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Building the Biomarker Tree of Life

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INTRODUCTION

It is the great challenge of geomicrobiology to study microorganisms in the context of their environments, both in Earth's distant past and in the present. Planet Earth and its biosphere have evolved together, and a chronicle of Earth's ecosystems and their geochemical cycles is recorded in sedimentary rocks spanning billions of years. A relatively new and very powerful approach to read these subtle microbial and environmental signatures in ancient rocks is the study of molecular fossils, or biomarkers, within the context of the biochemistry and phylogeny of their origins.

Biomarkers are organic compounds (primarily lipids) that have particular biosynthetic origins and can be preserved in sediments and sedimentary rocks. The most valuable biomarkers are taxonomically specific, i.e., they can be assigned to a defined group of organisms, and are resistant to degradation. Reading the biomarker signatures in rocks can give information about the ancient record of anoxic conditions in the water column (e.g., Summons and Powell 1986), the intensity of UV radiation penetrating lakes (Leavitt et al. 1997), hypersalinity in evaporitic environments (Grice et al. 1998), and the function of microbial communities at methane seeps (Hinrichs et al. 1999). Biomarkers have helped to reconstruct the first appearance of major groups of organisms (e.g., McCaffrey et al. 1994; Moldowan et al. 1994; Moldowan and Talyzina 1998; Brocks et al. 2005), elucidate events of global climate change (e.g., Brassell et al. 1986), record major perturbations and reorganization of geochemical cycles (e.g., Logan et al. 1995; Kuypers et al. 1999) and document catastrophic losses in biodiversity (e.g., Grice et al. 2005). They are even used as tools to help in the discovery of major new petroleum reservoirs (for a review see Peters et al. 2004). The field of biomarker research is young and many new applications wait to be discovered.

This review will explain how biomarkers form, how they are extracted from sedimentary rocks, and how they are used to reconstruct ancient and modern microbial ecosystems. It ends with a look to the future of biomarker research and the on-going efforts to reconcile the biomarker record with the tree of life. The review includes examples of the detection of important metabolic pathways, of the appearance of new biomarkers (and by inference new taxonomic groups) in the rock record, and of the reconstruction of geochemical processes. We will concentrate on biomarkers from bacteria, archaea and unicellular eukaryotes, excluding

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the molecular fossils of plants and other non-microscopic eukaryotes. We highlight biomarker research using outstanding recent examples with a pedagogic emphasis on concepts but with no claim for completeness. More encyclopedic reviews were given by Peters et al. (2004) and Brocks and Summons (2004). For readers who desire more background in organic geochemistry, excellent introductions to the nomenclature, chemistry, and biology of lipids can be found in textbooks by Killops and Killops (2005) and Madigan and Martinko (2005).

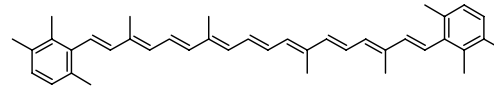
The biomarker principle

The origin of biomarkers. In lakes and oceans, the organic matter from dead organisms usually is almost quantitatively (> 99.9%) recycled back into carbon dioxide and water (Hedges and Keil 1995). The biological degradation of most proteins, nucleic acids and carbohydrates proceeds rapidly as dead biomass sinks through the water column, and it continues in the surface layers of the sediments. However, a small fraction of organic matter escapes the remineralization process and accumulates. Molecules that are especially recalcitrant, such as pigments, lipids and many structural macromolecules, will become concentrated (Tegelaar et al. 1989). With the onset of reducing conditions, the remaining sedimentary organic matter is degraded further by anaerobic heterotrophic organisms such as sulfate reducers, fermenters and methanogens (Megonigal et al. 2004); the chemical structure of the remains is altered by biological and chemical processes (Hedges and Keil 1995; Hedges et al. 1997; Rullkötter 1999). These alterations are referred to collectively as diagenesis. Smaller molecular units and degradation-resistant macromolecules are cross-linked and form kerogen, an amorphous and exceedingly complex structural network of biochemical subunits (e.g., Derenne et al. 1991; de Leeuw and Largeau 1993). During the formation of kerogen, vulcanization reactions mediated by sulfur and polysulphides often play an important role in connecting smaller molecular units, such as lipids, to the macromolecular aggregate, thus protecting them against further structural alterations (Sinninghe Damsté and de Leeuw 1990). Over millions of years, and with increasing burial depth and geothermal heat, most lipids will undergo structural rearrangement via cracking and isomerization reactions. These processes create a vast range of homologues and stereo- and structural isomers. Through reduction, elimination and aromatization the biomarkers typically lose all of their functional groups. The resultant products are geologically-stable hydrocarbon skeletons. Structures ***1*** and ***2***; ***3*** and ***4***; and ***5*** and ***6*** show examples of biolipids and their diagenetic hydrocarbon products.

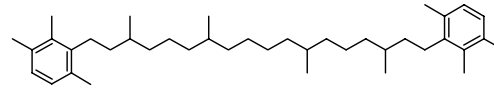
Bitumen is defined as the fraction of organic matter that can be extracted from sediments and sedimentary rocks using organic solvents, and it includes diagenetic components that have been thermally cracked, or released, from the kerogen. With increasing burial temperature and pressure, the thermal degradation of kerogen in organic-rich sedimentary rocks will generate enough liquid bitumen and natural gas for the expulsion of hydrocarbons in the form of petroleum. Petroleum reservoirs are, in fact, gigantic accumulations of biomarkers and other cracking products of sedimentary organic matter. However, at burial temperatures in the sedimentary unit exceeding 150–250 °C, most residual gas and liquid hydrocarbons have been expelled and the kerogen dehydrogenated to a highly aromatic, black carbon phase. This is the upper survival temperature for biological molecules over geologic time (Brocks and Summons 2004). The thermal destruction of biomarkers with deep burial is the primary complication in the search for biogenic molecular remains in very ancient, billion-year-old sedimentary rocks (Brocks et al. 2003a).

During the experimental analysis of biomarkers, organic-rich, black sedimentary rocks are crushed to powder, and the powder is extracted with solvents such as methanol and dichloromethane using conventional reflux extraction or automated solved extractors (ASE). The bitumen extracts are usually yellow to dark brown, highly complex mixtures, containing hundreds of thousands of compounds. To simplify further analyses, the bitumen is fractionated into saturated hydrocarbons, aromatics, and polar compounds (usually those containing the

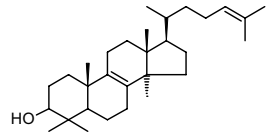
1 isorenieratene



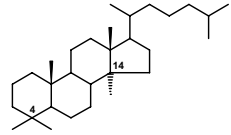
2 isorenieratane



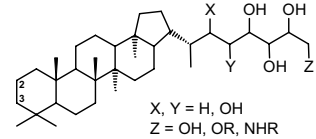
3 lanosterol



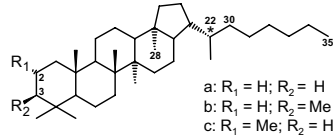
4 lanostane



5 bacteriohopanepolyols (BHP)



6 pentakishomohopane

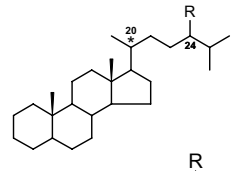


heteroatoms O, N, and S) using normal-phase (SiO_2-gel) chromatography. The fractions are then analyzed by gas chromatography-mass spectrometry (GC-MS).

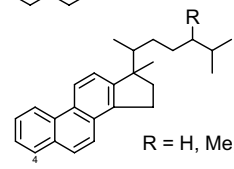
The ratios of different homologues and stereo- and structural isomers contained in a sample of bitumen may contain a plethora of information about conditions during burial and diagenesis, as well as the source organisms. For example, the relative abundance of stereoisomers such as 22S vs. 22R hopanes (see ***6***), 20S vs. 20R steranes (see ***7***), or triaromatic steroids ***8*** with intact and cleaved side chain, often helps to estimate relative burial temperatures (e.g., Brocks et al. 2003a). A high relative abundance of biomarkers such as aromatic carotenoids (e.g., ***2***), 28,30-bisnorhopanes (hopanes ***6*** lacking the C-28 and C-30 methyl groups) (Schoell et al. 1992), or the pentacyclic triterpane gammacerane ***9***, produced by ciliates grazing on bacteria in the anoxic zone (Sinninghe Damsté et al. 1995), may indicate anoxic and/or sulfidic conditions.

