ON THE PROBLEM OF AMBIGUITY IN EXTRATERRESTRIAL BIOMARKERS: IMPLICATIONS FOR MARS

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Abstract

One of the primary goals of martian studies is to determine if there ever was, or could still be, life on Mars. Many techniques for achieving this goal have been tried, and very many more have been proposed. However, there are flaws in virtually all of them. This paper initially illustrates the validity or otherwise of existing proposals for finding extant or extinct biological activity on Mars, and suggests an alternative method. It is argued that a class of bacterial membrane-lipid components known as hopanoids may provide a more potent and perspicuously biological signature, both on Mars and on other potentially life-bearing bodies. A sample of modern sediment from a river bed was analysed by GC-MS and shown to contain biohopanoid components that could be used to identify criteria for the unambiguous detection of these compounds by an experiment suitable for delivery to Mars on a lander. It is concluded that the wholly unambiguous detection of past or present biological activity on Mars (or any other planetary body) is likely to require the presence of human beings in situ.

Contents

1. INTRODUCTION

The field of exobiology - the study of life outside the Earth - has enjoyed a quite remarkable renaissance in recent years, spurred on in no small part by extraordinary discoveries in the Earth's oceans, ice sheets and deep lithosphere. Rising, phoenix-like, from the ashes of its former existence, exobiology has witnessed a resurgence of extraterrestrial search strategies and technologies which are critically reviewed together in this paper for the first time. In its earlier incarnation, exobiology was principally within the purview of theologians and philosophers, it being rather taken for granted that life - and more importantly thinking beings - existed 'elsewhere,' and it was in this naïve form that things fell into the willing arms of 19th and 20th century astronomers (see Dick, 1982). This is well illustrated in an encyclopaedia extract from the early 1890's:

"*…the telescope has revealed to us the existence on its surface of oceans, continents, mountains, arctic regions, hills, valleys and rivers, similar to ours. That animals, and perhaps beings superior to ourselves, inhabit Mars is conjectured as reasonable, for the planet seems fitted for such animated creations, and nothing, it is said, was ever made in vain.*"

(Ogilvie's Encyclopaedia of Useful Information. George W. Ogilvie. 1893)

Mounting evidence against the existence of animal or vegetable life on other solar system bodies during the 20th century sapped much of the life from the previously embonpoint field of study, and the Viking missions to Mars in 1976 marked, if not the death knell for the old exobiology, then a paradigm shift to a new way of thinking about, and looking for, alien life. Nevertheless, it has been the discoveries on Earth of life flourishing in the most extreme habitats possible; extremes of temperature, pressure, pH, salinity, and nutrient availability that has contributed most. Moreover, the identification of potentially suitable habitats in space for these extremophilic organisms, below the surfaces of Mars, Europa, and Titan, has fuelled the growth of exobiology into an edifice constructed from government money and buttressed by public interest. And, though the pinnacle of this structure is now within our grasp, will we know when we are there? By which criteria do we say that the evidence for alien life is definitive, particularly when the evidence may be highly degraded and ambiguous? To be brief, which biomarkers are we to employ?

The large numbers of potential biomarkers which have been suggested over the past fifty years represent a very ephemeral and highly mobile pair of goalposts to aim for. The degree of ambiguity inherent in the modern extraterrestrial biomarker suite is perhaps best exemplified in our study of meteorites, in particular the martian meteorite ALH84001. Although many biomarkers are undeniably present, ambiguity prevents their interpretation with sufficient rigour to indicate the presence or otherwise of biogenic material. It is for the purpose of addressing this problem that a detailed and critical assay of suggested biomarker schemes, and an objective score of their usefulness to exobiologists is essential. This is undertaken in section three, after which I shall go on to propose a novel extraterrestrial biomarker (though one of great familiarity to terrestrial biologists and petroleum geochemists) based on the hopane carbon skeleton, a suitable set of experimental procedures for identifying it, and criteria for its effective utilisation.

The discussion will combine my own biomarker work with that of previous investigators in an attempt to define a best working practice in the application of biogenic signatures to the search for both extant and extinct life primarily on Mars, but also on Europa and Titan. However, I shall begin with the era 1960-76, and look at the development of life-searching strategies for the Viking missions, focusing on the critical findings concerning the martian environment that led to the explosion in modern biomarkers discussed in section three.

2. A BRIEF HISTORY OF THE SEARCH FOR LIFE ON MARS

Ground based remote sensing of Mars prior to the 1960's had hinted at a dry world with a vanishingly thin atmosphere and few potential habitats for life (Lederberg and Sagan 1962: Rea 1964). Even with unpromising images being returned by the Mariner 4 spacecraft in 1965, and over the dissent of some biologists (Lovelock and Hitchcock 1967), the Viking project was begun in 1968. The life detection experiments on the Viking landers, based on work by Horowitz (1964) and Imshenetsky (1969), are described elsewhere (Klein *et al*. 1972: Oyama 1972: Levin 1972: Anderson *et al*. 1972: Rushneck *et al*. 1978), and were designed to ask three basic questions of any indigenous life: Does it breathe?, Does it eat?, and Is it alive? The experiments so fashioned were the Gas Exchange experiment, the Labelled Release experiment, and the Pyrolitic Release experiment, with a compact Gas-Chromatograph Mass-Spectrometer (GC-MS) included for the detection of organic molecules. Prior to launch, it was speculated that organisms were likely to be present on Mars, perhaps waiting in a state of 'cryptobiotic repose' for the end of the precessional winter (Sagan 1971: Sagan and Lederberg 1976: Klein 1976).

The results of the Viking life detection experiments were not positive (Margulis *et al.* 1979). Of the nine Pyrolitic Release experiments, seven showed evidence of carbon assimilation, even though one of these had been sterilised. The Gas Exchange experiment produced a very large effusion of O_2 gas that fell off rapidly, and which might have been attributed to microbial respiration. However, as with the Pyrolitic Release experiment, the same response was observed in the sterilised samples. The three Labelled Release experiments were perhaps the most contentious since initial releases of ^{14}C, possibly indicating metabolic activity, were not repeated in the sterilised samples. Most damning of all, though, was the complete absence of organic material in the martian soil, down to the detection limits of the GC-MS.

Analysis of the Viking results (Ballou *et al*. 1978: Biemann 1979: Klein 1979: Levin and Straat 1979, 1981) indicated that unusual chemical reactions in the martian soil samples were most likely responsible for the contradictory biogenic signatures, in particular reactions involving hydrogen peroxide (H_2O_2: Hunten 1979) and smectite clays (Banin and Rishpon 1979). Modelling of the photochemistry of the martian atmosphere (McElroy *et al*. 1977) had revealed that hydrogen peroxide would be a minor product condensing at the surface and that highly reactive atomic hydrogen, oxygen and hydroxyl radicals, would combine with iron minerals to form 'superoxides' (see Hartman and McKay 1995). Such oxidising materials were probably responsible for the large O_2 release in the Gas Exchange experiment and are far from conducive to the preservation of organic compounds in the near surface regolith (Oró and Holzer 1979). In addition, the high UV flux (2.6mW cm^{-2}: Kuhn and Atreya 1979) should contribute to the net organic

destruction rate (Biemann *et al*. 1977: Chun *et al*. 1978).

The Viking findings posed a small problem since organic material ought to be formed in the martian soil abiotically (e.g., Kobayashi *et al*. 1997: Zolotov and Shock 1999) and be delivered from exogenous sources by meteorite impacts (Pierazzo and Chyba 1999), as on the Moon, prompting further investigation into the effect of UV photolysis, modelling of soil-atmosphere interactions (Bullock *et al*. 1994), and study of the oxidative destruction of micro-organisms (Mancinelli 1989) and organic material (Kanavarioti and Mancinelli 1990: Zent and McKay 1994: Stoker and Bullock 1997: McDonald *et al*. 1998: Quinn and Zent 1999). They indicated that some organic matter could be preserved at the surface for periods exceeding 3.5Ga, with oxidative half-lives of order 10^{12} years for humic acids in the cold polar regions. McDonald *et al*. (1998) concluded that biotic polymers were considerably more likely to be preserved than abiotic polymers. Stoker and Bullock (1997), moreover, demonstrated that the rate of organic destruction was still consistent with the presence of microbial communities growing at or below the same rate as cryptoendolithic organisms in the Ross Desert of Antarctica.

These results have encouraged a reawakening of interest in identifying potential ecological niches in the martian environment, and survival strategies of micro-organisms (Imshenetsky 1980: Cabrol and Grin 1995: Arrhenius and Mojzsis 1996: Nealson 1997: Clark 1998: Fisk and Giovannoni 1999), which include endolithic habitats (Rothchild 1990: Boston *et al*. 1992), hypersaline environments below deep permafrost layers (Carr 1998: Litchfield 1998), and possible thermophilic associations with hydrothermal systems (Shock 1997: Farmer 1998).

I have identified a number of difficulties with these models. Firstly, the amount of free energy available to power putative biological systems is likely to be extremely small. Jakosky and Shock (1998) calculated the amount of energy available and determined a maximum productivity of just 5 microbes cm^{-3} $year^{-1}$, a factor of four million smaller than the figure for the mean terrestrial productivity. Jakosky and Shock (1998) considered such an ecosystem to be unsustainable. Secondly, any indigenous ecology will be associated with a volume of organic material (mostly decay products) at least four orders of magnitude greater in mass than the mass of cells. Even assuming a growth rate comparable to the slowest growing endolithic communities on Earth, the mass of additional biological waste could not be degraded fast enough to escape detection. Thirdly, a feature common to all organisms is the consumption and excretion of gas. As Lovelock and Hitchcock (1967) argued, an indigenous ecology will drive the mix of atmospheric gases towards a state of disequilibrium. On the grounds that the gases in the martian atmosphere *are* in equilibrium, they concluded - and I agree - that Mars is presently lifeless. Finally, life on Earth has adapted to every habitat that has been explored to date and, furthermore, flourishes in those habitats. It seems profoundly improbable that, in the ~3 Ga since Mars is thought to have lost its early atmosphere, life should not adapt to, and flourish in (to a detectable extent) the conditions at or near the martian surface, since there are many terrestrial organisms suitably equipped to endure the radiation and chemical hazards on Mars. The hypothesis of Lovelock (1988) that life is not in the habit of 'just hanging on' when the conditions are no longer favourable is most persuasive, and I find it unacceptable to arrive at any conclusions in this regard but the following: extant life is not difficult to find. It announces itself most dramatically via

the medium of the planetary atmosphere. I have previously contended (Fortes, submitted paper), by the same reasoning, that the atmosphere of Titan is wholly or partly of biological origin, and this has independently been asserted by Simakov (1999). I would also submit that the lack of an atmosphere about Europa (other than that resulting from particle sputtering) indicates that the proposed subsurface ocean (e.g., Pappalardo *et al*. 1999) is lifeless, contrary to recent work by Chyba (2000) - see Appendix 2. Karnaukov (1996) has postulated a closed martian ecosystem from which no waste products escape, but the lack of a precedent in terrestrial environments where such a strategy would have a remarkable survival value, and evolutionary edge, renders the idea untenable. Thus, at the risk of appearing foolish to future generations, I believe that the legacy of Viking is to show that Mars is not biologically active in the current epoch. Were it otherwise, it should be obvious.

Having said that, I find it acceptable to suppose that life arose on Mars in much the same way as it is considered to have done on Earth, when the conditions were more favourable (McKay *et al*. 1992: Brack 1996), and that it subsequently became extinct (Friedman and Konem 1989: Helfer 1990: Farmer 1995a). Our search is then for fossilised remains, trace, body, or chemical: Biomarkers.

The following section now concentrates on the variety of biomarkers that have been suggested in the post-Viking era, and analyses their usefulness since, as will become clear, many are ambiguous.

3. A CRITICAL ASSAY OF PROPOSED BIOMARKERS

Biomarkers are physical structures or chemical compounds that are either the remains of, or by-products of, living organisms. One of the problems that must be faced in martian exobiology is that the organisms we seek were probably microscopic, and they probably died several billion years ago. Thus the physical remains will be of comparable scale to many natural mineral textures, and the chemical remains dissociated into substances virtually indistinguishable from abiotic organic materials. How does this bear on proposed search strategies? The effect of small spatial scales and large temporal scales is manifested as doubt. The primary objective of this paper is to analyse the way in which this lack of certainty can be dealt with constructively and, in part, overcome. It is essential to be clear about just what can or cannot be gained from the use of given biomarkers, which is why I undertake the following assay as a prelude to introducing a solution.

