PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B

Phil. Trans. R. Soc. B (2006) **361**, 951–968
doi:10.1098/rstb.2006.1837
Published online 17 May 2006

Steroids, triterpenoids and molecular oxygen

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There is a close connection between modern-day biosynthesis of particular triterpenoid biomarkers and presence of molecular oxygen in the environment. Thus, the detection of steroid and triterpenoid hydrocarbons far back in Earth history has been used to infer the antiquity of oxygenic photosynthesis. This prompts the question: were these compounds produced similarly in the past? In this paper, we address this question with a review of the current state of knowledge surrounding the oxygen requirement for steroid biosynthesis and phylogenetic patterns in the distribution of steroid and triterpenoid biosynthetic pathways.

The hopanoid and steroid biosynthetic pathways are very highly conserved within the bacterial and eukaryotic domains, respectively. Bacteriohopanepolyols are produced by a wide range of bacteria, and are methylated in significant abundance at the C2 position by oxygen-producing cyanobacteria. On the other hand, sterol biosynthesis is sparsely distributed in distantly related bacterial taxa and the pathways do not produce the wide range of products that characterize eukaryotes. In particular, evidence for sterol biosynthesis by cyanobacteria appears flawed. Our experiments show that cyanobacterial cultures are easily contaminated by sterol-producing rust fungi, which can be eliminated by treatment with cycloheximide affording sterol-free samples.

Sterols are ubiquitous features of eukaryotic membranes, and it appears likely that the initial steps in sterol biosynthesis were present in their modern form in the last common ancestor of eukaryotes. Eleven molecules of O_2 are required by four enzymes to produce one molecule of cholesterol. Thermodynamic arguments, optimization of function and parsimony all indicate that an ancestral anaerobic pathway is highly unlikely.

The known geological record of molecular fossils, especially steranes and triterpanes, is notable for the limited number of structural motifs that have been observed. With a few exceptions, the carbon skeletons are the same as those found in the lipids of extant organisms and no demonstrably extinct structures have been reported. Furthermore, their patterns of occurrence over billion year time-scales correlate strongly with environments of deposition. Accordingly, biomarkers are excellent indicators of environmental conditions even though the taxonomic affinities of all biomarkers cannot be precisely specified. Biomarkers are ultimately tied to biochemicals with very specific functional properties, and interpretations of the biomarker record will benefit from increased understanding of the biological roles of geologically durable molecules.

Keywords: Archaean; biomarker hydrocarbons; steroids; sterols; triterpenoids; hopanes aerobic biosynthesis

1. INTRODUCTION

(a) *The advent of oxygenic photosynthesis and an oxygen-rich atmosphere*

The compositional and evolutionary history of the atmosphere–ocean system may be reconstructed from the chemistry and habit of sedimentary minerals, basalt weathering profiles and secular change in stable isotopes, along with numerical modelling and theoretical considerations (Cloud 1972). According to the paradigm of Cloud, Holland and Walker, oxygen was a trace component of the early atmosphere and rose, within weakly constrained bounds, to within one-tenth of the present atmospheric level (PAL) by about 540 Myr ago (Ma) (Cloud 1972; Walker *et al.* 1983). It is further hypothesized that oxygen played a role in the deposition of Archaean and Palaeoproterozoic banded iron formations, and that the fluxes of reduced minerals and volcanic gases into the ocean and atmosphere acted as a buffer to keep atmospheric oxygen concentrations low for a protracted period. An apparently 'sudden' development of oxidized soil profiles about 2300 Ma, together with carbon, sulphur and iron isotopic indicators, suggest that the oxygen rise was not uniform but occurred in a stepwise manner (Holland 1984; Holland & Beukes 1990; Des Marais *et al.* 1992;

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One contribution of 14 to a Discussion Meeting Issue 'Major steps in cell evolution'.

Karhu & Holland 1996; Rasmussen & Buick 1999; Des Marais 2000; Farquhar *et al.* 2000*a*,*b*; Holland 2002; Rouxel *et al.* 2005). The 'step' which occurred just prior to 2300 Ma was to greater than 10^{-5} PAL and accompanied by dramatic environmental changes indicated by large excursions in the $\delta^{13}C$ content of marine carbonates, and possible 'snowball Earth' events (Kirschvink *et al.* 2000; Bekker *et al.* 2004). A second very significant rise in the Late Neoproterozoic also probably took place in stages punctuated by multiple ice ages with dramatic swings in carbon and sulphur isotopes indicating a radical reorganization of the biogeochemical cycles (Hoffman & Schrag 2002; Rothman *et al.* 2003; Halverson *et al.* 2005; Hurtgen *et al.* 2005).

(b) *Isotopic and molecular evidence pertaining to timing*

Carbon and sulphur isotopes, molecular biomarkers, and possibly iron isotopes suggest that the advent of oxygenic photosynthesis preceded the Early Palaeoproterozoic 'Great Oxidation Event' by 400 Myr or more. Certain 2-methylhopanoid biomarkers that we associate with modern-day oxygen-producing cyanobacteria and steroids that require oxygen-utilizing enzymes for their biosynthesis can be found in rocks as old as 2715 Myr. This is consistent with the notion that oxygen production from oxygenic photosynthesis is indeed an ancient process. The inventory of organic carbon preserved in Middle to Late Archaean black shales and carbonates, indicating prolific primary productivity in diverse palaeoenvironments, is another signal that would be consistent with an early appearance of oxygenic photosynthesis since electron donors other than water might have been limiting in supply and/or spatial distribution in an anaerobic surface ocean (Walker *et al.* 1983; Des Marais 2000; Rosing & Frei 2004). Molecular hydrogen, a feasible alternative electron donor produced by sub-sea basalt weathering, was likely abundant in the deep ocean, available for carbon fixation and possibly for phototrophic carbon fixation (Sleep *et al.* 2004; Tice & Lowe 2004; Hayes & Waldbauer 2006). Sulphur isotopic data indicate that sulphate reducing bacteria were active by 2.7 Gyr ago (Ga) which, indirectly, suggest that oxygenic photosynthesis was extant (Shen *et al.* 2001; Rouxel *et al.* 2005).

Analyses of hydrocarbons from Fortescue and Hamersley group sediments of the Pilbara Craton, western Australia (Brocks *et al.* 1999; Summons *et al.* 1999; Eigenbrode *et al.* 2001, 2004; Eigenbrode 2004) have uncovered many of the same kinds of carbon skeletons that are prevalent in Phanerozoic sediments and petroleum (Peters *et al.* 2004). Of particular interest are the steroid and triterpenoid carbon skeletons that are apparently derived from sterols and bacteriohopanepolyols (BHP), respectively. The presence of these types of hydrocarbons in Late Archaean sediments, which are considered by many researchers to be diagnostic for Eukarya and Bacteria, has been further used as an evidence for the antiquity of both of these domains (Brocks *et al.* 1999).

(c) *Hopanoid biomarkers*

In bacteria, the functional forms of hopanoids are amphiphilic BHP. These comprise a C_{30} pentacyclic triterpene hydrocarbon skeleton, derived from squalene via the enzyme squalene–hopene cyclase, and are linked via a C–C bond to a C_5 sugar moiety derived from ribose. The polar moieties of BHP may be augmented with sugars, amino acids or other functionalized units. These substituents on BHP evidently serve a functional role and also provide a chemical mechanism for their preservation in the geological record. They facilitate intermolecular condensation reactions, and cross-linking by reduced sulphur compounds, that result in incorporation into kerogen (Wakeham *et al.* 1995). The apolar ring system of hopanoids may be modified by unsaturation or by addition of an extra methyl group at either position 2 or position 3 located in the A-ring. Although oxygen is not required for hopanoid biosynthesis, the vast majority of known hopanoid producers are aerobic or microaerophilic bacteria (Rohmer *et al.* 1984). These include the cyanobacteria and α- and β-proteobacteria. Notable exceptions include *Geobacter* sp. (Fischer *et al.* 2005) although, logically, there may be many more obligate or facultative anaerobes which make BHP, given that surveys of bacterial taxa have been limited. Recent studies have provided molecular isotopic evidence for biosynthesis of hopanoids in anaerobic environments (Thiel *et al.* 1999, 2003; Pancost *et al.* 2000).

Cyanobacteria are presently the only known bacteria to synthesize abundant 2-methylhopanoids with an extended polyhydroxylated side chain (i.e. 2Me-BHP) (Rohmer *et al.* 1984; Summons *et al.* 1999). Labelling experiments demonstrate that the 2-methyl substituent originates from L-methionine, presumably via *S*-adenosylmethionine, with preservation of all three hydrogens and consistent with methylation of a Δ^2-hopanoid (Zundel & Rohmer 1985). Other details of the biosynthesis and the specific functions of 2Me-BHP remain to be elucidated. Cyanobacteria containing 2Me-BHP are distributed broadly throughout cyanobacterial phylogeny including *Gloeobacter violaceus* (a deeply branching cyanobacterium lacking thylakoids) and the N_2-fixing *Nostoc* spp. (a heterocystous filament-former). While marine cyanobacteria were poorly represented in the initial survey (Summons *et al.* 1999), more recent work does not suggest that 2Me-BHP are widely represented in those marine cyanobacteria that have been brought into culture.

(d) *Steroid biomarkers*

Sterols are derived from the same squalene precursor as hopanoids but, in marked contrast to BHP, are known to have an oxygen-dependent biosynthesis beginning with the formation of the first intermediate, 2,3-oxidosqualene. Enzymes involved in sterol biosynthesis are highly specific in their substrate requirements and the mechanism by which oxidosqualene cyclase (OSC) converts 2,3-oxidosqualene to either of two protosterols, lanosterol or cycloartenol, depends on precise control of multiple intermediates along the cyclization cascade (Abe *et al.* 1993; Wendt *et al.* 1997, 2000). The unravelling of the intimate details of these exquisitely

stereospecific reactions, based on more than 50 years concerted research since the process was postulated (Woodward & Bloch 1953), is considered one of the classic accomplishments of molecular science.

Sterols are characteristic of all Eukarya. They are not found in Archaea and the proven occurrences in Bacteria are sparsely distributed and yield a limited array of products. *Methylococcus capsulatus*, *Gemmata obscuriglobus* and some members of the myxobacteria are proven steroid-producing bacteria (Bird *et al.* 1971; Kohl *et al.* 1983; Bode *et al.* 2003; Pearson *et al.* 2003; Volkman 2003, 2005).

There are at least 11 original studies and numerous reviews citing sterol occurrence in cyanobacteria. Prominent among these, a crystalline mixture of sterols was isolated from a culture of *Phormidium luridum* and identified by gas chromatography–mass spectrometry (GC–MS) to contain $C_{29}\Delta7$, $C_{29}\Delta5$, $C_{29}\Delta7,22$, $C_{29}\Delta5,7,22$ and $C_{29}\Delta5,22$ with minor amounts of cholesterol (DeSousa & Nes 1968). When it was conducted, this work stood in marked contrast to earlier studies asserting the absence of sterols from cyanobacteria (Levin & Bloch 1964) while other, more recent studies have failed to detect them (Rohmer *et al.* 1979, 1984).

The reports of cyanobacterial sterols apply to a taxonomically diverse range of cultured organisms including *Spirulina platensis*, *Nostoc* sp. and *Calothrix* sp. with $C_{29}\Delta5$, $C_{29}\Delta7$, $C_{29}\Delta5,22$ (Paoletti *et al.* 1976), *Anabaena* sp. (x3), *Nodularia* sp. and *Nostoc* sp. with $C_{29}\Delta5$, $C_{29}\Delta7$, $C_{29}\Delta5,22$, $C_{29}\Delta5,7,22$, $C_{29:0}$ and cholesterol (Kohlhase & Pohl 1988), and *Anabaena hallensis* with $C_{29}\Delta5$, $C_{28}\Delta5$, $C_{29:0}$ (Hai *et al.* 1996). Lanosterol has been identified in *Chlorogloeopsis fritschii* (Sallal *et al.* 1987) and there are numerous reports of sterols in natural samples such as cyanobacterial mats.

Given the variety of organisms investigated, and apart from the *C. fritschii* results, there is a striking similarity in the sterols identified as well as their relative abundances. It has been noted that the strong predominance of unsaturated C_{29} sterols—phytosterols—is similar to that found in green algae and vascular plants. Moreover, of the more than a dozen cyanobacterial genomes completed to date (ranging from *Gloeobacter* to *Nostoc*) none contains genes encoding sterol synthesis enzymes.

(e) *Alternative views about oxygen and biomarkers*

An alternate hypothesis concerning the history of environmental oxidation argues for a relatively late (just prior to 2.3 Ga) evolution of cyanobacteria whose oxygen-producing capability destroyed a methane greenhouse thereby directly triggering the 2.3–2.2 Ga Makganeyene glaciation. It was recently proposed (Kopp *et al.* 2005) that all the 'sudden' indicators of environmental oxidation, such as red bed appearance and the large attenuation in mass-independent sulphur isotope fractionation around this time period, record the inception of oxygenic photosynthesis, and that there was essentially no time lag between the origin of organisms capable of oxygenic photosynthesis and their rise to ecological dominance and impact on global geochemistry.

(f) *The present study*

The validity of biomarker methodologies for drawing inferences about biota and ocean redox in the Archaean rests upon a number of foundations.

(i) The fossilized lipids, hydrocarbons, reported in Archaean rocks must actually be 'Archaean' in age and indigenous to the samples in which they were found.
(ii) Membrane lipid compositions and biosynthetic pathways must be features of cell biology that are conserved over evolutionary time, such that information about past life can be drawn from knowledge of modern organisms.
(iii) The distribution of lipid biosynthetic pathways should be phylogenetically informative, such that the presence of particular molecules in the rock record provides information about contemporaneous biodiversity.

These premises all deserve critical appraisal. The issue of syngeneity has been addressed by Brocks *et al.* (2003) who concluded that Archaean biomarkers were 'probably syngenetic' with the host rocks. However, they could not totally exclude bitumen migration from younger sediments and, thus, additional research to address this point using freshly drilled and carefully curated drill cores is underway (Buick *et al.* 2004; Summons *et al.* 2004).

