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1 **Lost world of complex life and the late rise of the eukaryotic crown**

2
3 Jochen J. Brocks^{1*}, Benjamin J. Nettersheim^{1,2*}, Pierre Adam³, Philippe Schaeffer³, Amber J. M.
4 Jarrett^{1,4}, Nur Güneli¹, Tharika Liyanage¹, Lennart M. van Maldegem¹, Christian Hallmann⁵,
5 Janet M. Hope¹
6

7 ¹ Research School of Earth Sciences, The Australian National University, Canberra, ACT 2601, Australia.

8 ² MARUM – Center for Marine Environmental Sciences and Faculty of Geosciences, University of
9 Bremen, 28359 Bremen, Germany

10 ³ Université de Strasbourg, CNRS, Institut de Chimie de Strasbourg UMR 7177, F-67000 Strasbourg,
11 France

12 ⁴ Northern Territory Geological Survey, GPO Box 4550, Darwin NT 0801, Australia

13 ⁵ GFZ German Research Center for Geosciences, 14473 Potsdam, Germany

14 *These authors contributed equally to this work. e-mail: jochen.brocks@anu.edu.au;
15 bnettersheim@marum.de
16

17 **Keywords:** stem-group eukaryotes, cycloartenol, lanostane, protosterols, eukaryote evolution,
18 Proterozoic, Tonian, ursterols
19

20 **Eukaryotic life appears to have flourished surprisingly late in the history of our planet. This**
21 **view is based on the low diversity of diagnostic eukaryotic fossils in marine sediments of mid-**
22 **Proterozoic age (~1,600 to 800 million years (Ma) ago) and an absence of steranes, the**
23 **molecular fossils of eukaryotic membrane sterols^{1,2}. This scarcity of eukaryotic remains is**
24 **difficult to reconcile with molecular clocks that suggest that the last eukaryotic common**
25 **ancestor (LECA) already emerged ~1200 to >1,800 Ma ago. LECA, in turn, must have been**

26 preceded by stem-group eukaryotic forms by several hundred million years³. We report the
27 discovery of abundant protosteroids in sedimentary rocks of mid-Proterozoic age. These
28 primordial compounds remained unnoticed because their structures represent early
29 intermediates of the modern sterol biosynthetic pathway, as famously predicted by Konrad
30 Bloch⁴. The protosteroids reveal an ecologically prominent ‘Protosterol Biota’ that was
31 widespread and abundant in aquatic environments from at least 1,640 to ~800 Ma ago and
32 that likely comprised ancient protosterol-producing bacteria and deep-branching stem-
33 group eukaryotes. Modern eukaryotes started to rise in the Tonian period (1,000 to 720 Ma),
34 fuelled by the proliferation of red algae (rhodophytes) by ~800 Ma. This ‘Tonian
35 Transformation’ emerges as one of the most profound ecological turning points in our
36 planet’s history.

37

38 MAIN TEXT

39 All living eukaryotes evolved from a last common ancestor (LECA) that lived ~1,200 to >1,800
40 Ma ago^{3,5-7}. LECA and all its descendants form the crown of the eukaryotic tree including algae,
41 plants, fungi, animals and all extant, unicellular protists. Yet, the domain Eukarya has a much
42 deeper prehistory. The ancestral line leading towards LECA, and all its extinct side-branches, are
43 stem-group Eukarya (Fig. 1b). The genome and cell structure of living descendants only provide
44 limited insights into the evolution of LECA’s ancestors⁷, and almost nothing is known about their
45 abundance, ecology and habitats. To study the hundreds of millions of years of hidden eukaryote
46 evolution and ecology, we have to search for fossil and chemical remains directly in the geological
47 record⁸.

48 Body fossils with diagnostic eukaryotic features, such as processes, protrusions and cell-wall
49 ornamentation, emerge in the rock record ~1,650 Ma ago and are found throughout the
50 Mesoproterozoic (1,600 to 1,000 Ma) (Fig. 1c, specimens f-k). Yet, these fossils lack
51 characteristics that would reliably place them within the eukaryotic crown, and so it is possible
52 that they belonged to the stem^{1,8,9}. The oldest fossils that can be assigned to the eukaryotic crown
53 with confidence are the 1,050 Ma multicellular rhodophyte (red) alga *Bangiomorpha*¹⁰ and 1,000
54 Ma chlorophyte (green) alga *Proterocladus*¹¹ (Fig. 1c, specimens c,d). However, body fossils of
55 the crown only diversified and became abundant around 900 Ma with the emergence of fungi¹²
56 followed by possible rhizarians and testate amoebozoans¹³ (Fig. 1c, specimens a,b,e).

57 **The current molecular fossil record**

58 Biomarkers, the hydrocarbon fossils of biological lipids, provide complementary evidence on
59 eukaryote evolution. Almost all eukaryotes produce tetracyclic sterols that regulate membrane
60 physiology, while a wide range of bacteria synthesize the structurally related pentacyclic
61 hopanepolyols. An increasing number of bacteria is also known to possess genes for sterol
62 biosynthesis¹⁴. Sterols and hopanepolyols are relatively stable against degradation and may
63 become preserved in sedimentary rocks as saturated steranes and hopanes as well as aromatic
64 steroid and hopanoid derivatives (Fig. 2). The relative proportion of fossilized sterols over
65 hopanepolyols is regarded as a first order estimate of the relative fluxes of eukaryotic and bacterial
66 biomass to bottom sediments in ancient aquatic ecosystems^{15,16}. The most common sterols possess
67 cholestane (plotted in red in Fig. 1a), ergostane (blue) and stigmastane (green) carbon skeletons
68 distinguished by the degree of alkylation of the side chain (Fig. 2). Such ‘crown-sterols’ occur
69 across all major lineages of crown-group Eukarya, and LECA was presumably able to produce

70 sterols with the cholestane, ergostane, and possibly stigmastane skeleton¹⁷. The trajectory of the
71 relative abundances of the three saturated crown-steranes in the geological record reflects trends
72 of eukaryote evolution¹⁸. For example, increasing abundances of ergostane from the Mesozoic to
73 Caenozoic reflect the rise of secondary endosymbiotic algae in the oceans¹⁸, and the predominance
74 of stigmastane in the Ediacaran and Paleozoic suggests that chlorophyte algae were the prevalent
75 eukaryotic phototrophs^{18,19}. But, stepping back in time into the Cryogenian and Tonian periods,
76 the abundance of steranes relative to hopanes drops steeply, and steranes almost exclusively
77 comprise cholestane, with traces of ergostane but no stigmastane (Fig. 3a).

78 The combined record of saturated steranes and body fossils of the past 800 Ma presents a coherent
79 picture, with an initial increase in modern eukaryotic forms in the Tonian period, and a massive
80 expansion in the Ediacaran. However, deeper in time the records become contradictory. While
81 eukaryotic body fossils are found throughout the mid-Proterozoic interval, crown-steranes
82 persistently remain below detection limits (Fig. 3a). The absence of these steranes has been
83 explained by systematic preservation bias²⁰, prevalence of anaerobic eukaryotes that do not
84 produce sterols²¹⁻²³, or ecosystems pervasively dominated by prokaryotes^{2,22,24-27}. Yet, current
85 ecological hypotheses struggle to explain the apparent scarcity of aerobic eukaryotes over a time
86 interval of more than half a billion years and across all environments where biomarkers are
87 detectable, including apparently nutrient-rich and oxygenated habitats (Supplementary Text)²⁶.
88 One hypothesis remains untested, the prospect that mid-Proterozoic ecosystems were dominated
89 by eukaryotic stem-group forms that did not yet possess a full sterol biosynthetic pathway.

90 **Protosterols and ursterols**

91 Konrad Bloch, who deciphered the biosynthetic pathway of cholesterol, suggested that primordial
92 eukaryotes may not have possessed the full sterol biosynthetic pathway but instead generated
93 ancestral ‘ursterols’⁴. The modern sterol biosynthetic pathway commences with the cyclization of
94 oxidosqualene to one of two protosterols, lanosterol or cycloartenol (Fig. 2a). In energy and
95 oxygen intensive downstream modifications, three methyl groups are then removed, double bonds
96 modified, and new alkyl moieties added to the side chain. According to Bloch, each intermediate
97 in this pathway, called an ursterol, was hundreds of millions of years ago a fully adapted end-
98 product and an evolutionary improvement over its precursor. As LECA already possessed the
99 capacity to produce crown-sterols, the ursterol pathway must have been assembled before LECA
100 emerged. Cycloartenol in particular is a fully functional membrane molecule^{28,29}, and it may have
101 been the sole sterol in deep-branching stem-group eukaryotes. Yet, the molecular fossils of
102 protosterols and ursterols—saturated and aromatic protosteroids and ursteroids—remain largely
103 unrecorded or unknown. In this study we apply new molecular search templates to detect these
104 compounds in the deep geological record.

105 **A new Proterozoic biomarker record**

106 We obtained identification standards for fossil protosteroids and ursteroids through synthesis and
107 by subjecting lanosterol and mixtures of cycloartenol and 24-methylene cycloartenol, to artificial
108 maturation through pyrolysis. We then identified and quantified saturated and aromatic
109 protosteroids in bitumens and oils of Caenozoic to late Palaeoproterozoic age using gas
110 chromatography-mass spectrometry. All analyses were performed under strict exclusion of
111 contamination (Methods). The oldest biomarker assemblage from the 1,640 Ma Barney Creek
112 Formation revealed nearly 100 different protosterol derivatives, including lanostane, mono- and

113 diaromatic lanosteroids, and previously observed³⁰ triaromatic steroids. Also present in most
114 bitumens and oils—albeit not in the pyrolysis products—was pentacyclic ‘cyclosterane’,
115 tentatively interpreted as a geological transformation product of cycloartenol. Demethylated
116 derivatives of some di- and triaromatic steroids may, in principle, also stem from cholesteroloids or
117 other downstream products of the sterol biosynthetic pathway. However, absence of detectable
118 saturated counterparts such as cholestane and 4-methylcholestane, and absence of monoaromatic
119 cholesteroloids, makes such sources unlikely (Supplementary Text 2.8). Moreover, the presence of
120 saturated lanostane shows that the observed assemblage is unlikely to result from uncommon
121 diagenetic conditions that led to a quantitative aromatization of steroidal precursors. Thus,
122 biologically demethylated sterols, if they existed, could have only been a minor component of the
123 assemblage. Theoretically, the detected aromatic compounds may also derive from precursors of
124 the euphol/tirucallol family, distinguished from protosterols by 13 α ,14 β stereochemistry.
125 However, the absence of 13 α ,14 β -euphanes and presence of 13 β ,14 α -lanostanes in saturated
126 fractions excludes such sources (Extended Data Fig. 3b). Our laboratory experiments thus show
127 that all fossilized steroids in the mid-Proterozoic record can be explained as degradation products
128 of protosterols and 24-methyl ursterols (Fig. 2, Extended Data Fig. 1–9). Through the mid-
129 Proterozoic interval, these protosteroids were detected in deep and relatively shallow water
130 environments, microbial mats and pelagic habitats, shales and carbonates, as well as marine and
131 likely lacustrine basins (Supplementary Text).