3.1. ORGANIC MOLECULES

Interest in simple organic compounds as biomarkers began with the identification of potentially biological hydrocarbons in certain carbonaceous meteorites (Anders 1962), at a time when several investigators were reporting the detection of purported microfossils in the same meteorites (see 3.4 below). Subsequent characterisation of these materials concluded that the organic compounds offered evidence of extraterrestrial life on the grounds of 'similarity' to material found in terrestrial marine sediments (Meinschein *et al*. 1963) and the - more rigorous - detection of optical rotation (Nagy *et al*. 1964: Nagy 1966). This in turn was followed by the equally fatuous (but nevertheless published)

account relating the identification of sporopollenin in the insoluble organic fraction from two meteorites (Brooks and Shaw 1969). More credible contemporary work detected abundant amino acids (see Engel and Nagy 1982), nitrogen heterocyclics such as 4-hydroxypyrimidine (Folsome *et al*. 1971) and Uracil (Stoks and Schwartz 1979) organometallics including porphyrins (Hodgson and Baker 1969), and lipids (e.g. Deamer 1985). For an up to date account of meteoritic organic molecules see Shimoyama (1997).

In spite of the convincing demonstrations that all of these organic materials can be synthesized abiotically (e.g. Miller 1953: Simakov *et al*. 1997) both in space and on the surface of Mars (Kobayashi *et al*. 1997), the very same compounds have been mooted as biomarkers in several martian meteorites, most notably ALH84001 (McKay *et al*. 1996), and for Mars itself (Bada *et al*. 1997, 1998). The story of ALH84001 essentially repeats that of the carbonaceous meteorites studied in the 1960's with the exception that the organic material has been better characterised as low molecular weight polycyclic aromatic hydrocarbons (PAHs). However, PAHs are amongst those substances which are readily formed abiotically in space (comprising a significant component of interstellar dust: Léger *et al*. 1987), and quite probably on the surface of Mars (Zolotov and Shock 1999).

In all cases, the problem of terrestrial contamination features, in particular when samples were collected from Antarctica, where meltwater percolation may have concentrated organic material within the meteorites (Becker *et al.* 1997). Indeed, a large number of Antarctic chondrites can be demonstrated to contain terrestrial contamination (Brinton *et al*. 1998), though this is disputed as the origin of organics in ALH84001 and EETA79001 (Wright *et al*. 1997, 1998).

There are three ways of dealing with the amino acid problem. One is to discriminate on the basis of isotopic composition (e.g. Glavin *et al*. 1999: Jull *et al*. 1999), those containing isotopically 'light' carbon being presumed to be of biological origin. This fails because we have few hard constraints on fractionation mechanisms acting upon extraterrestrial organic carbon reservoirs and, can one distinguish between a terrestrial biogenic and an extraterrestrial biogenic fractionation signature, in particular against a background of terrestrial contamination? Another solution is to look at chirality (which is discussed in more depth later), classifying racemic mixtures as extraterrestrial and non-racemic mixtures as contaminants. As the arguments presented later illustrate, chirality is not a straightforward issue, and even where non-racemic amino acids have been 'demonstrated,' on the basis of isotopic composition to be extraterrestrial, they may still not be biological. Even less satisfactory is the search for proteinaceous amino acids. Only twenty of the seventy known amino acids are utilised in terrestrial organisms for protein synthesis, thus it might be contended that non-proteinaceous amino acids are abiological. Yet there is no reason to conjecture that alien life will use the same amino acids as terrestrial life, or the same number of amino acids.

But then perhaps we are being too prosaic. Does the detection of isotopically light, chiral, proteinaceous amino acids in meteorites (e.g. Engel and Macko 1997) imply that they are of no value as biomarkers, or does it mean that there was life in the meteorite parent body? Moreover, what are the implications of this for planetary - rather than meteoritic - exobiology? My own view, contrary to that of McDonald *et al*. (1999), is to regard the majority of simple organic molecules as either irrelevant to, or a hindrance to, exobiological search strategies; that their detection should be viewed with great caution,

and even then only employed as a guide to the application of more stringent tests. In other words, organics in martian meteorites, or at the martian surface, are not useful clues to life on Mars, as Brack and Pillinger (1998) extol.

However, in the course of discussing the scope of this paper, it was observed that there are simple tests for diagnosing the presence of a much more complex organic molecule - DNA. I will now discuss why even DNA is not a useful biomarker in the search for extinct life on Mars.

Genetic molecules

Both DNA and RNA are readily detected using fluorescent dyes and it has recently been suggested by Burde *et al*. (1999) and Kawasaki (1999) that epifluorescence microscopy could be applied to confirming the identity of extraterrestrial fossil microbes (see 3.4 below). The most frequently employed stains are 3,6-bis [dimethylamino] acridinium chloride (acridine orange: AO) and 4'6'-diamidino-2-phenylindole (DAPI) which typically fluoresce orange or red when attached to nucleic acids, though AO fluoresces green when bound to double-stranded DNA (Williams *et al*. 1998) which could be confused with *in situ* mineral and biological autofluorescence. Other commonly used stains include propidium iodide (PI), ethidium bromide (EB), ethidium monazide (EM), oxazole yellow (YO) and its dimer YOYO. Of these, PI, EB, and EM are only able to stain nucleic acids where the cell membrane is compromised (i.e., when the organism is dead). More recently, fluorescently labelled oligonucleotides have been used to target 16S rRNA components of cellular ribosomes (see Amann *et al*. 1997).

The problem in using nucleic acid stains in exobiology - and in terrestrial palaeontology - is the facility with which DNA breaks down. Hydrolysis of the N-glycoside bond liberates the purine bases adenine and guanine from the 2' deoxyribose group. This in turn leads to a b-elimination reaction at the phosphodiester bond of the apurinic sugar resulting in the complete disintegration of the DNA backbone (Lindahl and Nyberg 1972: Lindahl 1993a, 1993b). The rate of DNA depurination is comparable to the rate at which the most unstable amino acid - aspartic acid - racemizes (Bada *et al*. 1994: see 3.3 below) and the degree of amino acid racemization has been used to estimate the probability of extracting useful genetic material for analysis (Poinar *et al*. 1996). Thus, in most terrestrial habitats, depurination should destroy genetic material extremely rapidly (hundreds to thousands of years), though at low temperatures and - more importantly - under anhydrous conditions, depurination half-lives may be extended to millions of years. Special preservation conditions are believed to apply to organisms trapped in amber, where the amino acid racemization rate appears to be 10^4 - 10^5 times slower than under ambient conditions. The purported detection of DNA using the polymerase chain reaction (PCR: Pääbo *et al*. 1989) on insects trapped in amber has led to the suggestion that genetic material could be preserved for periods $>10^9$ years (Bada *et al*. 1994, 1999b).

In a planetary (and specifically martian) context, the probability of resinous substances - capable of maintaining biological material in an anoxic and anhydrous state favourable to the preservation of DNA - either occurring, or being found remotely, are minuscule. Even in permafrost layers or at depths in the regolith beyond the diffusive reach of superoxides there is likely to be sufficient grain boundary liquid to catalyse the hydrolytic

depurination of nucleic acids, and a single warm, wet episode in martian history will suffice to destroy DNA in the environment (Bada and McDonald. 1995). It should be noted, however, that there have been some remarkable reports of viral, microbial, and plant 'resurrection' following multi-million year periods of entrapment in ice (Gilichinsky *et al*. 1995: Vorobyova *et al*. 1996: Shi *et al*. 1997: Castello *et al*. 1999: Gilichinsky *et al*. 1999) and evaporites (Fredrickson *et al*. 1997), very clearly testifying to the preservation of genetic material.

Moreover, it is plausible that abiotically produced singe-stranded oligonucleotides could respond to nucleic acid dyes, yielding a false positive result for life. Much work is being done on the abiotic synthesis of nucleic acids (Kozlov *et al*. 1998: Orgel 1998: Echenmoser 1999) and it has proven possible to condense oligonucleotides up to 55-mer in length using a clay substrate as a catalyst (Ferris and Ertem 1993: Ferris *et al*. 1996: Ertem and Ferris 1999) in the laboratory. Abramo *et al*. (1998) and Simon *et al*. (1998) have reported fluorescent staining of oligonucleotides as short as 15-mer with YO and YOYO nucleic acid dyes. The use of PCR amplification would exacerbate this problem, and indeed PCR amplification of very tiny quantities of contaminant DNA - even single molecules - has been implicated in some of the reportedly successful extractions of DNA from very ancient terrestrial sources (see Lindahl 1993a).

Nucleic acid dyes alone cannot offer an unambiguous means of detecting biological activity, nor can it be used to corroborate the identification of proposed fossil structures of extinct martian life. However, the method might be useful in confirming the existence of extant life. The use of propidium iodide advocated by Burde *et al*. (1999) is only valid for cells with compromised membranes and can therefore only be applied to recently deceased martian microbes. As has already been observed though, extant life is unlikely to be hiding its light under such a weighty bushel that DNA testing is necessary. Another similar test, based on the photoluminescent reaction between ATP and the enzyme Luciferase, devised by undergraduates at Leicester University (Obousy *et al*. 2000), also relies on the existence of extant life for success.

3.2 MINERAL ASSEMBLAGES

3.2.1 Carbonate biomineralization

The use of widespread carbonate deposits in crater-lake floors (Scott *et al*. 1991: Wharton *et al*. 1995: Cabrol *et al*. 1999) has been proposed as a potentially useful biomarker on Mars, based on the occurrence of microbially precipitated calcium carbonate in terrestrial marine environments. On Earth, these typically take the form of stromatolites; layered carbonates with physical and chemical biological remains intercalated (Golubic and Lee 1999). Indeed, areas of exposed white material in Sabaea Terra and Juventae Chasma (Williams and Zimbelman 1994) have been suggested to be just such a stromatolitic association (Russell *et al*. 1999). Moreover, not only does study of Antarctic palaeolake sediments reveal abundant authigenic carbonate in combination with freeze-dried organic matter (Doran *et al*. 1998), but remote sensing techniques can now be used to quantify their abundance (Bishop *et al*. 1996: Davis *et al*. 1999).

However, disputes in the analysis, and possible biological significance, of the carbonates in martian meteorites ALH84001 (McKay *et al*. 1996) would appear to urge

caution. Although the carbonates date from a period when the martian climate was expected to have been warmer and wetter (Borg *et al*. 1999), it is unclear whether or not the mineral assemblage formed at high (Scott 1999) or low (Baker *et al.* 2000) temperatures. Furthermore, their association with a suite of biomarkers of questionable relevance highlights the difficulty in using mineral biomarkers. Until we have a better understanding of the thermal history of the martian meteorites, and the composition of the aqueous solutions flowing at the martian surface 3-4 billion years ago, then it would be unwise to depend on the remote or *in situ* detection of carbonate/organic complexes without further supporting evidence. Such evidence might be difficult to obtain without human intervention: Micrometer scale physical textures associated with microbial mats (Vali *et al*. 1997), and possible diagenetic alteration (Loisy *et al*. 1999) of aragonite and vaterite (Falini *et al*. 1998) precipitated in disequilibrium by membrane proteins, are features which require complex instrumentation to analyse, instrumentation which is unsuited to remote placement and operation.