To address (ii) and (iii) above, we draw on the inventory of protein sequences of key biosynthetic enzymes for fresh insights about the biosynthetic oxygen requirement for sterol synthesis and the status of sterols as markers that are specific for Eukarya. We also investigated the sterol contents of some cyanobacteria purported to contain them, including their capacity for biosynthesis. Lastly, we review studies of the biosynthesis of 2-methylhopanoids and the phylogenetic distribution that pertains to oxygen availability.

2. MATERIAL AND METHODS

(a) *Protein sequence and structure analysis*

Protein sequences and structures of sterol synthesis enzymes in various organisms were obtained from databases using the basic local alignment search tool (Altschul *et al.* 1997). Except as noted below, sequence and structure data were retrieved from databases searched through the National Center for Biotechnology Information and Joint Genome Institute Web servers. OSC sequences for *Acanthamoeba castellanii* and *Hartmannella vermiformis* were retrieved from the Protist EST Programme database (http://amoebidia.bcm.umontreal.ca/public/pepdb/welcome.php); for *Cyanidioschyzon merolae* from the *C. merolae* Genome Project (http://merolae.biol.s.u-tokyo.ac.jp/) and for *G. obscuriglobus* from the Institute for Genomic Research (http://www.tigr.org/tdb/ufmg/index.shtml). Sequences were aligned using CLUSTALX and alignments manually edited in GeneDoc, and protein crystal structures were visualized with Cn3D and RasMol.

(b) *Cyanobacterial lipid analysis*

(i) *Culture conditions*

Cyanobacteria were grown within an illuminated incubator (12–12 light–dark cycle) with 300 ml of BG-11 or D-media with addition of filter-sterilized cycloheximide (100 mg l^{-1}).

Anabaena cylindrica (ATCC 27899), *P. luridum* (UTEX 426), *Fischerella* sp. (ATCC 29538), *C. fristschii* (ATCC 27193), *Gloeobacter* sp. TS and *Gloeocapsa* sp. were grown at 30 °C, and the Yellowstone isolates *Phormidium* sp. RC and OSS4 at 35 °C. Cultures were harvested by centrifugation using Corex centrifuge bottles, which were solvent-rinsed with methylene chloride and methanol prior to use. Cell pellets were frozen, lyophilized and stored at −20 °C.

(ii) *Lipid analysis*

Total lipid was prepared from the stored cultures by a modified Bligh–Dyer extraction (Jahnke 1992) of lyophilized biomass. A 300 ml aliquot of BG-11 and medium D were processed in parallel as control blanks. After addition of an internal standard (epiandrosterone or cholesterol-D4), small aliquots (*ca* 0.1 mg) of the total lipid extracts were hydrolysed in 1 ml of 0.1 N HCl : methanol (1 : 1) at 60 °C for 2 h. After removal of solvent, and azeotropic drying with dichloromethane, the products were derivatized with 25 µl each of bis(trimethylsilyl)trifluoroacetamide and pyridine with heating at 70 °C for 30 min and analysed by GC–MS. Sterols were identified by comparison with literature spectra and the spectra of authentic compounds.

In a corollary set of analyses, lyophilized biomass from previous experimental protocols, which had been stored at 4 °C, were extracted in a similar manner. PCR amplification of DNA from *P. luridum*, *C. fristschii*, *Phormidium* RC and *Phormidium* OSS4 using a fungal primer (ITS-4B) specific to Basidomycetes showed positive bands. Laboratory maintained stock cultures of these same cyanobacteria were negative using this primer set (C. Raleigh & K. Cullings 2000, personal communication).

3. RESULTS AND DISCUSSION

We first discuss the sterol biosynthetic pathway, with particular emphasis on oxygen utilization and molecular evolution of the synthesis enzymes. Three phases of sterol biosynthesis are explored: the epoxidation of squalene, the cyclization of oxidosqualene to protosterols and modification of the sterol skeleton, principally by oxidative demethylation (figure 1). Second, we present evidence from lipid analyses of several cyanobacteria that previous reports of sterol synthesis by these organisms may have been compromised by contamination.

(a) *Squalene monooxygenase*

(i) *Mechanism and oxygen requirement*

The epoxidation of squalene is the first oxygen-dependent step in the sterol pathway, and the point at which the synthesis of steroids diverges from that of hopanoids. Early work showed that sterol biosynthesis in yeast does not occur in fermentative cells and only initiates at micromolar levels of O_2 (Jahnke & Klein 1983). The stereospecific conversion of squalene to (3*S*)-2,3-oxidosqualene is catalysed by the enzyme squalene monooxygenase (SQMO; also known as squalene epoxidase) (figure 2). SQMO is a flavoprotein that requires O_2 and flavine-adenine dinucleotide (FAD) to effect oxygenation, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)–cytochrome P450 reductase (itself a flavoprotein) to regenerate FAD and a soluble protein factor for squalene transport (Lee *et al.* 2004). Epoxidation proceeds by the reaction of oxygen with the bound dihydroflavin ($Fl_{red}H_2$) to give a 4a-hydroperoxyflavin (FlH(4a)-OOH). The oxygen transfer occurs by nucleophilic attack by the 2,3 double bond of squalene on the terminal oxygen of the 4a-hydroperoxide, yielding oxidized flavin (Fl_{ox}) and 2,3-oxidosqualene (Bruice *et al.* 1983; Torres & Bruice 1999). The FAD is regenerated by NADPH–cytochrome P450 reductase (Laden *et al.* 2000).

This reaction depends on the electrophilic character of the hydroxy group of the hydroperoxide, since the attack comes from the 2,3-olefin of squalene. The epoxidation also benefits from the relatively weak O–O bond in the hydroperoxide (47 kcal mol^{-1}; Blanksby & Ellison 2003), making the oxygen transfer energetically feasible. It has been suggested (Raymond & Blankenship 2004) that the squalene epoxide oxygen might be derived from a source besides O_2, such as water. Such a scheme would presumably proceed through hydroxylation of a cofactor followed by squalene epoxidation using the water-derived hydroxyl. This scheme is excluded on two grounds: first, such a hydroxyl would itself have a nucleophilic character, preventing attack by the squalene olefin; second, the C–O bond of the hydroxyl is much stronger (96 kcal mol^{-1}; Blanksby & Ellison 2003), providing a much higher energy barrier to oxygen transfer. Furthermore, the next enzyme in the pathway, OSC, requires the 3*S* (and rejects the 3*R*) form of 2,3-oxidosqualene, and hydroperoxide epoxidation affords the required stereoselectivity. In this chemical context, an O_2-independent route to oxidosqualene is highly disfavoured.

(ii) *Evolutionary conservation*

SQMO contains several motifs responsible for substrate and cofactor binding that are conserved in all known sequences of the protein, including those of prokaryotes (Lee *et al.* 2000, 2004; Pearson *et al.* 2003). All known SQMOs (and data are available for animals, fungi, plants, amoeboid and kinetoplastid protists, and bacteria) use the epoxidation mechanism described above. But might there be alternative, chemically and evolutionarily unrelated methods to produce oxidosqualene?

There are enzymes that catalyse the epoxidation of olefins without FAD. These are cytochrome P450 oxygenases that use Fe and O_2 and transfer oxygen to a variety of unsaturated substrates, such as arachidonate during eicosanoid biosynthesis. As discussed below with regard to sterol demethylases (which are P450 cytochromes), the O_2 requirement of such enzymes is at least as stringent as that of the flavoproteins. It is noteworthy that no organism is known to have replaced SQMO with a cytochrome P450, though there is no

Figure 1. (*Opposite*.) Generalized synthetic pathway of sterols. Sterol precursor squalene is oxidatively converted to oxidosqualene, which is cyclased to one of two protosterols: cycloartenol or lanosterol. The protosterol undergoes subsequent modifications including oxidative demethylations and desaturations to result in the terminal sterol product. Enzymes are labelled with EC number where available, or gene abbreviation. Terminal sterols yield derived steranes after burial and diagenesis. Enzymes labelled in bold are discussed in the text. Those requiring molecular oxygen are noted.

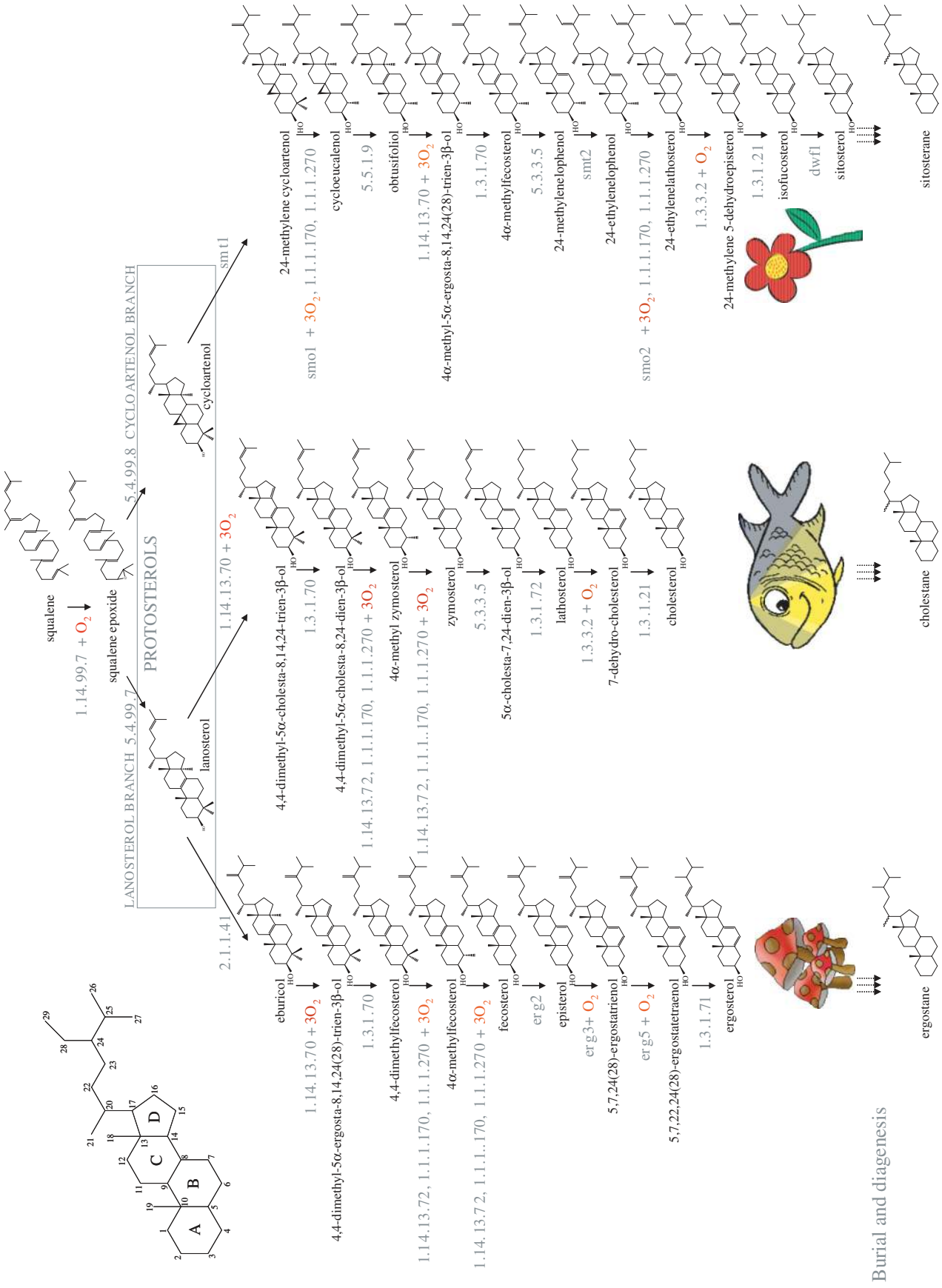

Figure 1. (*Caption opposite.*)

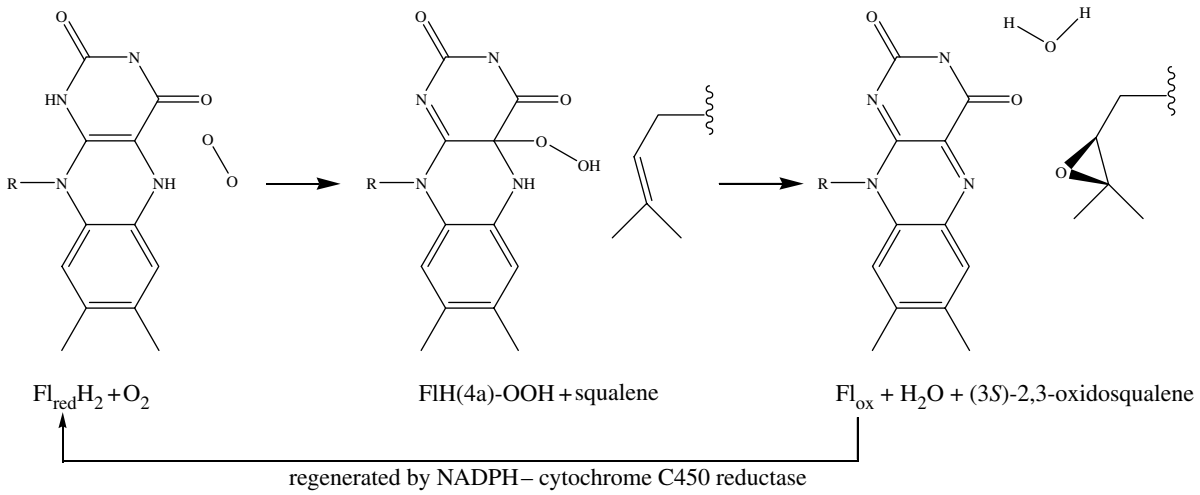

Figure 2. Mechanism for the epoxidation of squalene by squalene monooxygenase (SQMO).

recognized chemical or enzymological prohibition against this. In fact, a secondary squalene epoxidase activity for CYP17, a P450 cytochrome whose primary role is the hydroxylation of pregnenolone and progesterone in corticosteroid hormone synthesis in vertebrates, has recently been demonstrated in mouse tumour cells (Liu *et al.* 2005). Were lipid biochemistry sufficiently plastic, one might expect a gene replacement to have taken place, wherein the flavoprotein SQMO would be supplanted by a P450 cytochrome with multiple activities, thereby reducing the number of enzymes required to constitute the complete pathway. In fact, such gene replacement is not observed. The universal retention of the FAD-dependent oxygenase even when other (though equally O_2-requiring) mechanisms are available is an example of the evolutionary conservatism of biosynthetic pathways.

