

Branched aliphatic alkanes with quaternary substituted carbon atoms in modern and ancient geologic samples

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A pseudohomologous series of branched aliphatic alkanes with a quaternary substituted carbon atom (BAQCs, specifically 2,2-dimethylalkanes and 3,3- and 5,5-diethylalkanes) were identified in warm (65°C) deep-sea hydrothermal waters and Late Cretaceous black shales. 5,5-Diethylalkanes were also observed in modern and Holocene marine shelf sediments and in shales spanning the last 800 million years of the geological record. The carbon number distribution of BAQCs indicates a biological origin. These compounds were observed but not identified in previous studies of 2.0 billion- to 2.2 billion-year-old metasediments and were commonly misidentified in other sediment samples, indicating that BAQCs are widespread in the geological record. The source organisms of BAQCs are unknown, but their paleobiogeographic distribution suggests that they have an affinity for sulfides and might be nonphotosynthetic sulfide oxidizers.

Prokaryotes in ancient environments usually are detected by microscopy (1, 2) and geochemical evidence (e.g., stable isotope fractionation of carbon and sulfur; ref. 3). The fossil record of bacteria and archaea is meager and controversial because of their poor preservation potential (1, 4). However, some lipids have excellent preservation potential and are used to demonstrate the presence of archaea and bacteria in ancient sedimentary environments (2, 5–9). Here we identify branched aliphatic alkanes with quaternary substituted carbon atoms (BAQCs). These compounds, observed in hydrothermal waters at the Ocean Drilling Project (ODP) site 1026B on the flanks of the Juan de Fuca ridge (Northeast Pacific) are natural products and occur in nonhydrothermal sediments ranging in age from modern to at least 2.0 billion to 2.2 billion years (Ga).

Methods

Field Sampling. Particulate organic material was retrieved from hydrothermal waters by using a combusted glass-fiber filter (142 mm) affixed to a circulation obviation retrofit kit (10) of ODP borehole 1026B (Juan de Fuca Ridge, Northeast Pacific; ref. 11). The borehole, which penetrates 247 m of sediments and 48 m of basaltic oceanic crust, is sheathed by a steel liner. The glass-fiber filter was in place for 30.5 h. During that time, it collected particles from 8,200 liters of basaltic crust-derived waters. The filter was deployed and recovered by the remotely operated vehicle JASON. It was kept frozen (–15°C) until lyophilized.

The Cenomanian and Turonian (Late Cretaceous) samples were collected at outcrops along the Bainbridge river in the Pasquia Hills region of easternmost central Saskatchewan, Canada (12). They are thermally immature based on the dominant 17 β ,21 β (H) stereochemical configuration of the hopanes. The descriptions of other sediment samples analyzed can be found in the references listed in the Fig. 3 legend.

Laboratory Methods. Extraction, separation, and GC-MS were performed as described (13). The filter and sediment samples were Soxhlet-extracted by using dichloromethane and methanol

(7.5:1, vol/vol). The sediment extracts were further separated by column chromatography to obtain a hydrocarbon fraction. Linear alkanes were removed from the hydrocarbon fraction by adduction on US-Y zeolite to obtain a fraction of branched and cyclic hydrocarbons. The total extract and subfractions, as well as authentic standards, were analyzed by GC-MS on a Hewlett–Packard 6890 gas chromatograph coupled to a Hewlett–Packard 5973 mass selective detector operated in electron ionization mode at 70 eV, scanning a mass range of m/z 40–650 at 2.44 scans per s. A 30-m Hewlett–Packard HP-5MS fused silica capillary column (0.25 mm id, 0.25- μ m film thickness) was used with helium as carrier gas. Samples were injected at 60°C and held for 1.5 min, and the oven temperature was programmed to 130°C at 20°C/min, and then at 4°C/min to 315°C, at which it was held for 58 min.