3.2.2 Phosphate biomineralization

It is well established that apatite is formed in abundance in terrestrial marine phosphatic sediments by bacteria (Hirschler *et al*. 1990) and in other environments by nanobacteria (Kajander *et al*. 1998), in particular grains associated with, and occluding, isotopically light organic matter. Mojzsis and Arrhenius (1998) proposed the use of such chemofossils as biomarkers in the search for evidence of life on Mars. However, Mojzsis and Arrhenius (1998, p 28496, lines 20-25) confess that it is the isotopic composition of the occluded carbon that is essential to resolving ambiguity in the origin of the remains, and that our current values of $\delta^{13}C$ for various martian carbon reservoirs are based on uncertain extrapolations. I believe that a much improved understanding of carbon isotope systematics on Mars is essential to remove some of the ambiguity surrounding this biomarker. Furthermore, our knowledge relating to possible *prebiotic* carbon fractionation mechanisms is non-existent. For example, the carbonate carbon in the martian meteorites is inferred to have formed in equilibrium with isotopically heavy carbon dioxide ($\delta^{13}C$ » +40 ‰: Grady *et al*. 1994), whereas the organic carbon possesses $\delta^{13}C$ » -15 ‰ (Becker *et al*. 1999). Thus, the detection of isotopically light carbon in association with an abiological mineral assemblage indicates that the identification of isotopically light carbon occlusions in apatite as biogenic would have to be viewed with a certain amount of scepticism.

Mojzsis and Arrhenius (1998) go on to cite work showing that whitlockite and magnesium phosphate pentahydrate (MPP) are the stable phases precipitating in sterile solutions with pHs comparable to normal marine waters, and that apatite is precipitated in disequilibrium by microbial agents under such conditions. This is due to preferential nucleation of apatite by bacterial peptides (Lowenstam and Weiner 1989). However, above pH = 8.5, apatite can precipitate with whitlockite under sterile conditions, and might well be capable of occluding prebiotic organic matter. Strongly alkaline conditions were hypothesised to have pertained in standing bodies of water on Mars by Kempe and Kazmierczak (1997). It is also conceivable that diagenetic replacement of whitlockite by apatite (Krauskopf and Bird 1995) could destroy information relating to precipitation conditions and hence obfuscate arguments for biogenesis. Phosphates from the martian

meteorite EETA79001 indicate precipitation from low temperature, sterile, aqueous fluids (Mojzsis and Arrhenius 1998, and references therein).

There is no doubt that phosphates should play a role in exobiological search strategies, but their usefulness is restricted to gross identification (e.g., Banin 1996) of search regions (i.e., distinguishing potentially 'fertile' crater lake beds from barren ground) since, as on Earth, massive phosphates may be associated with upwelling and high microbial productivity (Burnett 1977). However, mineralogical textures in such formations suggestive of biological activity are likely to be too small to be detected by landers or rovers (e.g. Vali *et al*. 1997). A sample return strategy should aim to include phosphate bearing material, but much groundwork needs to be laid in order to inform effective interpretation.

3.2.3 Iron biomineralization: Magnetosomes

The fine grained magnetite, pyrrhotite and greigite found in association with the carbonate rosettes in martian meteorite ALH84001 was hypothesised to be of biogenic origin (McKay *et al*. 1996), being identified as possible microbial magnetosomes (see Vainshtein *et al*. 1998: Schuler and Frankel 1999). However, laboratory studies of the reaction sequences observed in the formation of bacterial magnetosomes (Pósfai *et al*. 1998a), and the work showing that the martian magnetite probably grew epitaxially at high temperature (Bradley *et al*. 1996, 1998) appears to rule out an intracellular origin. In addition, the 50nm cubes and 25nm teardrop shaped crystals are too small to be single domain magnets, and neither do they occur with phosphatic granules (Lins and Farina 1999), as in terrestrial magnetotactic bacteria.

However, genuine magnetosomes could persist in the martian environment over geological time (Pósfai *et al*. 1998b) and serve as biomarkers. The usefulness of magnetosomes as biomarkers is extremely limited, though, since features diagnostic of biological activity can only be elucidated via transmission electron microscopy (TEM), requiring either a serendipitous find in a return sample, or a concentrated search by humans on Mars.

It might also be the case that the evolution of terrestrial magnetosomes is the response to a temporally persistent, dipole dominated, global geomagnetic field, and we currently have no evidence hinting at longevity in the primitive areomagnetic field, or the relative importance or otherwise of multipole components. It is quite plausible that magnetosomes never evolved on Mars at all.

3.2.4 Manganese biomineralization: Rock varnish

Mancinelli and White (1996) proposed the use of desert varnish as a biomarker which could be identified by DTA/GC (Differential thermal analysis - Gas chromatography) on a future Mars lander, and which might have been detectable using the TEGA instrument (thermal and evolved gas analyser) on the defunct Mars Polar Lander (Boynton *et al*. 1999: Golden *et al*. 1999). Rock varnish is a finely laminated rind which occurs ubiquitously on exposed terrestrial rock surfaces but which is most often found in arid environments - composed of clay minerals cemented by Fe and Mn oxyhydroxides (Potter and Rossman 1979). It is the case that the modern view of rock varnish genesis

leans strongly towards a biological origin (Dorn and Oberlander 1981: Nagy *et al*. 1991: Krinsley 1998), favouring its use as an extraterrestrial biomarker. However, there are means of producing Mn-enriched clay coatings without the mediation of microbial agents (e.g., Smith and Whalley 1988), and so the remote chemical detection of rock varnish is not, of itself, an unambiguous signature of life. Examination of surface rinds by electron microscopy may yield evidence of Mn-Fe casts of relict cell walls or granular textures (see Krinsley 1998 for examples) which may be degraded bacterial cell casts or bacterial waste products, though making such an identification is fraught with problems (see 3.4 below). Furthermore, the use of rock varnish as an indicator of microbial activity is temporally limited by its fragility, being destroyed on timescales of 10^3 - 10^5 years in terrestrial environments by biogeochemical leaching and aeolian abrasion (Krinsley *et al*. 1990).

Thus the detection of rock varnish which could be demonstrated to be of biological origin, requiring at the very least the return of a sample to Earth for analysis, would almost certainly be indicative of extant life on Mars. Other factors (see §2 above) presently appear to militate against this contingency.

3.3 HOMOCHIRALITY

A significant feature of terrestrial biochemistry is the almost exclusive use of left-handed (laevorotary - L) enantiomers of amino acids in proteins, and of right handed (dextrorotary - D) sugars in RNA and DNA. In contrast, abiologically formed organic stereoisomers normally occur in racemic proportions (equimolar mixtures of D and L enantiomers). This property of homochirality might be construed as representing a particularly useful biomarker, therefore. Indeed MacDermott and Tranter (1994), MacDermott *et al*. (1996) and MacDermott (1997) considered the issue in some detail in designing an experiment to search for extraterrestrial homochirality - the SETH-Cigar.

Homochirality is a tempting proposition since, as argued by MacDermott *et al.* (1996), it is likely to be independent of biochemistry; all sufficiently complex molecules will be chiral and, in the interests of efficiency, must be homochiral. On Earth, chiral mixtures will racemize relatively quickly at a rate which is determined by environmental conditions (e.g. Bada and Schroeder 1975) and this is the basis of a commonly used dating method, AAR - Amino Acid Racemization dating. Under extremely cold and dry conditions, such as occur on the surface of Mars, the racemization half-lives of the most stable amino acids are in excess of 10^{13} years (Bada and McDonald 1995), and this might be taken as encouragement to send a polarimeter to Mars to look for an enantiomeric excess in any preserved organic molecules. However, warm (~300 K) and wet conditions (or, alternatively, microbial diagenesis: Child 1996), can reduce this racemization half-life to just a few hundred years, and only a single relatively brief warming episode would be required to completely erase any chiral signature.

In addition, it transpires that racemization reactions can be induced by ionising radiation (Bonner 1999). Thus, the cosmic ray flux to the martian surface may have significantly contributed to the thermodynamic racemization of chiral organic compounds, and perhaps eradicated any enantiomeric excess.

A further complication arises due to the formation of enantiomeric excesses from racemic mixtures by wholly abiological processes. Such processes include catalysis on a

chiral substrate (Iwamoto 1999a, 1999b), and asymmetric photolysis by circularly polarised light (CPL: Balavoine *et al*. 1974: Bonner 1991), though this mechanism has been the subject of some dispute (Bonner *et al*. 1999a, 1999b). It is also apparent that there must have been some prebiotic mechanism for the generation of enantiomeric excesses on the early Earth since, as Joyce *et al*. (1984) observe (though apparently contradicted by Schwartz 1997), polymerisation of L-polypeptides is precluded in a racemic solution. Certainly there are numerous mechanisms proposed to abiotically produce chiral solutions, typically involving the weak nuclear interaction and its manifestation in b-radiation (e.g. MacDermott 1991), the influence of the aforementioned CPL in combination with tidal cycles (Popa 1997), or radiolysis in a magnetic field (Pospelov 1996). Moreover, although the 2' aminoacylation of D-ribose RNA is not in itself a stereoselective process in free solution, the reaction automatically becomes stereoselective for L-amino acid enantiomers when constrained on a surface (Bailey 1998).

The presence of an extraterrestrial L-stereoisomer excess, such as are found in some carbonaceous meteorites (Engel and Macko 1997: Cronin and Pizarello 1999), could then be viewed in one of two ways: Either it represents abiotic chemogeny via the mechanisms referred to above, or it could be the relict of a steadily racemizing biochemical. Conversely, a racemic mixture could be taken at face value as the product of abiotic chemistry in the absence of any asymmetric enantiomer selection mechanism, or the end product of the complete racemization of biological material. In either case, the ambiguity is clearly unacceptable and must surely illustrate the specious nature of the argument that homochirality is a useful biomarker, as proposed.

3.4 BODY FOSSILS

The incidence of primary anaerobic metabolism in terrestrial eukaryotes is confined to only a very small number of unicellular protists (see Fenchel 1996 for a review of anaerobic metazoans), and so we may assume that it was most unlikely - though not impossible - for multicellular organisms ever to develop under anaerobic conditions on Mars. Hence, our search for body fossils is, of necessity, a search for single celled structures.

One of the most readily accessible lines of evidence for possible past life on Mars was the occurrence of what were purported to be microbial body fossils in martian meteorite ALH84001 (McKay *et al*. 1996: Benoit and Taunton 1997). An initial difficulty arising from this suggestion was the diminutive size of the objects in question (20-100nm), being an order of magnitude smaller than most known bacterial fossils, though of comparable size to similar structures seen in some terrestrial rocks which had been described as fossil 'nannobacteria' (Folk 1993).

Since then, however, there have been several reports documenting living nano-scale organisms. Uwins *et al*. (1998, 1999) found nanobes in the size range of 20-150nm growing in Triassic and Jurassic sandstones off Western Australia. These nanobes were shown to have a distinct cell wall and a possible nucleus, and responded positively to a number of DNA stains. Also noted was their extraordinary resistance to the hardships of electron microscopy, being able to survive gold and platinum plating, exposure to vacuum and electron irradiation. Bjorkland *et al*. (1999) and Çiftçioglu and Kajander

(1999) observed the endurance of nanobes when exposed to gamma radiation as well as UV, microwaves, and upon heating and drying, attributing this to the presence of biogenic apatite in the cell walls, as seen by Kajander *et al*. (1998). Nanobes were also shown by Bjorkland *et al*. (1999) to be resistant to a wide variety of disinfecting and sterilising chemicals, most notably hydrogen peroxide which is widely expected to occur at the martian surface (e.g., Mancinelli 1989). The occurrence of such minute organisms is still not generally accepted, however, as it remains to be demonstrated how nanobes compress the necessary genetic and metabolic molecular machinery into such a tiny volume (Kerr 1998). It has been argued that the appearance of purported nanobes is due to desiccation or to the electron microscopy preparation process.

While new evidence concerning terrestrial nanobes continues to emerge, and additional examples of possible alternative terrestrial analogues to the objects in ALH84001 are discovered (Thomas-Keprta *et al*. 1997: Folk and Lynch 1999), alternative abiological explanations for the features in ALH84001 have been presented (Bradley *et al*. 1997: McKay *et al*. 1997: Kerr 1997a, 1997b). Kirkland *et al*. (1999) showed that the precipitation of calcite under sterile conditions could produce spheres, ovoids, and rods between 25 and 300nm in length. The discovery in lunar meteorites of similar structures to those seen in ALH84001 (Sears and Kral 1998) also calls into question their proposed biological origin (although Benoit and Taunton, 1997, searched for, but failed to find, biomorphic microstructures in lunar meteorites), as does the evidence that nanometer scale rod-shaped carbonates in the Tatahouine meteorite were formed entirely by chemical alteration since the meteorite fell in 1931 (Padirac 1998). Even those examples which can be demonstrated to be biological may prove to be of terrestrial origin (Burckle and Delaney 1998, 1999).