132 In all investigated bitumens, proto- and ursterols are overwhelmingly preserved as aromatic
133 products, and Figure 3b summarizes the relative abundances of aromatic steroids and hopanoids
134 through time. This aromatic biomarker record provides a complementary view to the trends of
135 saturated steranes and hopanes described above (Fig. 3a), revealing unknown aspects of eukaryote

136 evolution. In the Palaeoproterozoic, the high relative abundances of aromatic protosteroids (shades
137 of purple) relative to hopanoids is striking, with proportions of steroids approaching Phanerozoic
138 levels. High proportions of protosteroids are observed to the end of the Mesoproterozoic, with the
139 additional appearance of side-chain methylated triaromatic ursteroids at ~1,300 Ma (cyan in Fig.
140 1a, 3b).

141 The new biomarker record thus reveals a ‘Protosterol Biota’ that was widespread and highly
142 abundant in the mid-Proterozoic (Fig. 1). Despite this profusion of protosteroids, the same mid-
143 Proterozoic formations failed to yield even traces of cholestane, ergostane and stigmastane,
144 bringing the absence of crown-steranes before 800 Ma into sharp focus (Fig. 3a). Our results show
145 that the lack of crown-steranes can no longer be dismissed as preservation artefact²⁰ but must
146 reflect extreme scarcity or absence of crown-group eukaryotes in open water habitat throughout
147 this interval.

148 The first crown-steroids emerge ~800 Ma ago (Fig. 1a). The record of saturated steranes in the
149 Tonian, Cryogenian and earliest Ediacaran is characterized by dominant cholestane, with sub-
150 percentage levels of ergostane in some samples, but a lack of stigmastane (Fig. 3a). However, in
151 contrast to earlier studies that exclusively focused on this saturated sterane record^{15,16,19}, the
152 aromatic hydrocarbon compilation reveals that steroids are far more abundant in the Tonian period
153 than previously appreciated (Fig. 3b). Moreover, ergosteroids constitute a one to two order of
154 magnitude larger proportion of total steroids, comprising up to 21% of monoaromatic steroids
155 (mean \pm stdv: $5.9 \pm 6.5\%$; n=16) and 33% of triaromatic steroids ($6.7\% \pm 11.6\%$, n=26) (Fig. 1a).
156 In modern eukaryotes, this steroid homolog abundance pattern is found in rhodophytes (red algae)
157 that have a cholesterol predominance of up to 100%, but typically contain ~5% ergosteroids¹⁵.

158 The data thus substantiate previous suggestions^{16,31,32} of an early expansion of rhodophytes and
159 suggest that algal primary production already started to rise in the Tonian period (Fig 3c). The
160 emergence of stigmasteroids after 635 Ma then heralds a second revolution at the base of the food
161 web, the rise of chlorophyte algae^{15,19}. In the Phanerozoic, the average abundance of aromatic
162 protosteroids relative to aromatic crown-steroids drops to $\sim 3.8 \pm 4.0\%$ (n=36), a proportion that
163 can be attributed to biosynthetic intermediates produced by crown-group eukaryotes plus unknown
164 contributions from bacteria (Supplementary Text).

165 **The origins of ancient protosteroids**

166 Apart from Eukarya, 94 bacterial taxa across twelve phyla are currently known to possess the genes
167 SQMO (squalene monooxygenase) and OSC (oxidosqualene cyclase) required for protosterol
168 biosynthesis³³. The bacterial and eukaryotic genes have a common ancestry in the
169 Palaeoproterozoic and were horizontally transferred between the domains. By the mid-Proterozoic,
170 stem-group eukaryotes and bacteria both likely had the capacity to produce sterols³⁴.

171 The importance of sterol producing bacteria in the mid-Proterozoic is difficult to assess. Based on
172 the shallow clade depths of SQMO and OSC and their sporadic phylogenetic distribution in extant
173 Bacteria^{34,35}, most taxa must have acquired sterol biosynthesis genes relatively recently via
174 horizontal gene transfer (Supplementary Text 8.2, Supplementary Fig. 1 and 2). The roots of
175 SQMO and OSC are deepest among the orders Methylococcales and Myxococcales³³, and these
176 groups are thus the most likely sources of bacterial sterols in the Proterozoic. Yet, most extant
177 Methylococcales and deep-branching (Group I) Myxococcales mainly produce 4-
178 methylcholesteroids and cholesterol, respectively³⁵. Since the corresponding saturated
179 hydrocarbons, 4-methylcholestane and cholestane, were below detection limits in all samples >800

180 Ma, crown-group Methylococcales and Group I Myxococcales were not likely major components
181 of the studied environments. However, contributions to the Protosterol Biota may have come from
182 stem-representatives of these two bacterial orders, or from unknown or extinct bacterial groups.
183 The importance of such contributions depends on the general abundance of sterol-producing
184 bacteria in aquatic environments. While bacterial SQMO and OSC are detected in a wide variety
185 of modern habitats³⁵, bacterial sterols themselves have only been reported from less common
186 environments such as hydrothermal springs and methane seeps (Supplementary Text). The mid-
187 Proterozoic likely witnessed elevated methane generation in marine sediments compared to the
188 present, providing potential substrate for Methylococcales³⁰. However, the carbon isotopic
189 signature of triaromatic steroids from the 1,640 Ma Barney Creek Fm ($\delta^{13}\text{C} = -19$ to -22‰)³⁶ is
190 inconsistent with a methanotrophic origin and suggests heterotrophic³⁶ or phototrophic sources.
191 Generally, however, bacterial sterol contributions to mid-Proterozoic environments are plausible.
192 A test for such contributions does not exist at present, and future research needs to evaluate the
193 importance and antiquity of sterol producing bacteria in marine environments. A solely bacterial
194 origin of the protosteroids would open the fascinating proposition that aerobic crown- as well as
195 stem-group eukaryotes remained extremely scarce for hundreds of millions of years after their
196 emergence.

197 Stem-group eukaryotic origins of protosteroids, on the other hand, can reconcile the continuous
198 record of eukaryote-like fossils with the scarcity of crown-steroids throughout the mid-Proterozoic
199 interval and may explain why all but two Mesoproterozoic species lack diagnostic crown-group
200 features. Early eukaryotic fossils already possessed complex cell-wall ornamentation and
201 symmetrically- or asymmetrically-arranged, cylindrical and occasionally branched processes,
202 structures that probably required a flexible endomembrane system³⁷ with active curvature control,

203 a capability aided by sterols in extant Eukarya³⁸. Moreover, large but simple fossils interpreted as
204 possibly eukaryotic, such as leiosphaerids, are widespread in mid-Proterozoic sediments³⁷ and may
205 have been a prominent source of protosteroids. A stem-group eukaryotic origin of protosteroids is
206 also supported by the detection of 24-methylated triaromatic sterols in Mesoproterozoic strata
207 that may derive from ursterols such as 24-methylenecycloartenol, obtusifoliol or 4-
208 methylfecosterol. Some bacteria possess SMT (sterol methyltransferase), a gene imparting the
209 hypothetical ability to methylate the side chain at C-24. However, most SMT gene trees^{33,39} do not
210 support a deep origin of side-chain methylation in bacteria, and side-chain methylated sterols have
211 never been detected in bacterial lipid extracts³⁵. By contrast, SMT was already present in
212 LECA^{17,33,40}. If a eukaryotic interpretation is correct, then the side-chain methylated ursteroids
213 may represent an evolutionary intermediate in the long biosynthetic pathway from cycloartenol to
214 ergosterol, assembled in the stem-lineage toward LECA (Fig. 1b, 2a). In ~1,300 Ma old sediments
215 these side-chain methylated steroids comprise ~20% of total steroids, pointing to notable
216 abundances of such intermediate organisms. In summary, we posit that the Protosterol Biota
217 partially or predominantly represents early stages of eukaryote evolution that did not yet possess
218 a complete sterol biosynthetic pathway.

219 **The late rise of the eukaryotic crown**

220 The paucity of fossils of crown-group eukaryotes before ~1,050 Ma and of crown-steranes before
221 ~800 Ma remains a conundrum in early-life research. Crown-group eukaryotes likely emerged
222 between ~1,200 to >1,800 Ma^{3,5,6} and rapidly diverged into major branches⁷. Based on all but the
223 youngest molecular clock estimates, the crown thus reached considerable morphological and
224 ecological diversity before 1,000 Ma, including sexually reproducing, multicellular algae¹⁰, and

225 protists capable of endocytosis, phagocytosis and locomotion⁴¹. Based on the predicted taxonomic
226 and ecological diversity of Mesoproterozoic crown-group eukaryotes, the scarcity of diagnostic
227 fossils and biomarkers seems difficult to explain.