Matrix isolation–Fourier transform IR (FTIR) spectra were acquired by using a conventional GC-MS (Agilent 6890/5973) coupled with a Thermo-Nicolet (Madison, WI) FTIR instrument. The carrier gas was doped with \approx 2% argon, and the column effluent was split \approx 30/70 between the MS and the matrix isolation cell. The analyte and argon were condensed at 10K on a gold-plated optical cylinder. IR data were collected via an IR microscope coupled with a Thermo-Nicolet FTIR. The system is an updated version of previous GC-matrix isolation-FTIR systems as described (14, 15).

Proton and ¹³C NMR spectra of synthesized standards were obtained on a Bruker (Billerica, MA) Avance DPX-400 spectrometer.

Synthesis. Authentic samples of 3,3-diethylpentadecane and 5,5-diethylpentadecane were obtained by atmospheric pressure hydrogenation at ambient temperature of 3,3-diethyl-4Z-pentadecene and 5,5-diethyl-2E-6Z-pentadecadiene, respectively, in ethyl acetate solution over 10% palladium/charcoal as catalyst. These alkenes were obtained by condensation under a nitrogen atmosphere in cold (–78°C) tetrahydrofuran solution of 2,2-diethylbutanal (16) and the ylid generated with butyllithium from commercial undecyltriphenylphosphonium bromide, and of 2,2-diethyl-4E-hexenal (17) with the ylid from nonyltriphenylphosphonium bromide, respectively. This process was followed by purification by chromatography over silver nitrate-impregnated silica gel, eluting with hexanes.

Coinjection Experiment. The authentic standards of 3,3- and 5,5-diethylpentadecane were coinjected with natural samples on

Abbreviations: BAQC, branched aliphatic alkane with a quaternary substituted carbon atom; DMA, dimethylalkane; DEA, diethylalkane; ODP, Ocean Drilling Project; Ga, billion years; FTIR, Fourier transform IR; Ma, million years.

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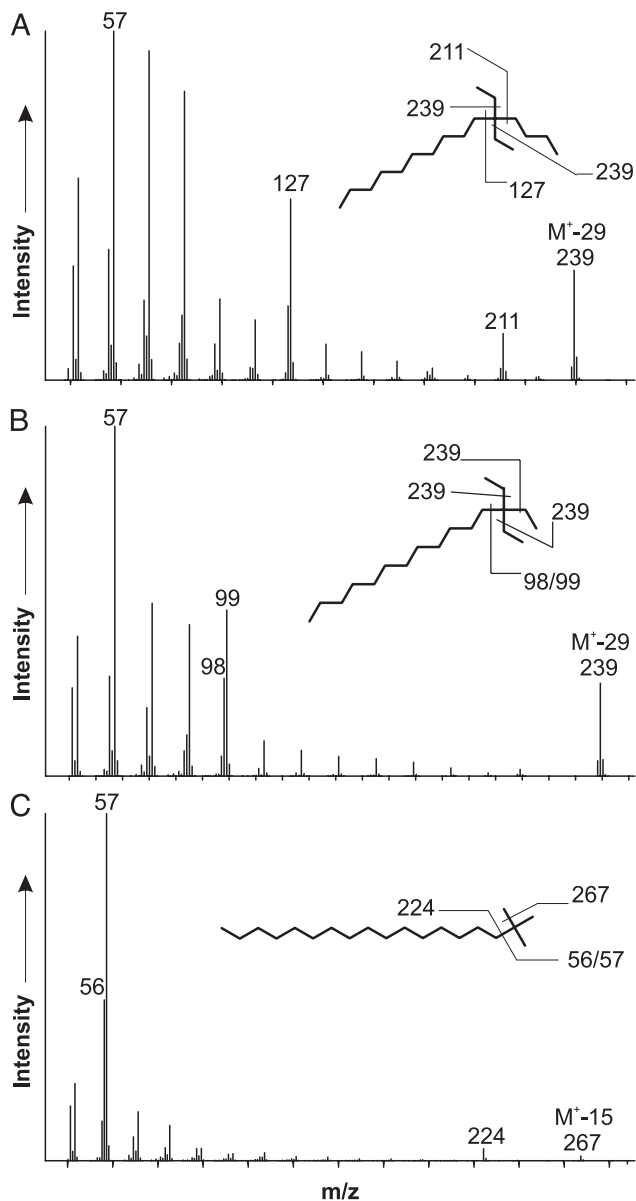