(b) *Oxidosqualene cyclase*

(i) *Structure and function of oxidosqualene cyclases*

The tetracyclic core that characterizes all sterols is created through the cyclization of squalene (3*S*)-2,3-epoxide by the enzyme oxidosqualene cyclase (OSC). This is the second step in sterol biosynthesis after the epoxidation of squalene, and one of the most complex biochemical reactions catalysed by a single enzyme. The cyclization is executed with a remarkable degree of specificity and stereochemical control. The products are either lanosterol or cycloartenol (figure 1), the two 'protosterols' that are subsequently modified to functional products such as cholesterol or phytosterols.

Several decades of work have elucidated much of the mechanism of cyclization by OSC and identified specific residues responsible for particular steps in the cyclization cascade. The detailed chemistry of this enzyme was recently reviewed (Wendt *et al.* 2000; Wendt 2005) and the crystal structure of human OSC determined at 2.1 Å resolution (Thoma *et al.* 2004) allowing visualization of the enzyme and interactions with the substrate in unprecedented detail. Figure 3 shows two views of the active site of human OSC with its product, lanosterol. Briefly, the key steps in the cyclization (and residues responsible) include:

(i) Positioning of squalene 2,3-epoxide in a prefolded configuration within the active site (Y98, Y704).
(ii) Protonation of the epoxide group by the catalytic acid (D455, C456, C533).
(iii) Ring formation, during which cation intermediates are stabilized by cation–π interactions with aromatic residues of the active site (W387, F444, W581, F696) and the cation migrates out to C20.
(iv) Skeletal rearrangement via hydride- and methyl-shifts (largely spontaneous) as the proton migrates back to a region of high π-electron density around the B/C rings.
(v) Deprotonation by basic residues to quench the final protosteryl cation (Y503, H232)—the position of the deprotonation determines the OSC product (lanosterol versus cycloartenol). D455 is ultimately reprotonated by E459.

The sequences of genes encoding OSCs are now available for many organisms, principally animals, plants and fungi, but also several protists. Alignment of these sequences shows very high degree of conservation across the breadth of eukaryotic diversity (at least five of the kingdom-level divisions of Adl *et al.* (2005)). The active-site residues mentioned above are absolutely conserved, i.e. 100% identity at the amino acid level. Alternative mechanisms for oxidosqualene cyclization do not appear to have arisen in any of the eukaryotic lineages sampled to date. Together with the conservation of function seen in the squalene epoxidases, this strongly suggests that, at least, the initial steps in sterol biosynthesis were present, generally in their modern form, in the last common ancestor of all eukaryotes.

(ii) *Oxidosqualene cyclase product profiles and eukaryote phylogeny*

There are two main types of OSCs, based on the end product of the cyclization: lanosterol synthases and cycloartenol synthases. The cyclization process in the two types of enzymes is identical until the final deprotonation step. A deprotonation from C9 forms

the 8,9-double bond of lanosterol whereas a deprotonation from C19 allows the cycloartenol cyclopropyl ring to close. Thus far, lanosterol synthase has been found only among the opisthokonts (animals+fungi+choanozoa), trypanosomatids (*Trypanosoma*, *Leishmania*) and dinoflagellates (Giner *et al.* 1991). All other eukaryotes that have been examined in this regard (at least members of the higher plants, green and red algae, amoebozoa, diatoms, euglenids and heterolobosea) make cycloartenol as their protosterol.

Site-directed mutagenesis experiments, notably those of Matsuda and co-workers (Meyer *et al.* 2000, 2002; Joubert *et al.* 2001; Segura *et al.* 2002; Lodeiro *et al.* 2004) have indicated the key residues that control the product profile of OSCs, including T381, C449 and V453. From analysis of the crystal structure, each appears to affect the position of the catalytic base dyad H232–Y503. By controlling the position from which the protosterol cation is deprotonated, these residues determine which product will be formed by the cyclase. The second-sphere residue C449 is particularly interesting: the H449N mutant of *Arabidopsis thaliana* OSC is the most efficient lanosterol synthase to be generated from a wild-type (WT) cycloartenol synthase (88% lanosterol yield). Given its distance from the substrate (approx. 7.4 Å), its control of the product profile is likely indirect. Interestingly, mutagenesis experiments have yet to induce cycloartenol formation from a WT lanosterol synthase. Natural OSCs generally conform to the residue–product relations found in mutagenesis experiments (figure 3*a*), with exceptions that may be evolutionarily informative. From the protein alignment, patterns in differential conservation of these product-controlling residues can be discerned: opisthokont lanosterol synthases are T381/C,Q449/V453, while cycloartenol synthases are all Y381/H449/I453. Two exceptions to this pattern emerge: the lanosterol-producing OSCs of the bacteria *M. capsulatus* and *G. obscuriglobus* (discussed further below) and the trypanosomatid lanosterol synthases.

The trypanosomatids make lanosterol as their protosterol, despite the presence of a tyrosine at position 381. Several lines of evidence indicate that the trypanosomatids ancestrally possessed a cycloartenol synthase. First, the T381Y mutation has been shown to decrease the efficiency of the *Saccharomyces cerevisiae* lanosterol synthase, and induce the formation of parkeol and lanostene-3,9-diol (and not cycloartenol) as secondary products. The fixation of a T381Y mutation in a lanosterol synthase is thus unlikely. Second, euglenids *Euglena gracilis* (Anding *et al.* 1971) and *Astasia longa* (Rohmer & Brandt 1973) and heterolobosea (*Naegleria* sp.; Raederstorff & Rohmer 1987), more deeply branching than the trypanosomatids within the Excavate kingdom (Simpson *et al.* 2005), have been shown to make sterols via the cycloartenol pathway. Third, post-cyclization modification of sterols in the kinetoplastids follows a cycloartenol-type route; the 14α-demethylase of *Trypanosoma brucei* has recently been shown to be specific for the cycloartenol pathway intermediate obtusifoliol (Lepesheva *et al.* 2004). Taken together, this evidence suggests the following hypotheses: the trypanosomatids began with a cycloartenol synthase (Y381/H449/I453) which underwent two mutations, H449Q and I453V (each requiring a single nucleotide change) to yield a lanosterol synthase. Downstream modification of the protosterol remained essentially as the cycloartenol pathway, but at least some of the enzymes must have adapted to different substrates; in particular smt1 and smo1 (figure 1). It is worth noting that the Y381T/H449Q/I453V triple mutant of *A. thaliana* OSC is a reasonably efficient lanosterol producer; second-sphere mutations may have further enhanced the specificity of the trypanosomatid OSC.

Lanosterol synthesis appears to have arisen at least twice among the eukaryotes: once in an ancestor of the opisthokonts and once in an ancestor of the trypanosomatids after the divergence of the euglenids. Dinoflagellates have also been reported to make lanosterol (Giner *et al.* 1991), but no sequence information is currently available for their cyclases; they may represent a third instance of innovation, or may have acquired a lanosterol synthase laterally. It is as yet unclear which type, lanosterol synthase or cycloartenol synthase, was the ancestral form of OSC. Given the hypothesized independent originations of lanosterol synthase and the apparent difficulty in mutating a WT lanosterol synthase to produce cycloartenol, it is tempting to infer cycloartenol synthase as the more ancient of the two forms. If, on the other hand, eukaryotic phylogeny is rooted in the branch leading to the opisthokonts (Arisue *et al.* 2005) there is no strong parsimony argument either way.

(iii) *Prokaryotic oxidosqualene cyclases*

While sterol synthesis is nearly ubiquitous among the Eukarya, only three instances are known among prokaryotes, all bacteria: *M. capsulatus* (a γ-proteobacterium), *G. obscuriglobus* (a planctomycete) and a paraphyletic group of myxobacteria (δ-proteobacteria). These organisms are not closely related and the reason for the sparse appearance of sterol synthesis in phylogenetically distant bacterial taxa remains unclear. If it is the result of vertical inheritance from a common, sterol-synthesizing bacterial ancestor, dozens of parallel losses of the entire pathway in many lineages would be required. Alternatively, sterol biosynthesis genes may have been acquired by bacteria via lateral transfer from eukaryotes, potentially followed by one or more intra-bacterial transfer events. Such a eukaryote-to-bacteria gene transfer has been proposed to account for the similarly sparse occurrence of glutaminyl-tRNA synthetase among the Bacteria (Lamour *et al.* 1994; Brown & Doolittle 1999). At present, however, there is not sufficient evidence to draw clear conclusions concerning the evolutionary relationships between eukaryotic and prokaryotic OSCs.

Of bacterial groups, sterol synthesis is most widely distributed among the myxobacteria. Of 11 genera (88 total strains) tested by Bode *et al.* (2003) only four were found to produce sterols. The sterol-producing genera do not form a monophyletic group in the myxobacterial phylogeny of Shimkets & Woese (1992), implicating some combination of multiple gains, multiple losses and transfer events to explain the distribution of this capability. The most complete bacterial sterol synthesis pathway is in the myxobacterium *Nannocystis excedens*,

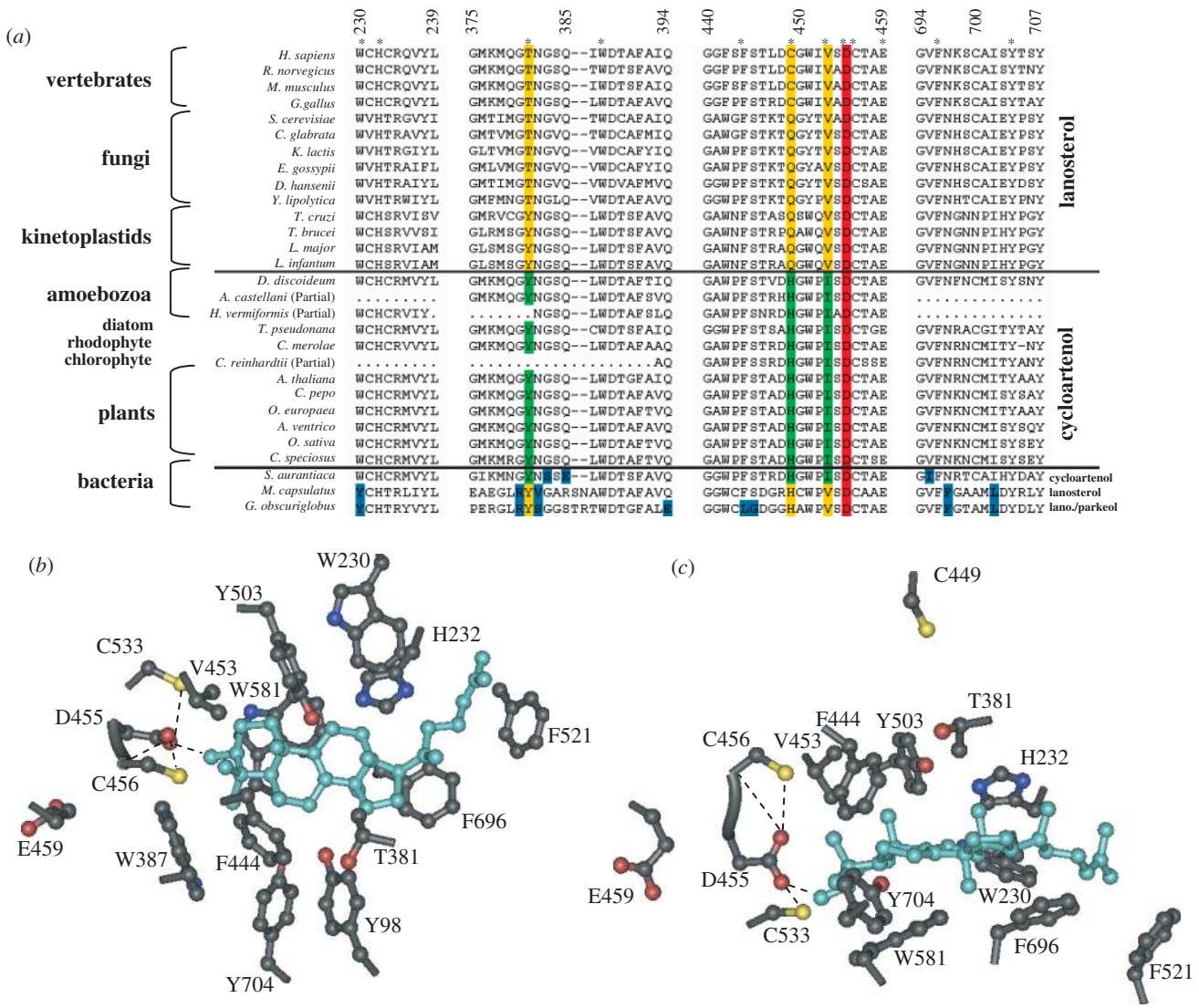

Figure 3. (*a*) Alignment of oxidosqualene cyclase (OSC) protein sequences. The catalytic acid, D455, is highlighted in red. Positions 381, 449 and 453 are differentially conserved between lanosterol synthases (yellow) and cycloartenol synthases (green). Highly conserved residues that are substituted in bacterial OSCs are highlighted in blue. Residues that are shown in the structures in (*b*) and (*c*) are indicated by asterisks. Numbering (throughout this figure and in text) refers to *Homo sapiens* OSC. (*b*) View of the active site of *H. sapiens* OSC with lanosterol shown in light blue. The hydrogen-bonding network around the catalytic acid D455 is indicated by dashed lines. H232 and Y503 constitute the catalytic base dyad and effect the final deprotonation. (*c*) View of the active site in the molecular plane of lanosterol. Note the distance of second-sphere residue C449 from the substrate. (*b*) and (*c*) are drawn from the crystal structure determined by Thoma *et al.* (2004).

which can demethylate lanosterol at C4 and C14 and progress as far as lathosterol (figure 1). Interestingly, the myxobacteria cyclize oxidosqualene to both lanosterol and cycloartenol; *Cystobacter* sp. produce both protosterols (Bode *et al.* 2003). If OSC was laterally acquired by the myxobacteria, it is unclear from the phylogenetic distribution alone which type they initially got from eukaryotes.