228 The discovery of the Protosterol Biota now suggests that the ecological success of early crown-
229 group eukaryotes may have been shaped by competition with early-diverging stem-group relatives
230 over several hundred million years of their co-existence (Fig. 1b and ref²³). As all stem-group
231 eukaryotes are now extinct, we remain oblivious to differences between crown-group and stem-
232 group eukaryotes that co-existed in the mid-Proterozoic. However, the fossil protosteroids indicate
233 that the composition of cell membranes was one of the physiological distinctions. The biosynthesis
234 of crown-sterols comes at immense metabolic cost and must have conferred distinct advantages to
235 the first eukaryotes adopting this pathway. Based on experiments on yeast, specific unsaturation
236 patterns of crown-sterols may protect cells against osmotic shock during desiccation and
237 rehydration⁴². Similarly, addition of an ethyl group to the sterol side chain adapts plant cells to
238 cold shock and heat as the ethyl group appears to enhance cohesion with other lipids, providing
239 better membrane ordering over large temperature scales^{29,43}. Moreover, through branch-points in
240 the sterol biosynthetic pathway, crown-group eukaryotes can regulate the proportion of different
241 sterols. This capacity further extends the temperature range of membrane-associated processes^{29,43},
242 may play a role in membrane repair during UV-B exposure⁴³ and promotes asymmetric cell
243 growth²⁹. Potentially important but more speculative is the role of down-stream modified sterols
244 in the protection of cell membranes against attack by membranolytic toxins⁴⁴⁻⁴⁶ and for the
245 response of cells to changing O₂ levels^{43,47}. These sterol-based mechanisms to adapt to physical
246 and chemical extremes were absent in organisms that solely produce protosterols. We thus posit
247 that crown-sterols enabled LECA's ancestors to radiate into marginal niches such as agitated

248 shoreline facies, periodically exposed mudflats, braided rivers and subaerial habitat, environments
249 that experience cycles of desiccation and rehydration, diurnal extremes of high and low
250 temperatures, and elevated UV-radiation. If correct, then early crown-group representatives were
251 eukaryotic extremophiles of their time.

252 Biomarkers are rarely preserved in sediments deposited in well-oxygenated, high-energy
253 environments, and an origin of LECA's ancestors in such habitats may explain why crown-steranes
254 have remained elusive in mid-Proterozoic sediments. This view is supported by the discovery of
255 the oldest accepted crown-group fossils, rhodophytes and chlorophytes, in shallow water
256 deposits^{10,11} (Fig. 1c).

257 The new biomarker record suggests that crown-sterol producers were not able to break into niches
258 inhabited by the Protosterol Biota in benthic and pelagic open-water settings. During the mid-
259 Proterozoic, atmospheric oxygen levels were still one to three orders of magnitude below the
260 present^{48,49} and surface waters may have become anoxic during the dark hours or experienced
261 incursions of sulphidic waters, toxic to crown-group Eukarya²⁴. Stem-group eukaryotes may have
262 possessed traits that allowed them to thrive in such conditions, and protosterol biosynthesis may
263 have provided an advantage due to its lower oxygen and energy requirements compared to
264 downstream sterols. Overall, the biomarker record is consistent with a facultatively anaerobic
265 origin of eukaryotes where the stem lineage moved from anoxic to increasingly oxygenated
266 environments in the course of the mid-Proterozoic^{50,51}.

267 The Tonian period eventually saw a rise in atmospheric oxygen levels, increased nutrient supply
268 to the oceans and changing abundances of bioessential trace elements^{48,52}. The ecological, physical
269 and chemical changes that caused, or permitted, the expansion of crown-group eukaryotes into

270 open marine environments remain unknown, but a preference for more oxygenated habitats and
271 their crown-sterol supported membranes may have provided crucial pre-adaptations for the
272 changing conditions of the Tonian. Exactly when the last branches of the eukaryotic stem became
273 extinct remains unknown, but competition from expanding crown-group eukaryotes may have
274 contributed to their demise. Their protosterol containing membranes may also have proved
275 maladapted to the cold of the Cryogenian Snowball Earth glaciations and the extreme global heat
276 in the aftermath⁵³.

277 **Konrad Bloch's Ursterols**

278 In the essay 'Evolutionary Perfection of a Small Molecule'⁴, Konrad Bloch suggested that the
279 discovery of fossil 'ursterols' would strengthen the idea of molecular evolution, the notion that
280 modern intermediates were once fully functional end products in ancient biosynthetic pathways.
281 Bloch was sceptical that fossilized proto- and ursterols could be discovered, yet we now find that
282 they are abundantly preserved in the oldest biomarker-bearing rocks. The detected compounds
283 represent at least three enzymatic steps (OSC, SQMO, SMT) in the biosynthetic pathway towards
284 modern eukaryotic ergosteroids. We posit that these fossil proto- and ursteroids are witnesses of a
285 lost world of ancient stem-group eukaryotes that were widespread and possibly abundant during
286 Earth's middle age. Our crown-group eukaryotic ancestors may have co-existed with these
287 primordial organisms for several hundred million years, unable to occupy their niches. With rising
288 atmospheric oxygen levels and changing marine chemistry, crown-group Eukarya started to
289 expand 1,000 to 800 Ma ago. This 'Tonian Transformation' is recorded in the body and molecular
290 fossil records and may be one of the most profound ecological transitions in the evolution of
291 complex life.

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426 **FIGURE LEGENDS**

427 **Fig. 1 | Geological time chart (in billion years, Ga) comparing the molecular fossil, microfossil**

428 **and phylogenetic records of early eukaryote evolution. a,** Relative abundances of aromatic

429 protosteroids (purple and cyan tones) and crown-steroids (reds, blues and greens), highlighting the

430 transition from a Protosterol Biota to a ‘Crown-sterol Biota’ in the Neoproterozoic. Each

431 horizontal colour bar represents one sample, and grey triangles assign data bundles to geological

432 units 1 to 11 (key provided in Methods). prot = protosteroids, meprot = 24-methyl protosteroids

433 (ursteroids), chol = cholesteroids, erg = ergosteroids, stig = stigmasteroids. Key to colours see

434 panel Figure 2 and Supplementary Table 1. For details on data assembly and geological formations

435 (1–11) see Methods. **b,** Phylogenetic tree of the domain Eukarya with white, red and green

436 highlighting crown-group branches. Stem-group branches (purple) are hypothetical only,

437 illustrating the notion that mid-Proterozoic ecosystems may have been dominated by extinct stem

438 forms that did not produce crown-sterols. LECA, the last common ancestor of all extant

439 eukaryotes, may have emerged between 1.2 and > 2.0 Ga (see text). **c,** Microfossils of early

440 eukaryotes. (a–e) Likely crown-group Eukarya (~1.1 to 0.7 Ga), (f–k) microfossils that are

441 possibly or certainly eukaryotic but lack diagnostic crown-group characteristics. See Methods for

442 detailed information and image credits. Tn, Cr, Ed are the Tonian, Cryogenian and Ediacaran

443 periods; P, M, C are the Palaeo-, Meso- and Caenozoic eras.

444 **Fig. 2 | Juxtaposition of biological and geological hopanoids and steroids, and the evolution**

445 **of the sterol biosynthetic pathway through time. a,** The pathway from squalene epoxide to

446 protosterols, ursterols and crown-sterols was likely assembled during the Proterozoic. ‘2x’

447 indicates number of successive enzymatic steps. **b,** Fossil steroids detected in the rock record that

448 correspond to intermediates and end-products in the evolving biosynthetic pathway. TAS =
449 triaromatic steroids, MAL = monoaromatic lanosteroids, DAL = diaromatic lanosteroids, 'x,4-
450 Me₂TAS' signifies a TAS methylated at C-4 and at an unknown position x. '?' indicates that
451 cyclosterane likely contains a bridge at an unknown position.

452 **Fig. 3 | The succession of fossil steroids and bacterial hopanoids through time. a,** The relative
453 abundances of saturated steranes (red, blue and green) and bacterial hopanes (hop, yellow). Note
454 absence of detectable steranes before 800 Ma, highlighting the paucity of crown-group eukaryotes.
455 **b,** A new view of biomarker evolution based on the relative abundances of aromatic protosteroids
456 (purple), 24-methyl protosteroids (ursteroids, cyan), aromatic crown-steroids (reds, blues and
457 greens) and aromatic hopanoids (yellow). The data reveal the existence of a protosterol-producing
458 biota that was ecological dominant in the Proterozoic. For (a) and (b), each horizontal colour bar
459 represents one sample. Key to colours, formations and abbreviations see Fig. 1. **c,** The geological
460 succession of dominant primary producers (PP) based on molecular fossils.

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464 **METHODS**

465 **Assembly of contamination-free biomarker data.** In biomarker research, identification of any
466 traces of hydrocarbon contamination is essential. The problem of contamination in Precambrian
467 biomarker research was discussed previously^{15,54}. In the present study, tests for hydrocarbon
468 syngeneity comprised cumulative laboratory system blanks and the quantitative comparison of
469 hydrocarbon contaminants on rock surfaces and rock interiors. The removal and quantification of
470 contaminants is based on well-established protocols⁵⁵⁻⁵⁸. Briefly, the laboratory system blanks
471 captured all procedures from rock crushing and powder preparation, extraction of bitumens with
472 solvents, column chromatography and instrumental analyses. Biomarker data was generally
473 discarded if blank levels of individual compounds reached 10% of the concentration in sample
474 extracts. Contaminants introduced into rock samples before analysis, for instance during drilling
475 and storage, were identified and eliminated using Exterior/Interior (E/I) experiments. In these
476 experiments, younger contaminant hydrocarbons are recognized by removing exterior rock
477 surfaces⁵⁵, followed by separate quantification of individual compounds in the exterior portions of
478 the rock ('E') and its interior ('I'). Indigenous hydrocarbons commonly yield $E/I \sim 1$, and surficial
479 contaminants are recognized by $E/I \gg 1$ and by E/I values that increase with rising carbon number
480 within homologous series, an effect caused by contaminant diffusion from rock surfaces into the
481 interior^{55,56,58,59}. For Precambrian biomarker data used to assemble Figures 1 and 3 and
482 summarized in Supplementary Tables 1 and 2, biomarker syngeneity was established using criteria
483 given in the references above, whereas samples that fulfilled the contamination criteria were not
484 included in our study.

485 **Rock sample preparation.** Provenance and lithology of all pre-Ediacaran rock samples are
486 summarized in Extended Data Table 1, and for all other samples in Supplementary Table 3. In

487 Exterior/Interior experiments (see above), rock surfaces were either trimmed using a solvent-
488 cleaned diamond wafering saw^{56,59} (Buehler Isomet™ 1000; Illinois, U.S.A) or, for fissile
489 mudstones and shales, surface ablation using a modified KG-1 Mini-Sonic tumbler⁵⁵ (Diamond
490 Pacific, USA). Exterior and interior rock fragments were ground to a < 240 mesh powder using a
491 Rocklabs iron puck mill. The mill was cleaned between samples by rinsing with methanol (MeOH)
492 and dichloromethane (DCM), and by grinding annealed (600°C, 9 h) quartz sand.