Fig. 1. Mass spectra and skeletal structure of authentic 5,5-diethylpentadecane (A) and 3,3-diethylpentadecane (B). (B) The fragment ion M^{+29} corresponds to the loss of one of the three ethyl substituents. The fragment-ion pair m/z 98 and 99 corresponds to the loss of the largest substituent of the quaternary carbon atom, with m/z 98 indicating a hydrogen rearrangement. (C) Skeletal structure and mass spectra of 2,2-dimethyloctadecane obtained from a peak, free of coeluting compounds, of the branched and cyclic hydrocarbon fraction of a Cenomanian shale (see Fig. 2B). Dominant ions m/z 56 and 57 correspond to a tertiary butyl fragment, and the ion m/z M^{+58} corresponds to the loss of a tertiary-butyl substituent. The minor ions M^{+15} correspond to the loss of one of the methyl substituents at C-2. The structure of 2,2-dimethyloctadecane was confirmed by the presence of tertiary butyl absorption bands in the GC-matrix isolation-FTIR spectra of that peak (see *Methods*).

two different capillary columns: HP-5 and HP-1, both 30 m, 0.25-mm internal diameter and 0.25- μ m film thickness.

Mass Spectra. The mass spectra of 5,5-diethylalkanes (DEAs), for example, that of 5,5-diethylpentadecane (Fig. 1A), are characterized by three fragment ions corresponding to the cleavage of the carbon-carbon bonds α to the quaternary carbon atom at C-5. The fragment ions M^{+29} and M^{+57} correspond to the loss

of an ethyl and a butyl substituent, respectively. The predominance of m/z M^{+29} over m/z M^{+57} is explained by the presence of two ethyl substituents. The fragment-ion pair at m/z 126 and 127 corresponds to the loss of the largest substituent on the quaternary carbon atom, with m/z 126 indicating a hydrogen rearrangement.

NMR of Authentic 3,3-Diethylpentadecane. The ^1H and ^{13}C NMR spectra of 3,3-diethylpentadecane are fully consistent with the expected structure. Three identical ethyl groups are indicated in the ^1H spectrum. Consistent with the presence of a 3,3-diethyl group, only 15 unique signals appear in the ^{13}C spectrum, including a quaternary carbon. ^1H -NMR (400 MHz, CDCl_3): δ 0.71 (*t*, $J = 7.5$ Hz, 9 H), 0.88 (*t*, $J = 7.0$ Hz, 3 H), 1.16 (*q*, $J = 7.5$ Hz, 6 H), 1.26 (br. s, 22 H); ^{13}C -NMR (100 MHz, CDCl_3): δ 7.47, 14.13, 22.70, 22.90, 27.50, 29.36, 29.66–29.75 (broad envelope containing five overlapping signals), 30.72, 31.93, 35.05, 37.00 (quaternary C).

FTIR Spectroscopy of 2,2-Dimethylalkane (DMA). FTIR spectra of all 2,2-DMAs are typical of saturated hydrocarbons, with no indication of the presence of unsaturated or heteroatom-containing structures. C-H bending modes for the compounds identified as 2,2-DMAs are characterized by absorption at 1,255, 1,395, and 1,370 cm^{-1} , with absorption at 1,370 cm^{-1} being considerably stronger than at 1,395 cm^{-1} . This pattern, which arises because of coupling of the in-phase and out-of-phase symmetrical CH_3 deformations of methyl substituents attached to a common carbon, is characteristic of tertiary butyl structures, and hence strongly supports the structural assignments for these compounds.