Three bacterial OSC sequences are presently available: the lanosterol synthase of *M. capsulatus*, the OSC of *G. obscuriglobus*, which produces a mixture of lanosterol and parkeol and the cycloartenol synthase of the myxobacterium *Stigmatella aurantiaca*. Overall, these enzymes are quite similar to the eukaryotic cyclases and make use of the same catalytic groups to effect the same chemistry. Of the three, the *Stigmatella* OSC is most like eukaryotic enzymes (figure 3). It shows the standard Y381/H449/I453 pattern of eukaryotic cycloartenol synthases, and fewer differences among highly conserved residues than the other two known sequences. The *M. capsulatus* and *G. obscuriglobus* OSCs are another exception to the pattern of differential conservation of residues 381, 449 and 453. These cyclases are Y381/H449/V453, making 453 the only position to be consistently differentially conserved between WT lanosterol and cycloartenol synthases. Both the *M. capsulatus* and *G. obscuriglobus* OSCs do, however, have modifications to other active-site residues (not yet explored in mutagenesis experiments) that may contribute to their unusual product profiles. In particular, the W230Y, G380R and N382 (V,S) substitutions (residues otherwise conserved across the Eukarya and in *Stigmatella*) could all affect the configuration of the active site near the deprotonating base, altering the enzyme product. Further, *Gemmata* alone has F444L and S445G substitutions; these are residues positioned close to T381 and C449 and may contribute to the *Gemmata* OSC's high yield

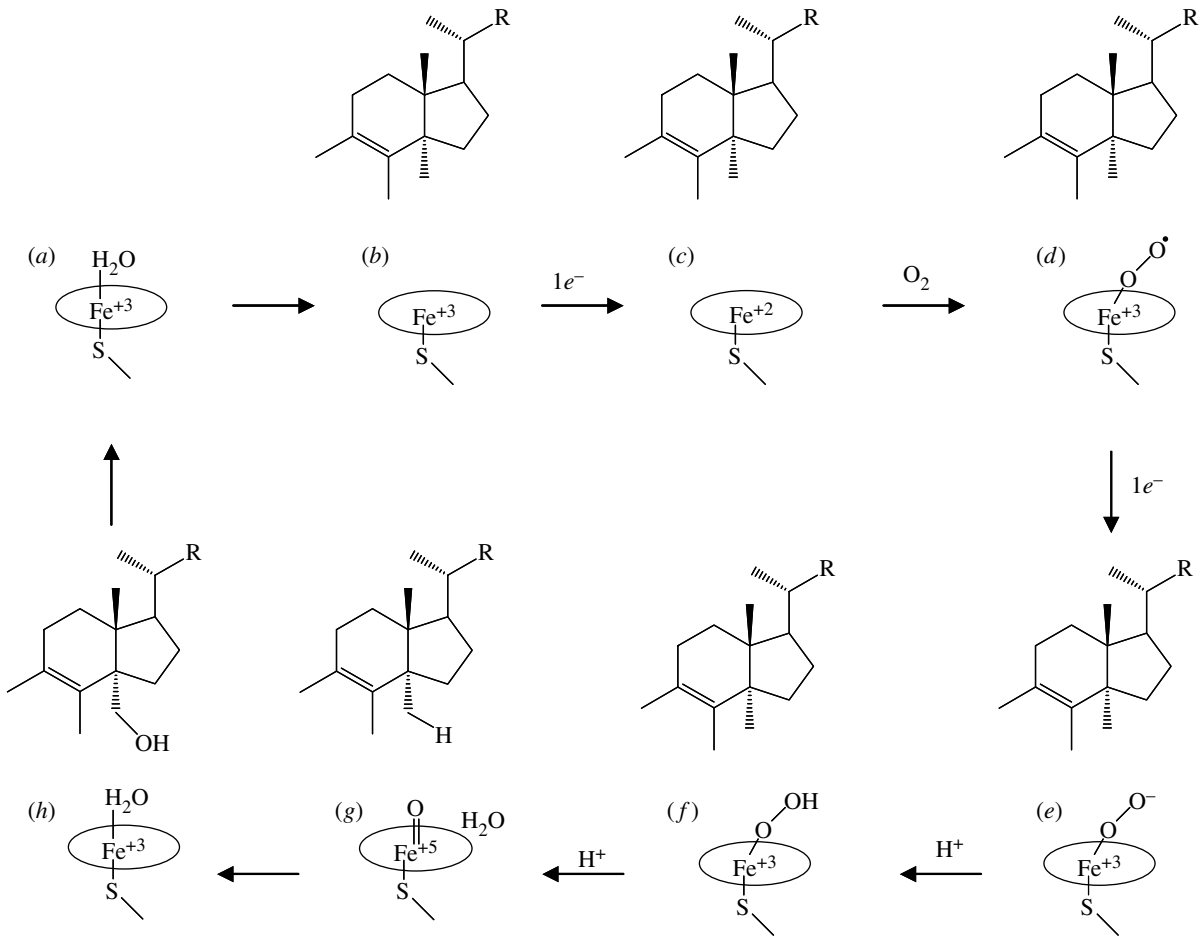

Figure 4. Steps in the oxidation of the 14α angular methyl group of sterols by the CYP51 active site and molecular oxygen. The methyl group is oxidized three times by the cycle (*a–h*), each using one molecule of O_2. Note that the Fe (IV) + Por+ complex is written Fe(V) for simplicity. After the third oxidation the methyl group is removed.

of parkeol, a protosterol otherwise very minor among WT cyclases.

(iv) *Oxygen requirements of cyclization*

The cyclization of oxidosqualene to the sterol hydrocarbon skeleton is not oxidative and does not require an external electron acceptor. OSC is, however, highly specific for (3*S*)-2,3-oxidosqualene; neither (3*R*)-2,3-oxidosqualene, squalene epoxidized at other positions, nor unoxygenated squalene are suitable substrates. OSC has likely always acted on oxidosqualene, so the ability to epoxidize squalene was a prerequisite for production of the 6,6,6,5-ring structure. Indeed, the catalytic acid group of OSC (the conserved DCxxE motif) may not be acidic enough to protonate the olefin of squalene, but can manage the epoxide. That the sterol cyclization chemistry was present in its modern (oxidosqualene-dependent) form in the eukaryotic last common ancestor is supporting evidence that early eukaryotic life had the means to oxygenate squalene.

(c) *Sterol demethylases*

According to Bloch's (1987) postulate, the sequential departure of the three nuclear methyl groups from the protosterol lanosterol, in the order 14α-methyl, 4α-methyl, and 4β-methyl, leads to an improvement in the fitness of the molecule, reaching a maximum with cholesterol. While it is now known that the order of removal of the methyl groups does vary in plants, compared to the above order in fungi and animals, there are some lines of experimental evidence that functional fitness improves (Bloch 1983).

Demethylation at the 14α carbon is catalysed by an oxidative demethylase of the cytochrome P450 superfamily in plants, animals and fungi, and a few bacteria. Oxidation of each of the two C-4 methyl groups is carried out by an unrelated enzyme of the sterol desaturase family. The functions of these enzymes are essential for eukaryotes, perhaps because demethylation of α face of sterols is required to allow proper sterol–fatty acid interaction to achieve the maximum membrane microviscosities (Dahl *et al.* 1980). In animals, removal of the 14α-methyl group is the first step in the sterol synthetic pathway following cyclization, and the CYP51 substrate is lanosterol (figure 1). In filamentous fungi, lanosterol is methylated at C-24 before being demethylated at C-14 and the CYP51 substrate is eburicol (24-methylene-24,25-dihydrolanosterol). Several steps occur along the cycloartenol pathway in plants before 14α-demethylation, including one demethylation at C-4 and the opening of the cyclopropyl ring so that plant CYP51 operates on obtusifoliol.

(i) *C-14 demethylases*

The removal of the 14α-methyl group of sterols (figure 4) is performed by sterol 14α-demethylase (CYP51). CYP51 belongs to the cytochrome P450 enzyme

superfamily, which includes more than 2000 members in all three domains of life. All P450s are used in oxidative reactions on various molecules and all require molecular oxygen as a substrate. The active site of P450s contains a Fe-protoporphyrin IX (haem). CYP51 is the only cytochrome P450 that performs the same function in different biological domains. Some researchers have suggested that CYP51 is the ancestral P450 (Nelson & Strobel 1989; Aoyama *et al.* 1994; Rezen *et al.* 2004). CYP51 operates on one of four substrates—lanosterol (in animals and fungi), dihydrolanosterol (animals), obtusifoliol (plants and kinetoplastids), or eburicol (fungi). These substrates differ only in the nature of the side chain and the presence or absence of a second methyl group at C-4.

The small differences between substrates probably account for a general lack of substrate specificity among the demethylases. Most CYP51s can demethylate any of the four substrates, although a few plant CYP51s are obtusifoliol-specific. In humans, a defect in CYP51 causes Antley–Bixler syndrome. In yeast, 14α-demethylase inhibition is fatal, and this makes the enzyme an attractive target for fungicides. Fungicides may take advantage of the differences between fungal and animal CYP51 active sites to selectively inhibit the fungal enzyme.

Rezen *et al.* (2004) found that CYP51s separate phylogenetically into plant (obtusifoliol), animal (lanosterol) and fungal (eburicol) groups. Bacterial CYP51s including those of *M. capsulatus* and *Mycobacterium tuberculosis* both fall within the plant lineage. The purified *M. tuberculosis* protein will demethylate lanosterol *in vitro* although obtusifoliol is preferred. However, despite the phylogenetic placement of its CYP51, *M. capsulatus* synthesizes lanosterol. It has yet to be demonstrated whether lanosterol is the substrate for *M. capsulatus* CYP51. Jackson *et al.* (2002) note that the *M. capsulatus* CYP51 is novel in that it is linked to a ferredoxin domain, but that the CYP51 in *M. tuberculosis* is part of an operon in which it is followed by ferredoxin. The *M. capsulatus* gene may be the result of a lateral transfer event from *M. tuberculosis* followed by a mutation. Perhaps the best example of the broad substrate specificity of these enzymes is *Streptomyces coelicolor*, which contains a gene with low-level homology to bacterial CYP51s and that demethylates eburicol but not lanosterol (Lamb *et al.* 2003). However, this gene is not a CYP51, and the conserved sites differ significantly from CYP51. *Streptomyces coelicolor* does not contain sterols, and the *in vivo* function of this protein is unknown.

The particulars of amino acid residue participation in substrate binding and catalysis of CYP51 are unknown. Podust and co-workers (Podust *et al.* 2001*b*) noted that two channels with access to the haem may serve to transport substrate and product to and from the active site. They (Podust *et al.* 2001*a*) also performed modelling experiments based on the crystal structure of *M. tuberculosis* CYP51 in the presence of the azole inhibitors fluconazole and 4-phenylimidazole. They found that the inhibitors coordinate themselves with the haem iron in the large (2600 $Å^3$) cavity opposite the cysteine (C394) that binds the haem to the protein. Amino acids surrounding this cavity were considered likely to be involved in binding and/or catalysis and showed a high degree of conservation across CYP51s from many organisms. Only approximately 10% (41 conserved residues) of CYP51s are absolutely conserved across all domains of life (with the exception of five residues in the kinetoplastids) and most of these are not at the active site. Half of these sites have been examined by site-directed mutagenesis experiments, which have shown that most of them are essential for CYP51 function. Naturally occurring azole-resistant strains of *Candida albicans* contain the mutation Y132H, which does not directly interact with substrate and may reflect a more complicated interaction between protein and substrate, and Bellamine *et al.* (2004) report from several site-directed mutagenesis experiments that azole and substrate binding are uncoupled. Among the residues identified by Podust *et al.* (2001*a*,*b*) as participating in the active site, there are several positions which differ between CYP51 subfamilies operating on different substrates.

Other site-directed mutagenesis studies on 10 residues that were believed to be strictly conserved among CYP51s (Lepesheva & Waterman 2004) found that most mutants lost all demethylase activity, although one (A197G) showed enhanced demethylase activity. Based on a combination of mutagenesis experiments and the crystal structure of CYP51 they postulated potential substrate binding sites: D90 as a binding site for the sterol 3β-OH, L172 and R194 associating with the side chain, and F82 binding the sterol A or B ring. Kinetoplastid genomes revealed differences from the 'strictly conserved' at five of the CYP51 residues in which mutations produced total or near total loss of function in *M. tuberculosis* (D90A, L127M, G175S/C, R194C, D195H/R). Lepesheva *et al.* (2004) showed that the kinetoplastid CYP51s were obtusifoliol-specific and that mutation of these five residues back to the conserved state did not improve the ability of the kinetoplastid enzyme to metabolize lanosterol. A clearer understanding of the chemical role of these conserved residues will be necessary to discern the evolutionary implications of these changes.

The general mechanism of substrate oxidation by P450 enzymes is known from several decades of work on the camphor-oxidizing P450 of *Pseudomonas putida*. It consists of eight steps (Groves & Han 1995; Meunier *et al.* 2004):

(i) substrate binding and subsequent displacement of the haem Fe(III);
(ii) electron transfer to Fe(III) from a reductase cofactor to yield Fe(II) and a haem with a negative charge;
(iii) binding of molecular oxygen to the ferrous iron yielding a Fe(III)–dioxygen bond;
(iv) transfer of a second electron to the haem to yield a negatively charged Fe(III)–peroxo complex which is a very strong nucleophile;
(v) protonation of the Fe(III)–peroxo complex yielding a P450–Fe(III)–OOH which also behaves as a nucleophile. This protonation likely involves the action of an acidic residue (D251 in P450cam) near the active site;

(vi) a second protonation of the distal oxygen atom and cleavage of the O–O bond, yielding a molecule of water and the formation of the reactive electrophilic iron–oxo species Fe(IV);
(vii) transfer of the oxygen atom from the iron–oxo complex to the substrate;
(viii) dissociation of the product.