One might feel less chastened were this the first instance of confusion over the identification of extraterrestrial fossils, but it is not. Claus and Nagy (1961) discovered so-called 'organised' structures in the Murchison carbonaceous chondrite, and there were further suggestions of microfossils in the Alais and Orgueil carbonaceous meteorites (Staplin 1962: Palik 1962: Nagy *et al*. 1963: Timofejev 1963: VanLandingham 1965). These microstructures were the subject of considerable debate at the time; being identified as instrumental, mineralogical or organic artefacts (Briggs 1962: Gregory 1962: Mueller 1962: Pearson 1962: Rossignol-Strick and Barghoorn 1971). Remarkable though it may appear, it is still contended in some quarters that apparently biological structures seen in the Murchison, Orgueil, Allende, Mighei and Efremovka meteorites are the fossilised remains of an ecology indigenous to the chondrite parent body (Hoover 1997: Pflug and Heinz 1997: Zhmur *et al*. 1997: Hoover *et al*. 1998: Hoover and Rozanov 1999: Rozanov and Hoover 1999: Zhmur and Gerasimenko 1999).

Thus, the occurrence of objects similar in appearance to fossilised microbes (Westal 1999), or even viruses (Ksanformality 1999), may be a useful guide to the detection of relict life but it is by no means an equivocal test, unless intracellular structures are well preserved and observable, or traces of DNA remain (*cf*. Burde *et al*. 1999: see 3.1 above).

The most concise method of summarising the foregoing analysis is to present a set of scores for each biomarker scheme based on its usefulness to exobiology in *positively identifying ancient microbial remains on Mars*. Subjectivity is obviated by a set of scoring criteria, which are set out in more detail in Appendix I. This method is widely

used, for example, in forensic sciences for the statistical analysis of fatal injury data (e.g., Baker *et al*. 1974).

	Biological or not?	Preservability	Facility of analysis†	TOTAL (max score =9)
<u>Organic molecules</u>				
Amino acids	0	3	2	**5**
Polycyclics*	0	3	2	**5**
Genetic molecules**	1	2	1	**4**
<u>Mineral assemblages</u>				
Carbonates	2	3	0	**5**
Phosphates	2	3	0	**5**
Iron minerals	2	3	0	**5**
Manganese minerals‡	2	2	0	**4**
Homochirality	2	2	2	**6**
Body fossils	0	3	1	**3**

† Applies to the *martian* surface and subsurface environment.
* Includes PAH's, nitrogen heterocyclics, porphyrins and other metallocenes.
** Includes DNA, RNA, and mono- and polynucleotides.
‡ Specifically, rock varnish.

Note that none of the suggested schemes scores highly, and it need not be a *sine qua non* that use of one or more of these schemes in conjunction would yield a more favourable result. The following section proposes a novel extraterrestrial biomarker that does score highly by the criteria discussed.

4. A NOVEL CLASS OF BIOMARKERS

An ideal biomarker would necessarily be stable against decay or diagenesis for periods of several billion years, be of unambiguously biological origin, and be easy to detect at the martian surface with standard flight instrumentation (e.g., GC-MS, FT-IR) as part of the current exploration strategy (i.e., single complex landers). I have previously advocated the use of membrane lipids, such as sterols and hopanoids, in the search for life on Titan (Fortes, submitted paper), though without qualifying my reasons for doing so. Lipid biomarkers appear to have been entirely overlooked as extraterrestrial biomarkers (in spite of their remarkable success as terrestrial biomarkers), with the exception of the remark by Burlingame *et al*. (1965) that 'isoprenoid alkanes could serve as "biological markers" in our quest for evidence of life.....specifically in carbonaceous meteorites.' Hopanoids have the advantage of being biologically distinctive (as a result of the complexity of the cyclization reaction which forms them - see below), and being extraordinarily resistant to microbial degradation and high temperature diagenesis: Hopanoids, for example, have been extracted from the 2715 million year old Maddina Formation of the Australian Pilbara Craton (Brocks *et al*. 1999).

Although the work of McDonald *et al*. (1998) indicates that complex biopolymers may be stable against chemical oxidation in the surface layers near the martian poles, it is more probable that a lander would need to dig below the H_2O_2 diffusion depth (~3 metres) to locate suitable organic residues. Thus, by the criteria outlined in Appendix I, hopanoid biomarkers score 8 out of 9. If they are accessible in surface materials, they score nine, making them ideal for identifying extinct biology on Mars.

4.1 NATURE AND OCCURRENCE OF TERRESTRIAL HOPANOIDS

Hopanoids are a family of triterpenoid (C_{3x} isoprenoid) hydrocarbons having a primary pentacyclic hopane skeleton (illustrated below) of four cyclohexane rings and one cyclopentane ring. Over a hundred members of the hopane family occur naturally (Ourisson *et al*. 1979), variants being methylated at either C_2 or C_3, oxidised at C_3 or elongated with side chains attached at C_{29}. Hopanoids are subdivided into geo- and biohopanoids.

Biohopanoids (Ourisson and Rohmer 1992) are found in many plants, ferns, mosses, and fungi, typically being 3-oxyhopanoids without side chains, whose function is unclear, though they are widely believed to be metabolic by-products. 3-deoxyhopanoids with C_{29} side chains have been demonstrated to play an important role, however, in the cell membranes of many prokaryotes, most common being bacteriohopanetetrol and aminobacteriohopanetetrol (reduced, in an effort to avoid excessive sesquipedalianism, to BHT and ABHT respectively). The most abundant bacterial non-elongated hopanoids are diploptene (hop-22[29]-ene) and diplopterol (22-hydroxyhopane).

Geohopanoids are ubiquitous in crude oils, coals, slates, and sediments of all ages from all environments (Moldowan *et al*. 1991) and are the most abundant natural products on Earth, massing approximately 10^9 kg of carbon (Ourisson and Albrecht 1992: Damste and Schouten 1997). They are typically elongated at C_{29}, which is taken to indicate a bacterial origin via diagenesis of biohopanoid precursors (Tritz *et al*. 1999), and the variability in observed structures has been used extensively as an indication of

maturation processes in sediments and oil reservoirs (e.g., Mycke *et al*. 1987). They have also been used in palaeobiological research to study probable metabolic pathways in Archaean prokaryotes: 2α-methylhopanes, for example, are taken to be indicative of cyanobacteria (Brocks *et al*. 1999: Summons *et al*. 1999).

The most widely accepted function for hopanoids in bacteria is as a replacement for the sterols (pentacyclic hydrocarbons of five cyclohexane rings) found in eukaryote cell membranes and which play a crucial role in controlling the rigidity and permeability of the cell wall (Kannenberg and Poralla 1999). That they represent the phylogenetic precursors of eukaryotic sterols is indicated by several factors in the production of BHT. Firstly, the production of hopanoids from a squalene precursor (an acyclic triterpenoid) requires less intermediate steps, whereas the production of cholesterol must proceed via squalene epoxide and lanosterol. The hydration reaction in the production of diploptene is anaerobic whereas the formation of cholesterol is aerobic. Finally, the cyclization reaction of squalene (itself the most complex single-step reaction in the biological world) is easier to achieve in the production of hopanoids since the molecular conformation is more energetically favourable (Ourisson *et al*. 1979).

These factors have some useful implications for exobiology. To begin with, molecules constructed from isoprene units are biologically universal on Earth, from bacterial lipids to mammalian hormones. Although there is no *a priori* reason why martian organisms should employ isoprene building blocks in their molecular construction kits, it is surely reasonable to invoke parallel evolution based on the remarkable, and repeated, coevolution of isoprenoid structures on Earth. Secondly, the formation of hopanoids over steroids is favoured in the, presumably, anaerobic conditions throughout martian history. Thirdly, there appears to be a correlation of hopanoid production in bacterial cell membranes with strains having high G + C contents (Taylor 1984), itself identified as a response to environmental stress. Thus, increasing stressful environmental conditions as Mars became colder and drier may well have fostered the use of hopanoid lipids in the stabilisation of progressively strained phospholipid membranes.

However, a problem to be faced is that hopanoid use is not universal amongst bacteria, and there is little apparent correlation of its expression even within taxonomically similar groups. Hopanoids have not been identified in any Archaean micro-organisms, and it is probable that even more primitive terpenoids (e.g., β-carotene) may be employed by those groups. A problem in understanding hopanoid distribution in bacteria is that some strains contain such tightly bound hopanoids that they cannot be detected by conventional means. In addition, many bacteria contain genes coding for hopanoid biosynthesis yet have not been observed to produce them. Other strains are believed to strictly regulate their production of hopanoids, so evading detection. Again, this might be a response to environmental stress (Kannenberg and Poralla 1999). There is nevertheless, I believe, a strong possibility that any organisms which could have arisen on Mars would have had the ability to make hopanoids and would have used that ability to an extent which would be detectable today.

4.2 DETECTING HOPANOIDS

We have good evidence for the existence of river systems on Mars at some time in the past, strong (though indirect) evidence that certain craters held large standing bodies of

water (Scott *et al*. 1991), and hints of an ocean occupying the northern lowlands (Malin and Edgett 1999). The martian palaeohydrosphere was the most probable habitat of any organisms that might have arisen and the sink for all of the resultant biological detritus. Therefore, fluvial, lacustrine, and marine sediments on Mars are the best place to search for hopanoids (*cf*., Masursky *et al*. 1979). Terrestrial geohopanoids are largely the result of high temperature and pressure diagenetic processes, coupled with chemical and microbial degradation, acting on biohopanoid precursors (e.g., Damste *et al*. 1995). In the absence of any significant geological activity during post-bombardment martian history, and the probable limited depth of accumulated sedimentary piles (extensive vertical sections of layered rocks exposed in Valles Marineris are almost certainly volcanic), then geohopanoids might be expected to be scarce, with immature biohopanoids predominating. In certain locations where organic materials in sediments have been heated by proximity to magmatic fluids, or buried beneath large volumes of volcanic material, then a degree of maturation in favour of geohopanoids is plausible: Such locations are likely to be inaccessible to sampling for some considerable time. Given also the reduced degree of erosion at the martian surface relative to the Earth (Golombek and Bridges 2000), then biohopanoids should remain accessible *in situ*, and relatively unaltered (with the exception of oxidative destruction by H_2O_2 in the uppermost regolith), in stream, river, and lake beds.

With these conditions in mind, I have conducted an experiment which aims to identify appropriate sampling, preparation and analysis techniques that could, with suitable miniaturisation, be conducted remotely by a lander on Mars (or any other potentially life-bearing body). The sampling strategy recognises that the most likely landing site will be a random section of a river valley or crater-lake bed (Farmer *et al*. 1999: Crisp and Golombek 1999), and so the sample used here was collected from an arbitrary stretch of the nearest river using a simple scoop. The organic extraction, described shortly, used well established, straightforward, wet chemical methods which are amenable to miniaturisation and automation. Portable analogues of the analysis equipment employed exist and indeed have flown in space.

The thrust of the experiment is two-fold. Firstly, the detection of biohopanoids in a recent river sediment, wherein they are expected to be ubiquitous (Rohmer *et al*. 1989), might encourage us to hope that biohopanoids could be readily found - if present - in the types of sediments that Mars landers will be exploring in the coming decade. Secondly, the extracted organic fraction can be analysed, using a variety of other procedures, so as to identify alternative signatures of hopanoid molecules which can be used in tandem with the GC-MS results described below to yield a more robust and unambiguous characterisation of possible martian biochemicals. Gas chromatography - Fourier transform infrared spectroscopy (GC-FTIR), for example, has developed over the past decade into a useful complement to GC-MS analysis, offering significant advances in the discrimination of complex mixtures of low concentration PAHs (Doumenq *et al*. 1990: Smyrl *et al*. 1992: Vredenbregt and Visser 1994). The recent fabrication of infrared spectrometers occupying < 2 cm^3 (Keraenen *et al*. 1999), and ultra miniaturised GC columns etched onto silicon wafers (Matzke *et al*. 1998), suggests that GC-IR could be readily achieved within the scope of a Mars lander science package.

4.2.1 Experimental methods.

Sampling and preliminary analysis.