Molecular fossils as markers for biosynthetic pathways. For many geobiologists, the most interesting application of biomarkers is the reconstruction of ancient microbial ecosystems and the concurrent environmental conditions, at time-scales of millions to billions

7 steranes

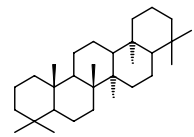


8 triaromatic steroids



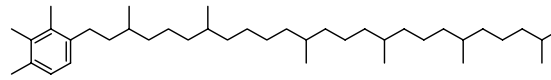
R = H, Me, Et

9 gammacerane

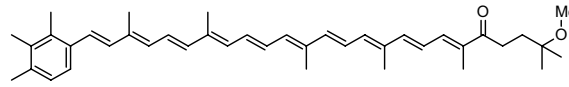


of years. Although lipids usually lose their functional groups during diagenesis, and often also stereochemical specificity, the remaining hydrocarbon skeletons retain useful biological and ecological information. For instance, the aromatic carotenoid okenane ***10***, a molecule detected in 1,640 million-year-old sedimentary rocks of the McArthur Basin in northern Australia, is regarded as a biomarker for purple sulfur bacteria of the family Chromatiaceae (Brocks et al. 2005). The only known biological precursor of okenane is the red-colored phototrophic pigment okenone ***11***, and okenone is found exclusively in Chromatiaceae. As the Chromatiaceae have a very specific ecology, the presence of okenane has been used to predict that the waters of the McArthur Basin 1,640 million years ago were sulfidic up into the photic zone.

10 okenane



11 okenone

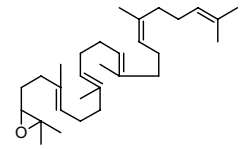


The distribution of carotenoids in the biosphere is comparatively well known due to their intense color. Therefore, the interpretation of okenane as a biomarker for purple sulfur bacteria appears to be robust. However, the interpretation of many other biomarkers is more complex. Strictly speaking, biomarkers are not markers for taxonomic groups or for environmental conditions. Biomarkers are the products of biosynthetic pathways that may occur in unrelated organisms. An example of potential misinterpretation is the carotenoid isorenieratane ***2***. Isorenieratane ***2*** is commonly interpreted as a specific biomarker for green sulfur bacteria (Chlorobiaceae). However, the precursor of isorenieratane ***2***, isorenieratene ***1***, also occurs in the gram positive bacterial group Actinomycetales (Krügel et al. 1999; Phadwal 2005), and a genome library search for genes involved in isorenieratene biosynthesis suggests that cyanobacteria may have this capacity as well (Woodward Fischer, personal communication).

In general, the distribution of genes for carotenoid biosynthesis in Bacteria is characterized by horizontal gene transfer and gene duplication events (Phadwal 2005). Thus the capacity for biosynthesis of isorenieratene ***1*** might appear in other lineages as well. Additionally, the aromatic end-group of isorenieratene also may form by abiotic, diagenetic aromatization of cyclohexyl (ionene) end-groups (Koopmans et al. 1996). Thus, isorenieratane may form in sedimentary environments by alteration of a wide range of carotenoids, including β-carotene. Therefore, isorenieratane ***2*** is not a biomarker solely for Chlorobiaceae. Isorenieratane is a product of all biosynthetic and abiotic pathways that can produce and alter suitable precursors.

A second example of the ambiguities in the interpretation of biomarkers is the phylogenetic distribution of the biosynthetic pathway leading to sterols. The C_{30} steroid hydrocarbon lanostane ***4*** has two common diagenetic precursors, lanosterol (produced by animals and fungi) and cycloartenol (biosynthesized by plants). The enzyme oxidosqualene cyclase (OSC) catalyzes the formation of lanosterol ***3*** and cycloartenol from the precursor compound oxidosqualene ***12***. These compounds are the initial steroidal products that feed the long, complex biosynthetic pathways that produce all known eukaryotic steroids. However, although most biology textbooks state that steroid biosynthesis is one of the defining characteristics of eukaryotes, lanostane ***4*** is not always a biomarker for Eucarya. OSC also is expressed in at least three groups of bacteria: the *Methylococcales* (Bird et al. 1971), *Myxococcales* (Kohl et al. 1983), and *Planctomycetales* (Pearson et al. 2003). Several of these species produce detectable amounts of lanosterol, as well as down-stream (modified) sterols. Therefore, the compound lanostane is a biomarker for eukaryotes and bacteria. However, fossil steroids that have an additional alkyl substituent at position C-24 (see ***7***) in the side chain must still be regarded as diagnostic for eukaryotes, as no group of bacteria is known (yet) to possess the biosynthetic capacity to alkylate the steroid side chain (Brocks et al. 2003b; Volkman 2003).

12 oxidosqualene



The carbon isotopic composition of biomarkers

Fractionation of the stable isotopes of carbon, ^{12}C and ^{13}C, occurs in association with biological reactions and remains imprinted in the isotopic signatures of biomarker molecules. In addition to the intrinsic taxonomic utility of certain lipids, the isotopic ratios of these compounds can provide insight about the environmental conditions and metabolic capabilities of the source organisms. As such, compound-specific isotopic analysis is a valuable additional tool for understanding the modern and ancient geologic record.

Carbon occurs naturally as three isotopes, ^{12}C, ^{13}C, and ^{14}C; with fractional abundances of 0.989, 0.011, and 10^{-12}, respectively. The last, ^{14}C, is radioactive, and its half-life of 5730 years yields useful radiocarbon chronologies only over the most recent few tens of thousands of years. Therefore, most isotopic analyses of individual biomarkers focus primarily on the two stable isotopes of carbon. Isotopic composition is expressed most commonly as the ratio of ^{13}C to ^{12}C in the substance, relative to the ratio in a standard material. The units of isotopic fractionation are parts per thousand, or "permil" (‰), and the standard reference material is a carbonate rock (VPDB; Vienna Pee Dee Belemnite), which by definition has a $\delta^{13}C_{VPDB}$ value of 0‰.

$$\delta^{13}C = \left(\frac{^{13}C/^{12}C_{Sample}}{^{13}C/^{12}C_{Standard}} \right) \bullet 1000$$

There are numerous factors that determine the final $\delta^{13}C$ values of total biomass and of individual biomarker compounds. Some of the principles most fundamental to geobiological interpretations are summarized below; however, the reader is encouraged to examine the more comprehensive reviews by Hayes (1993, 2001), from which most of this material was adapted.

The isotopic composition of the carbon source. The $\delta^{13}C$ values of biomarkers depend in complex ways on the pathway(s) and condition(s) of carbon fixation, as described below; but the baseline for these values necessarily is that of the inorganic carbon source from which the biomass is produced. The speciation of inorganic carbon is governed by acid-base equilibria between CO_2, HCO_3^-, and CO_3^{2-} species.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \leftrightarrow CO_3^{2-} + 2H^+$$

The relative abundance of each component is dependent on the pH of the local environment and varies among fresh-water, marine, fluvial, hydrothermal, and other systems of geologic interest. The isotopic distribution, however, is a function primarily of temperature, and in the case of CO_3^{2-}, also of the partitioning between dissolved and mineral [M[II]CO_3(s)] phases (Bottinga 1969; Emrich and Vogel 1970). These equilibrium isotope exchange reactions favor the incorporation of ^{13}C into the more stable (lower energy) bonding environment; the primary result is a relative isotopic depletion of ^{13}C in CO_2(aq) and CO_2(g) relative to HCO_3^- or CO_3^{2-}. The equilibrium fractionation factor, $\alpha_{A\text{-}B}$, is defined as the $^{13}C/^{12}C$ ratio of **A** (R_A) divided by the $^{13}C/^{12}C$ ratio of **B** (R_B):

$$\mathbf{A} \leftrightarrow \mathbf{B}$$

$$\alpha_{A-B} \equiv \frac{R_A}{R_B} = \frac{(1000 + \delta_A)}{(1000 + \delta_B)}$$

Equilibrium fractionation factors at common biosynthetic or environmental temperatures were summarized by Falkowski and Raven (1997). However, in many systems of interest to geobiology, such as thermal springs, temperatures may exceed the values presented here. Bottinga (1969) and Richet et al. (1977) have presented equations to extrapolate values of α for the reaction CO_2(aq) $\leftrightarrow$ HCO_3^- to higher temperatures. Values of $\alpha_{A\text{-}B}$ decrease at higher temperatures, resulting in smaller isotopic differences between the carbon species.

Typical values of $\delta^{13}C$ for inorganic carbon substrates in geological settings are −6 to −8‰ for atmospheric CO_2; −2 to +2‰ for dissolved HCO_3^- and CO_3^{2-}; and a range of values for CO_2(aq) dependent largely on the relative amount of *i*) respiration-derived CO_2 (having lower $\delta^{13}C$ values), or *ii*) residual, isotopically heavy CO_2 such as is found in strongly methanogenic systems.

