta$: 80–600 $\mu\text{g/g}$, $\alpha\beta$: 60–300 $\mu\text{g/g}$ (Innes et al., 1997), and peat (up to 78 $\mu\text{g/g}$ for the total C_{32} compounds (Quirk et al., 1984).

The *bis*-homohopanoic acids in the Black Sea mats show a strong preference of the ‘geological’ $\alpha\beta$ - over the biological $\beta\beta$ -configuration ($\alpha\beta/\beta\beta$ ratios of between 1.0 and 30.7, Table 2). They possess the 22*R* stereochemistry which is characteristic of living bacteria. Unlike the seep deposits, the control site yields almost

exclusively the $\beta\beta$ (22*R*) epimer (Fig. 2), as is common for modern marine sediments. The differences in spatial distributions and stereochemistry indicate that the $\alpha\beta$ -hopanoids are indigenous to the methane seep environment.

In the microbial mats, where reliable carbon isotope values could be obtained, we found the *bis*-homohopanoic acid to be strongly depleted in ^{13}C ($\delta^{13}\text{C}$ as low as -78.4%) compared to normal marine lipids ($\delta^{13}\text{C}\approx-30\%$). Little isotopic difference occurs between the $\alpha\beta$ - and $\beta\beta$ -isomers (Table 2), consistent with a common autochthonous source for both. The low $\delta^{13}\text{C}$ values of the hopanoids and of seep methane in the study area (-62.4 to -68.3% ; Michaelis et al., 2002) strongly indicate an incorporation of methane carbon into the biomass of the hopanoid producers.

Hopanoic acids and other monofunctionalized hopanoids with an extended side chain are ubiquitous in sediments and oils (e.g. Farrimond et al., 2002; Jaffé et al., 1988) and are generally considered as sedimentary decomposition products of BHP (Innes et al., 1997) which are, notably, thought to be formed under oxic to suboxic conditions (Peters and Moldowan, 1993). As a degradation product, the $\alpha\beta$ -*bis*-homohopanoic acid can be expected to occur as a free compound that should be easily solvent-extractable. Indeed, only little difference occurred between the yields of $\alpha\beta$ -*bis*-homohopanoic acid obtained from the DCM:MeOH raw extract of the microbial mat 10–10 (389 $\mu\text{g/g}$) and upon alkaline hydrolysis (433 $\mu\text{g/g}$), thus giving no evidence for true chemical (ester-) bonding of these compounds in the cellular matter.

In the acetylated raw extract of the microbial mat, we found only trace amounts of GC-amenable BHP derivatives. These compounds were not further characterized due to their very low concentrations, except one, which we identified by comparison with an authentic standard as bacteriohopanetetrol tetraacetate (<0.1 $\mu\text{g/g}$; m/z 191, 493, M^+ 714). This compound showed the $\beta\beta$ -stereochemistry and is therefore unlikely to represent a direct lipid precursor of the $\alpha\beta$ -hopanoids observed. To confirm the original presence of composite BHP, we

Table 2
Concentrations ($\mu\text{g/g}$ dry wt) and $\delta^{13}\text{C}$ values ($\%$ vs. PDB) of *bis*-homohopanoic acids

Sample	$\alpha\beta$ $\mu\text{g/g}$	$\beta\alpha$ $\mu\text{g/g}$	$\beta\beta$ $\mu\text{g/g}$	$\alpha\beta/\beta\beta$ ratio	$\alpha\beta$ $\delta^{13}\text{C}$	$\beta\beta$ $\delta^{13}\text{C}$	Main fatty acid	$\mu\text{g/g}$
86-1 control site	TR	n.d.	2.2	<0.01	n.a.	n.a.	<i>n</i> - C_{16}	1.0
10-10 pink mat	433	TR	14	30.7	-69.6 ± 0.2	n.a.	<i>ai</i> -15	190
118-1 pink mat (sed.)	5.4	n.d.	0.4	13.8	-69.3 ± 0.2	-65.9 ± 0.4	<i>ai</i> -15	22
55-4B pink mat ^a	214	n.d.	86	2.5	-78.4 ± 0.5	-78.4 ± 0.1	<i>ai</i> -15	1243

The concentrations of the respective main fatty acids are given for comparison. *ai*-15 = 12-methyltetradecanoic acid. TR = trace; n.d. = not detected; n.a. = not analyzed.