493 **Extraction and fractionation of bitumens.** Bitumens were extracted from rock powders using a
494 Dionex Accelerated Solvent Extractor (ASE 200) using 100% DCM or a mixture of 90% DCM
495 and 10% MeOH. Solvent was reduced to ~100 µl under a stream of purified nitrogen gas. Rock
496 extracts and petroleum samples were fractionated into saturated, aromatic, and polar fractions
497 using micro column chromatography over annealed and dry packed silica gel. Saturate
498 hydrocarbons were eluted with 0.5 dead volumes (DV) of *n*-hexane, aromatic hydrocarbons with
499 2 DV of *n*-hexane : DCM (1:1 or 4:1 v/v) and polars with 3 DV DCM : MeOH (1:1 v/v). As
500 internal standard for full scan and selected ion recording (SIR) experiments, 1 to 2 µg 18-MEAME
501 (18-methyl-eicosanoic acid methyl-ester; Chiron Laboratories AS) were added to the saturated and
502 aromatic fractions and/or 50 ng D10 (Pyrene-d₁₀, Chiron) to the aromatic fraction. For metastable
503 reaction monitoring (MRM) experiments, 25 to 50 ng of D4 (d₄-C₂₉-ααα-ethylcholestanol; Chiron
504 Laboratories AS) were added to the saturated hydrocarbon fraction.

505 **Characterization of protosteroids.** Saturated and aromatic protosteroids were identified by their
506 mass spectra, and co-injection and co-elution experiments with authentic standards and/or
507 pyrolysis products of lanosterol, cycloartenol and 24-methylenecycloartenol (Supplementary
508 Methods). Structures, mass chromatograms and mass spectra are given in Extended Data Figures

509 1 to 9. ^1H and ^{13}C -NMR chemical shifts of synthetic monoaromatic lanosteroid (20R MAL) are
510 provided in Supplementary Table 4.

511 **Gas chromatography-mass spectrometry (GC-MS).** Steroids and hopanoids were evaluated and
512 quantified using GC-MS analyses on an Agilent 6890 GC coupled to a Micromass Autospec
513 Premier double sector MS (Waters Corporation, Milford, MA, USA). The GC was equipped with
514 a 60 m DB-5 capillary column (0.25 mm i.d., 0.25 μm film thickness; Agilent J&W Scientific,
515 Agilent Technologies, Santa Clara, CA, USA), and helium was used as the carrier gas at a constant
516 flow of 1 ml/min. Samples were injected in splitless mode into a Gerstel PTV injector at 60°C
517 (held for 0.1 min) and heated at 260°C min^{-1} to 300°C. The MS source was operated at 260°C in
518 EI mode at 70 eV ionization energy and 8000 V acceleration voltage. All samples were injected in
519 *n*-hexane to avoid deterioration of chromatographic signals by FeCl_2 build-up in the MS ion source
520 through use of halogenated solvents⁶⁰. The GC oven was programmed from 60°C (held for 4 min)
521 to 315°C at 4°C min^{-1} , with a total run time of 100 min. Saturated steranes and hopanes were
522 quantified using metastable reaction monitoring (MRM), and precursor \rightarrow product transitions and
523 MS parameters are given in Supplementary Tables 5 and 6. Aromatic (proto)steroids and
524 hopanoids were quantified using selected ion recording (SIR) of base or molecular ions under
525 magnet control. Recorded ions and MS parameters are given in Supplementary Table 7. All ratios
526 and abundance proportions are reported uncorrected for differences in MS-response. Mass spectra
527 were collected using full scan experiments at 1,000 resolution and with a total cycle time of 1.28
528 sec. The identity of 24-methylated triaromatic steroids was confirmed by elution time experiments
529 on a VF-200ms GC capillary column (60 m, 0.25 mm i.d., 0.25 μm film thickness; Agilent J&W
530 Scientific, Agilent Technologies, Santa Clara, CA, USA) using the same parameters as above.

531 **Assembly of Figure 3a.** Figure 3a shows the evolution of the relative abundances of saturated
532 hopanes (hop, in yellow) and saturated steranes (cholestanes, chol in red; ergostanes, erg in blue;
533 stigmastanes, stig in green) through geological time. Each horizontal bar represents one bitumen
534 or oil. The proportion of each colour in the horizontal bars is proportional to the relative
535 abundance of the respective compound class (expressed in %). For example, 100% yellow in the
536 Palaeo- and Mesoproterozoic indicates that these samples contain hopanes but no detectable
537 steranes; and predominant yellow, variable amounts red and nearly invisible traces of blue in the
538 Tonian indicate that hopanes predominate and that steranes are overwhelmingly dominated by
539 cholestanes with sub-percent traces of ergostanes but no detectable stigmastanes. A key to the
540 colours and all molecules included in each compound class is supplied in Supplementary Table 2.
541 In Figure 3a, samples are bundled into packages that represent broad geological time units or
542 geological formations. For the Neoproterozoic to present, samples are bundled into the Tonian,
543 Cryogenian, earliest Ediacaran, late Ediacaran, Paleozoic, Mesozoic and Caenozoic. The exact age
544 of individual samples is given in Supplementary Table 3. Palaeo- and Mesoproterozoic bitumens
545 were bundled into individual geological formations or groups labelled (1) to (11) in the time bar
546 of Figure 1 and 3. Information about each geological unit is given in Methods ‘Key to geological
547 formations in Figures 1 and 3’. Grey shaded triangles to the left of Figure 1a and 3a assign these
548 geological units to sample bundles. Care thus needs to be taken not to misread the colour charts as
549 a continuous record of biomarker data through time. Biomarker bearing units are rare in the
550 Proterozoic, and the record is temporally sparse and fragmented. Major temporal gaps in the record
551 are represented by grey background.

552 **Assembly of Figures 1a and 3b.** Figure 3b shows the evolution of the relative abundances of
553 aromatic hopanoids (yellow), aromatic protosteroids (shades of purple), aromatic 24-methyl

554 protosteroids or ‘ursteroids’ (cyan and light blue-green), and aromatic crown-steroids including
555 aromatic cholesteroloids (shades of red), ergosteroids (shades of blue) and stigmasteroids (shades of
556 green). Figure 1a shows the same data but excludes hopanoids. Different colour shades represent
557 mono-, di- and triaromatic steroids as well as different degrees of ring-system methylation. A key
558 to the colours of all molecules included in each compound class is supplied in Supplementary
559 Table 1, and the corresponding mass chromatograms, mass spectra and chemical structures are
560 given in Extended Data Figures 1 to 9. As described in Methods ‘Assembly of Figure 3a’,
561 individual samples are bundled into geological units, formations or groups, and the figure should
562 not be read as a continuous time record. See Supplementary Text for information about how
563 individual mass chromatographic signals were assigned to protosteroid, ursteroid or crown-steroid
564 sources. How different diagenetic triaromatic steroids (TAS) that lost methyl groups were assigned
565 to protosterol or crown-sterol sources is described in detail in Supplementary Text 2.8.

566 **Key to geological formations in Figures 1 and 3.** **1** = 1,640 Ma Barney Creek Fm, McArthur
567 Basin. **2** = 1,460 Ma Hongshuizhuang, **3** = 1,439 Ma Tieling, **4** = 1,392 Ma Xiamaling Fms, North
568 China Craton. **5** = 1,308 Ma Velkerri and Kyalla fms, McArthur Basin. **6** = 1,100 Ma Nonesuch
569 Fm, Keweenawan Rift. **7** = 1,100 Ma En Nesoar and Tourist fms, Taoudeni Basin. **8** = 1,000 Ma
570 Ui and Lakhanda groups, Sette-Daban fold belt. **9** = 750 Ma Visingsö Gr, Lake Vättern Basin; 729
571 Ma Walcott Mm, Grand Canyon; 777–725 Ma Hussar Fm, 734 Ma Kanpa Fm, 717–725 Ma
572 Steptoe Fm, Officer Basin; < 820 Ma Loves Creek and Johnnys Creek fms, > 750 Ma Wallara Fm,
573 Amadeus Basin. **10** = 659 Ma Aralka Fm, Amadeus Basin. **11** = 635 Ma Guia and Mirassol d'Oeste
574 fms, Araras Group.

575 **Key to fossils in Figure 1c.** (a) *Melicerion poikilon*, a possible testate rhizarian with an age of
576 ~ 742 Ma⁶¹. Image courtesy S. Porter, Fig. 15 (panel 3) in ref⁶¹. (b) *Bonniea dacruchares*, a testate
577 amoebozoan with an age of ~ 742 Ma⁶¹. Image courtesy S. Porter, Fig. 9 (panel 9) in ref⁶¹. (c)
578 *Bangiomorpha pubescens*, a likely bangiacean rhodophyte alga⁶² with an age of $1,047 \pm 13/-17$
579 Ma⁶³. Image courtesy N. Butterfield, Fig. 9A in ref⁶⁴. (d) *Proterocladus antiquus*, a likely
580 multicellular, benthic, siphonocladalean chlorophyte alga with an age of $1,056$ to 947.8 Ma¹¹.
581 Image courtesy Q. Tang, Fig. 2g in ref¹¹. (e) *Ourasphaira giraldae*, a likely fungus with an age of
582 $1,010$ to 890 Ma¹². Image courtesy C. Loron, Fig. 1b in ref¹². (f) *Trachyhystrichosphaera aimika*,
583 a microfossil with diagnostic eukaryotic features from the $1,100$ Ma En Nesoar Formation. Image
584 courtesy J. Beghin, Fig. 4a in ref⁶⁵. (g) *Leiosphaeridia jacutica*, a microfossil of possible
585 eukaryotic origin from the $1,100$ Ma En Nesoar Formation. Image courtesy J. Beghin, Fig. 2e in
586 ref⁶⁵. (h) *Satka favosa*, a microfossil with diagnostic eukaryotic features from the $\sim 1,300$ Ma Roper
587 Group⁹. Image courtesy E. Javaux, Fig. 5(9) in ref⁹. (i) *Valeria lophostriata*, a microfossil with
588 diagnostic eukaryotic features from the $\sim 1,300$ Ma Roper Group⁹. Image courtesy E. Javaux, Fig.
589 7(2) in ref⁹. (j) *Tappania plana*, a microfossil with diagnostic eukaryotic features from the $\sim 1,600$
590 Ma Ruyang Formation¹. Image courtesy Y. Leiming, Fig. 4A in ref¹. (k) *Shuiyousphaeridium*
591 *macroreticulatum*, a microfossil with diagnostic eukaryotic features from the $1,600$ Ma Ruyang
592 Formation¹. Image courtesy Y. Leiming, Fig. 4B in ref¹. [Permissions pending]

593 **Data availability.** All processed data generated during this study are included in this published
594 article and its supplementary information files. Raw data is available from the corresponding
595 author on reasonable request.