Fractionation associated with fixation of inorganic carbon to biomass. Carbon isotope fractionation in biological reactions most commonly results from unidirectional kinetic isotope effects (KIE). These effects result from the effectively slower rate of reaction for a ^{13}C-containing bond relative to the same bond in which the carbon atom is ^{12}C. Thus, isotopic fraction ultimately is controlled at the atomic level by the reaction rates at individual sites within molecules.

$$^{13}\mathbf{A} \rightarrow {}^{13}\mathbf{B}, \text{ rate } k_1$$

$$^{12}\mathbf{A} \rightarrow {}^{12}\mathbf{B}, \text{ rate } k_2;\ k_2 > k_1$$

When inorganic carbon is fixed into biomass, these reactions necessarily are controlled by the activity of specific enzymes, each of which has an associated KIE. The biological transformation of substrate (**A**) to product (**B**) is accompanied by a fractionation factor that most commonly is written using the ε notation, for the subsequent convenience of relating $\varepsilon_{A\text{-}B}$ to δ_A and δ_B when ε is a small number. Because ^{13}C preferentially remains in **A**, $\varepsilon_{A\text{-}B}$ always is a positive number and products are "lighter" than reactants.

$$\varepsilon_{A\text{-}B} \equiv (\alpha_{A\text{-}B} - 1)1000$$

$$\varepsilon_{A\text{-}B} \approx \delta_A - \delta_B$$

The enzymes utilized by organisms to fix carbon are categorized most easily by the metabolic pathways in which they are used and by the species of inorganic carbon substrate for which they are specific (see Bott and Thauer 1989; and Hayes 2001, for compilations of substrates and isotopic fractionations, respectively). The latter is important, as it is clear that organisms utilizing pathways specific for HCO_3^- necessarily begin with a substrate that is heavier isotopically than organisms that fix CO_2 directly. The recent discovery of novel metabolic pathways in some species of microbes (e.g., the newly discovered 3-HP pathway; Strauss and Fuchs 1993; Raymond, this volume)—and the possibility that mixotrophic or hybrid metabolisms are expressed by species in the environment—also complicates the picture considerably. However, the currently available information can be divided into pathways that typically yield isotopically "heavy" biomass (rTCA and 3-HP); pathways yielding isotopically intermediate biomass (Calvin-Benson cycle and some methanogens); and pathways yielding isotopically light biomass (homoacetogens, some methanogens, other Acetyl-CoA pathway organisms, and all methanotrophs).

The above discussion does not include strict heterotrophs, because the carbon isotopic composition of their lipids and biomass broadly reflects their carbon sources. The $\delta^{13}C$ values of biomass of heterotrophic microbes and macroscopic heterotrophs appear to be united by the principle "you are what you eat, plus 1‰" (DeNiro and Epstein 1978). This rule of thumb is based on the weak carbon isotopic fractionation associated with the glycolytic respiratory metabolism of most heterotrophs. Methanotrophy and methylotrophy, on the other hand, may be considered here as special cases of autotrophy rather than heterotrophy, as they involve fixation of C_1 metabolites and thus are accompanied by large ε values. For further discussion of fractionations associated with aerobic methanotrophy see Summons (1994); and for separate treatment of methylotrophic metabolism see Summons (1998). Anaerobic methanotrophy has not yet been explained mechanistically, although the first *in situ* isotopic measurements (Hinrichs et al. 1999) immediately indicated large values of $\varepsilon_{CH_4\text{-}biomass}$.

Application of $\delta^{13}C$ analyses at the molecular level. Isotopic analysis of lipid biomarker molecules can provide valuable information about the geobiology and biogeochemistry of contemporary and ancient systems. Compound-specific measurements usually employ the "continuous-flow," isotope-ratio mass spectrometric methods developed by Hayes (Matthews and Hayes 1978; Hayes et al. 1990). Initial studies focused on lipids extracted from samples of ancient geologic age (e.g., Freeman et al. 1990) and showed the utility of this approach to describe a diversity of biomarkers and their origins. Such molecular-level analyses rely on the intrinsic advantages provided by lipids: their volatility permits separation by gas chromatography, and lipids are thermally and diagenetically stable.

An important, but complicating, factor in the interpretation of $\delta^{13}C$ values of individual lipids is the extent to which the lipids themselves are fractionated isotopically relative to the total biomass of the source. Intracellular fractionations are the consequence of the diversion of metabolic intermediates such as pyruvate and acetate, which are necessary for the biosynthesis of lipids, into other pathways such as the citric acid cycle and/or for the biosynthesis of amino

acids. A detailed mathematical treatment of the isotopic consequences of such branched pathways is given in Hayes (2001), but in general, the fractionation between biomass and lipids ($\varepsilon_{biomass\text{-}lipid}$) results in a more negative value of $\delta^{13}C$ for the lipid due to fractionation during the decarboxylation of pyruvate to acetate. This is true for organisms expressing the Calvin cycle, but it is not necessarily true for species that synthesize acetate directly or for those that have alternative biosynthetic routes to acetate (e.g., van der Meer et al. 2001) or isoprenoids (Schouten et al. 1998a). Organisms that have unusual metabolisms have not yet been studied thoroughly, and the range of variability in expression of $\varepsilon_{biomass\text{-}lipid}$ needs further exploration.

BIOMARKERS IN GEOMICROBIOLOGY

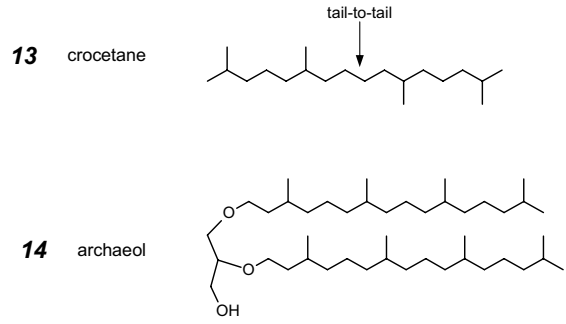
There are far too many examples of the informative use of biomarkers in geobiology to review all of them in this chapter. Therefore, in the section that follows we present a series of case studies and outstanding examples of biomarker geochemistry. These examples are grouped by metabolic pathway, biogeochemical process, and/or by the phylogeny of the source organism. Each is an attempt to illustrate the unique contributions that the analysis of lipid biomarkers can make to the field of geomicrobiology.

Biomarkers as indicators of metabolism

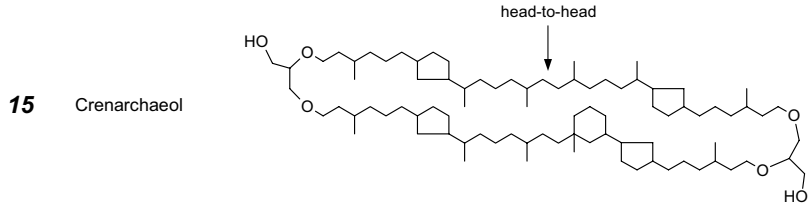
Methanotrophic methanogens: the anaerobic oxidation of methane. The need for an anaerobic sink for the methane produced in marine sediments was recognized by geochemists as early as the 1970s-1980s (e.g., Reeburgh 1976; Alperin and Reeburgh 1985). Profiles of dissolved CH_4 in sedimentary pore waters indicated that in most environments it did not reach the sediment-water interface and therefore was not oxidized by O_2 in the deep ocean. Zehnder and Brock (1979) first proposed—and later Hoehler et al. (1994) expanded upon—the hypothesis that the anaerobic oxidation of methane (AOM) could be achieved by methanogenic archaea utilizing a specialized metabolism designed to run "in reverse." They suggested that the energetic expense of this reaction could be overcome by consumption of the H_2 by-product or other reducing equivalents by syntrophic, sulfate-reducing bacteria (SRB). Such a process would require a close physical association in the form of an aggregate or consortium. However, it was only recently that the microorganisms were identified that putatively are involved in this process. Currently they are classified broadly among three new categories of Euryarchaeota, ANME-1, ANME-2, and ANME-3 (Boetius et al. 2000; Orphan et al. 2001, 2002; Knittel et al. 2005). At least one of these groups (ANME-2) indeed appears to live in consortia with SRB. Much still remains to be learned about the biology of AOM, including the possibility that there are ANME-group archaea or SRB that are capable of oxidizing CH_4 independently, without a syntrophic partner.