Results and Discussion

Lipids extracted from particulate matter collected at ODP hole 1026B (11) are dominated by families of previously unknown BAQCs, specifically, 2,2-dimethyl and 5,5-DEAs. Lesser amounts of 3,3-DEAs were also observed (Fig. 2A). These compounds, with 16–27 carbon atoms, were identified on the basis of their mass spectral fragmentation and their precise coelution with authentic standards of 3,3- and 5,5-diethylpentadecane on two different capillary columns (see *Methods*). The structure of the 3,3-diethylpentadecane was confirmed by ^1H and ^{13}C NMR, and the structure of 2,2-DMAs was confirmed by the presence of tertiary-butyl absorption bands in the GC-matrix isolation-FTIR spectra of 2,2-DMAs (see *Methods*).

Pseudohomologous families of BAQCs, such as 2,2-DMAs, 3,3-DEAs, and 5,5-DEAs, are preponderant in the branched- and cyclic-hydrocarbon fractions of the lipid extracts of thermally immature Cenomanian and Turonian black-shales (Cretaceous, Saskatchewan, Canada; Fig. 2B). Most of the less abundant components (Fig. 2B) appear to represent pseudohomologous families of BAQCs. Structures await verification by synthesis of authentic standards.

In both sample sets, the 5,5- and 3,3-DEAs occur as C_{15} to C_{29} components with odd carbon numbers. The 2,2-DMAs, which range from C_{16} to C_{26} , have even carbon numbers. These distinct carbon-number distributions are characteristic of biosynthetic products (18).

Paleobiogeographic Distribution. The distribution of 5,5-DEAs was surveyed at the University of Illinois (Chicago) in samples spanning the last ≈ 800 million years (Ma) of the sedimentary record (Fig. 3). The identification of 5,5-DEAs in modern to Holocene shelf sediments of the Barents Sea, the St. Anna Trough (Arctic Russia), and Abu Dhabi (United Arab Emirates), as well as in several Mesozoic, Paleozoic, and Neoproterozoic shales, indicates that organisms biosynthesizing BAQCs