^a The $\delta^{13}\text{C}_{\text{org}}$ value of the bulk 55-4B sample is -72.2% (Michaelis et al., 2002).

replicately applied the periodate method after Rohmer et al. (1984) on the microbial mat 10-10, which showed the highest concentration of $\alpha\beta$ -bis-homohopanoic acid. The chemical degradation generated 105 $\mu\text{g/g}$ $\alpha\beta$ -bis-homohopanol (as acetate). This implies the existence of composite $\alpha\beta$ -BHP in the microbial mat, albeit in much lower concentration than found for the $\alpha\beta$ -bis-homohopanoic acid. A common autochthonous source and, possibly, a genetic relationship between these components is indicated by the isotopic composition of the $\alpha\beta$ -bis-homohopanol released by periodate treatment ($\delta^{13}\text{C} = -68.8\text{‰}$).

The finding of ^{13}C depleted, extended $\alpha\beta$ -hopanoids in an anoxic methane-related environment promotes the idea of an additional microbial component participating in the anaerobic cycling of methane carbon. The great quantitative variability observed between different mat samples (Table 2) may reflect a patchy distribution of these organisms, or variations in their hopanoid biosynthesis with growth stage or trophic conditions.

The situation found at the Black Sea gas seeps is at odds with the opinion that hopanoids do not occur in strictly anaerobic bacteria and are not generated in anoxic settings. Our findings also suggest a formation of bis-homohopanoic acids under anoxic conditions. Furthermore, the results argue against the still common view that $\alpha\beta$ -epimers form only in the geological environment. In fact, the latter opinion had been challenged by observations of $\alpha\beta$ -geohopanooids in Holocene peats and modern lacustrine sediments (Dehmer, 1992; Innes et al., 1997; Pancost et al., 2000b; Quirk et al., 1984; Rohmer et al., 1980). These occurrences were explained by rapid catalytic epimerization of $\beta\beta$ -hopanoids under acidic conditions (van Dorsselaer et al., 1977) or by microbial mediation (Quirk et al., 1984). However, the recent discovery of $\alpha\beta$ -BHP in the nitrogen fixing soil bacterium *Frankia* spp. (Rosa-Putra et al., 2001) demonstrated the potential for a primary biosynthesis of $\alpha\beta$ -hopanoids and raised unprecedented problems with respect to the possible mechanisms of triterpenoid biosynthesis from squalene (see Rosa-Putra et al., 2001, for a detailed discussion). Interestingly, a 16S rDNA sequence of an actinobacterium, with *Frankia* as the closest relative, was recently recovered from massive gas hydrate in the Gulf of Mexico (Lanoil et al., 2001).

Although the biological origin of the ^{13}C -depleted Black Sea hopanoids remains to be elucidated, our results indicate that hopanoids with the 'geological' $\alpha\beta$ -configuration may be generated in a recent marine environment. As our findings pertain to an extreme, highly localized setting, we do not believe that they will affect the overall applicability of the $\beta\beta/(\beta\alpha + \alpha\beta)$ maturity parameter. However, the phenomenon may help to interpret inconsistencies in the abundance and isotopic signatures of hopanoids and their stereoisomers in the sedimentary record.

4. Conclusions

- High concentrations of $\alpha\beta$ -hopanoids in microbial mats from methane seeps in the euxinic Black Sea strongly suggest that certain microorganisms produce hopanoids in anoxic environments.
- The absence of $\alpha\beta$ -hopanoids in the surrounding non-seep sediments indicates that these compounds are indigenous to the methane-rich environment rather than being sourced from the overlying water column.
- Extremely low $\delta^{13}\text{C}$ -values found for the $\alpha\beta$ -hopanoids reveal that the source biota incorporate methane carbon and may comprise a yet unrecognized microbial component in the anaerobic methane cycle.
- The presence of hopanoids with the 'geological' $17\alpha(\text{H}), 21\beta(\text{H})$ configuration in a living microbial mat implies that $\alpha\beta$ -isomers can be generated in modern marine environments.

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