Prior to the discovery of the consortia that mediate AOM, however, there was evidence from $\delta^{13}C$ values of biomarker lipids that archaea were involved in AOM. The history behind the discovery of this process represents an outstanding case in which isotopic analysis of biomarkers led directly to conclusions about geomicrobiology and environmental metabolisms. Bian (1994) first observed (but could not yet explain) depleted values of $\delta^{13}C$ for crocetane ***13***, a C_{20} isoprenoid isomer of phytane, in sediments of the Kattegat Strait between Denmark and Sweden. Subsequent work by Hinrichs et al. (1999) on sediments from coastal California, USA, showed similarly extreme isotopic depletions for both archaeal and bacterial biomarkers from a CH_4-rich, anaerobic sediment. Hinrichs et al. (1999) showed that the lipids archaeol ***14*** and *sn*-2-hydroxyarchaeol, which are glycerol diethers typical of methanogenic euryarchaeota, had $\delta^{13}C$ values $\leq -100‰$. Such light values indicated that consumption of isotopically-depleted CH_4 must serve as the primary carbon source for the archaea from which the lipids were derived. The earlier $\delta^{13}C$ values for crocetane ***13*** and the related isoprenoid

hydrocarbon 2,6,10,15,19-pentamethylicosane (PMI), could finally be explained as products of similar CH_4-consuming archaea (Bian et al. 2001).



Recent work has suggested that crocetane and C_{20} isoprenoids derived from archaeal diethers may be biomarkers specific for the archaea associated with ANME-2-type consortia. The ANME-1 archaea (frequently observed as individual cells and filaments; Orphan et al. 2002) may be typified by the C_{40} isoprenoids associated with tetraethers ***15*** (Blumenberg et al. 2004). There may be further taxonomic potential to be discovered within archaeal biomarkers, and more may be learned if ANME archaea are brought into pure or enrichment culture.



After the early reports of the assimilation of CH_4 by relatives of the normally methanogenic archaea, numerous other cases of AOM were documented using lipid biomarkers. Isotopically-depleted isoprenoid ethers have been found in association with Mediterranean mud volcanoes (Pancost et al. 2000); cold-temperature CH_4 seeps (Pancost et al. 2001; Zhang et al. 2003); hot-temperature CH_4 seeps (Teske et al. 2002; Schouten et al. 2003b); in the Black Sea (Thiel et al. 2001; Michaelis et al. 2002; Wakeham et al. 2003); and in the spectacular carbonate chimneys of the serpentinite-hosted hydrothermal system of the Lost City (Kelley et al. 2005). There is also considerable evidence for the association of bacterial groups with the AOM process. These microbes are probably involved in the metabolism of the organic end-products of AOM and/or in the incorporation of ^{13}C-depleted DIC. However, it remains possible that some unidentified groups of bacteria assimilate CH_4 directly. Hopanoids (Elvert et al. 2000; Pancost et al. 2000; Thiel et al. 2001, 2003) and fatty acids derived from phospholipids (e.g., Hinrichs et al. 2000) with highly depleted $\delta^{13}C$ signatures are frequently found in the same samples as the archaeal biomarkers for AOM.

Anammox: the discovery of autotrophic denitrification. In a now-classic paper entitled "2 kinds of lithotrophs missing in nature", Broda (1977) postulated the anaerobic oxidation of ammonia by nitrate or nitrite-reducing bacteria. Oxidation of NH_4^+ with subsequent reduction

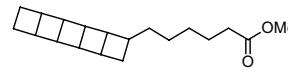
of NO_2^- would represent both a means to provide energy for lithoautotrophic fixation of CO_2 to biomass *and* a form of denitrification with associated effects on the global nitrogen cycle.

$$NH_4^+ + NO_2^- \rightarrow N_2 + 2H_2O \qquad \Delta G^\circ = -360 \text{ kJ/mol}$$

The elegance and simplicity of Broda (1977) make the paper virtually required reading for the student of geobiology; however, the predicted "anammox" reaction and more specifically the associated organisms remained undiscovered for a further 20 years. Anammox was finally revealed in association with nitrogen-rich wastewater reactors (Mulder et al. 1995; Strous et al. 1997; van de Graaf et al. 1997), and the organisms responsible for the reaction were identified as members of an unusual bacterial group, the *Planctomycetales* (Strous et al. 1999). Recently it has been suggested that anammox may be responsible for up to 30–50% of the denitrification occurring in the global ocean (Dalsgaard et al. 2003, 2005; Kuypers et al. 2005), all of which had been previously assigned to the activity of denitrifying, anaerobic heterotrophs.

Not all members of the *Planctomycetales* are capable of anammox metabolism; most members of this group appear to be heterotrophs or chemoorganotrophs. However, it is within the anammox genera (*Brocadia, Kuenenia, Scalindua*) that the unique lipids known as ladderanes ***16*** are found (Sinninghe Damsté et al. 2002). Ladderanes are believed to be critical components of the anammox organelle, the "anammoxosome" (van Niftrik et al. 2004). Specifically, ladderanes may provide a diffusional barrier to the toxic intermediate of ammonium oxidation, hydrazine (N_2H_4). For this purpose, ladderanes possess a unique 'ladder' of concatenated cyclobutane rings (see ***16***) that can be stacked into a dense membrane; and due to a lack of branched methyl groups, they are apparently biosynthesized from acetate rather than from isoprene. The C_{20} moieties may be connected via ether or ester linkages to a glycerol backbone (Sinninghe Damsté et al. 2002).

16 [5]-ladderane



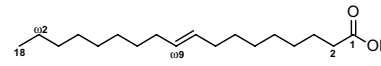
It is unknown at present to what extent intact ladderanes, or more likely, their degradation products, are preserved in the geologic record. Because of the highly strained nature of cyclobutane rings, these high-energy structures should be unstable and prone to rapid degradation. However, the ring-opening products of the cyclobutane groups may yield diagnostic and geologically stable biomarker products. Searching for these products, it may eventually be feasible to determine how far back into the geologic record the anammox process persists. Simple consideration of biogeochemical cycles suggests that anammox would not have developed as a significant process until after the advent of oxygenic photosynthesis, since the reaction is dependent on sufficient quantities of NO_2^-. Nitrite is a product of the aerobic oxidation of NH_4^+ by O_2 (nitrosification, by genera such as *Nitrosomonas* or *Nitrosobacter*).

The limited $\delta^{13}C$ data that are currently available for ladderane lipids are consistent with an autotrophic metabolism for the anammox bacteria. Schouten et al. (2004) observed $\delta^{13}C$ values between −55‰ and −58‰ for samples of ladderanes from the water column of the Black Sea. The isotopic fractionation of lipid relative to substrate (CO_2) was 32‰ to 49‰ (lipids depleted relative to substrate) for the samples from the Black Sea and for samples taken from laboratory enrichment cultures. These data could be consistent with a metabolic pathway dependent on autotrophic synthesis of Acetyl-CoA. Such metabolism is also broadly consistent with an ancient origin of these species and of this chemoautotrophic pathway.

Ladderanes are not the only unusual lipids to be found within the Planctomycetales. Species of *Pirellula* and *Planctomyces* (Kerger et al. 1988) contained abundant $C_{18:1\omega9}$ fatty

acid ***17***, more commonly a component of eukaryotes (in the above nomenclature for fatty acids, C_{18} refers to the total number of carbon atoms in the molecule, ':1' to the number of C=C double bonds in the chain, and 'ω9' designates the position of the double bond counted from the last (ω = omega) carbon atom in the chain). They also contained unique distributions of 3-hydroxy-fatty acids, which were suggested to be sufficiently diagnostic biomarkers for planctomycetes in some environmental settings. Unlike most bacteria, the *Planctomycetales* lack peptidoglycan in their cell walls and as such contain no muramic acid (e.g., König et al. 1984; Stackebrandt et al. 1986). Although many planctomycetes, including the anammox genera, contain "nucleoids" which confine the cellular DNA, *Gemmata obscuriglobus* is the only species known to contain a "nucleoid" which is *double* membrane-bound as found in eukaryotes (Fuerst and Webb 1991; Lindsay et al. 2001). *G. obscuriglobus* also is among the few bacterial species that biosynthesize sterols, and it is the only species (prokaryotic or eukaryotic) in which those sterols are not subsequently demethylated at position C_{14} (see lanostane ***4***) (Pearson et al. 2003). It remains unknown what intracellular roles all of the unusual planctomycete lipids serve, or how far back in the geologic record the molecular fossils of this group may extend.