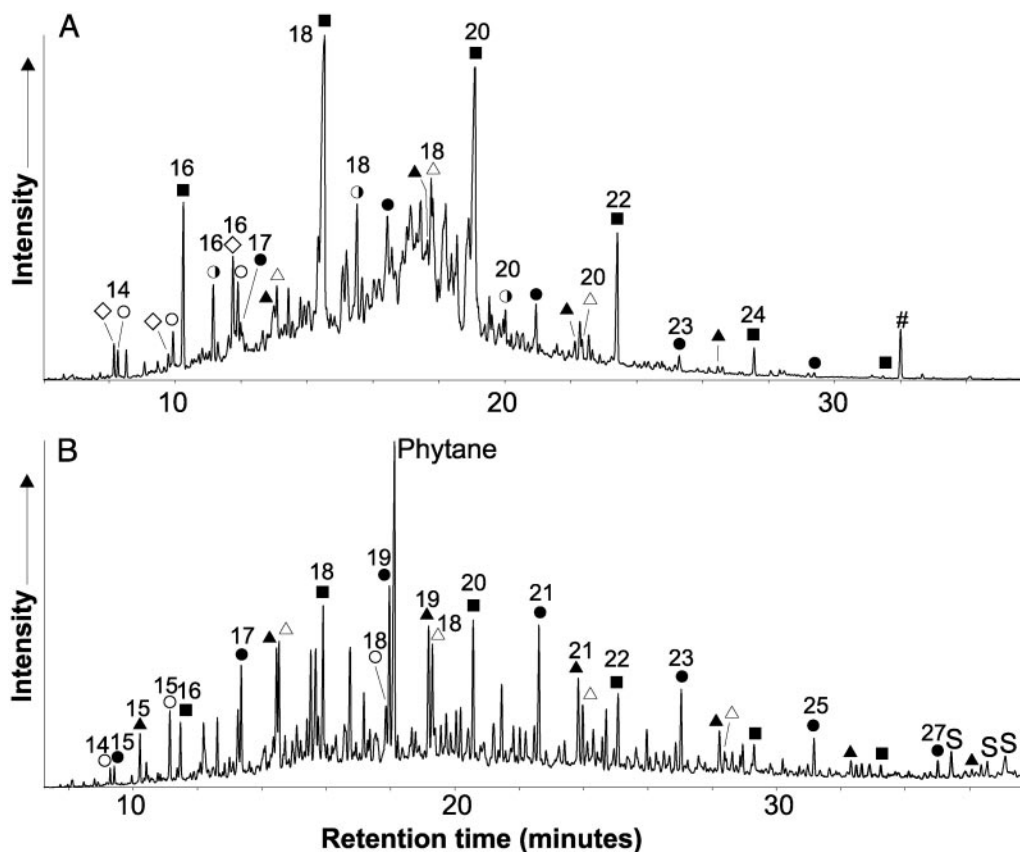


Fig. 2. Total ion chromatograms showing the distribution of BAQCs. (A) Total extract of a glass-fiber filter used for *in situ* filtration of water escaping from ODP borehole 1026B on the Juan de Fuca Ridge (Eastern Pacific). (B) Branched and cyclic hydrocarbon fraction of a Cenomanian shale from the Bainbridge River section (Pasquia Hill, Saskatchewan, Canada). Numbers on peaks refer to carbon atoms. ●, 5,5-DMAs; ▲, 3,3-DEAs; ■, 2,2-DMAs; ○, 2-methylalkanes; △, *n*-alkylcyclopentanes; ○, *n*-alkanes; ◇, *n*-alkenes; S, steroidal hydrocarbons.

are not limited to deep-sea hydrothermal environments or black shales (Fig. 3).

The distributions of 2,2-DMAs in fluids escaping from the borehole at ODP site 1026B (C_{16} – C_{26} , with predominant C_{18}) and in the Cenomanian shales (C_{16} – C_{28} , with predominant C_{18} ; Fig. 2B) are similar. However, differences in the relative abundances of 2,2-DMAs, 5,5-DEAs, and 3,3-DEAs between these two samples and among others analyzed, where 2,2-DMAs and 3,3-DEAs are sometimes absent, suggest that BAQCs do not derive from a single organism but from a group of related organisms. Alternatively, changes in relative abundance of BAQCs may be caused by a biosynthetic response by source organism(s) to different environmental conditions.

Data available in prior reports show that the occurrence of 5,5-DEAs extends to 2.0–2.2 Ga (Fig. 3). Until now these compounds have been either mistaken for 3,7-DMAs or unidentified. Mycke *et al.* (19) discuss the presence of 3- and 5-methylalkanes, *n*-alkylcycloalkanes, and an unknown pseudohomologous series of branched compounds in organic matter released by hydrogenolysis of extracted sediments from the 2- to 2.2-Ga Shungit coal (Russia) and the 1.5- to 1.6-Ga Mount Isa massive sulfide and black shales of Australia. The mass spectrum of one of the unknown branched compounds (19) is identical to that of the 5,5-diethylnonadecane identified in waters escaping the ODP 1026B borehole and in extracts of the Pasquia Hill black shales. Moreover, the carbon-number distribution (C_{17} – C_{31} ; odd carbon numbers, exclusively) and retention times (immediately after the *n*-alkane with one less carbon) of these unknown compounds are identical to those observed for the 5,5-DEAs in the Pasquia Hill

samples. Very probably, the unknown branched alkanes in these 2-Ga- to 2.2-Ga-old and 1.5-Ga- to 1.6-Ga-old samples are 5,5-DEAs.