596

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655 **Author Contributions**

656 J.J.B. and B.J.N. interpreted the data and wrote the paper with contributions from C.H. and all co-
657 authors. B.J.N., A.J.M.J., N.G., T.L. L.M.v.M, J.H. and J.J.B. conducted the biomarker analyses.
658 B.J.N. and J.H. conducted pyrolysis and ring opening experiments. P.A. and P.S. synthesized
659 standards, conducted RuO₄ oxidation experiments and assisted with compound identification.
660 J.J.B. conceived the project and compiled data and figures.

661

662 **Competing interests statement**

663 The authors declare no competing interests.

664

665 **Additional Information**

666 Supplementary Information is available for this paper.

667 Correspondence and requests for materials should be addressed to J.J.B. and B.J.N.

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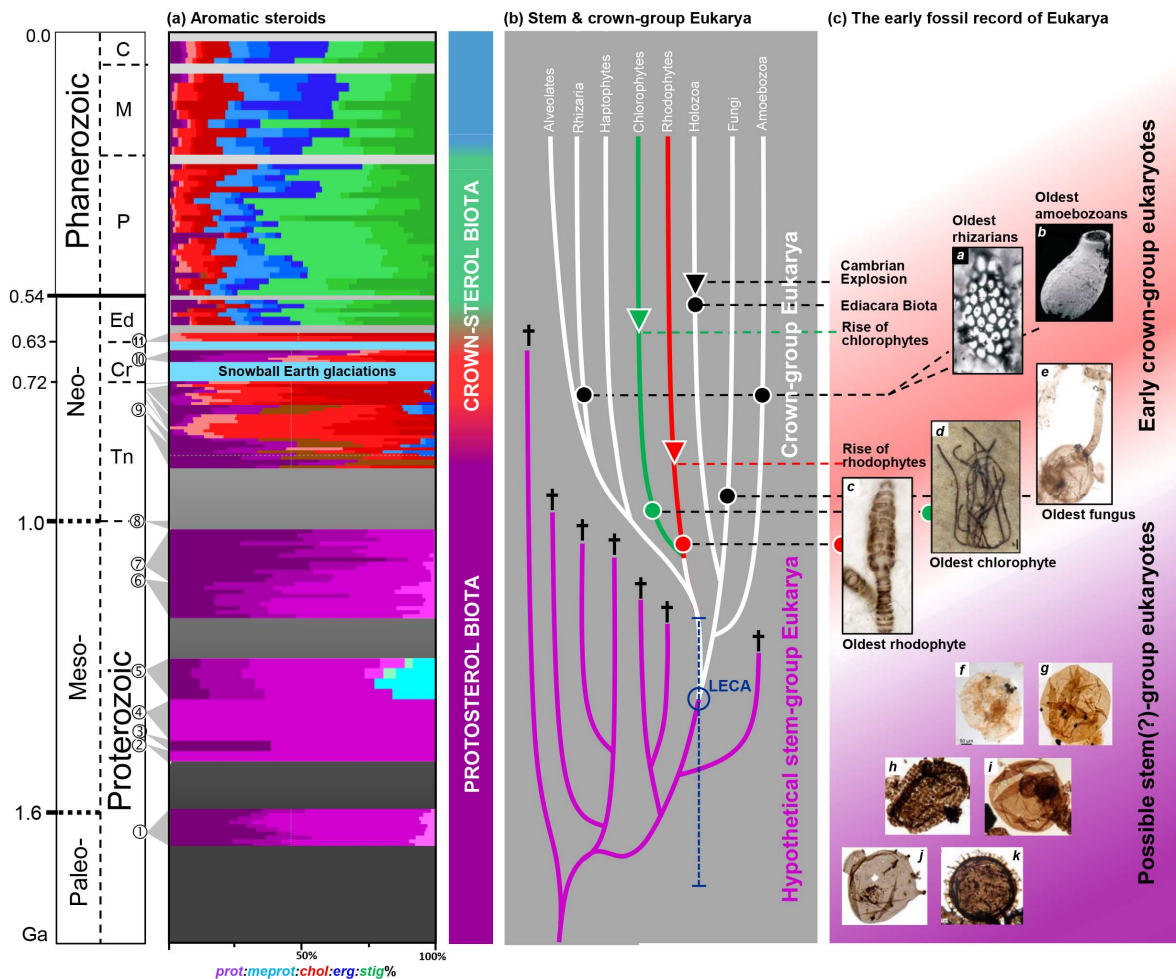


Fig. 1 | Geological time chart (in billion years, Ga) comparing the molecular fossil, microfossil and phylogenetic records of early eukaryote evolution. a, Relative abundances of aromatic protosteroids (purple and cyan tones) and crown-steroids (reds, blues and greens), highlighting the transition from a Protosterol Biota to a ‘Crown-sterol Biota’ in the Neoproterozoic. Each horizontal colour bar represents one sample, and grey triangles assign data bundles to geological units 1 to 11 (key provided in Methods). prot = protosteroids, meprot = 24-methyl protosteroids (ursteroids), chol = cholesteroloids, erg = ergosteroids, stig = stigmasteroids. Key to colours see panel Figure 2 and Supplementary Table 1. For details on data assembly and geological formations (1–11) see Methods. **b,** Phylogenetic tree of the domain Eukarya with white, red and green highlighting crown-group branches. Stem-group branches (purple) are hypothetical only, illustrating the notion that mid-Proterozoic ecosystems may have been dominated by extinct stem forms that did not produce crown-sterols. LECA, the last common ancestor of all extant eukaryotes, may have emerged between 1.2 and > 2.0 Ga (see text). **c,** Microfossils of early eukaryotes. (a–e) Likely crown-group Eukarya (~1.1 to 0.7 Ga), (f–k) microfossils that are possibly or certainly eukaryotic but lack diagnostic crown-group characteristics. See Methods for detailed information and image credits. Tn, Cr, Ed are the Tonian, Cryogenian and Ediacaran periods; P, M, C are the Palaeo-, Meso- and Caenozoic eras.

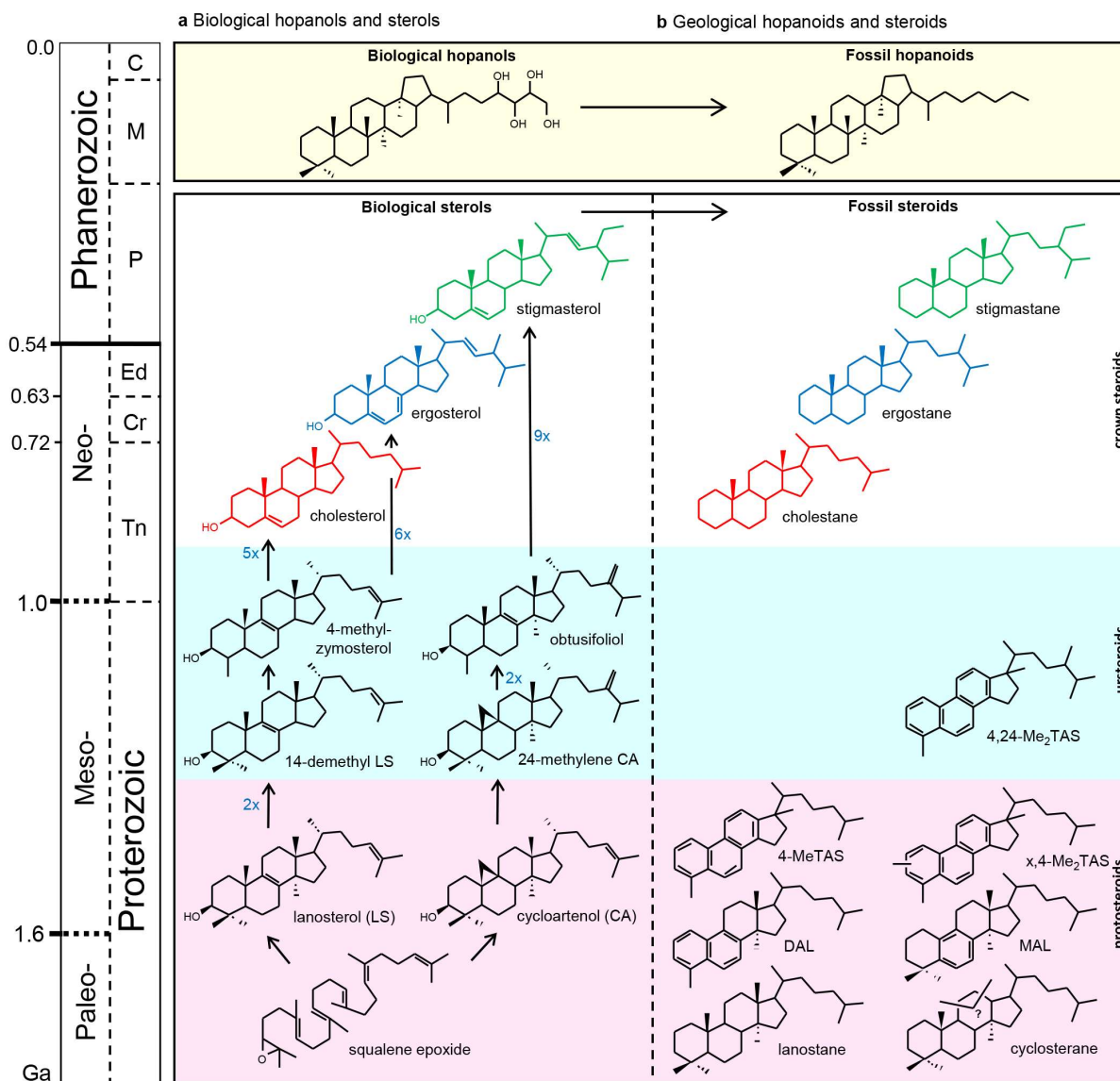


Fig. 2 | Juxtaposition of biological and geological hopanoids and sterols, and the evolution of the sterol biosynthetic pathway through time. a, The pathway from squalene epoxide to protosterols, ursterols and crown-sterols was likely assembled during the Proterozoic. ‘2x’ indicates number of successive enzymatic steps. **b,** Fossil sterols detected in the rock record that correspond to intermediates and end-products in the evolving biosynthetic pathway. TAS = triaromatic steroids, MAL = monoaromatic lanosteroids, DAL = diaromatic lanosteroids, ‘x,4- Me₂TAS’ signifies a TAS methylated at C-4 and at an unknown position x. ‘?’ indicates that cyclosterane likely contains a bridge at an unknown position.