17 $C_{18:1\omega9}$ fatty acid

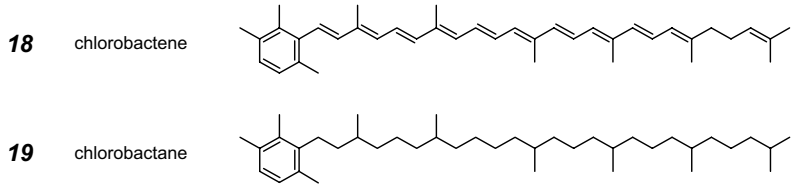


The Planctomycetales represent an unusual and divergent microbial lineage which traditionally has been difficult to place within the tree of life. Using approaches based on alternative phylogenetic treeing methods for 16S rRNA genes (Brochier and Philippe 2002) and on the relationships among C_1 metabolic pathway genes (Chistoserdova et al. 2004), it has been suggested that the *Planctomycetales* may be the most deeply branching of the bacterial taxa. By these analyses, the planctomycetes are basal to the rest of the bacteria and are the closest bacterial relatives to the archaea and eukaryotes.

Aromatic carotenoids, biomarkers for phototrophic oxidation of sulfide. Anoxic conditions in aquatic systems, and particularly the enigmatic Oceanic Anoxic Events such as OAE1b in the mid-Cretaceous, have become critical research areas for the geosciences. Periods of prevailing anoxia in large basins might be responsible for the widespread deposition of black shales, increased accumulation of petroleum source rocks, changes in global biogeochemical cycles, extreme shifts in climate, major mass extinctions, and concomitant biological radiations. Currently the only biomarker proxies available to study the most extreme form of anoxia, photic zone euxinia, are biomarkers of the phototrophic green and purple sulfur bacteria (Summons and Powell 1986; Requejo et al. 1992; Brocks et al. 2005; Grice et al. 2005).

Green sulfur bacteria (family Chlorobiaceae) are only distantly related to other phototrophic bacterial groups (Fig. 1). Chlorobiaceae only use photosystem I (PS I) and are strictly anaerobic, exploiting reduced sulfur species, such as hydrogen sulphide, as electron sources. In microbial mats, the requirement for sulfide and light restricts their habitat to the anoxic zone millimeters below the mat surface. In planktonic environments they live in a layer below the anoxic-oxic boundary, but within the photic zone. To adjust to the wavelength distribution and attenuated intensity of light at depth, Chlorobiaceae commonly possess an abundance of accessory carotenoid pigments. Green-pigmented species of planktonic Chlorobiaceae grow in a thin layer at water depths up to ~13 m, and their major carotenoid pigments are chlorobactene ***18*** and hydroxychlorobactene (Imhoff 1995), both of which yield the sedimentary biomarker, chlorobactane ***19.*** Brown-pigmented Chlorobiaceae inhabit a zone deeper than the green-pigmented species; they are usually found at water depths up to 18 m,

but were also observed in the Black Sea at 80 m (Repeta et al. 1989). Their pigment system is dominated by the carotenoids isorenieratene ***1*** and β-isorenieratene, the precursors for the hydrocarbon biomarkers isorenieratane ***2*** and β-isorenieratane (Liaaen-Jensen 1965).



Purple sulfur bacteria of the family Chromatiaceae are a sub-group of the γ-proteobacteria and utilize the PS II photosynthetic reaction center. Although unrelated to Chlorobiaceae, the Chromatiaceae have similar environmental requirements. Their preferred physiology is phototrophic oxidation of reduced sulfur under anoxic conditions. However, they are generally more tolerant to oxygen and can exploit a more versatile range of electron donors, including hydrogen. In microbial mats, as well as in planktonic environments, they grow in a thin layer directly above the zone of green sulfur bacteria but below the oxycline. Several species of planktonic Chromatiaceae have an accessory pigment system based on the red-

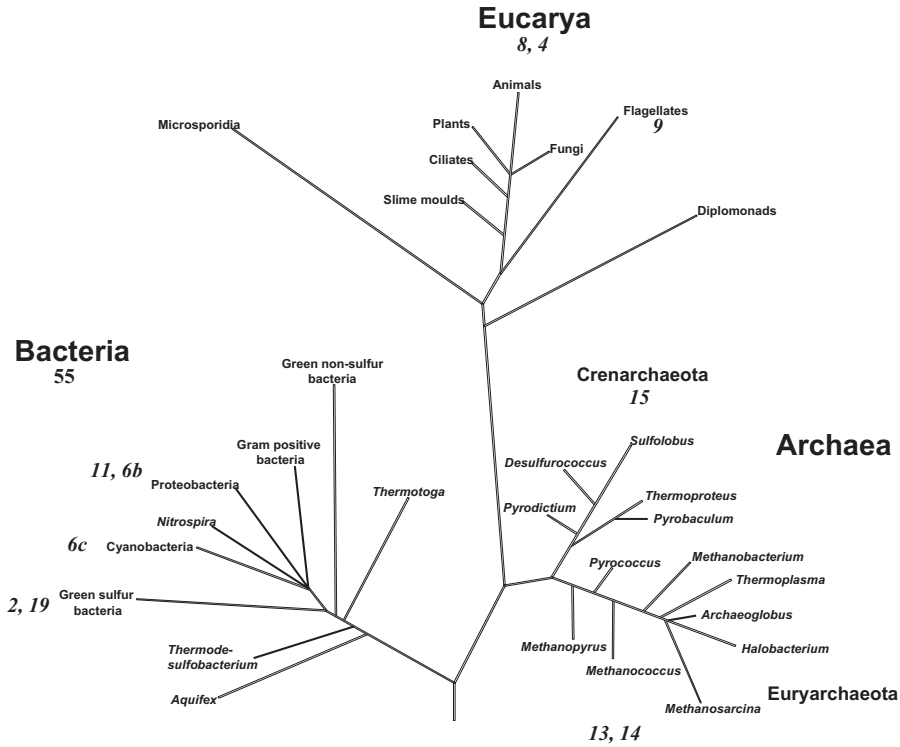


Figure 1. SSU rRNA phylogenetic tree annotated with structure numbers of biomarkers discussed in the text (adapted after Brocks and Summons 2004).

colored monoaromatic carotenoid okenone ***10***, which has a 2,3,4-trimethylaryl end-group. Planktonic species are commonly observed at water depths around 10 meters or less, and very rarely deeper than 20 meters (Van Gemerden and Mas 1995). Okenone ***11*** is the only known precursor for the hydrocarbon biomarker okenane ***10***, which represents the sole known proxy for purple sulfur bacteria in the fossil record. Biomarkers of green and purple sulfur bacteria are particularly valuable tracers for the study of marine anoxic conditions in the Precambrian (> 542 million years (Ma) ago), as body fossils of animals that give evidence about anoxic conditions in younger rocks do not exist in this period.

Biomarkers as indicators of the evolution of life and the environment

Phototrophic sulfur bacteria and a sulfidic ocean in the Proterozoic. Today, Earth's oceans are teeming with complex life, and even deep marine trenches contain enough oxygen to support macroscopic organisms. However, oceans in the distant past were fundamentally different. For the first two billion years of its existence, the ocean-atmosphere system was almost entirely anoxic (Fig. 2) (Holland 1994), but around 2450 to 2320 Ma ago, the disappearance of mass-independent fractionation of sulfur isotopes indicates that the concentration of atmospheric oxygen rose from previously trace levels to at least $10^{-5}\times$ the present level (Farquhar et al. 2000; Bekker et al. 2004; Holland 2004). Soon after this initial rise of oxygen, fossil soils (paleosols) begin to show typical oxic weathering patterns that suggest atmospheric O_2 quickly may have reached 15% of its present value (Rye and Holland 1998). However, the deep oceans remained mostly or entirely anoxic until at least ~1,800 Ma ago, the point in geological history when the last Paleoproterozoic banded iron formations (BIFs; iron silicates and iron carbonates) disappeared (Fig. 2).

Surprisingly, the state of the ocean in the following "mid-Proterozoic" interval (~1,800 to ~800 Ma) remains particularly mysterious. One model suggests that deposition of BIFs ceased ~1,800 Ma ago because Fe^{II} emitted from mid-oceanic ridges was precipitated immediately on the oxygenated sea-floor as Fe^{III}-hydroxides (Holland 1994). However, according to a competing model (Canfield 1998), Fe^{II} was not removed as oxidized rust but precipitated as Fe^{II}-sulfides in sulfidic ocean waters. Evidence is accumulating from the isotopic composition and distribution of sulfides (Canfield 1998; Shen et al. 2003; Poulton et al. 2004), sulfates (Kah et al. 2001) and molybdenum (Arnold et al. 2004), that appears to support Canfield's model. It is also possible that a hybrid of both models existed, resembling a 'marble cake' ocean (A. H. Knoll, personal communication).