The literature contains numerous other reports (20–24) of pseudohomologous families of compounds with mass spectra and retention times described as equivalent to those of Mycke *et al.* (19). In most cases, the compounds have been tentatively identified as 3,7-DMAs on the basis of their mass spectral fragmentation (20–24). However, the mass spectra of dimethyl alkanes are commonly dominated by fragmentation α to the tertiary centers (25). In a 3,7-dimethyl compound, fragmentation at C-7 should yield ions at $M^+ - 99$ and m/z 127. In the specific case of the 3,7-dimethylhentriacontane, these appear with intensity that are 28% and 49% of the base peak, respectively (26). The generality of this pattern is demonstrated by the mass spectra of authentic 3,9-dimethyltricosane (25), in which fragmentation at C-9 prominently yields ions at $M^+ - 127$ (25%) and m/z 155 (52%). The putative 3,7-DMAs (15–20) have thus been reassigned on two bases. Their spectra (i) lack the peak at $M^+ - 99$ that would arise from fragmentation at C-7 and (ii) are instead consistent with the spectrum of 5,5-DEAs (Fig. 1A).

In sum, the analyses reported here and numerous earlier reports indicate that 5,5-DEAs are commonly present in modern and ancient sediments (Fig. 3). Notably, there is very little variation in the carbon number distributions of 5,5-DEAs (minimum C_{15} and maximum C_{35} , odd number of carbon atoms exclusively; Fig. 3) in both the samples processed at the University of Illinois (Chicago) and earlier reports, suggesting that the biosynthetic pathway for these compounds has been conserved for at least ≈ 2.0 Ga.