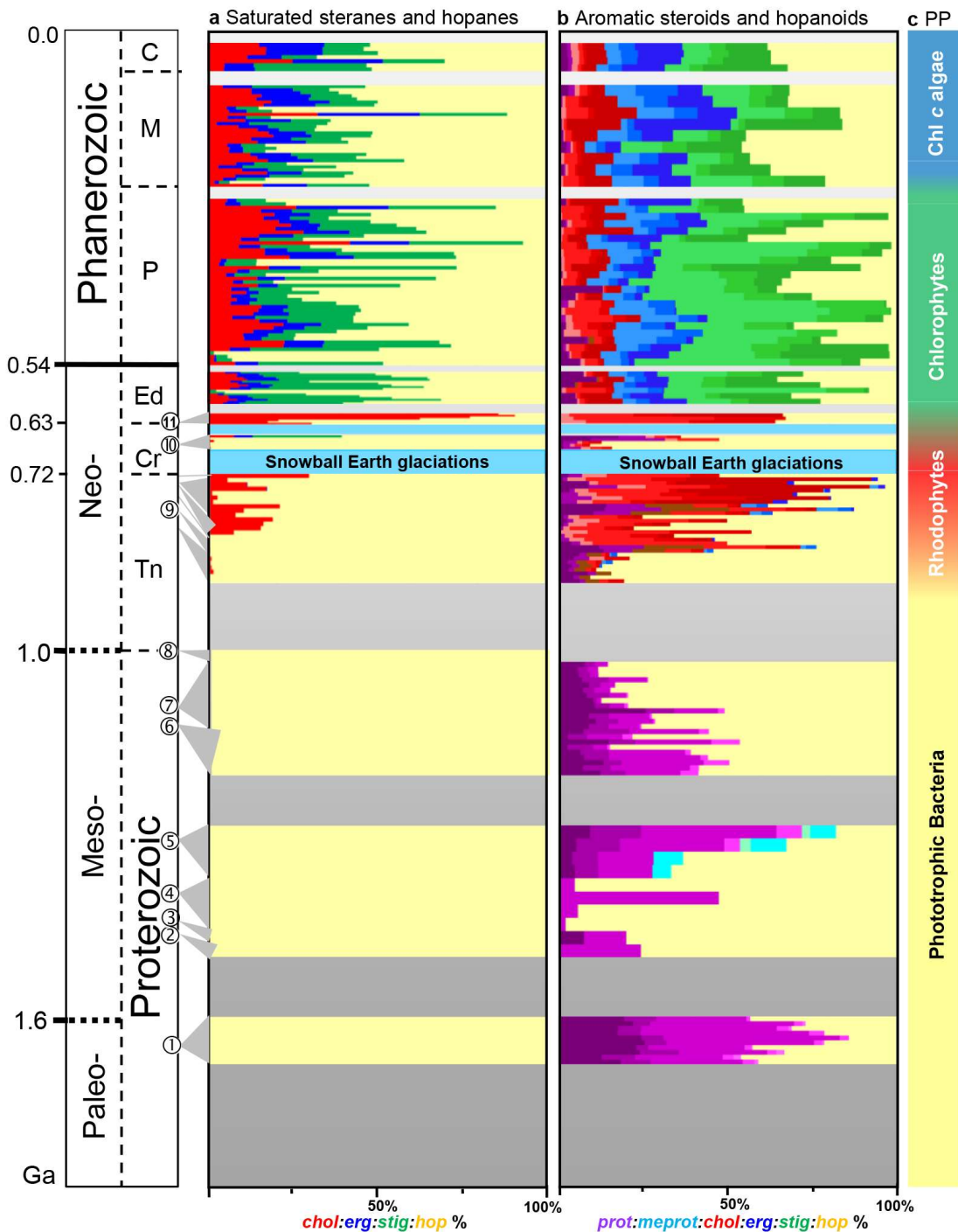


Fig. 3 | The succession of fossil steroids and bacterial hopanoids through time. a, The relative abundances of saturated steranes (red, blue and green) and bacterial hopanes (hop, yellow). Note absence of detectable steranes before 800 Ma, highlighting the paucity of crown-group eukaryotes. **b,** A new view of biomarker evolution based on the relative abundances of aromatic protosteroids (purple), 24-methyl protosteroids (ursteroids, cyan), aromatic crown-steroids (reds, blues and greens) and aromatic hopanoids (yellow). The data reveal the existence of a protosterol-producing biota that was ecological dominant in the Proterozoic. For (a) and (b), each horizontal colour bar represents one sample. Key to colours, formations and abbreviations see Fig. 1. **c,** The geological succession of dominant primary producers (PP) based on molecular fossils.

670 **EXTENDED DATA TABLE & FIGURE LEGENDS**

671 **Extended Data Table 1.** Information on Cryogenian to Palaeoproterozoic geological samples.

672 * Alternate sample names 14B311 = (#1-14); 14B316 = (#3-14); 14B313 = (#2-16) in refs^{66,67}.

673

674 **Extended Data Fig. 1 | Diagenetic and pyrolysis products of ur- and protosterols.** Chemical

675 structures in purple and cyan are biogenic precursors. Structures in black are fossil lipids detected

676 in mid-Proterozoic sedimentary rocks and generated in pyrolysis experiments of the respective

677 biolipids. Green arrows signify biosynthetic reactions, black dashed arrows point to products of

678 diagenesis (and laboratory pyrolysis). Note that the dashed arrows do not imply direct product-

679 precursor relationships as diagenetic reactions commonly involve numerous intermediates and

680 complex reaction networks. All structures in black, apart from **X**, were found in mid-Proterozoic

681 bitumens. TAS = triaromatic steroids, MAL = monoaromatic lanosteroids, DAL = diaromatic

682 lanosteroids. 'x,4,24-Me₃TAS' signifies a TAS methylated at C-4, C-24 and at an unknown

683 position x.

684 **Extended Data Fig. 2 | Mass spectra and elution behaviour of cyclosterane and its cleavage**

685 **products in a severely biodegraded migrabitumen (12Z083, drill core MY4, 103.3 m). a, *M/z***

686 412 to 300 partial ion chromatograms of cyclosterane and successive side-chain cleavage products.

687 'x' indicate absence or low concentration of pseudohomologs indicative of side-chain branching

688 positions. R is the pentacyclic core of cyclosterane, '*' denotes a chiral centre. **b**, Total ion current

689 (TIC) of the saturated hydrocarbon fraction of the biodegraded oil, highlighting the biodegradation

690 resistance of the two cyclosterane isomers *k1* and *k2*. '~' marks truncated signal of internal

691 standard. **c**, Mass spectra of the cycloartane side-chain cleavage products. See text for explanation

692 of red and blue dashed lines. **d**, Juxtaposition of the mass spectra of cyclosterane isomer *k2* (upper
693 panel) and cycloartane from the NIST 95 library (lower panel) (the mass spectrum of *k1* is not
694 shown as it is nearly identical to *k2*). **e**, Suggested major MS fragmentation of hypothetical
695 cyclosterane structure **I** and cycloartane.

696 **Extended Data Fig. 3 | Mass chromatograms and spectra of protosteranes.** **a**, *M/z* 259 partial
697 mass chromatograms of a co-injection experiment of Eocene bitumen Y2 containing 8 β (H),9 α (H)-
698 lanostane *l4* (see text) and 1,640 Ma Barney Creek Fm (B03178). **b**, MRM 414 \rightarrow 259 showing
699 the elution positions of lanostane isomers *l1* to *l4*, and the results of pyrolysis experiments on
700 cycloartenol, lanosterol and euphenol (3 β ,13 α ,14 β ,17 α -lanost-8-en-3-ol). **c**, Mass spectra of signal
701 *m3* of euphenol pyrolysis, *l4* in BCF sample B03178 and lanostane in the Y2 standard. **d**, **e**, MRM
702 chromatograms highlighting the relative elution positions of stigmastanes, lanostane,
703 cyclosteranes and C₂₇ hopanes in (d) the 1,440 Ma Tieling Fm (sample 17B101) and (e) an
704 Ordovician oil from Australia (GA#299). TTP1 and 2 are Tetracyclic Terpene isomers⁶⁸. Note that
705 8 β (H),9 α (H)-lanostane *l4* co-elutes with TTP2, but that its presence can be recognized by an
706 elevated TTP2 peak or a shoulder trailing the peak. The chromatograms are identified by MRM
707 precursor \rightarrow product transitions and relative signal heights in % relative to the highest signal.

708 **Extended Data Fig. 4 | Mass chromatograms and spectra of monoaromatic lanosteroids**
709 **(MAL).** **a**, *M/z* 379 mass chromatogram identifying the 20S and 20R isomers of C₂₉ MAL in
710 sample B03163 from the 1,640 Ma Barney Creek Fm (black) and coinjection experiment with an
711 authentic MAL standard on a DB-5MS capillary column (red). **b**, Authentic C₂₉ MAL standard. **c**,
712 C₂₈ MAL of sample 14B211 from the 725 Ma Kanpa Fm showing an immature isomer distribution
713 (S \ll R). **d**, C₂₈ MAL generated through pyrolysis of lanosterol (sample BEX20150624). **e**, C₂₈

714 MAL of sample B03163 from the 1,640 Ma Barney Creek Fm showing a mature isomer
715 distribution ($S \approx R$). seco-hop = monoaromatic 8,14-secohopanoids (see Extended Data Fig. 8a).
716 **f**, Mass spectra of signals labelled in (a) to (e). **a1** to **a4** are chromatographic signal identifiers for
717 geological bitumens, and **A1** to **A4** identifiers for corresponding authentic standards and pyrolysis
718 products. Ancient bitumen chromatograms are in black, standard chromatograms in blue.