If large areas of the world ocean were euxinic (anoxic and sulfidic) in the mid-Proterozoic, then our understanding of more than one fifth of Earth history would change

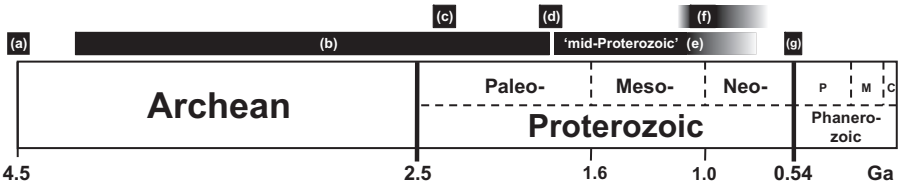


Figure 2. Geological time chart beginning with (a) the formation of Earth ~4.6 billion years ago (Ga); (b) anoxic, non-sulfidic oceans; (c) onset of oxygenation of the atmosphere (Bekker et al. 2004; Holland 2004); (d) disappearance of banded iron formations as indicator of changing ocean chemistry (Holland 2004); (e) the informally defined "mid-Proterozoic" interval with possible widespread, anoxic and sulfidic marine conditions (Canfield 1998); (f) major radiation of eukaryotic algae (Knoll 1992); (g) first appearance and major radiation of multicellular organisms and animals. 0.54 Ga marks the Precambrian-Cambrian boundary. P = Paleozoic, M = Mesozoic, C = Cenozoic.

radically. Geochemical cycles would have been altered, and many bioessential elements, such as nitrogen, molybdenum, and copper would have been rare. Trace-metal limitation may explain why familiar forms of life, such as modern algae and animals, arose so late in Earth history (Anbar and Knoll 2002). In the euxinic ocean, organisms requiring oxygen would have been marginalized, restricted to surface waters and shorelines. The ocean would have promoted extensive growth of green and purple sulfur bacteria wherever sulfidic conditions rose into the photic zone. The earliest evidence for the existence of phototrophic sulfur bacteria comes from a biomarker study on the 1,640 Ma Barney Creek Formation in the McArthur Basin, northern Australia (Brocks et al. 2005). Well preserved, organic-rich dolostones of the Barney Creek Formation were deposited in deep waters of the rift basin. The lipids extracted from these sedimentary rocks contain some of the oldest, clearly indigenous biomarkers known to date (Summons et al. 1988b). Significantly, the samples contain relatively high concentrations of isorenieratane ***2***, chlorobactane ***19***, and okenane ***10***. This indicates that the basin was stratified, and euxinic conditions extended—at least episodically—into the photic zone of the water column.

This ancient assemblage of biomarkers had two further characteristics which were radically different from any younger bitumen. Steroids alkylated at C-24 in the side chain and diagnostic for eukaryotic organisms were present at levels close to or below detection limits. In contrast, aromatic steroids without side chain alkylation but which were methylated at C-4 (see ***8***) were very abundant. These biomarkers, together with high relative concentrations of 3β-methyl-hopanes, suggest aerobic type-I methanotrophic bacteria were abundant members of the population. Aerobic methanotrophs are typically found in sulfate-starved environments (<0.5 mM) (Hoehler et al. 1998). Thus, these biomarkers corroborate isotopic evidence for marine sulfate concentrations below 0.5–2.5 mM in the mid-Proterozoic (Kah et al. 2004), far below present levels of about 28 mM. The paucity of diagnostic eukaryotic steroids in these bitumens, despite the abundance of typical bacterial steroids, is unique in Earth history. It suggests that eukaryotic algae were insignificant in the off-shore habitat of the McArthur Basin, possibly because they were asphyxiated by hydrogen sulfide (Martin et al. 2003), and/or because permanent sulfidic conditions depleted the pool of bioessential transition metals (Anbar and Knoll 2002). This scenario agrees with the distribution of algal fossils observed in the mid-Proterozoic Roper group, where algal diversity and fossil abundance were highest in the agitated, presumably nutrient-rich shore-line facies, but radically declined towards increasingly deeper waters (Javaux et al. 2001).

Apparent radiation of crenarchaeota associated with a Cretaceous OAE. Membrane lipids of crenarchaeota typically are composed of C_{86} compounds having glycerol dialkyl glycerol tetraether (GDGT; e.g., ***15***) structures (e.g., De Rosa and Gambacorta 1988; Koga et al. 1993). The alkyl chains of such molecules are acyclic, mono-, or poly-cyclic tetraterpenoids with the characteristic head-head linkage (as in ***15***) found in archaeal isoprenoids. These intact, parent lipids can be found in a diverse range of contemporary environmental settings, from the coldest lakes and oceans to the hottest thermal springs (e.g., Schouten et al. 2000, 2003b; Pearson et al. 2004; Powers et al. 2004; Pancost et al. 2005). The GDGTs and their C_{40}-isoprenoid hydrocarbon degradation products also are common throughout recent geologic history (e.g., Chappe et al. 1982; Kohnen et al. 1992; Hoefs et al. 1997).

The archaea thought to be responsible for the production of these compounds in marine settings have been classified as Marine Group I Crenarchaeota (DeLong 1992; Fuhrman et al. 1992), although some GDGTs also may derive from the Marine Group II Euryarchaeota. Enumeration of archaeal cells by FISH (fluorescent *in situ* hybridization) has revealed that these groups comprise as many as 40% of the free-living prokaryotic cells in the sub-euphotic zone and as many as 20% of the total prokaryotic cells in the open ocean today (Karner et al. 2001). This finding of a major archaeal population in the global ocean represents a major shift

from previous ecological views, which had held archaea to be the dominant species only in "extreme" environments.

The GDGTs of marine archaea hold promise not only as taxonomic biomarkers, but also as paleotemperature indicators and as isotopic tracers of changes in the ^{13}C-concentration of dissolved inorganic carbon (DIC) in the ocean. The GDGT paleotemperature proxy, TEX_{86} (Schouten et al. 2002), shows promise to reconstruct ocean temperatures in the past. TEX_{86} is a parameter calculated in a fashion similar to the $U^{k'}_{37}$ alkenone paleotemperature proxy (Brassell et al. 1986; Prahl and Wakeham 1987); it uses the ratio of the number of pentacyclic rings contained in the GDGT homologues that contain 1, 2, 3, and 5 rings. TEX_{86} has been approximated as a linear function of temperature: $TEX_{86} = 0.015T(°C) + 0.028$ (Schouten et al. 2002). Temperature reconstructions based on this calibration appear to be accurate over a wide range of environmental settings (including lakes; Powers et al. 2004) and apply to temperature regimes from 0°C to ~35°C. Recently, TEX_{86} has been used to determine that sea-surface temperatures were warm and the pole-equator temperature gradient was small in the Cretaceous (Schouten et al. 2003a; Jenkyns et al. 2004).

A fundamental goal of biogeochemistry is to understand major reorganizations in the global carbon cycle. This requires the ability to accurately reconstruct the carbon isotopic composition of dissolved inorganic carbon (DIC) in the oceans. Paleoproxies based on the $\delta^{13}C$ values of calcium carbonate can be subject to diagenetic recrystallization, and often they are not continuous through time due to the high solubility of $CaCO_3$. Hence, the archaeal GDGTs may play a critically important role for biogeochemistry: they can be used to reconstruct the $\delta^{13}C$ value of DIC. The isotopic fractionation expressed between GDGTs and DIC ($\varepsilon_{lipid\text{-}DIC}$) appears to be constant over a wide range of environmental settings and geologic age. Values of $\delta^{13}C$ for these lipids were first measured by Kohnen et al. (1992) and in more detail by Hoefs et al. (1997). The latter paper reported $\delta^{13}C$ values for archaeal C_{40} hydrocarbons from suspended particulate matter, contemporary marine sediments, and Pleistocene, Pleiocene, and Miocene-age materials. The samples represented both oxic and anoxic environments with a range of depositional settings, yet the range in $\delta^{13}C$ values of $C_{40:0}$ (0-ring) to $C_{40:3}$ (3-ring) isoprenoids was only −23.4‰ to −18.7‰, and the average value was −21 ± 1‰ (data averaged from: Hoefs et al. 1997; Schouten et al. 1998b; Pearson et al. 2001). This value is isotopically heavier than most sedimentary lipids, and Hoefs et al. (1997) suggested that it might reflect the utilization of an autotrophic pathway with a small fractionation factor, $\varepsilon_{biomass\text{-}DIC}$. Apparent confirmation of autotrophy in these marine archaea was obtained by Pearson et al. (2001) who showed compound-specific ^{14}C evidence that the archaea utilize DIC from below the euphotic zone; and also by Wuchter et al. (2003), who showed the uptake of ^{13}C-labeled DIC into archaeal biomass in a mesocosm incubation experiment.