In CYP51, this process operates three times on the 14α-methyl group of the sterol which successively converts the 14α-methyl group to 14α-hydroxymethyl, 14α-carboaldehyde and 14α-formyl intermediates, subsequently eliminating formic acid and leaving a Δ14,15 double bond in the sterol. It is significant that the initial hydroxylation of the methyl, which requires the abstraction of $H^{\cdot}$, is achieved with the high redox potential associated with the Fe(IV) complex, which is achieved only through the action of molecular oxygen. This complex has an effective oxidation state of Fe(V) due to the additional charge on the porphyrin. See Meunier *et al.* (2004) for a detalied review.

(ii) *C-4 demethylases*

Demethylation of a C-4 methyl group requires the action of a suite of three enzymes working sequentially (Gachotte *et al.* 1998, 1999; Benveniste 2004):

(i) C-4α-methyl oxidase (ERG25 in yeast and smo1/smo2 in plants), which operates on the 4α-methyl carbon three times with molecular oxygen to produce a 4α-carboxylic acid (Darnet & Rahier 2003, 2004);
(ii) 4α-carboxysterol-C-4-dehydrogenase/C-4-decarboxylase (4α-CD or ERG26 in yeast) which decarboxylates the 4α-acid and produces a 3-oxosteroid (Gachotte *et al.* 1998; Rondet *et al.* 1999);
(iii) NADPH-dependent sterone reductase (ERG27) which stereospecifically reduces the 3-oxosteroid to a 3β-OH.

These enzymes always act on the 4α-methyl group. In the first demethylation, the 4α-methyl is removed, and the 4β-methyl group rearranges to the 4α position. In both animals and fungi, the two methyl groups are sequentially removed following 14α-demethylation. The C-4 demethylase enzymatic suite converts 4,4-dimethyl-5α-cholesta-8,14,24-3β-ol to 4α-methyl zymosterol, which is subsequently converted by the same suite of enzymes to zymosterol (Benveniste 2004). In plants, the first methyl group is removed at the level of 24-methylene cycloartenol, which is converted to cycloeucalenol. This pathway goes through several subsequent steps, including the removal of the 14α-methyl group before being demethylated at C-4 a second time at the level of 24-ethylenelophenol, which is converted to 24-ethylene-lathosterol. Plant genomes show the presence of two distinct sterol methyl oxidases (smo1 and smo2) (Darnet & Rahier 2004), and gene silencing experiments in *A. thaliana* have demonstrated that each of these operate with high substrate specificity—smo1 on 24-methylene cycloartenol and smo2 on 24-ethylene-lophenol (Benveniste 2004).

Other taxa which synthesize C-4 desmethyl sterols include the bacteria *M. capsulatus* and *N. excedens*, and the kinetoplastids. Genome sequences of *M. capsulatus* and three kinetoplastid species are available in GenBank and a comparison of the sequenced genome to known sequences for erg25, smo1 and smo2 revealed no significant homologues to any of these genes among these organisms. It is possible that these organisms are using an alternative enzyme for C-4 sterol demethylation.

(iii) *Energetic constraints and oxygen usage*

The mechanism of sterol demethylation functionalizes a methyl group attached to a quaternary carbon by attaching an oxygen atom. CYP51 achieves this by the homolytic cleavage of a C–H bond to create a methyl radical, which subsequently reacts with the oxygen distally attached to the haem of the P450 (Meunier *et al.* 2004). This is an energetically expensive process which is overcome in part by using the most powerful oxidizing agent available: molecular O_2.

The C–H bond dissociation energy (D) of a methyl group attached to a quaternary carbon is 401 kJ mol^{-1} (March 1992). However, bond energies of the transition state in the demethylase enzymes are unknown and so this does not indicate actual activation energy of the methyl group, but gives an indication of the stability of the radical. This dissociation energy is higher (indicating a less stable radical) than that of secondary (401 kJ mol^{-1}) and tertiary (401 kJ mol^{-1}) carbons, but not as high as that of the C–H bond in primary (419 kJ mol^{-1}) carbons, methane (438 kJ mol^{-1}) or benzene (464 kJ mol^{-1}).

Microbial degradation of both benzene and methane takes place readily in the presence of oxygen, but is also possible under many conditions as mildly exergonic processes with sulphate as a terminal electron acceptor. This indicates that abstraction of C–H bonds stronger than those in sterol methyl groups is possible without molecular oxygen. Although thermodynamically feasible, an enzyme that demethylated sterols anaerobically would be energetically much more expensive.

Sterol demethylases have evolved at least twice, and in each case require three molecules of molecular oxygen to catalyse the reaction. Constructed phylogenetic trees of CYP51 cluster those demethylases into groups that parallel the families of sterol cyclases, suggesting that oxidative sterol demethylation is an ancient and conserved pathway that has existed at least since the time of the split between plants, kinetoplastids and opisthokonts (Rezen *et al.* 2004). Nature may contain at least one undescribed sterol demethylase (the C-4 demethylase in *M. capsulatus* and kinetoplastids). As this undescribed enzyme occurs in obligate aerobes, it is likely to require molecular oxygen.

On strict chemical grounds it may be possible to devise biosynthetic routes to sterols that proceed anaerobically as has been recently proposed (Raymond & Blankenship 2004). However, any postulated anaerobic pathway for sterol synthesis must replace five enzymes which use a combined 11 or 12 molecules of O_2 with anaerobic enzymes capable of performing the equivalent process, and further postulate that all of these enzymes have been lost or are unknown. Any

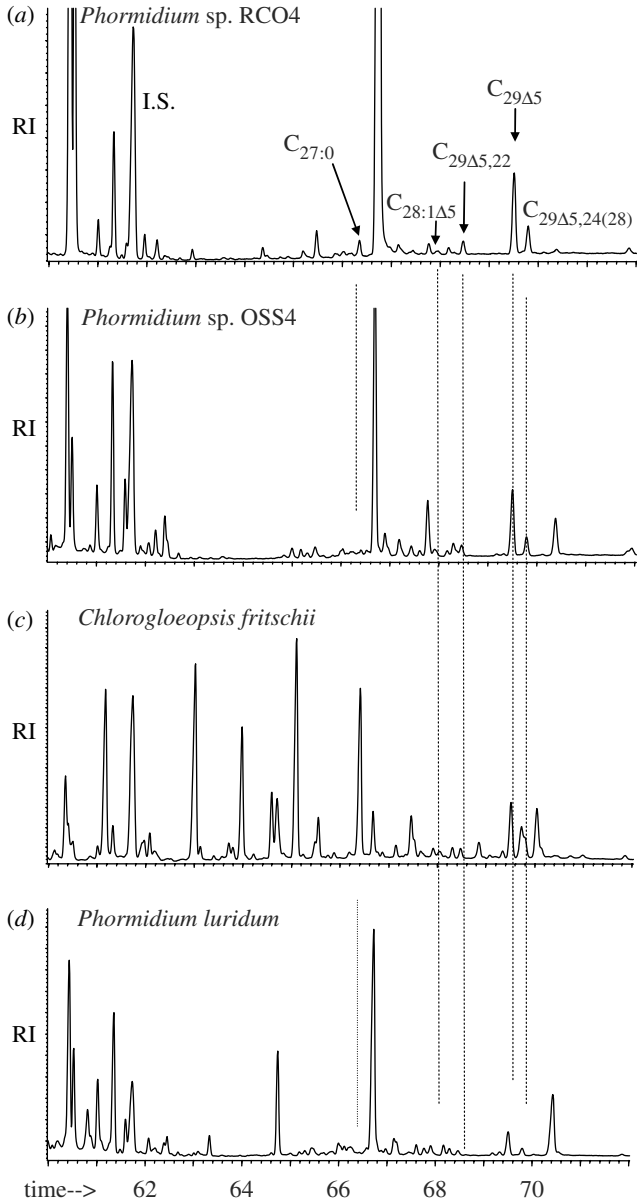

Figure 5. Total ion chromatograms for showing sterols in the total lipid extracts of four of the investigated cyanobacterial cultures (*a–d*). *Phormidium* sp. RCO4 and OSS4 are isolates from Yellowstone National Park. All organisms were grown for a biomarker and isotopic investigation of mat-forming communities (Jahnke *et al.* 2004). RI, relative intensity; I.S., internal standard.

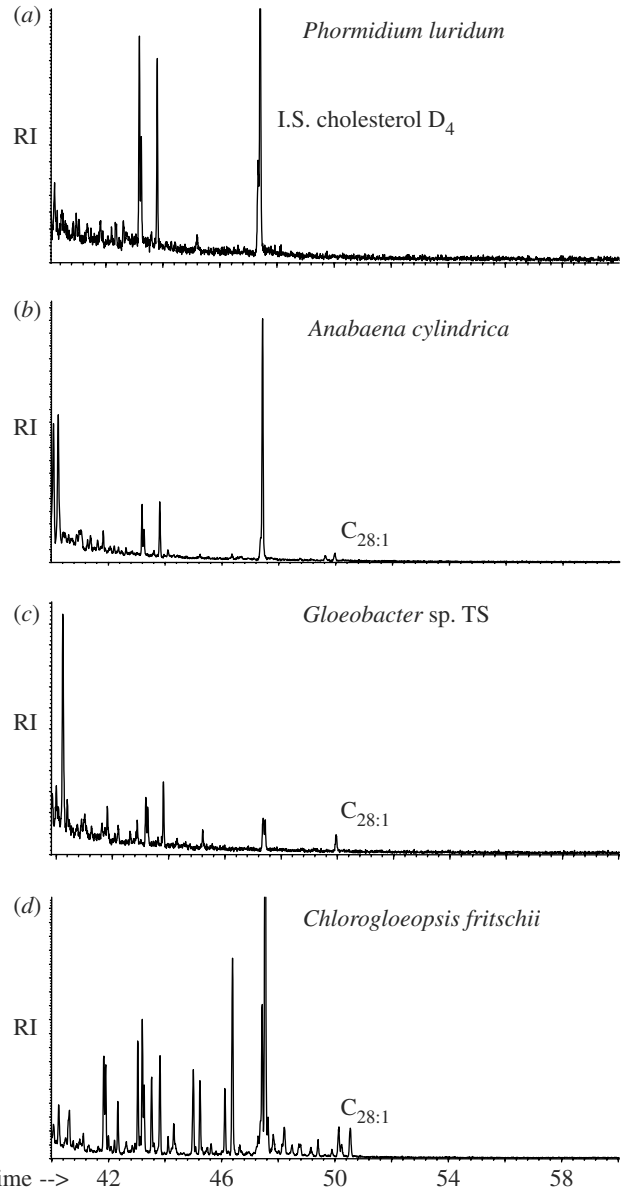

Figure 6. Gas chromatography–mass spectrometry data, depicted as the *m*/*z* 129 ion which is diagnostic for trimethylsilyl (TMS) sterols, for some cyanobacteria cultured in the presence of cycloheximide (*a–d*). The only detectable sterol is ergosterol which was also present in blank analyses of the BG-11 culture medium and attributable to that source.

hypothesis that proposes enzymes existed in the past, and for which all evidence has been lost, is not testable.

(d) *On the occurrence of sterols in cyanobacteria*

In connection with earlier studies of 2-methylhopanoids and other biomarkers in strains of cultured cyanobacteria (Summons *et al.* 1999; Jahnke *et al.* 2004), we checked for the presence of sterols in the total lipid extracts of some of the genera previously reported to contain them. Samples were hydrolysed with acid in order to render conjugated sterols in the free form and then converted to trimethylsilyl derivatives for GC–MS. As figure 5 shows, our cultured samples contained an abundance of sterols. Moreover, the distributions were similar in all samples with many of the same compounds reported by previous workers including a strong predominance of $C_{29}\Delta5$, $C_{29}\Delta5,22$ and C_{29} $\Delta5,24(28)$ (figure 5).

These results prompted us to examine other options, one of which was to re-culture the organisms in media with defined sterol contents and in the presence of cycloheximide, a compound known to inhibit the growth of eukaryotes by blocking protein synthesis. After several sub-cultures in the presence of cycloheximide we could only identify traces of a C_{27} and a C_{28} sterol that were subsequently found to be components of the BG-11 medium (figure 6). After re-culturing of these cyanobacteria in the original media, and in the absence of cycloheximide, they continued to be free of detectable sterols.

The results of these experiments indicated that our original cultures were contaminated by an organism that produced C_{29} sterols in abundance. The source of contamination was investigated using, firstly,

a universal gene probe for eukaryotes which was positive and, secondly, a probe specific for Basidomycetes (rust fungi) which was also positive. Unlike other fungi which produce ergosterol ($C_{28}\Delta5$) or cholesterol ($C_{27}\Delta5$) as their major sterols, Uredospores such as *Uromyces phaseoli* (bean rust), flax rust and *Cronartium fusiforme* (fusiform rust) all produce C_{29} sterols like their host plants. In these organisms, the principal sterols are $C_{29}\Delta7$ and $C_{29}\Delta7,24(28)$ (Jackson & Frear 1968; Lin *et al*. 1972; Carmack *et al*. 1976).

Lack of sterols in cyanobacteria is further evidenced by their genome sequences, which reveal that the only genes significantly homologous to sterol synthases are squalene–hopene synthases. As microbial genome sequencing progresses, diverse new sterol producers may be discovered on the basis of gene content; the utility of this approach has already been demonstrated (Pearson *et al*. 2003). Such searches will be aided by the high degree of conservation among sterol synthesis genes, which makes them readily recognizable on the basis of sequence similarity. It is worth noting that, among the 258 prokaryotic genomes sequenced and available as of this writing, only one previously unknown sterol producer, *G. obscuriglobus* (Pearson *et al*. 2003), has been discovered.