719 **Extended Data Fig. 5 | Mass chromatograms of diaromatic lanosteroids (DAL). a, d, g, M/z**
720 **404, 379 and 361** mass chromatograms identifying isomers of C_{28} , C_{29} and C_{30} DAL generated
721 through pyrolysis of cycloartenol. **b, e, h**, C_{28} , C_{29} and C_{30} DAL of sample 14B211 from the 725
722 Ma Kanpa Fm showing an immature isomer distribution ($20S \ll 20R$). **c, f, i**, C_{28} , C_{29} and C_{30}
723 DAL of sample B03162c from the 1,640 Ma Barney Creek Fm with a mature isomer distribution
724 ($20S \approx 20R$). **b1** to **b10** are chromatographic signal identifiers for geological bitumens, and **B1** to
725 **B10** identifiers for pyrolysis products of authentic standards in blue.

726 **Extended Data Fig. 6 | Mass spectra of DAL** given in Extended Data Figure 5. **b1** to **b10** are
727 chromatographic signal identifiers for geological bitumens, and **B1** to **B10** identifiers for pyrolysis
728 products of cycloartenol. Vertical labels '13 Aug 18 35' are unique identifiers for individual GC-
729 MS experiments. Signals marked 'x' are from coeluting compounds.

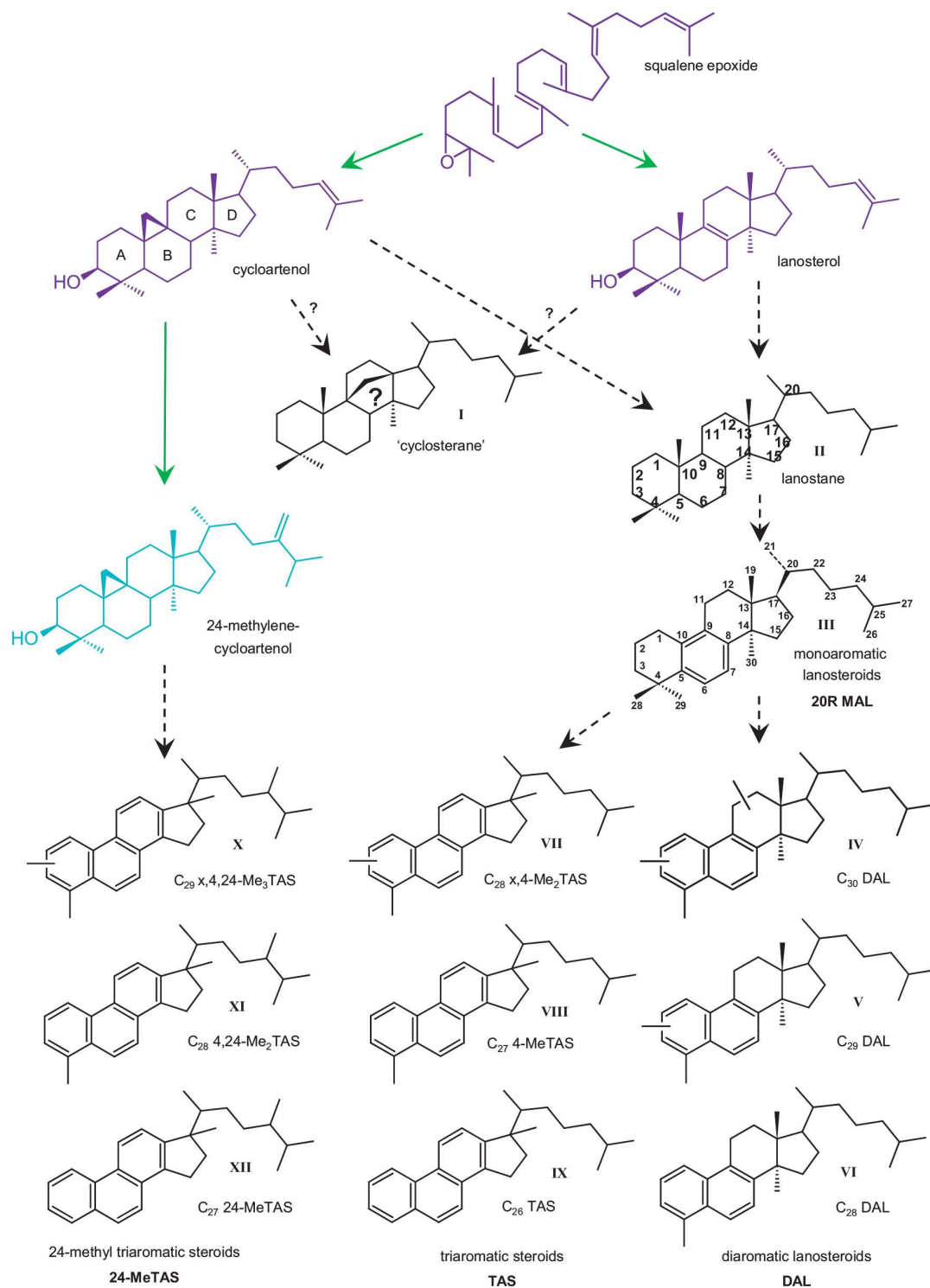
730 **Extended Data Fig. 7 | Mass chromatograms of triaromatic steroids (TAS). a, M/z 259** mass
731 chromatogram of TAS with two methylations in the ring system (Me_2TAS) in sample 13N14 from
732 the 1,100 Ma El Mreïti Group, and **b**, in the pyrolysate of an enrichment mixture of cycloartenol
733 (CA) and 24-methylene cycloartenol (MCA). **c, M/z 245** chromatogram of A-ring methylated TAS
734 ($MeTAS$) of the pyrolyzed CA and MCA mixture, **d**, of sample 10J093 of the ~750 Ma Chuar
735 Group, **e** the El Mreïti Gr (as above), and **f**, the Phanerozoic-based AGSOSTD oil reference

736 standard. TAS dinosteroids in grey. **g**, M/z 231 mass chromatogram of TAS without methylations
737 in the ring system of the Chuar Gr, **h**, the El Mreïti Gr and **i**, the AGSOSTD (samples as above).
738 **j**, M/z 253 chromatogram of monoaromatic steroids (MAS) of sample 14B212 of the ~725 Ma
739 Kanpa Fm and **k**, the AGSOSTD. Colour coding as in panel (e). MAS compound identification
740 and roman numeral nomenclature follows Figure 13.107 and 13.108 in ref⁶⁹.

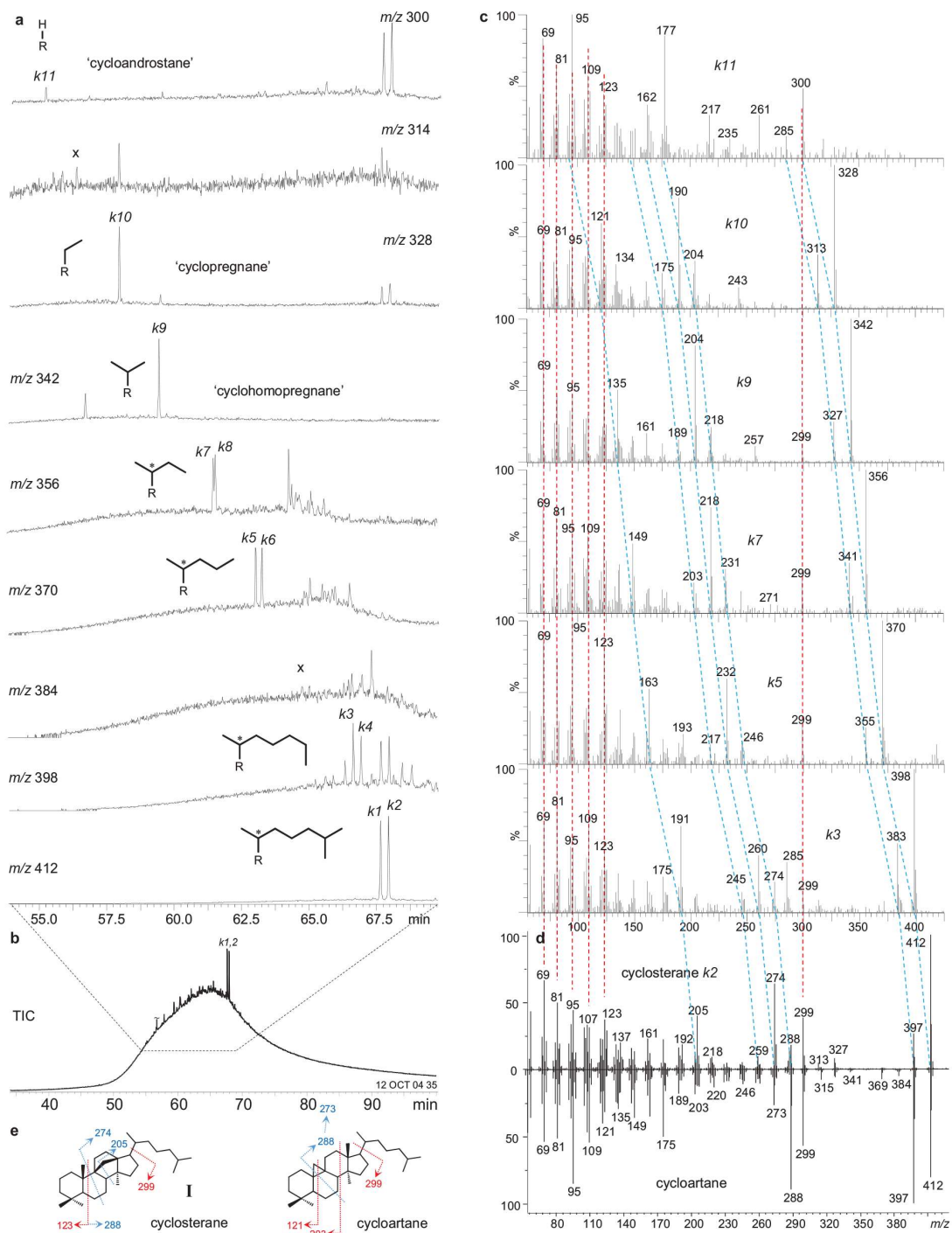
741 **Extended Data Fig. 8 | Mass chromatograms of aromatic hopanoids in the 1,640 Ma Barney**
742 **Creek Fm (12B117), and thermal maturity evaluation of the sample set.** Mass chromatograms
743 of **a**, m/z 365 identifying regular aromatic 8,14-secohopanoids, **b**, m/z 351 regular 28-nor-8,14-
744 secohopanoids, **c**, m/z 414 8,14-secohopanoids with a fluorene moiety, **d**, m/z 416 and **e**, m/z 402
745 8,14-secohopanoids with an acenaphthene moiety, and **f**, m/z 191 benzohopanoids. **g, h**, Evaluation
746 of possible bias in the aromatic steroid and hopanoid record caused by thermal maturity based on
747 cross plots of thermal maturity indicator $R_c(\text{MPR})$ against (g) sample age, and (h) the relative
748 abundance of aromatic hopanoids and steroids ($H/(H+S)$). $R_c(\text{MPR})$ = computed vitrinite
749 reflectance (R_c) based on the Methyl Phenanthrene Ratio (MPR, see Supplementary Table 1).