If it is assumed that the offset between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{GDGT}$ is consistent throughout geologic time, the $\delta^{13}C_{DIC}$ values of past oceans can be reconstructed. This proxy is not limited to intervals of time or locations in which carbonate (e.g., in the form of foraminifera) is preserved, and potentially extends throughout much of the Phanerozoic. However, as yet it is unknown how far back in geologic time the marine archaea that produce these lipids extend. Recently, Kuypers et al. (2001) suggested the major radiation of this group occurred in association with Cretaceous OAE 1b (~112 Ma). Values of $\delta^{13}C$ for archaeal isoprenoids throughout the OAE were ~ −18‰, which is 3‰ heavier than observed in contemporary oceans. This is consistent with the increased burial of organic matter in marine sediments during the OAE, which would drive the $\delta^{13}C$ of oceanic DIC to heavier values. Significantly, the $\delta^{13}C$ value of total buried organic carbon (TOC) in these sediments also increased from values of ~ −25‰ to ~ −20‰ over the duration of the OAE. Kuypers et al. (2001) argue that deposition of isotopically-heavy archaeal biomass accounts for this change, and that up to 80% of the TOC in these sediments is derived from the biomass of archaea.

Thus, the warm, stratified OAE conditions may have been the trigger that permitted the radiation of archaea throughout the world ocean (Kuypers et al. 2001). However, it is possible that in select cases the record of marine archaeal lipids may be extended farther back in time. Peckmann and Thiel (2004) recently reviewed data for fossilized CH_4 seeps extending back to the late Jurassic. $C_{40:0}$ and $C_{40:1}$ archaeal isoprenoids were found in the oldest samples (Peckmann et al. 1999), suggesting that the marine crenarchaeota could predate the Cretaceous, although they may have been confined to sedimentary rather than pelagic origins. Regular acylic isoprenoids with 25 carbon atoms even date back to the 1,640 Ma Barney Creek Formation in northern Australia, and they are likely derived from unspecific Archaea (Summons et al. 1988b). However, crenarchaeol ***15***, the cyclohexane ring-containing diagnostic compound for marine pelagic archaea, has not been found prior to the Cretaceous.

Orphan biomarkers and unknown pathways

Hopanes, formerly orphans. More than one thousand billion tons of hopanes ***6*** are stored in sedimentary rocks and oil reservoirs, a mass that is almost as high as the combined mass of carbon in all living organisms (Ourisson 1994). Although hopanes are among the most abundant organic molecules on Earth, their biological source remained obscure for a long time (Ourisson 1994). A search for the biological origins of these "orphan biomarkers" led to the discovery of their parent compounds, the bacteriohopanepolyols ***5*** (for an overview see Rohmer et al. 1984). Hopanoids now are recognized as one of the most important and abundant classes of lipids in bacteria, although not all—indeed, not even a majority of—bacteria contain hopanoids. The main function of hopanoids is usually thought to be modification of membrane properties similar to the role of sterols in eukaryotes. The search for the biological origins of hopanoids also led to the discovery of the fundamentally new methylerythritol phosphate (MEP) pathway responsible for the biosynthesis of the C_5 isoprenoid building block in bacteria (Rohmer et al. 1993).

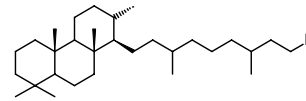
Pentacyclic terpenoids with the C_{30} hopane skeleton have also been detected in some plants, but the bacteriohopanepolyols ***5,*** with an extended side chain and 35 carbon atoms, appear to be diagnostic for bacteria. However, below the level of Domain, the taxonomic value of hopanes appears somewhat limited. Hopanoids occur in some, but not all, groups of bacteria and their distribution among these groups does not appear to follow a clear rule (Rohmer et al. 1984). Hopanes that have an additional methyl group at ring-A, however, can be used as taxonomic markers. These products include the 2-methyl and 3-methylhopanoids. The 3β-methylhopanoids ***6b*** appear to be diagnostic for microaerophilic methanotrophs, methylotrophs, and acetic acid bacteria (Zundel and Rohmer 1985; Summons and Jahnke 1992). The values of $\delta^{13}C$ measured for 3β-methylhopanoids are often strongly depleted in ^{13}C (Jahnke et al. 1999), confirming that aerobic methanotrophs are an important source of these lipids in sedimentary environments where they metabolize ^{13}C-depleted methane. The second class of ring-A methylated hopanoids, the 2-methylhopanepolyols, are believed to be products exclusively of cyanobacteria, and 2α-methylhopanes ***6c*** in ancient sedimentary rocks were, thus, interpreted to be indicators for oxygenic photosynthesis (Summons et al. 1999).

The biological origin of the formerly parentless hopanes is solved. However, the sources of many other orphan biomarkers remain mysterious, and their discovery may yield profound insights into unknown biosynthetic pathways or even lead to the discovery of unnoticed organisms. Among these classes of enigmatic and elusive orphans are cheilanthanes and BAQCs.

Cheilanthanes. One group of particularly abundant, orphan biomarkers in sedimentary rocks and petroleums of all ages, which is found from the Precambrian (Summons et al. 1988a) to the present (Simoneit et al. 2004), is the cheilanthanes ***20*** (Aquino Neto et al. 1982). This family of tricyclic terpanes contains homologues having from 19 to at least 45 carbon atoms (Moldowan and Seifert 1983). Natural products bearing the cheilanthane skeleton are

known from sponges (Manes et al. 1988; Buchanan et al. 2001), nudibranchs (Miyamoto et al. 1992) and ferns (Khan et al. 1971). However, these organisms do not contribute high mass of organic matter to sediments and they are certainly not the biological sources of the ubiquitous and abundant cheilanthanes.

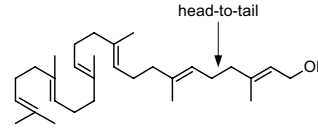
20 cheilanthane



R = isoprenyl

Cheilanthanes ***20*** are particularly interesting for scientists studying the earliest history of life and the evolution of biosynthetic pathways. (Ourisson 1994) recognized that the biosynthesis of membrane lipids based on the cheilanthane skeleton could follow a "primitive" pathway that might have predated the evolution of C_{40} isoprenoids (e.g., ***15***) in archaea, bacteriohopanepolyols ***5*** in bacteria, and sterols (e.g. ***3***) in eukaryotes. The biosynthesis of the acyclic precursors to cheilanthanes involves, from a chemical point of view, a less complex process than the complicated syntheses required for these other classes of lipids. First, hexaprenol ***21*** or hexaprene, plausible acyclic precursors of cheilanthanes, are solely constructed of head-to-tail linked C_5 isoprene units. The generation of head-to-tail linkages is relatively simple, either chemically (abiotic) or enzymatically. In contrast, the biosynthesis of C_{40} isoprenoids of archaea requires head-to-head (as in ***15***) condensation of two C_{20} units; and squalene (***12*** without the oxygen atom), the precursor of sterols and hopanoids, requires tail-to-tail condensation (as in ***13***) of two C_{15} units. Both of these linkages are more difficult to achieve from a chemical standpoint, and they include enzymatic reactions which are not well understood. Second, the cyclization of hexaprenol to the cheilanthane skeleton follows a chemically favorable stereochemistry that could, in principle, even proceed by simple acid catalysis without the aid of enzymes (Ourisson 1994). Third, in contrast to the synthesis of sterols, the production of cheilanthanes probably follows an anaerobic pathway and could have evolved early in Earth history before the rise of oxygen. These observations led Ourisson to speculate that cheilanthanes might have a very ancient biological origin as membrane lipids.

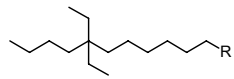
21 hexaprenol



However, there is also evidence that the biological precursor compounds that eventually degrade to form cheilanthanes do not occur as free membrane lipids. The origin of cheilanthanes may be from the cell walls of eukaryotic algae. Evidence comes from the Late Carboniferous to Early Permian Tasmanite Oil Shale in Tasmania, which contains very high concentrations of cheilanthanes. It also contains abundant, preserved cells of *Tasmanites* (Simoneit et al. 1986). Laser pyrolysis experiments on the cell walls of *Tasmanites* (Greenwood et al. 2000), as well as chemical degradation of the kerogen with subsequent determination of the $\delta^{13}C$ values of the generated lipids (Simoneit et al. 2005) suggests that cheilanthanes are derived directly from the algal cell wall material. However, the data could also be consistent with an independent biological source of cheilanthanes from microorganisms feeding on decaying cells of *Tasmanites*. Thus, the cheilanthanes will remain orphans until they are detected in living organisms.