A single sample of sediment was collected from the River Cherwell near Banbury in Oxfordshire (52º 04' 38.6"N: 1º 19' 40.8"W) on January 17. 2000. The material was acquired using an ordinary trowel from a shallow (10-50 cm deep) pool containing only a small amount of vegetation, sampled muds extending to depths of ~20 cm below the bottom. The sample was sieved to remove macroscopic vegetable matter and allowed to settle for one week, after which much of the extraneous water was drained off.

The following preparatory and analytical procedures were carried out in the Wolfson Geochemistry Laboratory at UCL. The sediment was dried for three days at 45ºC and ground to a fine powder. Two aliquots of sediment (~600 mg each) were treated with 10% HCl in a Leco filtering crucible for ~45 minutes to remove any inorganic carbon-bearing material, followed by a few drops of concentrated HCl and a rinse in distilled water, before being dried overnight at 45ºC. The treated sediment samples were placed in a CS-125 Leco Carbon-Sulfur Analyser in order to determine the acid insoluble (syn: organic) carbon content. Both samples contained 6.2wt% organic carbon and variable quantities of sulfur (0.0282 wt. % and 0.0017 wt. %).

Extraction of organics.

Following the example of Innes *et al*. (1997), one gram of the dried sediment was measured into a cellulose thimble and extracted using a Soxhlet apparatus with a chloroform-methanol mixture (150 ml; 2:1 v/v) for 18 hours. Elemental sulfur, which is harmful to the mass spectrometer, was removed by the addition of activated copper turnings to the solution prior to the extraction. The resulting extract (~200 ml following rinsing with 50ml of dichloromethane) was evaporated to a concentrate in a Büchi R-114 Rotoevaporator and transferred to a vial for further drying in a Techne DB.3A Dri block. The vial was gently warmed in the Dri block to ~30ºC and blown completely dry under nitrogen gas. The remaining material was then redissolved in 2 ml of dichloromethane for injection into the GC-MS.

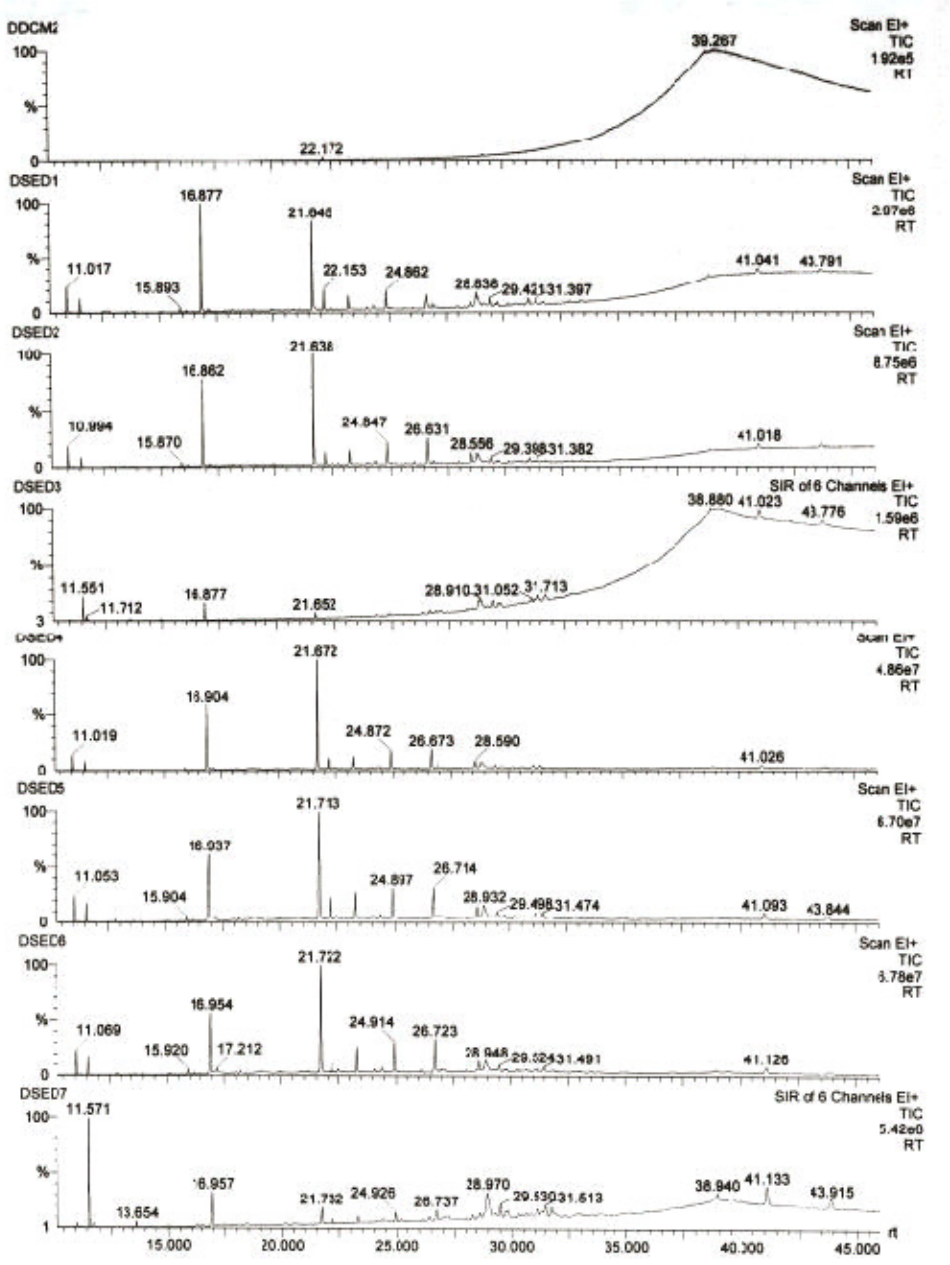
GC-MS Analysis

A Fisons GC8060 gas chomatograph, coupled to a Fisons MD800 quadrupole mass spectrometer was used for the analysis of the organic extract. The GC system was fitted with a 20m x 0.32 mm ID fused silica BP5 capillary and helium at a pressure of 30kPa was used as a carrier gas. Samples were injected directly at 50ºC and the oven was programmed from 50º to 275ºC at 10ºC per minute, 275º to 350ºC at 5ºC per minute, and then held at 350ºC for eight minutes. The mass spectrometer was operated in electron impact mode, scanning from 50 to 818 a.m.u. every 0.45 seconds with a one minute solvent delay. The interface between the GC and MS was maintained at 350ºC, and the detector was held at 300V.

Each GC-MS run lasted 47 minutes, and the results of the seven complete runs are shown below. The first run (DDCM2) consisted of an injection of the DCM solvent to

define a baseline. DSED1 shows the total ion current for an injection of 1ml of the sediment extract taken from the 2ml concentrate. The solution was evaporated down to 1ml and a further 1ml was injected for a third run (DSED2). The extract was sequentially evaporated down to 0.8, 0.5 and 0.2ml, with 1.2 ml injections from the first two concentrates, and 1 ml from the third, yielding DSEDs 3 to 5. DSED6 is a repeat of DSED5, illustrating the high degree of reproducibility. DSED7 shows the selected ion record (SIR), counting only those ions with m/z of 190 - 193 and 339, and hence shows which peaks might contain the characteristic m/z 191 breakdown ion of hopanoids.

Fig. 3. Chromatograms DDCM2 and DSED's 1-7



4.2.2 Experimental Results

Mass spectra were acquired for forty-one of the peaks in the chromatogram from GC run DSED6, shown in figure 4 below. At no time throughout the extraction and analysis process were any steps taken to quantify the concentrations of individual components in the GC-MS output, and any references to concentrations in the ensuing discussion are based on the *relative* heights of the GC peaks. Although the objective of the experiment was to identify biohopanoids in the sedimentary extract, attempts were made to name compounds associated with as many peaks as possible. The table below lists for each GC peak, the retention time, the molecular weight of the largest detected ion, the main metastable ion peaks, and the tentative identification.

Several features of the mass spectra need to be addressed before attempting identification.

- Many of the compounds occur in such low concentrations that the molecular ion may be below the detection threshold of the mass spectrometer, making positive identification virtually impossible.

- Nearly all of the spectra contain a contaminant ion at m/z 207 (and possibly at m/z 281 as well). This is unfortunate since many methyl steranes and methyl hopanes have important metastable peaks at m/z 205 which are likely to be masked by the 207 peak.

- There is a drift of two mass units in the m/z values of metastable ions in some of the spectra. These are most obvious in spectra where the contaminant peak at m/z 207 is shifted to 205, and corresponding diagnostic peaks for steranes (217 ± 215 and 231 ± 229) and hopanoids (191 ± 189 and 177 ± 175) are affected. This drift appears to be restricted to m/z < 240, and was fully accounted for in identifying compounds in the sedimentary extract.

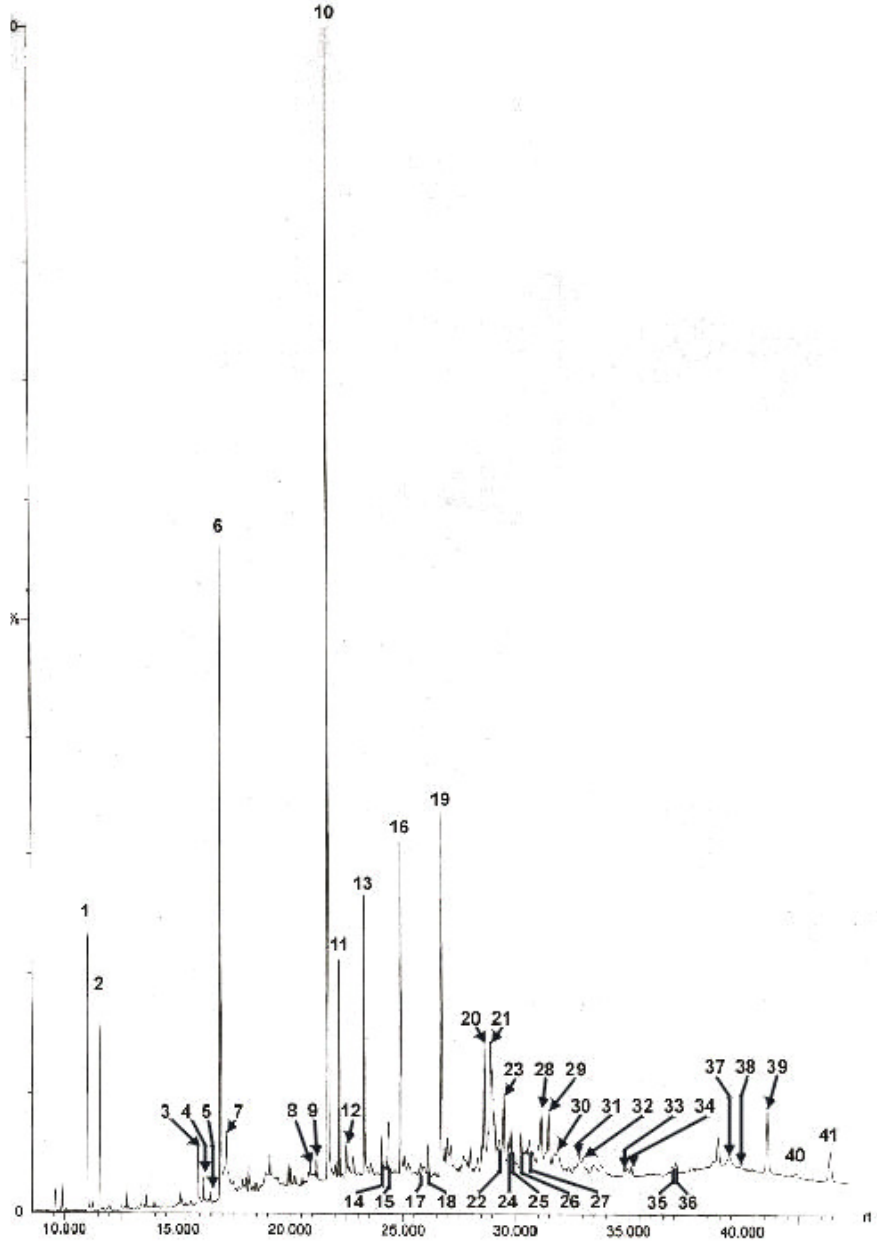
Summary table of the properties of peaks 1-41