4. RELATING BIOMARKERS TO BIOLOGICAL AND GEOCHEMICAL EVOLUTION

(a) *Membrane function of sterols and evolutionary and ecological adaptation*

The function of sterols in cellular membranes has been a topic of long-standing interest among biochemists and cell biologists. Fifty years after the central steps in the sterol synthesis pathway were elucidated by Bloch and co-workers, understanding of the structural and functional roles of these cardinal eukaryotic lipids remains incomplete and an active area of research. It was recognized early on that sterols modulate the micro-scale fluidic properties of the membrane—its density, viscosity, and so on—and that sterols that differ by only the addition of a methyl group or a double bond can produce measurably different effects. The principal mechanism for this structural effect of sterols is their induction of a liquid-ordered phase in membranes, a state intermediate between high-temperature liquid-disordered and low temperature solid-ordered (gel) phases. Further, the liquid-ordered and liquid-disordered phases can coexist in the same membrane, allowing for spatial heterogeneity and the notion of the membrane as a 'fluid mosaic' with discrete domains (lipid rafts) (Simons & Vaz 2004). A functional role for sterols has been suggested in the reduction of permeability of membranes to cations, particularly protons and sodium, hence assisting in energy conservation (Haines 2001). This is likely due to enhanced exclusion of water clusters or chains from the membrane, though the precise mechanism remains under investigation (Tepper & Voth 2005).

The foregoing discussion of the structural and functional characteristics of steroids could apply essentially equally well to hopanoids, which are membrane constituents of many bacteria. The two classes of lipids share the basic polycyclic skeleton side chain structure and are of nearly identical molecular dimensions (approx. 8 Å by 19 Å). Hopanoids have been demonstrated to influence membrane ordering and fluid properties similarly to steroids (Kannenberg *et al*. 1983). On the basis of such comparisons, hopanoids have been termed 'sterol surrogates' and 'functionally equivalent' in membranes (Ourisson *et al*. 1987). This begs the question: why should functionally equivalent molecules be so strongly differentially conserved across the breadth of the diversity of cellular life?

While steroids and hopanoids are structurally similar, they differ in a key respect: steroids have their polar group attached directly to the ring structure at C3, while the polar functions in hopanoids are attached to the side chain. As a result, the ring structure of steroids sits near the edge of the lipid bilayer, but that of hopanoids is nearer the centre. This suggests that the two types of terpenoid may move quite differently in membranes, particularly with regard to their ability to translocate from one leaflet of the bilayer to the other, a phenomenon known as 'lipid flip-flop'.

Flip-flop is an important property of membrane lipids because in order for a membrane to deform—i.e. to curve inward or outward—one leaflet of the bilayer must become longer while the other becomes shorter. This curvature is effected by flipping lipid molecules from one leaflet to the other. Sterols have among the shortest $t_{1/2}$ values for transbilayer flip-flop of any membrane lipid (Holthuis & Levine 2005), meaning that steroid-containing membranes are readily deformed. This was elegantly demonstrated (Bacia *et al*. 2005) in a cell-free system of giant unilamellar vesicles, where it was found that not only does the addition of sterols to lipid vesicles induce domain formation and budding, but the *type* of sterol controls the *direction* of curvature. Cholesterol and lophenol induce positive (outward) curvature, while lanosterol and cholesteryl sulphonate cause inward (negative) budding. Varying the proportions of a mixture of cholesterol and cholesteryl sulphonate controlled both domain size and budding behaviour. Eukaryotic cells have highly specific structural requirements for their membrane sterols: changing even the position of unsaturation in the ring system or the stereochemistry of the hydroxyl group attachment can result in an incompetent cell envelope (Xu *et al*. 2005). Taken together, this evidence suggests that a membrane with a well-regulated sterol composition is a powerful tool for export and import across the cell membrane.

Eukaryotes, both unicellular and multicellular, make extensive use of endo- and exocytosis. The innovation of sterol biosynthesis, in allowing rapid membrane deformation, may have been a key step in the evolution of these processes. In eukaryotes, phagocytosis and membrane biogenesis are closely coupled. When part of a membrane is drawn in to engulf a particle, lipid synthesis is stimulated (through the sterol regulatory element binding protein transcriptional regulators) to just the degree to replace the consumed membrane segment (Castoreno *et al*. 2005). With a flexible, deformable membrane, many mechanisms to generate curvature are possible, including protein scaffolding, helix insertion and active

cytoskeletal remodelling (McMahon & Gallop 2005). Much of the dynamic character of the eukaryotic membrane system can be attributed to these curvature mechanisms. Though experimental quantification of the transbilayer movement of hopanoids (particularly their flip-flop half-time) is lacking, the absence of endo- and exocytosis among the bacteria may indicate that hopanoids are not functionally equivalent to sterols in this regard.

The ability to perform this type of transmembrane transport had profound evolutionary and ecological consequences. In essence, the invention of endocytosis is the dawn of predation. Prior to endocytosis, heterotrophy proceeded largely through the dissolved phase, and no stratified trophic relationships existed. Once large particles (including other cells) could be imported and enzymatically degraded intracellularly, the predator–prey dynamic, that shaped much of evolutionary history, was established.

(i) *Possible role of O_2 in the biosynthesis of 2-methylbacteriohopanepolyols*

Precise details of the biosynthetic pathway leading to 2-methylbacteriohopanepolyols (2Me-BHP) are not known although the methyl group is known to be transferred intact from L-methionine (Zundel & Rohmer 1985). Genomes of sequenced cyanobacteria and *M. capsulatus* (which produces 3Me-BHP) reveal that they contain homologues of sterol-methyltransferases found in plants. The transfer of a methyl group to the hopanoid ring structure is presumably preceded by desaturation at the 2–3 position. Aerobic sterol desaturases are present in the genomes of several cyanobacteria, with unknown function. It has yet to be demonstrated that either sterol-methyltransferases or sterol desaturases are involved in the methylation of the hopanoid A-ring, but their potential should be investigated.

(ii) *Alternative oxidants on the early Earth*

Oxidizing power may have been scarce on the Earth's surface before the oxygenation of the atmosphere. Under such conditions, it is possible that cellular life made use of oxidants that have since been supplanted by nearly ubiquitous O_2. Postulated scenarios for the use of such alternative oxidants should uphold criteria of geochemical and biochemical viability; nitrogen oxides fail the first test, while water, as discussed above for SQMO, generally fails the second. One feasible alternative is hydrogen peroxide. Significant H_2O_2 is generated by the reaction of water with pyrite under anaerobic, UV-illuminated conditions (Blankenship & Hartman 1998; Borda *et al.* 2003), a plausible scenario on the early Earth. The use of peroxide as a 'transitional' redox partner (both as an oxidant and as a reductant) in biogeochemical evolution has been discussed previously (Kasting *et al.* 1985; Blankenship & Hartman 1998; Borda *et al.* 2003). Hydrogen peroxide may have been a suitable oxidant for biosynthetic oxygenation reactions, such as those described above in sterol synthesis. Such a scenario has, at minimum, three prerequisites to be fulfilled: (i) H_2O_2 must have been produced in geochemically significant quantities and have been available to micro-organisms in a variety of habitats. (ii) The enzymes of the pathway of interest must be able to use H_2O_2 as an oxidant, and themselves be stable in concentrations of peroxide thought likely to arise. (iii) Cells must be able to use exogenous peroxide anabolically to produce the biochemicals at question. Even if only the first condition can be demonstrated, further chemistry (such as the iron-catalysed Haber–Weiss reaction) should be considered as sources for redox partners for metabolism. Exploration of these possibilities will likely lead to new insights into the coevolution of the redox chemistry of the geosphere and biosphere, and highlight the importance of understanding the evolutionary biochemistry of reactive oxygen species.

5. SUMMARY AND FUTURE DIRECTIONS

The sequences of key enzymes in steroid biosynthesis are very highly conserved within the eukaryotic domain and it appears likely that the initial steps of the pathway were present in their modern form in the last common ancestor of eukaryotes.

Steroid biosynthesis is an oxygen intensive process with, for example, 11 molecules of O_2 being required for the synthesis of one molecule of cholesterol. It is also energetically expensive. While one can postulate anaerobic alternatives to some steps in the pathway, these would be even more energy intensive. Any postulate for an ancestral anaerobic pathway to sterols must explain the replacement of five enzymes with anaerobic equivalents and, further, that all of these have been lost or are unknown.

Previous reports of sterol biosynthesis in cyanobacteria appear to be erroneous. It seems that cyanobacterial cultures are easily contaminated by fungi related to rusts. The sterol biosynthetic capability of other Bacteria is patchily distributed, characterized by pathways that are either anomalous or incomplete, and likely gained from Eukarya by lateral gene transfer.

The generally accepted hypothesis that hopanoids are sterol surrogates in bacteria deserves re-visiting with investigations of the localization and functional roles of BHP. In particular, studies of the phylogenetic distribution, biosynthesis and functional role of 2Me-BHP in cyanobacteria would be particularly valuable. As a starting point, one could hypothesize that the biosynthesis of 2Me-BHP in cyanobacteria involves an oxygen-dependent desaturase and a methyltransferase analogous to those employed in sterol biosynthesis.

Understanding early steps in cellular evolution will be aided by more detailed studies of hydrocarbons in Archaean and Proterozoic rocks. Further studies of the membrane function of sterols and their role in evolutionary and ecological adaptation will also be valuable.

We thank Tsegereda Embaye (NASA) and Janet Hope (Geoscience Australia) for technical assistance in the analysis of cyanobacterial lipids. We also thank Yanek Hebting (MIT), Ken Cullings and Chris Raleigh (NASA Ames) for helpful discussion. Lee Kump, Katja Meyer, Ann Pearson and John Volkman provided insightful and constructive reviews that improved the manuscript. This work was supported by the NASA Exobiology Program (Award NNG05GN62G) and NSF (Award EAR 0418619).

REFERENCES

Abe, I., Rohmer, M. & Prestwich, G. D. 1993 Enzymatic cyclization of squalene and oxidosqualene to sterols and triterpenes. *Chem. Rev.* **93**, 2189–2206. (doi:10.1021/cr00022a009)

Adl, S. M. *et al.* 2005 The new higher level classification of eukaryotes with emphasis on the taxonomy of protists. *J. Eukaryot. Microbiol.* **52**, 399–451. (doi:10.1111/j.1550-7408.2005.00053.x)

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. 1997 Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl. Acids Res.* **25**, 3389–3402.

Anding, C., Brandt, R. D. & Ourisson, G. 1971 Sterol biosynthesis in *Euglena gracilis* Z. Sterol precursors in light-grown and dark-grown *Euglena gracilis* Z. *Eur. J. Biochem.* **24**, 259–263. (doi:10.1111/j.1432-1033.1971.tb19679.x)

Aoyama, Y., Funae, Y., Noshiro, M., Horiuchi, T. & Yoshida, Y. 1994 Occurrence of a P450 showing high homology to yeast lanosterol 14-demethylase (P450(14DM)) in the rat liver. *Biochem. Biophys. Res. Commun.* **201**, 1320–1326. (doi:10.1006/bbrc.1994.1848)

Arisue, N., Hasegawa, M. & Hashimoto, T. 2005 Root of the Eukaryota tree as inferred from combined maximum likelihood analyses of multiple molecular sequence data. *Mol. Biol. Evol.* **22**, 409–420. (doi:10.1093/molbev/msi023)

Bacia, K., Schwille, P. & Kurzchalia, T. 2005 Sterol structure determines the separation of phases and the curvature of the liquid-ordered phase in model membranes. *Proc. Natl Acad. Sci. USA* **102**, 3272–3277. (doi:10.1073/pnas.0408215102)

Bekker, A., Holland, H. D., Wang, P. L., Rumble, D., Stein, H. J., Hannah, J. L., Coetzee, L. L. & Beukes, N. J. 2004 Dating the rise of atmospheric oxygen. *Nature* **427**, 117–120. (doi:10.1038/nature02260)

Bellamine, A., Lepesheva, G. & Waterman, M. 2004 Fluconazole binding and sterol demethylation in three CYP51 isoforms indicate differences in active site topology. *J. Lipid Res.* **45**, 2000–2007. (doi:10.1194/jlr.M400239-JLR200)

Benveniste, P. 2004 Biosynthesis and accumulation of sterols. *Ann. Rev. Plant Biol.* **55**, 429–457.

Bird, C. W., Lynch, J. M., Pirt, F. J. & Reid, W. W. 1971 Steroids and squalene in *Methylococcus capsulatus* grown on methane. *Nature* **230**, 473. (doi:10.1038/230473a0)

Blankenship, R. E. & Hartman, H. 1998 The origin and evolution of oxygenic photosynthesis. *Trends Biochem. Sci.* **23**, 94. (doi:10.1016/S0968-0004(98)01186-4)

Blanksby, S. J. & Ellison, G. B. 2003 Bond dissociation energies of organic molecules. *Accounts Chem. Res.* **36**, 255–263. (doi:10.1021/ar020230d)

Bloch, K. 1983 Sterol structure and membrane function. *CRC Crit. Rev. Biochem.* **14**, 47–92.

Bloch, K. 1987 Summing up. *Annu. Rev. Biochem.* **56**, 1–18. (doi:10.1146/annurev.bi.56.070187.000245)

Bode, H. B., Zeggel, B., Silakowski, B., Wenzel, S. C., Reichenbach, H. & Muller, R. 2003 Steroid biosynthesis in prokaryotes: identification of myxobacterial steroids and cloning of the first bacterial 2,3(*S*)-oxidosqualene cyclase from the myxobacterium *Stigmatella aurantiaca*. *Mol. Microbiol.* **47**, 471–481. (doi:10.1046/j.1365-2958.2003.03309.x)

Borda, M. J., Elsetinow, A. R., Strongin, D. R. & Schoonen, M. A. 2003 A mechanism for the production of hydroxyl radical at surface defect sites on pyrite. *Geochim. Cosmochim. Acta* **67**, 935–939. (doi:10.1016/S0016-7037(02)01222-X)

Brocks, J. J., Logan, G. A., Buick, R. & Summons, R. E. 1999 Archean molecular fossils and the early rise of eukaryotes. *Science (Washington, DC)* **285**, 1033–1036. (doi:10.1126/science.285.5430.1033)

Brocks, J. J., Buick, R., Logan, G. A. & Summons, R. E. 2003 Composition and syngeneity of molecular fossils from the 2.78 to 2.45 billion-year-old Mount Bruce Supergroup, Pilbara Craton, Western Australia. *Geochim. Cosmochim. Acta* **67**, 4289–4319. (doi:10.1016/S0016-7037(03)00208-4)

Brown, J. R. & Doolittle, W. F. 1999 Gene descent, duplication, and horizontal transfer in the evolution of glutamyl- and glutaminyl-tRNA synthetases. *J. Mol. Evol.* **49**, 485. (doi:10.1007/PL00006571)

Bruice, T. C., Noar, J. B., Ball, S. S. & Venkataram, U. V. 1983 Monooxygen donation potential of 4a-hydroperoxyflavins as compared with those of a percarboxylic acid and other hydroperoxides. Monooxygen donation to olefin, tertiary amine, alkyl sulfide, and iodide ion. *J. Am. Chem. Soc.* **105**, 2452–2463. (doi:10.1021/ja00346a057)

Buick, R., Anbar, A. D., Mojzsis, S. J., Kaufman, A. J., Kieft, T. L., Lyons, T. W. & Humayun, M. 2004 The case for scientific drilling of Precambrian sedimentary sequences: a mission to early Earth. *American Geophysical Union, Fall Meeting 2001*, abstract #P22B-0544.