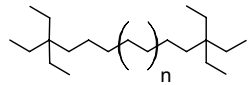
BAQCs (Branched Alkanes with Quaternary Carbon). In 1988, Mycke et al. detected a suite of previously unknown molecules in a shale and a massive sulfide from the 1,653 Ma (Page and Sweet 1998) McNamara Group, Mount Isa Basin, Australia. The same set of compounds was also found in a ~2,000 Ma coal from the Zaonezhskaya Formation in Russia. According to gas chromatograms and mass spectra, the molecules appeared to be branched alkanes having exclusively odd numbers of carbon atoms in the range of C_{17} to C_{31}. Alkanes with the same 100%-odd distribution and identical mass spectra later were discovered in several other Precambrian and Phanerozoic sedimentary rocks and preliminarily (and wrongly) identified as 3,7-dimethylalkanes (for overviews see Brocks and Summons 2004; Greenwood et al. 2004; Kenig et al. 2005). Using a chemically synthesized standard, Kenig et al. (2003) later identified the branched compounds as a homologous series of 5,5-diethylalkanes ***22***; this group of compounds possesses a quaternary carbon atom (a central carbon with four carbon substituents) and as such they are called BAQCs. Kenig identified a total of 12 different series of BAQCs with one or two quaternary carbon centers, including 2,2-dimethyl- and 3,3,ω3,ω3-tetraethylalkanes ***23***. Each homologous series contains exclusively even or exclusively odd numbers of carbons. Once identified, BAQCS were recognized to occur in many ancient sedimentary rocks, in recent sediments, and even in crustal fluids emanating from a borehole in the sea floor (Kenig et al. 2003, 2005).

22 5,5-diethylalkanes



R = *n*-alkyl

23 3,3,ω3,ω3-tetraethylalkanes



BAQCs are orphan biomarkers, and their biological source and the entire biosynthetic pathway leading to their quaternary carbon atoms remains unknown, although they would appear to be produced from acetate, rather than from isoprene. Generally, acyclic biochemicals with quaternary carbon atoms are exceedingly rare in nature. The only notable exceptions appear to be the highly branched isoprenoids from algae of the genus *Botryococcus* and fatty acid-based toxins found in one species of cyanobacteria (Orjala et al. 1995), although both sources are unlikely precursors to the BAQCs described here.

What is the physiology and habitat of the BAQC organisms? Kenig et al. (2003), appraising the absence and presence of BAQCs in different recent and palaecological settings, suggested that the unknown source organisms are non-photosynthetic, thermophilic prokaryotes that oxidize sulfide at benthic redox boundaries. They may use oxygen or nitrate as electron sources, and these BAQC organisms might have inhabited sedimentary environments dating back to at least 2,000 Ma. However, this assessment can not be entirely correct. The 1,653 Ma McNamara Group and ~2,000 Ma Zaonezhskaya Formation, in which the BAQCs were first detected, have both experienced greenschist facies metamorphisms at temperatures above 200 to 250 °C. These temperatures are conclusively inconsistent with the preservation of 100% odd over even carbon number predominances; degradation of the homologous series to include a measurable odd:even carbon number ratio would have occurred. The carbon number ratios of biomarkers are modified during thermal alteration, due to generation of shorter products by thermal cracking. Therefore, the BAQCs must have entered the samples

after metamorphism. There is additional evidence that the mysterious BAQC organisms may inhabit rock surfaces and fissures but are not always part of the original environments where the sedimentary rocks were deposited. For instance, in a recent appraisal of Paleoproterozoic rocks from the McArthur Basin in Australia and a Neoproterozoic carbonate from China (J. J. Brocks, unpublished results), BAQCs were detected on surfaces of rock samples but appeared to be absent from the interior. They were particularly abundant in decaying shales containing oxidized sulfides. Thus, it is at least plausible that BAQCs are often derived from organisms inhabiting recent environments, such as rock surfaces, where they might be involved in the aerobic decomposition of sulfide minerals or organic matter.

BUILDING THE BIOMARKER TREE OF LIFE

One of the most fascinating aims of geobiology is to reconstruct the co-evolution of life and environment throughout the history of Earth. When did the major metabolic pathways, such as oxygenic photosynthesis and anaerobic and aerobic oxidation of methane, first appear in the geologic record? What were the impacts of these pathways on environmental conditions and on geochemical cycles? How did the associated global change, in turn, affect subsequent biological evolution?

Biomarkers extracted from sedimentary rocks have the potential to tie together information about these historical events with the advent of specific microbial metabolisms. Biomarkers can help to detect the activity of sulfide-oxidizing phototrophs, anaerobic methanotrophs, and oxygenic phototrophs, to name only a few examples. However, utilizing these biomarker proxies to understand Earth history is not equivalent to reconstructing the origin of individual microbial species or groups. Such notions of the temporal stability of taxonomic groups are not well defined, and taxonomic lineages (Fig. 1) may be quite incongruent with a time-line of Earth history (Fig. 2) if metabolic pathways can be transferred horizontally between unrelated organisms. What the chemical structures of hydrocarbon biomarkers and their associated carbon isotopic compositions do provide are records of the *metabolic pathways* of carbon assimilation and of lipid biosynthesis. As such, phylogenetic trees based not on 16S rRNA sequences, but on the amino acid sequences of enzymes critical to the metabolic pathways, may yield a record of the origin and distribution of some of these fundamental processes. Biomarkers extracted from the rock record represent a means to validate any hypotheses resulting from these phylogenetic reconstructions. We must view the traditional 16S rRNA-based tree of life as only one possible template on which to superimpose information about the evolution of biosynthetic capabilities.

Therefore, future investigations of the origins of biomarkers in the context of Earth history are likely to include more emphasis on the phylogenetic distribution of the pathways of lipid biosynthesis. This approach has been enabled recently by the revolution in genomic sequencing. Now organic geochemists have the ability to investigate molecular markers not only as found in post-depositional settings that integrate the associated, complex ecosystems, but also within the enzymatic and phylogenetic contexts of their biosynthetic pathways. As such, the "biomarker tree of life" may eventually be constructed and used as a tool in the quest to interpret the record of biomarkers in sedimentary rocks.

A phylogenetic approach to the origin and distribution of biomarkers

Early attempts to define the phylogenetic distribution of particular classes of biomarkers relied on the broad survey—or screening—approach. An example is the seminal paper by Rohmer et al. (1984) on the distribution of bacteriohopanepolyols ***5*** in prokaryotes. In this work, more than 90 bacterial and archaeal taxa were grown in pure culture and their lipids were analyzed for the abundance and diversity of hopanoid products. Several fundamental

conclusions resulted from this study, namely that hopanoids are absent in archaea (then called "archaebacteria"), and that hopanoids are not universally or even systematically distributed among the bacteria. In addition, this work also suggested that obligate anaerobes did not make hopanoids, despite the absence of a requirement for O_2 in hopanoid biosynthesis. The only anaerobes shown to make hopanoids (Rohmer et al. 1984; Neunlist et al. 1985; Ourisson et al. 1987) were facultative anaerobes belonging to the purple non-sulfur bacteria (α-proteobacteria). Only more recently have hopanoids been found in additional species growing anaerobically, including anammox planctomycetes (Sinninghe Damsté et al. 2004), *Geobacter metallireducens*, *Geobacter sulfurreducens*, and *Magnetospirillum magnetotacticum* (Fischer et al. 2005; Härtner et al. 2005). Significantly, the *Geobacter* and *Magnetospirillum sp.* were initially identified as producers of hopanoids based solely on their genome sequences, which contain genes for a central enzyme in the production of hopanoids, squalene-hopene cyclase (Fischer et al. 2005).

This approach suggests that future investigations of the source-specificity of biomarkers will focus on simultaneous characterization both of the lipid products and of the genes that produce them (e.g., Bode et al. 2003; Pearson et al. 2003; Fischer et al. 2005). To extend the application of biomarker analysis to geobiology, it is critical to use a comprehensive genomic, molecular, biochemical, and isotopic approach to understand the distribution of lipids in environmental samples. With this information, more will be learned about the evolutionary and taxonomic roles these lipids can illuminate. In particular, it is only through analysis of the amino acid sequences of lipid biosynthesis genes that questions about the origins of particular biomarkers will be answered. Such information is the key to resolving questions of the antiquity of biosynthetic pathways, vertical inheritance of these genes, and/or evolutionary scrambling via horizontal gene transfer.

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