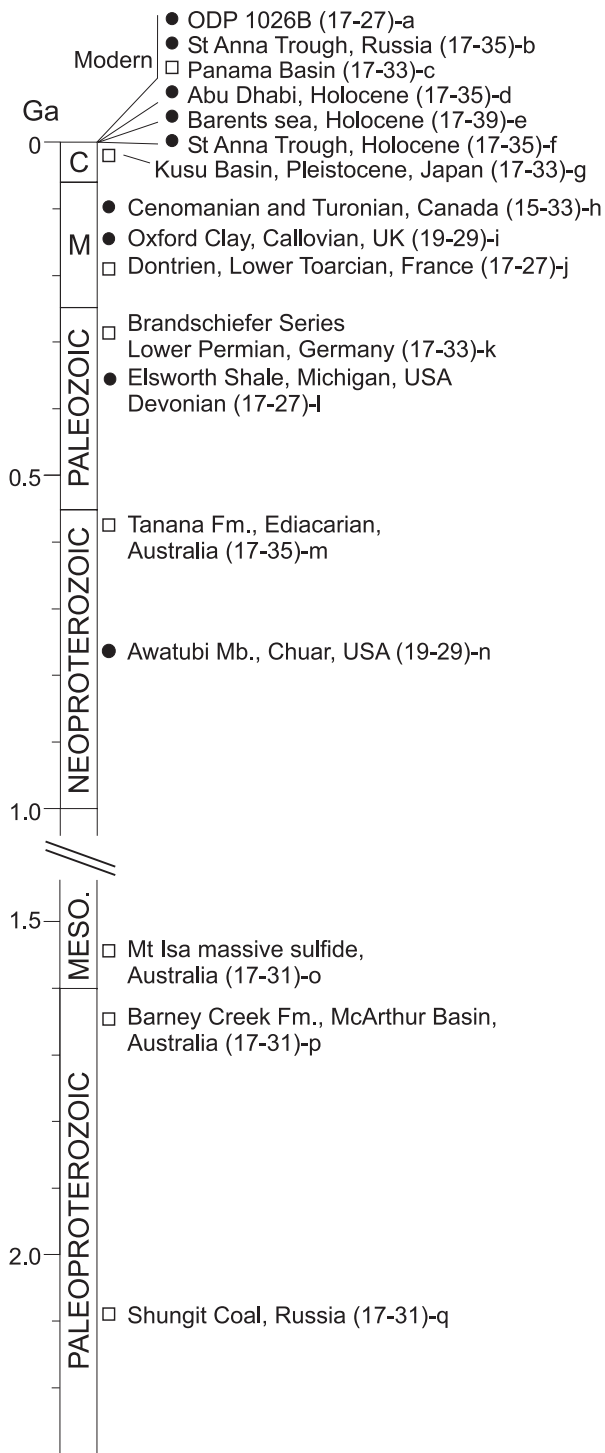


Fig. 3. Occurrence of 5,5-DEAs in modern and ancient sediments. ●, samples studied at the University of Illinois (Chicago); ■, reinterpretation of MS data from the literature (see *Methods*). Numbers in parentheses indicate the carbon number distribution of 5,5-DEAs. All samples have isomers with odd carbon numbers, exclusively. a, Eastern Pacific, basaltic fluids escaping from ODP borehole 1026b (11). b, PL94-67, 0–5 cm (37). c, Abu Dhabi lagoon, carbonate sediments (United Arab Emirates), 40–50 cm deep; d, ≈8,000 years B.P. (37). e, PL94–67, ≈7,800 years B.P. (37). f, Hydrothermally reworked lacustrine phosphorite nodules in diatomaceous mudstone, 0.5–0.2 Ma (22). g, ODP 677A (24). h, Black shales (12). i, Black shales, Bed 10 at Peterborough (38). j, Black shales, released on kerogen pyrolysis (21). k, Shales (20). l, Gray shale, core Statechester 18. m, Microbial mat facies (23). n, Black mudstone. o, 1.5–1.6 Ga, released on hydrogenolysis of massive sulfide (19). p, 1,640 Ma (35). q, 2.0–2.2 Ga, released on hydrogenolysis (19).

Origin of BAQCs. Quaternary substituted carbon atoms are extremely rare in aliphatic natural products, with the exception of the isoprenoid-based botryococenes from the alga *Botryococcus braunii* (27). These rare exceptions also include the tert-butyl lipidic side chains of the feeding deterrents ypaamide and antillatoxin of the cyanobacterium *Lyngbia majuscula* (28, 29). The latter reports indicate that a biosynthetic pathway for tert-butyl aliphatic structures, although still unclear, is present in the bacterial domain.

Given the scarcity of BAQCs in biological samples, we have to consider first the possibility that BAQCs in geological samples are contaminants. BAQCs are encountered in synthetic lubricants (e.g., ref. 30) and pharmaceutical waste waters (e.g., ref. 31). However, the components of these lubricants do not match even remotely the specific structures and distributions of BAQCs in our samples. In addition, BAQCs were not found in laboratory analytical blanks, and not all samples analyzed contained BAQCs. For example, black shales from the Cenomanian/Turonian boundary of Kansas, extracted and separated following the same analytical protocol as used for Pasquia Hill samples, do not contain BAQCs (13). Additionally, only some samples of the Oxford Clay and Abu Dhabi contain BAQCs. Lastly, researchers working in at least five different laboratories located in as many countries (Japan, France, Germany, Australia, and Belgium; see Fig. 3) have found the same 5,5-DEAs in diverse geological samples. Therefore, these compounds are not derived from contamination of the samples during extraction and analyses.

Accordingly, BAQCs are likely to be the products of organisms contributing to sedimentary organic matter and the deep-sea hydrothermal waters. The environments in which these compounds have been found provide some clues about the source organisms. The presence of BAQCs in warm hydrothermal fluids suggests that the source organisms are thermophilic. The absence of sunlight at ODP borehole 1026B (water depth is 2,600 m) further indicates that the source organisms are not photosynthetic. Mycke *et al.* (19) considered that the “unknown branched alkanes” observed in the 1.5- to 1.6-Ga Mount Isa massive sulfide (Australia) and the graphitic 2.0- to 2.2-Ga Shungit coal (Karelia, Russia), here recognized as 5,5-DEAs, were relicts of Proterozoic bacteria that inhabited sites of sulfide deposition, suggesting that organisms producing BAQCs favor sulfidic environments. This hypothesis is further supported by the occurrence of BAQCs in Paleozoic and Mesozoic shales that all were deposited under stratified water columns with recurrently euxinic (anoxic and sulfidic) bottom waters (12, 32).