Carmack, C. L., Weete, J. D. & Kelly, W. D. 1976 Hydrocarbons, fatty acids and sterols of *Cronartium fusiforme*. *Physiol. Plant Pathol.* **8**, 43–49.

Castoreno, A. B., Wang, Y., Stockinger, W., Jarzylo, L. A., Du, H., Pagnon, J. C., Shieh, E. C. & Nohturfft, A. 2005 Transcriptional regulation of phagocytosis-induced membrane biogenesis by sterol regulatory element binding proteins. *Proc. Natl Acad. Sci. USA* **102**, 13 129–13 134. (doi:10.1073/pnas.0506716102)

Cloud, P. E. 1972 A working model of the primitive Earth. *Am. J. Sci.* **272**, 537–548.

Dahl, C., Dahl, J. & Bloch, K. 1980 Effect of alkyl-substituted precursors of cholesterol on artificial and natural membranes and on the viability of *Mycoplasma capricolum*. *Biochemistry* **19**, 1462–1467. (doi:10.1021/bi00548a031)

Darnet, S. & Rahier, A. 2003 Enzymological properties of sterol-C4-methyl-oxidase of yeast sterol biosynthesis. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids* **1633**, 106–117.

Darnet, S. & Rahier, A. 2004 Plant sterol biosynthesis: identification of two distinct families of sterol 4a-methyl oxidases. *Biochem. J.* **378**, 889–898.

Des Marais, D. J. 2000 Evolution: when did photosynthesis emerge on Earth? *Science* **289**, 1703–1705.

Des Marais, D. J., Strauss, H., Summons, R. E. & Hayes, J. M. 1992 Carbon isotope evidence for the stepwise oxidation of the Proterozoic environment. *Nature* **359**, 605–609. (doi:10.1038/359605a0)

DeSousa, N. J. & Nes, W. R. 1968 Sterols: isolation from a blue-green alga. *Science* **162**, 363–364.

Eigenbrode, J. L. 2004 Late Archean microbial ecology: an integration of molecular, isotopic and lithological studies. Ph.D. thesis, Earth Sciences, the Pennsylvania State University.

Eigenbrode, J. L., Freeman, K. H., Brocks, J. J., Summons, R. E. & Logan, G. A. 2001 Late Archean biomarkers of carbonate and shale lithofacies from the Hamersley Basin, Pilbara Craton, Western Australia. In *11th V.M. Goldschmidt Conference, Hot Springs, VA, USA*, p. 3461.

Eigenbrode, J. L., Freeman, K. H. & Summons, R. E. 2004 Biogeochemical processes in Late Archean marine biosphere revealed by isotopic and molecular records. *American Geophysical Union, Fall Meeting 2004*, abstract #B33B-0260.

Farquhar, J., Bao, H. & Thiemens, M. 2000*a* Atmospheric influence of Earth's earliest sulfur cycle. *Science* **289**, 756–758. (doi:10.1126/science.289.5480.756)

Farquhar, J., Savarino, J., Jackson, T. L. & Thiemens, M. 2000*b* Evidence of atmospheric sulphur in the Martian regolith from sulphur isotopes in meteorites. *Nature* **404**, 50–52. (doi:10.1038/35003517)

Fischer, W. W., Summons, R. E. & Pearson, A. 2005 Targeted genomic detection of biosynthetic pathways: anaerobic production of hopanoid biomarkers by a common sedimentary microbe. *Geobiology* **3**, 33–40. (doi:10.1111/j.1472-4669.2005.00041.x)

Gachotte, D., Barbuch, R., Gaylor, J., Nickel, E. & Bard, M. 1998 Characterization of the *Saccharomyces cerevisiae* ERG26 gene encoding the C-3 sterol dehydrogenase (C-4 decarboxylase) involved in sterol biosynthesis. *Proc. Natl Acad. Sci. USA* **95**, 13794–13799.

Gachotte, D., Sen, S. E., Eckstein, J., Barbuch, R., Krieger, M., Ray, B. D. & Bard, M. 1999 Characterization of the *Saccharomyces cerevisiae* ERG27 gene encoding the 3-keto reductase involved in C-4 sterol demethylation. *Proc. Natl Acad. Sci. USA* **96**, 12655–12660.

Giner, J.-L., Wünsche, L., Andersen, R. A. & Djerassi, C. 1991 Dinoflagellates cyclize squalene oxide to lanosterol. *Biochem. Syst. Ecol.* **19**, 142–145.

Groves, J. T. & Han, Y.-Z 1995 Models and mechanisms of cytochrome P450 action. In *Cytochrome P450: structure, mechanism, and biochemistry* (ed. P. R. Ortiz de Montellano), 2nd edn, pp. 1–45. New York: Plenum Press.

Hai, T., Schneider, B., Schmidt, J. & Adam, G. 1996 Sterols and triterpenoids from the cyanobacterium *Anabaena hallensis*. *Phytochemistry* **41**, 1083. (doi:10.1016/0031-9422(95)00778-4)

Haines, T. H. 2001 Do sterols reduce proton and sodium leaks through lipid bilayers? *Prog. Lipid Res.* **40**, 299–324. (doi:10.1016/S0163-7827(01)00009-1)

Halverson, G. P., Hoffman, P. F., Schrag, D. P., Maloof, A. C. & Rice, A. H. N. 2005 Toward a Neoproterozoic composite carbon-isotope record. *Geol. Soc. Am. Bull.* **117**, 1181–1207. (doi:10.1130/B25630.1)

Hayes, J. M. & Waldbauer, J. R. 2006 The carbon cycle and associated redox processes through time. *Phil. Trans. R. Soc. B* **361**, 931–950. (doi:10.1098/rstb.2006.1840)

Hoffman, P. F. & Schrag, D. P. 2002 The snowball Earth hypothesis: testing the limits of global change. *Terra Nova* **14**, 129–155. (doi:10.1046/j.1365-3121.2002.00408.x)

Holland, H. D. 1984 *The chemical evolution of the atmosphere and oceans*. Princeton, NJ: Princeton University Press.

Holland, H. D. 2002 Volcanic gases, black smokers, and the Great Oxidation Event. *Geochim. Cosmochim. Acta* **66**, 3811–3826. (doi:10.1016/S0016-7037(02)00950-X)

Holland, H. D. & Beukes, N. J. 1990 A palaeoweathering profile from Griqualand West, South Africa: evidence for a dramatic rise in atmospheric oxygen between 2.2 and 1.9 bybp. *Am. J. Sci.* **290-A**, 1–34.

Holthuis, J. & Levine, T. 2005 Lipid traffic: floppy drives and a superhighway. *Nat. Rev. Mol. Cell Biol.* **6**, 209–220. (doi:10.1038/nrm1591)

Hurtgen, M. T., Arthur, M. A. & Halverson, G. P. 2005 Neoproterozoic sulfur isotopes, the evolution of microbial sulfur species, and the burial efficiency of sulfide as sedimentary pyrite. *Geology* **33**, 41–44. (doi:10.1130/G20923.1)

Jackson, L. L. & Frear, D. S. 1968 Lipids of rust fungi—II. Stigmast-7-enol and stigmasta-7,24(28)-dienol in flax rust uredospores. *Phytochemistry* **7**, 651. (doi:10.1016/S0031-9422(00)88242-4)

Jackson, C. J., Lamb, D. C., Marczylo, T. H., Warrilow, A. G. S., Manning, N. J., Lowe, D. J., Kelly, D. E. & Kelly, S. L. 2002 A novel sterol 14α-demethylase/ferredoxin fusion protein (MCCYP51FX) from *Methylococcus capsulatus* represents a new class of the c cytochrome P450 superfamily. *J. Biol. Chem.* **277**, 46 959–46 965. (doi:10.1074/jbc.M203523200)

Jahnke, L. L. 1992 The effects of growth temperature on the methyl sterol and phospholipid fatty acid composition of *Methylococcus capsulatus* (Bath). *FEMS Microbiol. Lett.* **15**, 209–212.

Jahnke, L. L. & Klein, H. P. 1983 Oxygen requirements for formation and activity of the squalene epoxidase in *Saccharomyces cerevisiae*. *J. Bacteriol.* **155**, 488–492.

Jahnke, L. L., Embaye, T., Hope, J., Turk, K. A., Van Zuilen, M., Des Marais, D. J., Farmer, J. D. & Summons, R. E. 2004 Lipid biomarker and carbon isotopic signatures for stromatolite-forming, microbial mat communities and *Phormidium* cultures from Yellowstone National Park. *Geobiology* **2**, 31–47. (doi:10.1111/j.1472-4677.2004.00021.x)

Joubert, B. M., Nguyen, L. N., Matsuda, S. P. T. & Buckner, F. S. 2001 Cloning and functional characterization of a *Trypanosoma brucei* lanosterol 14α-demethylase gene. *Mol. Biochem. Parasitol.* **117**, 115–117. (doi:10.1016/S0166-6851(01)00337-1)

Kannenberg, E., Blume, A., McElhaney, R. N. & Poralla, K. 1983 Monolayer and calorimetric studies of phosphatidylcholines containing branched-chain fatty acids and of their interactions with cholesterol and with a bacterial hopanoid in model membranes. *Biochim. Biophys. Acta* **733**, 111–116.

Karhu, J. A. & Holland, H. D. 1996 Carbon isotopes and the rise of atmospheric oxygen. *Geology* **24**, 867–870. (doi:10.1130/0091-7613(1996)024<0867:CIATRO>2.3.CO;2)

Kasting, J. F., Holland, H. D. & Pinto, J. P. 1985 Oxidant abundances in rainwater and the evolution of atmospheric oxygen. *J. Geophys. Res.* **90**, 10 497–10 510.

Kirschvink, J. L., Galdos, E. J., Bertani, L. E., Beukes, N. J., Jens, G., Maepa, L. N. & Steinberger, R. E. 2000 Paleoproterozoic snowball Earth: extreme climatic and geochemical global change and its biological consequences. *Proc. Natl Acad. Sci. USA* **97**, 1400–1405. (doi:10.1073/pnas.97.4.1400)

Kohl, W., Gloe, A. & Reichenbach, H. 1983 Steroids from the myxobacterium *Nannocyctis excedens*. *J. Gen. Microbiol.* **129**, 1629–1635.

Kohlhase, M. & Pohl, P. 1988 Saturated and unsaturated sterols of nitrogen-fixing blue-green algae (cyanobacteria). *Phytochemistry* **27**, 1735–1740. (doi:10.1016/0031-9422(88)80434-5)

Kopp, R. E., Kirschvink, J. L., Hilburn, I. A. & Nash, C. Z. 2005 The Paleoproterozoic snowball Earth: a climate disaster triggered by the evolution of oxygenic photosynthesis. *Proc. Natl Acad. Sci. USA* **102**, 11 131–11 136. (doi:10.1073/pnas.0504878102)

Laden, B. P., Tang, Y. & Porter, T. D. 2000 Cloning, heterologous expression, and enzymological characterization of human squalene monooxygenase. *Arch. Biochem. Biophys.* **374**, 381–388. (doi:10.1006/abbi.1999.1629)

Lamb, D. C., Ikeda, H., Nelson, D. R., Ishikawa, J., Skaug, T., Jackson, C., Omura, S., Waterman, M. R. & Kelly, S. L. 2003 Cytochrome P450 complement (CYPome) of the avermectin-producer *Streptomyces avermitilis* and comparison to that of *Streptomyces coelicolor* A3(2). *Biochem. Biophys. Res. Commun.* **307**, 610–619. (doi:10.1016/S0006-291X(03)01231-2)

Lamour, V., Quevillon, S., Diriong, S., N'Guyen, V. C., Lipinski, M. & Mirande, M. 1994 Evolution of the Glx-tRNA synthetase family: the glutaminyl enzyme as a case of horizontal gene transfer. *Proc. Natl Acad. Sci. USA* **91**, 8670–8674.

Lee, H. K., Denner-Ancona, P., Sakakibara, J., Ono, T. & Prestwich, G. D. 2000 Photoaffinity labeling and

site-directed mutagenesis of rat squalene epoxidase. *Arch. Biochem. Biophys.* **381**, 43–52. (doi:10.1006/abbi.2000.1966)

Lee, H. K., Zheng, Y. F., Xiao, X. Y., Bai, M., Sakakibara, J., Ono, T. & Prestwich, G. D. 2004 Photoaffinity labeling identifies the substrate-binding site of mammalian squalene epoxidase. *Biochem. Biophys. Res. Commun.* **315**, 1–9. (doi:10.1016/j.bbrc.2004.01.012)

Lepesheva, G. I. & Waterman, M. R. 2004 CYP51—the omnipotent P450. *Mol. Cell. Endocrinol.* **215**, 165–170. (doi:10.1016/j.mce.2003.11.016)

Lepesheva, G. I., Nes, W. D., Zhou, W., Hill, G. C. & Waterman, M. R. 2004 CYP51 from *Trypanosoma brucei* is obtusifoliol-specific. *Biochemistry* **43**, 10 789–10 799. (doi:10.1021/bi048967t)

Levin, E. Y. & Bloch, K. 1964 Absence of sterols in blue-green algae. *Nature* **202**, 90–91.