Peak no'	Retention time (minutes)	Max m/z	Major ions	Possible ID
1	11.069	195	194-5, 163-4, 133-5	C_{13} alkene
2	11.578	504	327, 281-3, 147	Unknown
3	15.920	243	223-4, 205, 167, 149, 132, 121, 104, 93, 76, 65, 57	C_{16} alcohol?
4	16.179	268	268, 197, 169, 141, 127, 113, 99, 85, 71, 57	$C_{19}H_{40}$ pristane
5	16.470	270	270, 239,227, 204, 199, 185, 171, 162, 157, 143, 129, 115, 97-101, 87, 74, 55	C_{18} alcohol?
6	16.954	323	323, 278-9, 223-4, 205, 167, 149-50, 122-3, 104-5, 93, 76, 57	Branched alkane?
7	17.212	282	282, 256-7, 239, 227, 213, 185, 171, 157, 149, 129, 112, 97-9, 85, 71, 57	$C_{20}H_{42}$ phytane
8	19.938	270	268-70, 257, 192-5, 139, 125,111, 97, 83, 69, 55-7	C_{22} alkane?
9	20.030	324	230, 207, 183, 169, 155, 141, 127, 113, 99, 85, 71, 57	C_{23} alkane
10	21.722	309	308-9, 280, 252, 238, 224, 210, 195, 181, 167, 153, 139, 125, 111, 97, 83, 70, 55	C_{22} alkene

11	22.197	368	368, 351, 279, 180, 167, 149, 132, 113, 104, 83, 70-1, 57	Branched alkane?
12	22.533	341	340-1, 297, 253, 231, 215, 207, 165, 149, 140, 125, 111, 97, 83, 69, 57	C_{24} sterane?
13	23.314	280	239, 225, 207, 183, 169, 155, 139-41, 125, 111, 97, 85, 71, 57	C_{20} alcohol / acid (phytol?)
14	24.075	350	350, 327, 281, 252-3, 225, 169, 154, 141, 125-7, 111-3, 97-9, 85, 71, 57	Unknown
15	24.389	402	264, 251, 220, 208, 191-3, 166, 148, 137-8, 123-5, 109-11, 96-7, 82, 67, 57	Unknown
16	24.914	332	267, 251, 239, 225, 211, 197, 178, 169, 153-5, 139, 125, 111, 97, 85, 71, 57	C_{24} alkane
17	25.756	396	396, 385, 355, 343, 253, 239, 224, 189, 141, 127, 113, 99, 94, 85, 71, 57	$C_{28\text{-}29}$ hopane?
18	26.140	404	280, 207-10, 153, 139, 123-5, 109-11, 96-7, 82, 69, 57	C_{25} alkane?
19	26.723	382	250, 236, 223, 208, 181, 167, 153, 139, 125, 111, 97, 83, 69, 57	C_{26} alkane
20	28.657	447	446-7, 412, 397, 355, 341, 281-3, 207, 178, 131, 125, 111, 97, 83, 69, 57	C_{30} ββ hopane
21	28.948	424	424, 414, 399, 396, 381, 329-30, 303, 273, 255, 231, 213, 205, 189, 173, 159-61, 145-7, 133, 119, 105-9, 95, 69, 57	C_{29} friedelane
22	29.310	429	429, 410-12, 397-9, 383, 355, 327, 314, 298, 267-9, 257, 250, 240, 219, 205-7, 189-90, 176, 169, 159-60, 147, 130-3, 119, 107, 93, 85, 75, 69	C_{31} αβ hopane
23	29.524	426	425-6, 409, 400, 383, 368, 355, 341, 327, 313, 299, 281, 265, 253, 245, 229, 215, 205, 202, 189-91, 175, 161, 145-9, 133, 121, 105, 93, 79, 69	C_{31} gammacerane
24	29.740	430	430, 426, 411-3, 397, 383, 369, 355, 341, 327, 315, 281, 253-5, 229, 218, 207, 203, 189, 175, 161, 147, 131-5, 119, 105-7, 95, 81, 69	C_{31} αβ hopene (diploptene?)
25	29.840	412	412, 397, 369, 355, 341, 327, 313, 281, 274, 259, 253, 245, 229, 215, 207, 189-91, 173-5, 161, 147, 133, 119, 105-9, 95, 81, 69	C_{29} serratane
26	30.274	426	426, 412-3, 400, 385, 370, 356, 329, 308, 300, 290, 281, 271, 257, 250, 229-30, 211, 207, 203, 187-9, 173-5, 163, 159, 146-9, 135, 124, 119, 105, 91, 81, 69, 57	C_{29} 5β stigmastane
27	30.641	430	397, 355, 327, 296-7, 281, 268, 259, 253, 229, 215, 207, 189-91, 173-5, 161, 147, 133-5, 119-21, 105-9, 95, 81, 69	ββ hopanoid
28	31.149	451	451, 443, 424, 409, 397, 394, 355, 341, 329, 313, 301, 298, 281, 267, 253, 245, 229, 217, 205-7, 189, 175, 161-3, 147, 133-5, 121, 109, 95, 81, 69	αβ hopanoid
29	31.491	470	470, 451, 430, 412-3, 397, 359, 355, 341, 327, 316, 301, 281, 267, 253, 245, 221, 215, 207, 205, 189-91, 175-7, 163, 147, 135, 123, 107-9, 95, 81, 69	C_{33} αβ 2-methyl hopane
30	31.882	429	428-9, 426, 412, 398, 384, 355, 341, 327, 313, 299, 285, 281, 273, 267, 233, 245, 232, 221, 207, 191, 177, 161-3, 147, 133-5, 119, 105-9, 95, 81, 69	C_{31} ββ hopanoid
31	32.824	430	429, 405, 397, 355-7, 341, 327, 313-5, 296, 281, 267, 259, 249, 237, 221, 207, 191, 177, 163-5, 147, 133, 119, 109, 95, 81, 71, 67	C_{30} diplopteral
32	32.991	451	451, 429, 405, 377, 355, 341, 327, 313, 299, 295, 281, 267, 253, 239, 221, 207, 205, 191, 177, 163, 147, 133, 123, 107-9, 95, 81, 71, 69	C_{32} αβ hopanol
33	34.833	503	503, 451, 429, 405, 377, 355, 341, 327, 313, 295, 281, 267, 253, 249, 221, 207, 191, 177, 163, 147, 135, 119, 109, 95, 81, 73, 69	C_{33} αβ hopanol
34	35.168	451	451, 429, 405, 377, 355, 341, 327, 313, 295, 285, 281, 267, 257, 253, 239, 221, 207, 191, 177, 163, 147, 133-5, 109-11, 95-7, 83, 73, 69, 57	C_{32} αβ hopanol
35	36.950	503	503, 489, 451, 429, 416, 405, 389, 377, 355, 341, 327, 313, 295, 281, 267, 253, 249, 239, 221, 207, 191, 177, 165, 147, 135, 119, 109, 95, 81, 73	C_{33} αβ hopanol
36	37.092	517	517, 503, 479, 451, 429, 415, 405, 384, 369, 377, 355, 341, 327, 313, 295, 281, 267, 257, 253, 249, 239, 221, 207, 191, 177, 163, 147, 135, 119, 111, 97, 83, 73, 69	C_{34} αβ hopanol
37	39.468	530	530, 429, 405, 369, 355, 341, 327, 315, 281-2, 267, 253, 249, 221,	C_{35} αβ hopanol

			207-9, 191, 177, 163, 147, 133-5, 119, 97, 81-3, 73, 57	
38	39.959	622	620-22, 503, 461, 429, 377, 355, 341, 327, 313, 281-3, 267, 253, 249, 221, 207-9, 191, 177, 163, 147, 133-5, 111, 97, 83, 73, 57	Hopanepolyol
39	41.126	650	650, 551, 529, 503, 479, 429, 405, 355, 341, 327, 313, 285, 281, 267, 253, 249, 221, 207-9, 191, 177, 163, 147, 133-5, 111, 97, 83, 73, 57	Hopanepolyol
40	42.410	535	535, 503, 479, 429, 405, 355, 341, 327, 315, 281, 267, 253, 249, 221, 207-9, 191, 177, 163, 147, 133-5, 119, 97, 91, 81-3, 73, 57	Hopanepolyol
41	43.902	677	677, 537, 503, 465, 429, 405, 369, 355, 341, 327, 313, 281, 267, 253, 249, 221, 207-9, 191, 17.7, 163, 147, 133-5, 111, 97, 83, 73, 57	Hopanepolyol

Fig. 4. Enlarged version of DSED6 showing the peak numbering used.



Prior to the GC-MS analysis, it was expected that the earliest eluting compounds, and by far the largest peaks, would be due to the isoprenoid hydrocarbons pristane ($C_{19}H_{40}$) and phytane ($C_{20}H_{42}$). These two substances result from the breakdown of dihydrophytol, a side chain of the chlorophyll molecule. A significant component of steranes was anticipated, dominated by C_{21} cholestane and C_{28} methylcholestane (derived from cholesterol), and a very small contribution from a variety of triterpanes including hopane.

The results of the GC-MS analysis differed from the aforementioned expectations in many respects, as the table below illustrates. While (probable) isoprenoid hydrocarbons, steranes, and triterpanes (including hopanoids) are all present in the sedimentary extract, their relative concentrations are most surprising. The abundances of pristane and phytane proved to be considerably lower than expected, and lower even than most of the other n-alkanes and alkenes which appear to be present. Contrary to expectation, the relative abundance of steranes seems to be lower than that of the hopanoids, with the variety of hopanoid structures greatly outnumbering those of the steranes. These findings suggest that that the source of the organic carbon in this river mud is primarily bacterial rather than vegetable.

Hydrocarbons

Initial identification was attempted using the searchable database on the PC operating the GC-MS. However, the 'best fit' spectra were rather poor matches and yielded results more characteristic of a human blood sample than a river sediment, the database comprising largely pharmaceutical and toxicological mass spectra. The hydrocarbons eluting in the first 25 minutes of the GC run were therefore identified as closely as possible from consideration of the observed fragmentation pattern. Since these compounds appear to be extremely abundant in the sample, most of the spectra are very clean and easy to read. Lower concentration peaks (e.g., peaks 8, 9, and 12) have visibly lower signal to noise ratios and the molecular ion may well be absent from these spectra.

Spectra for several compounds (the best examples being 4, 7, 16, and 19) show peaks systematically separated by 14 mass units, indicating fragmentation into units of M - $[CH_2]^+$, and hence identifying them with a high degree of certainty as n-alkanes, n being determined (or guessed) from the mass of the molecular ion. Peaks 4 and 7 have exactly the correct molecular masses to be pristane (mass = 268) and phytane (mass = 282) respectively, though, as noted before, these are in markedly lower concentrations than expected.

Other spectra show peaks separated by 30 mass units (e.g., peak 1) and 45 mass units (e.g., peak), which could be explained by loss of $[C_2H_5]^+$ ions and $[C_2H_5OH]^+$ or $[COOH]^+$ functional groups, respectively. Identifications of these compounds is considerably more tentative than for the alkanes.

Certain peaks fall short by two mass units of the correct molecular weight for the corresponding alkane. Peak 10, for example, shows a probable molecular ion at m/z 308 and M^+ at 309, whereas a C_{22} alkane (as is indicated by the lower mass peaks) should have a molecular mass of 310. The difference is interpreted as a single degree of unsaturation, and the elution of this compound *after* the C_{23} alkane is consistent with the lower volatility of olefins relative to the corresponding paraffins.

The mass spectrum of peak 2 appears to contain fragments of ions up to m/z 504, many of which are more typical of compounds eluting after 35 minutes, and so this compound (if it is a single substance) is ignored.