However, the presence of sulfide is not the sole environmental requirement. BAQCs are absent from sediments such as the Messinian marl beds of the Gessoso-solfifera Formation (33), which were deposited under a permanently stratified water column with euxinic bottom waters. The Ellsworth Shale (Devonian, MI) contains BAQCs in its dominant gray shale facies (deposited under intermittently euxinic waters), but not in the interbedded laminated black shale beds (deposited under a persistently stratified water column with sulfidic bottom waters). Black shales from the Cenomanian/Turonian boundary of Kansas accumulated under an intermittently euxinic and fully oxygenated water column (13) do not contain BAQCs. In contrast, their stratigraphic equivalents from the Cenomanian/Turonian of Saskatchewan (Canada, Fig. 2B), which were deposited under a more persistently stratified water column with euxinic bottom waters alternating with dysoxic bottom waters (34), contain very abundant BAQCs. BAQCs are also present in open shelf areas, such as the Barents Sea sediments and the carbonate sediments of Abu Dhabi lagoon (Fig. 3) where the sediments, but not the water column, are sulfidic. Considering the above examples, the most parsimonious explanation for the variable occurrence of BAQCs is the occurrence of their source organisms at benthic

redox boundaries, between anoxic and sulfidic interstitial waters and dysoxic bottom waters.

Logan *et al.* (18, 35) summarized morphological, chemical and isotopic evidence for the presence of sulfide-oxidizing bacteria in studies of Australian 1.64-Ga sediments of the Barney Creek Formation and midshelf benthic mats of the Neoproterozoic Centralian Superbasin. They suggested that the extant γ -proteobacterial, benthic, sulfide-oxidizing *Beggiatoa* and *Thioploca* are possible modern equivalents to the Proterozoic microbes and that the geological record of sulfide-oxidizing bacteria extends to 1.64 Ga B.P. These Proterozoic sediments have an unusual biomarker distribution including monomethylalkanes with even carbon numbers, exclusively, and compounds with carbon-number distributions and mass spectra similar to those of 5,5-DEAs (odd carbon numbers, C₁₇-C₃₁). Our study of the paleobiogeographical distribution of 5,5-DEAs in ancient sediments (see above) supports this interpretation. However, rRNA gene sequence data from borehole 1026B fluids (11) do not indicate the presence of any γ -proteobacteria and the strictly anoxic and warm conditions in 1026B fluids would not allow long-term survival of γ -proteobacterial microaerophilic sulfide oxidizers that are not thermophiles. Moreover, molecular-clock data suggest that sulfide-oxidizing bacteria of the γ -proteobacterial subdivision originated during the Late Proterozoic (36). Thus, γ -proteobacteria may not be the source of the BAQCs of 1026B fluids and Paleoproterozoic samples. Alternatively, the source organisms of BAQCs may be part of a more ancient lineage of nonphotosynthetic sulfide oxidizers.

Implications and Conclusions. BAQCs have routinely been overlooked in prior analyses of geological samples (19–24, 35). Thus,

it seems likely that their occurrence extends to many other sediment formations and extends further back in time. The latter possibility would be consistent with the reducing environments prevalent in the Archaean ocean and with the presence, at least locally, of low concentrations of free oxygen as indicated by evidence from sulfur isotopes (3) and the occurrence of steroids in 2.7-Ga samples (6). BAQCs released by catalytic hydrogenolysis of Mesoproterozoic Mount Isa shales have undergone lower greenschist metamorphism (19), requiring that they have withstood temperatures in excess of 250°C for millions of years. Thus, BAQCs can be preserved in metasediments, enlarging significantly the applicability of molecular paleontology to the study of ancient sediments.

The physiological role of BAQCs is unknown, and neither the acetyl-CoA nor the mevalonate pathways can easily explain their biosynthesis. The identification of BAQCs and the recognition of their widespread occurrence in the geological record prompt investigation of their biosynthetic pathway and their evolutionary origins.

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