Lin, H.-K., Langenbach, R. J. & Knoche, H. W. 1972 Sterols of *Uromyces phaseoli* uredospores. *Phytochemistry* **11**, 2319–2322. (doi:10.1016/S0031-9422(00)88399-5)

Liu, Y., Yao, Z.-X. & Papadopoulos, V. 2005 Cytochrome P450 17α-hydroxylase/17,20 lyase (CYP17) function in cholesterol biosynthesis: identification of squalene monooxygenase (epoxidase) activity associated with CYP17 in Leydig cells. *Mol. Endocrinol.* **19**, 1918–1931. (doi:10.1210/me.2004-0271)

Lodeiro, S., Segura, M., Stahl, M., Schulz-Gasch, T. & Matsuda, S. 2004 Oxidosqualene cyclase second-sphere residues profoundly influence the product profile. *ChemBioChem* **5**, 1581–1585. (doi:10.1002/cbic.200400086)

March, J. 1992 *Advanced organic chemistry: reactions, mechanisms, and structure*, 4th edn. New York: Wiley.

McMahon, H. T. & Gallop, J. L. 2005 Membrane curvature and mechanisms of dynamic cell membrane remodelling. *Nature* **438**, 590–596. (doi:10.1038/nature04396)

Meunier, B., de Visser, S. P. & Shaik, S. 2004 Mechanism of oxidation reactions catalyzed by cytochrome P450 enzymes. *Chem. Rev. (Washington, DC, United States)* **104**, 3947–3980.

Meyer, M., Segura, M., Wilson, W. & Matsuda, S. 2000 Oxidosqualene cyclase residues that promote formation of cycloartenol, lanosterol, and parkeol. *Angew. Chem. Int. Ed.* **39**, 4090–4092. (doi:10.1002/1521-3773(20001117)39:22<4090::AID-ANIE4090>3.0.CO;2-8)

Meyer, M. M., Xu, R. & Matsuda, S. P. T. 2002 Directed evolution to generate cycloartenol synthase mutants that produce lanosterol. *Org. Lett.* **4**, 1395–1398. (doi:10.1021/ol0257225)

Nelson, D. & Strobel, H. 1989 Secondary structure prediction of 52 membrane-bound cytochromes P450 shows a strong structural similarity to P450cam. *Biochemistry* **28**, 656–660. (doi:10.1021/bi00428a036)

Ourisson, G., Rohmer, M. & Poralla, K. 1987 Prokaryotic hopanoids and other polyterpenoid sterol surrogates. *Annu. Rev. Microbiol.* **41**, 301–333. (doi:10.1146/annurev.mi.41.100187.001505)

Pancost, R. D., Sinninghe Damste, J. S., de Lint, S., van der Maarel, M. J. E. C., Gottschal, J. C. & Party, M. S. S. 2000 Biomarker evidence for widespread anaerobic methane oxidation in Mediterranean sediments by a consortium of methanogenic Archaea and Bacteria. *Appl. Environ. Microbiol.* **66**, 1126–1132. (doi:10.1128/AEM.66.3.1126-1132.2000)

Paoletti, C., Pushparaj, B., Florenzano, G., Capella, P. & Lerker, G. 1976 Unsaponifiable matter of green and blue-green algal lipids as a factor of biochemical differentiation of their biomass. II. Terpenic alcohol and sterol fractions. *Lipids* **11**, 266–271.

Pearson, A., Budin, M. & Brocks, J. J. 2003 Phylogenetic and biochemical evidence for sterol synthesis in the bacterium *Gemmata obscuriglobus*. *Proc. Natl Acad. Sci. USA* **100**, 15 352–15 357. (doi:10.1073/pnas.2536559100)

Peters, K. E., Walters, C. C. & Moldowan, J. M. 2004 *The biomarker guide*, 2nd edn. Cambridge, UK: Cambridge University Press.

Podust, L. M., Poulos, T. L. & Waterman, M. R. 2001*a* Crystal structure of cytochrome P450 14α-sterol demethylase (CYP51) from *Mycobacterium tuberculosis* in complex with azole inhibitors. *Proc. Natl Acad. Sci. USA* **98**, 3068–3073. (doi:10.1073/pnas.061562898)

Podust, L. M., Stojan, J., Poulos, T. L. & Waterman, M. R. 2001*b* Substrate recognition sites in 14α-sterol demethylase from comparative analysis of amino acid sequences and X-ray structure of *Mycobacterium tuberculosis* CYP51. *J. Inorg. Biochem.* **87**, 227–235. (doi:10.1016/S0162-0134(01)00388-9)

Raederstorff, D. & Rohmer, M. 1987 Sterol biosynthesis via cycloartenol and other biochemical features related to photosynthetic phyla in the amoebae *Naegleria lovaniensis* and *Naegleria gruberi*. *Eur. J. Biochem.* **164**, 427–434. (doi:10.1111/j.1432-1033.1987.tb11075.x)

Rasmussen, B. & Buick, R. 1999 Redox state of the Archean atmosphere: evidence from detrital heavy minerals in *ca* 3250–2750 Ma sandstones from the Pilbara Craton, Australia. *Geology* **27**, 115–118. (doi:10.1130/0091-7613(1999)027<0115:RSOTAA>2.3.CO;2)

Raymond, J. & Blankenship, R. E. 2004 Biosynthetic pathways, gene replacement and the antiquity of life. *Geobiology* **2**, 199–203. (doi:10.1111/j.1472-4677.2004.00037.x)

Rezen, T., Debeljak, N., Kordis, D. & Rozman, D. 2004 New aspects on lanosterol 14α-demethylase and cytochrome P450 evolution: lanosterol/cycloartenol diversification and lateral transfer. *J. Mol. Evol.* **59**, 51–58.

Rohmer, M. & Brandt, R. 1973 Sterols and their precursors in *Astasia longa* Pringsheim. *Eur. J. Biochem.* **36**, 446–454. (doi:10.1111/j.1432-1033.1973.tb02929.x)

Rohmer, M., Bouvier, P. & Ourisson, G. 1979 Molecular evolution of biomembranes: structural equivalents and phylogenetic precursors of sterols. *Proc. Natl Acad. Sci. USA* **76**, 847–851.

Rohmer, M., Bouvier-Nave, P. & Ourisson, G. 1984 Distribution of hopanoid triterpanes in prokaryotes. *J. Gen. Microbiol.* **130**, 1137–1150.

Rondet, S., Taton, M. & Rahier, A. 1999 Identification, characterization, and partial purification of 4 alpha-carboxysterol-C3-dehydrogenase/C4-decarboxylase from Zea mays. *Arch. Biochem. Biophys.* **366**, 249–260.

Rosing, M. T. & Frei, R. 2004 U-rich Archaean seafloor sediments from Greenland—indications of >3700 Ma oxygenic photosynthesis. *Earth Planet. Sci. Lett.* **217**, 237–244. (doi:10.1016/S0012-821X(03)00609-5)

Rothman, D. H., Hayes, J. M. & Summons, R. E. 2003 Dynamics of the Neoproterozoic carbon cycle. *Proc. Natl Acad. Sci. USA* **100**, 8124–8129. (doi:10.1073/pnas.0832439100)

Rouxel, O. J., Bekker, A. & Edwards, K. J. 2005 Iron isotope constraints on the Archean and Paleoproterozoic ocean redox state. *Science* **307**, 1088–1091. (doi:10.1126/science.1105692)

Sallal, A. K., Ghannoum, M. A., Al-Hasan, R. H., Nimer, N. A. & Radwan, S. S. 1987 Lanosterol and diacylglycerophosphocholines in lipids from whole cells and thylakoids of the cyanobacterium *Chlorogloeopsis fritschii*. *Arch. Microbiol. (Hist. Arch.)* **148**, 1–7. (doi:10.1007/BF00429638)

Segura, M., Lodeiro, S., Meyer, M., Patel, A. & Matsuda, S. 2002 Directed evolution experiments reveal mutations at

cycloartenol synthase residue His477 that dramatically alter catalysis. *Org. Lett.* **4**, 4459–4462. (doi:10.1021/ol0269897)

Shen, Y., Buick, R. & Canfield, D. E. 2001 Isotopic evidence for microbial sulphate reduction in the Early Archaean era. *Nature* **410**, 77–81. (doi:10.1038/35065071)

Shimkets, L. & Woese, C. R. 1992 A phylogenetic analysis of the myxobacteria: basis for their classification. *Proc. Natl Acad. Sci. USA* **89**, 9459–9463.

Simons, K. & Vaz, W. L. C. 2004 Model systems, lipid rafts and cell membranes. *Annu. Rev. Biophys. Biomol. Struct.* **33**, 269–295. (doi:10.1146/annurev.biophys.32.110601.141803)

Simpson, A. G. B., Inagaki, Y. & Roger, A. J. 2005 The evolutionary relationships amongst excavates: a concatened protein analysis. *J. Eukaryot. Microbiol.* **52**, 7S–27S. (doi:10.1111/j.1550-7408.2005.05202003_1_68.x)

Sleep, N. H., Meibom, A., Fridriksson, T., Coleman, R. G. & Bird, D. K. 2004 H2-rich fluids from serpentinization: geochemical and biotic implications. *Proc. Natl Acad. Sci. USA* **101**, 12 818–12 823. (doi:10.1073/pnas.0405289101)

Summons, R. E., Jahnke, L. L., Hope, J. M. & Logan, G. A. 1999 2-Methylhopanoids as biomarkers for cyanobacterial oxygenic photosynthesis. *Nature* **400**, 554–556. (doi:10.1038/23005)

Summons, R. E., Waldbauer, J. R., Buick, R., Dunlop, J. S. & Bonser, L. C. 2004 Hydrocarbon analysis of Hamersley Basin deep drill cores: preliminary results. *American Geophysical Union, Fall Meeting 2004*, abstract #B32B-05.

Tepper, H. L. & Voth, G. A. 2005 Protons may leak through pure lipid bilayers via a concerted mechanism. *Biophys. J.* **88**, 3095–3108. (doi:10.1529/biophysj.104.056184)

Thiel, V., Peckmann, J., Seifert, R., Wehrung, P., Reitner, J. & Michaelis, W. 1999 Highly isotopically depleted isoprenoids: molecular markers for ancient methane venting. *Geochim. Cosmochim. Acta* **63**, 2959–3966. (doi:10.1016/S0016-7037(99)00177-5)

Thiel, V., Blumenberg, M., Pape, T., Seifert, R. & Michaelis, W. 2003 Unexpected occurrence of hopanoids at gas seeps in the Black Sea. *Org. Geochem.* **34**, 81–87. (doi:10.1016/S0146-6380(02)00191-2)

Thoma, R., Schulz-Gasch, T., D'Arcy, B., Benz, J., Aebi, J., Dehmlow, H., Hennig, M., Stihle, M. & Ruf, A. 2004 Insight into steroid scaffold formation from the structure of human oxidosqualene cyclase. *Nature* **432**, 118. (doi:10.1038/nature02993)

Tice, M. M. & Lowe, D. R. 2004 Photosynthetic microbial mats in the 3164-Myr old ocean. *Nature* **431**, 549–552. (doi:10.1038/nature02888)

Torres, R. A. & Bruice, T. C. 1999 Theoretical investigation of the [1,2]-sigmatropic hydrogen migration in the mechanism of oxidation of 2-aminobenzoyl-CoA by 2-aminobenzoyl-CoA monooxygenase/reductase. *Proc. Natl Acad. Sci. USA* **96**, 14 748–14 752. (doi:10.1073/pnas.96.26.14748)

Volkman, J. K. 2003 Sterols in microorganisms. *Appl. Microbiol. Biotechnol.* **60**, 495–506.

Volkman, J. K. 2005 Sterols and other triterpenoids: source specificity and evolution of biosynthetic pathways. *Org. Geochem.* **36**, 139–159. (doi:10.1016/j.orggeochem.2004.06.013)

Wakeham, S. G., Sinninghe Damste, J. S., Kohnen, M. E. L. & De Leeuw, J. W. 1995 Organic sulfur compounds formed during early diagenesis in Black Sea sediments. *Geochim. Cosmochim. Acta* **59**, 521–533. (doi:10.1016/0016-7037(94)00361-O)

Walker, J. C. G., Klein, C., Schidlowski, M., Schopf, J. W., Stevenson, D. J. & Walter, M. R. 1983 Environmental evolution of the Archean–Early Proterozoic Earth. In *Earth's earliest biosphere: its origin and evolution*, pp. 260–290.

Wendt, K. U. 2005 Enzyme mechanisms for triterpene cyclization: new pieces of the puzzle. *Angew. Chem. Int. Ed.* **44**, 3966–3971. (doi:10.1002/anie.200500804)

Wendt, K. U., Poralla, K. & Schulz, G. E. 1997 Structure and function of a squalene cyclase. *Science* **277**, 1811–1815. (doi:10.1126/science.277.5333.1811)

Wendt, K. U., Schulz, G. E., Corey, E. J. & Liu, D. R. 2000 Enzyme mechanisms for polycyclic triterpene formation. *Angew. Chem. Int. Ed.* **39**, 2812–2833. (doi:10.1002/1521-3773(20000818)39:16<2812::AID-ANIE2812>3.3.CO;2-R)

Woodward, R. B. & Bloch, K. 1953 The cyclization of squalene in cholesterol synthesis. *J. Am. Chem. Soc.* **75**, 2023–2024. (doi:10.1021/ja01104a535)

Xu, F., Rychnovsky, S. D., Belani, J. D., Hobbs, H. H., Cohen, J. C. & Rawson, R. B. 2005 Dual roles for cholesterol in mammalian cells. *Proc. Natl Acad. Sci. USA* **102**, 14 551–14 556. (doi:10.1073/pnas.0503590102)

Zundel, M. & Rohmer, M. 1985 Prokaryotic triterpenoids. 3. The biosynthesis of 2 beta-methylhopanoids and 3 beta-methylhopanoids of *Methylobacterium organophilum* and *Acetobacter pasteurianus* ssp. *pasteurianus*. *Eur. J. Biochem.* **150**, 35–39. (doi:10.1111/j.1432-1033.1985.tb08984.x)