Steranes and non-hopanoid triterpanes

The classification system of Wardroper *et al*. (1977) was used in conjunction with published spectra and relative retention times of the expected steranes (Burlingame *et al*. 1965: Anderson *et al*. 1969: Gallegos 1971) in an effort to locate and identify any steranes in the sediment extract. Steranes should have a dominant metastable peak at m/z 217 and a second diagnostic peak at m/z 231. There are no compounds in the extract with a large peak at m/z 217, and the relative intensity of the 217 and 231 ions in many of the spectra is wholly consistent with the fragmentation of pentacyclic triterpanes rather than tetracyclic steranes. Only peak 26 contains a sufficiently intense mass spike at m/z 231 (actually 229 since this spectrum exhibits the 2 a.m.u drift mentioned earlier). Taken in conjunction with the occurrence of peaks at m/z 400, 385, 370, 290, 203, 175, and 151 (again, allowing for drift at m/z <240), I believe that this compound offers a reasonable match to the model spectrum of $C_{29}H_{52}$ stigmastane. Furthermore, the strength of the m/z 151 peak identifies the isomer 5β stigmastane. Ions at 412 and 426, and contributions at lower molecular weights, may be fragments of a co-eluting compound.

The complete absence of large m/z 217 peaks, molecular ions at 288, 372, and 386, or metastable ions at 262+14n (n = 0, 1, 2, 3, etc.) rules out categorically the occurrence of pregnane, ergostane, and cholestane, the most abundant steranes which were expected to be present.

Pentacyclic triterpanes were equally conspicuous by their absence. Applying the criteria of Wardroper *et al*. (1977) I was able to tentatively identify peak 25, from its diagnostic ions at m/z 259, 274, 231, 191, and 123, as the C_{29} triterpane serratane, and peak 21 as a candidate for friedelane. Peak 23, with its molecular ion at m/z 426 and peaks at 191 and 177, has the correct molecular mass and approximate retention time to be C_{31} gammacerane, though the intensity of the m/z 191 peak is much lower than it should be.

Hopanoids

As with the steranes, the classification system of Wardroper *et al*. (1977) was applied to the identification of hopanoids in the sediment extract, along with published spectra of bio- and geohopanoids and relative retention times (Gallegos 1971: Volkman *et al*. 1983: Moldowan *et al*. 1991). Pentacyclic triterpanes with a five membered E-ring (i.e., hopanoids) typically display diagnostic metastable ions at m/z 191 and 177 (the ratio of intensities of the two indicating the isomer of the epimer with a stereocenter at C_{17}). C_{30} hopanes have molecular ions at m/z 412 (hopenes at 410, and hopanols at 429) and signature peaks at 398, 383-4, and 369-70.

Nearly half of the GC peaks contain some or all of the diagnostic peaks of hopanoids, in complete contradiction of what was anticipated prior to the experimental analysis. Although most of the hopanoid components appear to occur at very low relative concentrations, and so have rather noisy mass spectra, I believe I have been able to

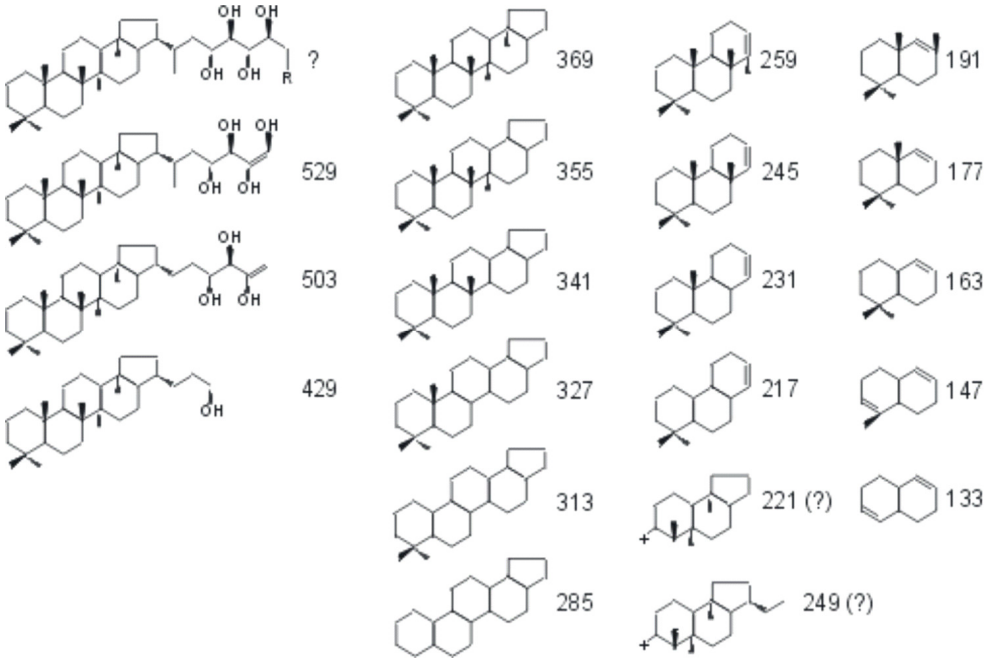
identify most of them with sufficient accuracy to be able to confirm that biohopanoids are present, and that the compounds eluted in approximately the correct order.

Although there is a possibility that peak 17 represents a C_{28-29} hopane, peak 20 is the first to show the molecular ion at m/z 412, with very small peaks at 191 and 178. The relative intensities of the latter two ions shows this to be a 17β isomer of C_{30} hopane (and hence 17β, 21β, since the 21α isomer is vanishingly rare). Biosynthesised hopanes are exclusively formed with the ββ conformation and are altered to the more thermodynamically stable αβ variety with time, so this first hopane peak is almost certain to be the result of biological activity. In contrast, the rest of the hopanoids in this sample (with the exception of peaks 27 and 30) show evidence of being αβ isomers. The identification of αβ hopanes in the sediment sample might be taken to indicate contamination from a hydrocarbon source, such as petroleum, engine oil, or asphalt, or (since the rate at which the ββ → αβ transformation takes place in fluvial and lacustrine environments is not presently constrained) the rapid maturation of biohopanoids *in situ*. The location from which the sample was collected is ~10 metres downstream of the main London to Birmingham rail link, and ~1km downstream from the M40 motorway, so the potential for contamination from surface runoff does exist. The only way to address this problem is to characterise the branched hydrocarbons in fluids draining the road surface and rail bed. However, it is worth noting that Rohmer *et al.* (1980) found αβ hopanes in modern pond muds which were demonstrated to be free of contamination, and attributed the finding to acid-catalysed isomerization and/or 'microbiological processes.'

GC peaks 31-41 are particularly interesting in the context of looking for biohopanoids. The mass spectra for these compounds are essentially identical below m/z 429 but differ markedly for ions of higher molecular weight. This implies that they share a common carbon skeleton but vary in the details of the functional groups attached to that skeleton. In spite of their possessing the αβ conformation, it is my opinion that the degree of functionalisation of these high molecular weight hopanoids favours their formation *in situ* from bacterial precursors. My interpretation of the fragmentation pattern of the side chains (which, along with the main skeleton fragments, is illustrated in detail further down the page) indicates that the precursor molecule was a C_{35} hopanepolyol with a pentafunctionalised side chain bearing at least four alcohol functional groups and an unknown, though high molecular weight, moiety on the terminal carbon, typical of bacterial membrane lipids (Sahm *et al*. 1993). Positive identification of these compounds requires a rather more involved extraction process than was employed in this experiment due to the high polarity and concomitant low solubility in organic solvents. Hopanepolyols have been extracted with a chloroform-methanol solution previously, but normally require derivatisation with periodic acid and sodium borohydride (see, for example, Innes *et al*. 1997) to yield more readily detectable hopane-acetates. The extremely low concentration of hopanepolyols in this sedimentary extract may mean that the molecular ion is below the detection threshold of the mass spectrometer, and thus it is unlikely that an improved characterisation could be achieved from the data presented here. Another interesting feature is the elution of pairs of hopanols with the same molecular masses two to three minutes apart. The spectra for these compounds are practically identical, and the only explanation that presents itself is that these are different isomers of the hopanoid epimers with stereocenters at Ch or at carbons located in the side

chains. If that is the case, then the earlier eluting compounds are the 22S isomers, and the later, the 22R isomers.

Fig. 8. The structure of the m/z 191 ion has been widely published. The illustration above contains my own interpretation of the ion structures associated with metastable peaks in the mass spectra of hopanoids. Note that peaks at m/z 259, 245, 231, and 217 are all absent from the spectra of most of the hopanes and all of the hopanols, but do occur in the spectra of pentacyclic triterpanes with a six-membered E-ring (e.g., gammacerane). The identity of metastable ions with masses of 405 and 281 in the spectra of these compounds is unknown.



5. DISCUSSION

The detection of small quantities of substances which I am confident in identifying as bacteriohopanepolyols supports my previous arguments that biohopanoids could be detected in martian fluvial sediments by a suitably miniaturised and automated equivalent of the extraction and analysis equipment used in the Wolfson Laboratory. What I believe I have shown is that:

- Martian biota may have used hopanoids in an analogous fashion to terrestrial prokaryotes.
- These hopanoids are unambiguously biological.
- They are preserved for multi-billion year periods.
- They are ubiquitous in the types of river sediment that might occur on Mars.

These arguments lend considerable weight to my contention that hopanoids are excellent biomarkers in the search for signs of extinct life on Mars, and are superior in a number of respects to many existing biomarker schemes. Having thus appeared to rubbish virtually every biomarker scheme ever proposed to the apparent advantage of my own, permit me to qualify the arguments that I presented in the first half of this paper.

In defining a best working practice for seeking out an extinct martian biota there is, within the framework of the present program, a place for each of the biomarkers discussed, but we should be aware that, even in concert, they cannot bear the great burden of certainty that are wont to be piled upon them. Some less so than others. Clearly, the best way forward involves an integrated exobiology strategy (e.g., Brack *et al*. 1999) which includes remote sensing of potential ecological niches (Farmer 1997: Sims *et al*. 1999), in situ characterisation of surface mineralogy consistent with terrestrial life bearing habitats (e.g., Mössbauer detection of hydrothermal vent minerals: Walter and Des Marais 1993: Wade *et al*. 1999), and the detailed analysis of remnant organic materials. A search for hopanoids should be a part of such an integrated strategy.

In order to move forward from the current position it is essential to determine at least one, and preferably more, independent signatures produced by hopanoid molecules which can be used to complement existing GC-MS results. Only when such information is in hand can criteria be developed for the wholly unambiguous detection of hopanoids. Such work could form the basis of a rewarding piece of fundamental research. As was indicated in the introduction to section 4.2, GC-IR analysis would be particularly valuable, and though the resources were not available within the scope of this project, this is just one of several methods for characterising organics in small quantities (see Zolensky *et al*. 2000). Several fluorescence techniques have proven ability to characterise complex mixtures of polycyclic aromatic hydrocarbons; those of Stasiuk and Snowdon (1997) and Swaminadham *et al.* (1999) have been applied to crude oil mixtures, Lewitska *et al*. (1999) employed solid state UV lasers to study aromatic molecules in water, and Storrie-Lombardi *et al*. (1999) have demonstrated a high spatial resolution UV raman spectrometer. Clearly, a determination of the fluorescence spectrum and UV raman spectrum of hopanoids could be useful if such instruments were to be sent to Mars.

Since hopanoids have structural similarities to many floral scent molecules (which are mono- and diterpenoids) then it might be possible to develop a derivatisation technique which would render opanes sufficiently volatile to be amenable to analysis by an artificial nose (e.g., Lang *et al*. 1999).

A future piece of work building on what has been achieved here can progress in one of two directions. The first would involve continued characterisation of the complex hopanepolyol component of the river sediment, and further analysis by GC-IR or fluorescence spectroscopy. GC-IR is still relatively uncommon, but several commercial laboratories offer such a service, with costs being typically five to ten times greater than for GC-MS. The second path would be to buy purified hopanes from which additional diagnostic signatures could be derived. A selection of hopanes can be purchased from the Norwegian chemicals company Chiron AS (see www.chiron.no) for between US$260-520 per 0.1mg vial, with 'hopane kits' (containing five different varieties of hopanoid) available for US$1500-2000. Needless to say, the former program of research is the most cost effective, but I also feel it is the most useful since it is the complex polyols that are of most interest to the study of biomarker degradation and diagenesis under possible

martian conditions. The most appropriate first step would be to repeat the experiments performed here, including derivatisation to characterise the heavily functionalised side chains of the bacteriohopanes, perhaps backing up this work with NMR studies to confirm the structures.

There is evidently a great deal of scope for further study of the signatures of biohopanoids, but I believe that it is important first to overhaul the whole means by which we acquire scientific data from other planets, and that is the topic of the following brief discussion, which caps the arguments on biomarker search strategies.

6. FUTURE DIRECTION ON MARS

The present exploration strategy is moderately well suited to the preliminary geochemical and geophysical characterisation of Mars, but not to the identification of relict biological activity. The future of NASA's Mars program is currently in doubt following the untimely destruction of the Mars Climate Orbiter and the subsequent loss of the Mars Polar Lander, since the 2001 lander is of the same design as the Polar Lander. Even if the program remains on track, then the prospects for exobiology are not great. Whilst it is true that high resolution orbital imagery and spectroscopy will be able to identify potential locations where biogenic remains might be preserved, and that landers and rovers will probably be successful in assessing the chemistry and petrology of restricted parts of the surface and subsurface materials, and even returning samples, none have the capacity to retrieve organic compounds from deep in the regolith, if they are present (Farmer 1999). The Italian Space Agency is presently developing a science package for the 2003 NASA lander (Angrilli *et al*. 1999) which incorporates a drill for sampling the uppermost 50cm of the regolith. The so-called DeeDre (Deep Drill) *may* have the capacity to penetrate to depths of up to 5m on the 2005 or 2008 sample return missions (Di Pippo 1999).

The European Space Agency is currently designing its own Mars mission, the Mars Express (Kolbe 1999: Schmitt *et al*. 1999) and its lander, Beagle 2 (Pillinger *et al.* 1999: Sims *et al*. 1999). Beagles' exobiology program aims to identify potential biogenic fractionation of atmospheric methane, and hence is a search for extant life.

For incorporation into landers beyond 2005, advanced instrumentation is being developed, much of it greatly miniaturised, including organics detectors (Bada *et al*. 1999a), microfabricated capillary electrophoresis chirality analysers (Hutt *et al*. 1999), laser Raman spectrometers (Israel *et al*. 1997: Wang *et al*. 1998: Storrie-Lombardi *et al*. 1999), GC-MS (Kojiro *et al*. 1996, 1997, 1998: Raulin *et al*. 1999: Sternberg *et al*. 1999), and XRD/XRF spectrometers (Vaniman *et al*. 1998), which will all contribute to the exobiological exploration of Mars.

However, the strategy of delivering a single, technologically complex, lander to a single locality on the martian surface is an outmoded remnant of 1960's space science principles. It should not take the loss of a $165 million lander to illustrate this point. Furthermore, the concentration on developing rovers, without wishing to disparage the achievements of Sojourners' engineers and mission scientists, is expensive and needless. A more appropriate use of resources should be to fabricate large numbers of microlanders, incorporating the newly miniaturised instruments, or even emulating the 'lab on a chip' concept (Jacobson and Ramsey 1998), ultimately driving towards the

MEMS (MicroElectroMechanical System: Santoli 1999) design concept and nanotechnology (Laval *et al*. 1999) for the analysis of progressively smaller samples (Muller 1998). Utilising mass-produced (or even grown), lightweight, components, it would be possible to deliver a large number of microlanders to Mars for either an equivalent, or smaller, cost of a conventional lander.
The benefits are numerous:

- Risk of mission failure is reduced significantly by the redundancy inherent in a distributed multi-component lander network.
- A large number of sample sites dramatically increases the probability of locating a signature associated with past life on Mars.
- Multiple landers impact all areas of martian studies, potentially providing widespread and rapid characterization of heterogeneous surface geochemical provinces, and providing the foundation for a global seismic and climatological network.

Even this is a poor substitute for the, admittedly more expensive, alternative of sending humans to Mars. Humans could achieve all of the objectives of the present exploration program far more quickly and thoroughly. However, a microlander strategy would yield greater dividends than the current program, even in the absence of humans on Mars, since they could be deployed more cost effectively to locations such as Europa and Titan where, again, present thinking predicates the use of expensive 'one-shot' options.

7. CONCLUSION

Modern exobiology is on the brink of being able to locate evidence of life on other bodies in our solar system, but many of the tools applied in that search lack the finesse to provide good evidence, least of all conclusive proof. An experiment designed to find the signature of prokaryotic triterpenoids, such as hopane, would provide unambiguous evidence (though not proof) for relict biological activity, using well established and robust GC-MS and FT-IR techniques. Although this evidence is most likely to be found at depths of several metres in the martian regolith, the retrieval of suitable samples is within the capabilities of a lander.

Converting strong evidence into incontrovertible proof will require widespread, detailed, sampling of the target area, characterization of geological context *in situ*, and consideration of additional biomarkers. This is beyond the scope of current robotic technologies, and will require the presence of suitably equipped humans on the surface of Mars.

8. ACKNOWLEDGMENTS

The experimental work for this dissertation was carried out at the Wolfson Geochemistry Laboratory at University College London. I should particularly like to recognise the efforts of Tony Osborn, who conducted the preparatory work and TOC analysis on the original sediment sample, and Sarah Houghton, who worked so hard on

my behalf on the extraction and GC-MS characterisation of the organic component in the sediment. I should also like to thank Dr Karen Hudson-Edwards for organising the laboratory work and giving permission for the use of equipment. Without their contributions, this work would have been impossible. Of course, any misrepresentation of the lab procedures, or misinterpretations of the data, are entirely my own.

APPENDIX I: BIOMARKER USEFULLNESS

	Biological or not?	Preservability	Facility of analysis†	TOTAL (max score =9)
Organic molecules				
Amino acids	0	3	2	**5**
Polycyclics*	0	3	2	**5**
Genetic molecules**	1	2	1	**4**
Mineral assemblages				
Carbonates	2	3	0	**5**
Phosphates	2	3	0	**5**
Iron minerals	2	3	0	**5**
Manganese minerals‡	2	2	0	**4**
Homochirality	2	2	2	**6**
Body fossils	0	3	1	**3**
Hopanoids	**3**	**3**	**2**	**8**

† Applies to the *martian* surface and subsurface environment.
* Includes PAH's, nitrogen heterocyclics, porphyrins and other metallocenes.
** Includes DNA, RNA, and mono- and polynucleotides.
‡ Specifically, rock varnish.

SCORE CRITERIA

Biological or not?

I have assigned values of zero to three in this category based on the extent to which a given substance, property, or texture could be said to be the result of biological activity. Thus amino acids and polycyclics score zero since they can produced by wholly abiotic mechanisms at or near the martian surface. While mononucleotides and polynucleotides can be produced abiotically (e.g., Simakov *et al*. 1997), it is felt that the majority of such materials occurring naturally are likely to be found in association with living organisms, and so a score of two (mostly biological in origin) was assigned. However, the score for genetic molecules is reduced to one by the very strong possibility of contamination - in particular if PCR is employed.

The scores assigned to the various mineral biomarkers are probably the most open to debate. Clearly, biological agents do act as a mediator in the formation of many mineral

deposits, but distinguishing biological from abiological mechanisms requires a greater consideration of geological and petrographic context. Moreover, many of the features of these minerals which are least equivocal in making the distinction are of such small size that the analysis necessitates sample return (hence, the low scores in column three - see below).

Homochirality scores two since the balance of probability is that a large enantiomeric excess will be of biological origin, though this is militated against by a variety of symmetry breaking mechanisms and racemization reactions.

Microfossils score very poorly in light of the difficulty surrounding their use as a biomarker in several carbonaceous meteorites, and most notably in martian meteorite ALH84001. This score is lowered by the potential for false positive identification of instrumental artefacts, particularly in the field of electron microscopy.

Hopanoids have no known abiological source and so their occurrence may be taken as an unambiguously biogenic signature.

Preservability

Biomarkers are assigned a score relating to their preservation potential in the martian surface and subsurface environment from zero (destroyed in hundreds of years), through one (thousands of years), two (millions of years), and three (stable for billions of years).

The low temperatures and largely anhydrous conditions allow most organic molecules to survive for several billion years, certainly in the martian polar regions. However, the strongly oxidising surface environment indicates that they are likely to be preserved - if present at all - at depths of several metres in the regolith, or in permafrost. DNA is the least stable of the organic materials, but dramatic examples of preservation under certain isolated terrestrial conditions yields a score of two.

All of the mineral biomarkers are likely to be stable against destruction for the entire history of Mars. Manganese rock varnishes on Earth are known to be relatively fragile, but have been assigned a score of two here in view of the limited abrasive power of aeolian erosion on Mars relative to Earth (Golombek and Bridges 2000) and the limited potential for either chemical or biological degradation.

The score of two (million year preservation times) for homochirality is based on the work of Bada *et al*. (1995). Microfossils score three (billion year preservation times) on the strength of their occurrence in early Archaean terrestrial rocks and, if the notion is to be given any credence, in ancient meteoritic bodies.

Hopanoids have been preserved in terrestrial environments for up to 2.7 Ga and there is no reason to suspect that they could not survive for an equal period of time in the arguably less demanding martian geosphere.

Facility of analysis

Biomarkers were assigned a score in this category between zero and three depending on how difficult the biomarker would be to properly evaluate. A score of three indicates that a given biomarker could yield an unambiguous result (within the scope of its own criteria) by analysis - GCMS, XRD, XRF, optical microscopy, etc., - of surface soils or the atmosphere. A score of two is applied if more technologically ambitious means are

required; this might include drilling to depths of several metres, careful sectioning of rocks, or complex chemical separation techniques. A score of one employs the same criteria as the former category but includes the possibility that even the smallest amount of contamination could yield a false result. A score of zero is applied if a biomarker can only be properly evaluated in a terrestrial laboratory, or by suitably equipped humans on the surface of Mars.

Hence, most organic molecules (including hopanoids) receive a score of two since they are readily analysed if recovered from sufficient depth in the regolith by a drill core or from the interior of a surface boulder. DNA is the exception, scoring one based on the very strong possibility of PCR amplification of single-molecule DNA contamination.

All mineralogical biomarkers score zero since the features which might be used to unequivocally categorise them as biogenic are micron to nanometre scale, requiring the use of electron microscopy for study. Fossils likewise score zero since they require the use of electron microscopy for proper evaluation.

Homochirality is very easily studied but is subject to the same strictures as apply to organic molecules, in as much as samples would need to be extracted either from depth in the regolith or from the interiors of rocks. This biomarker thus receives a score of two.

APPENDIX II: EUROPA'S ATMOSPHERE

The issue of why Europa's lack of an atmosphere has a bearing on its biological character deserves a brief expansion since it is an original argument and is important to understanding why I believe that Titan is, and Mars is not, biologically active at present.

The idea is based on the work of Vondrak (1974) who calculated the impact of human contamination on the tenuous lunar atmosphere (each Apollo landing effectively doubled the mass of the lunar atmosphere). Vondrak (1974) constructed a very simple isothermal model which indicated that a mass flux - assuming pure oxygen - of ~100 kg s^{-1} would be sufficient to provide the Moon with a dense, long-lived atmosphere. Europa is very much similar in size to the Moon, though far colder, and so could sustain a dense atmosphere at a much lower mass flux. However, if we assume that the gas being emitted by organisms living in a subsurface ocean is methane (which has half the molecular mass of O_2), then 100 kg s^{-1} is perhaps a useful figure. Taking the lower limit of terrestrial biogenic methane output (itself only a tiny fraction of all biogenic gas emission) as 3.0×10^{14} g yr^{-1}, it is straightforward to show that 100 kg s^{-1} is ~1% of this figure. Outgassing efficiency must be relatively large since only a small proportion of the gas can go into solution, and the accumulation of gas pockets beneath an ice crust is gravitationally unstable. The observation of fissures and chaos regions in Europa's ice shell indicate that routes for potential gas escape are plentiful. That we clearly do not see a significant atmosphere about Europa, or any hint of past activity in the neutral gas or plasma composition of the Jovian magnetosphere has to rule out all but the most paltry ecology on Europa.

This is supported by the calculations of Jakosky and Shock (1998) who show that free-energy for life on Europa is likely to be negligible, and I fail to see that Chyba (2000), who favours the impact mixing of radiolytically altered ices into the ocean as an energy source, alters that conclusion.

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