750 **Extended Data Fig. 9 | Confirmation of the elution position of S and R 4,24-dimethyl**
751 **triaromatic cholesterol in SIR m/z 245 traces on DB-5MS (left) and VZ-200 (right) gas**
752 **chromatographic columns.** **a, c**, The AGSOSTD standard, **b, e**, the ~1,300 Ma Velkerri
753 Formation, Roper Group (sample 230692, drill core Atree-2, 410.55 m). Note that the 20S-isomer
754 of the 4,24-dimethyl triaromatic cholesterol coelutes with the 20R-isomer of 4-methyl triaromatic
755 cholesterol on the DB-5MS capillary column, but is resolved on VZ-200. **d**, A-ring methylated
756 24-methyl triaromatic cholesterol standard formed by pyrolysis of ergosterol (Supplementary

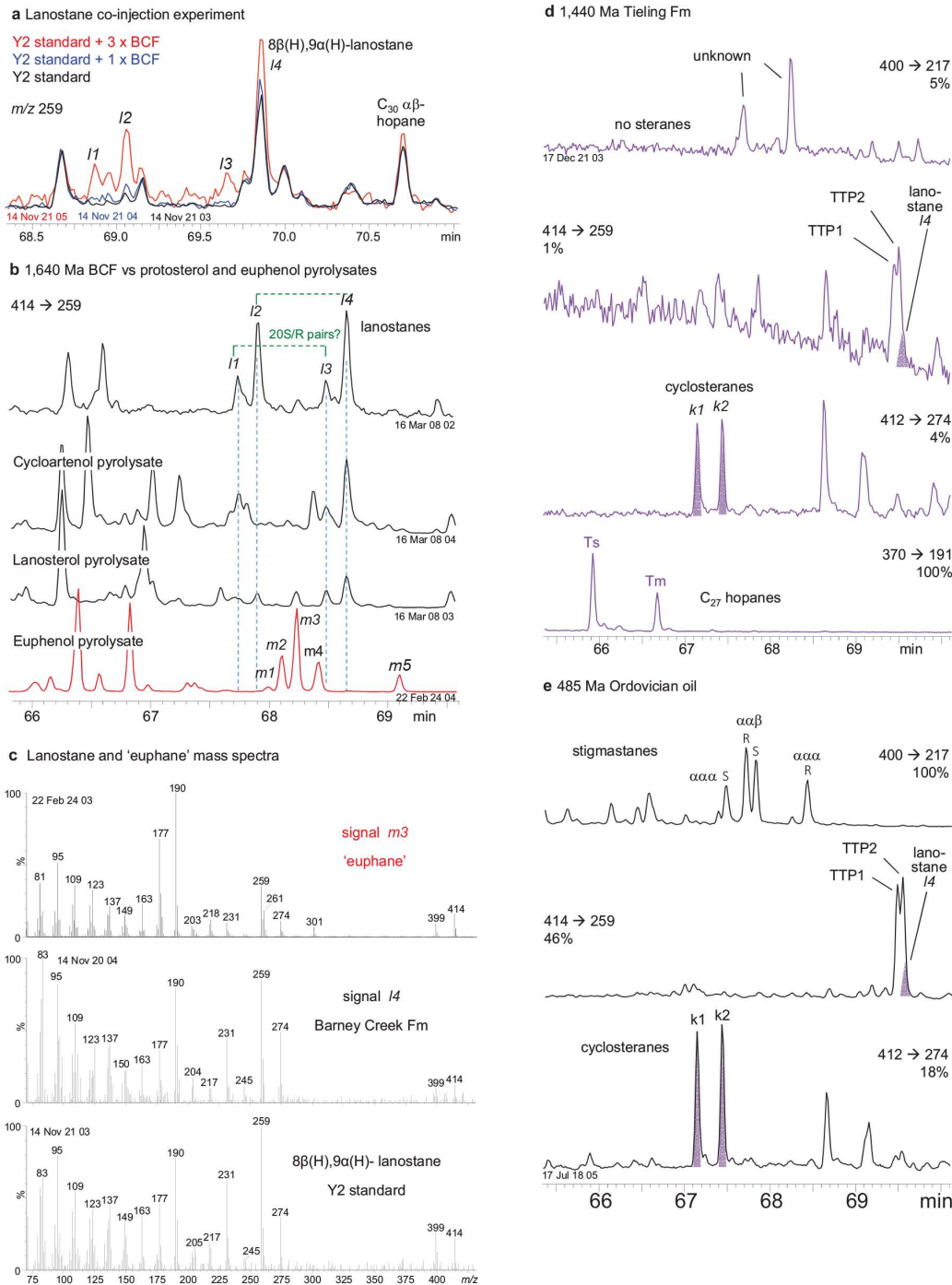
757 Methods). 4,24-dimethyl triaromatic cholesteroids were also generated through pyrolysis of the
758 C₃₁ ursterol 24-methylene cycloartenol (Extended Data Fig. 7c).



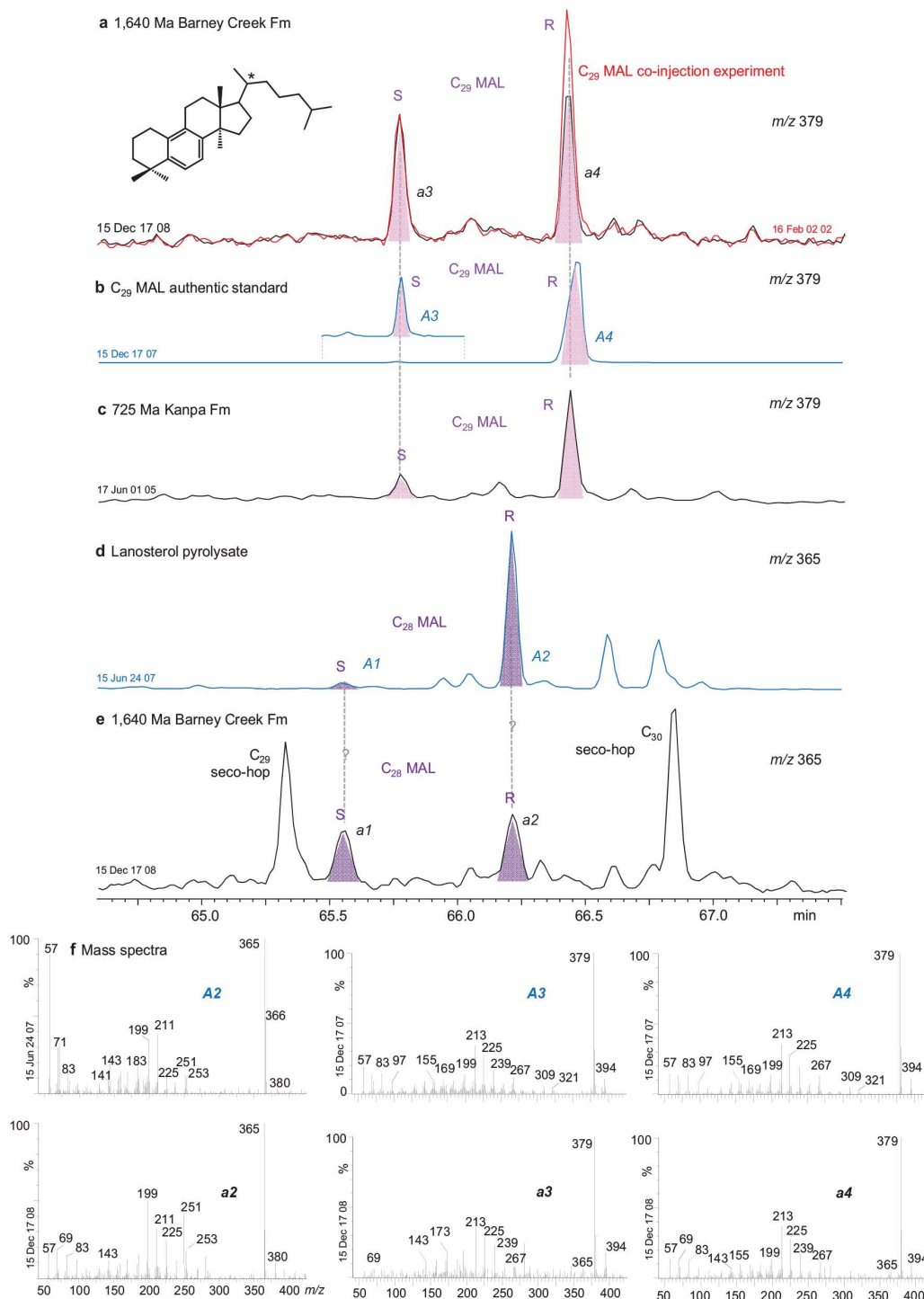
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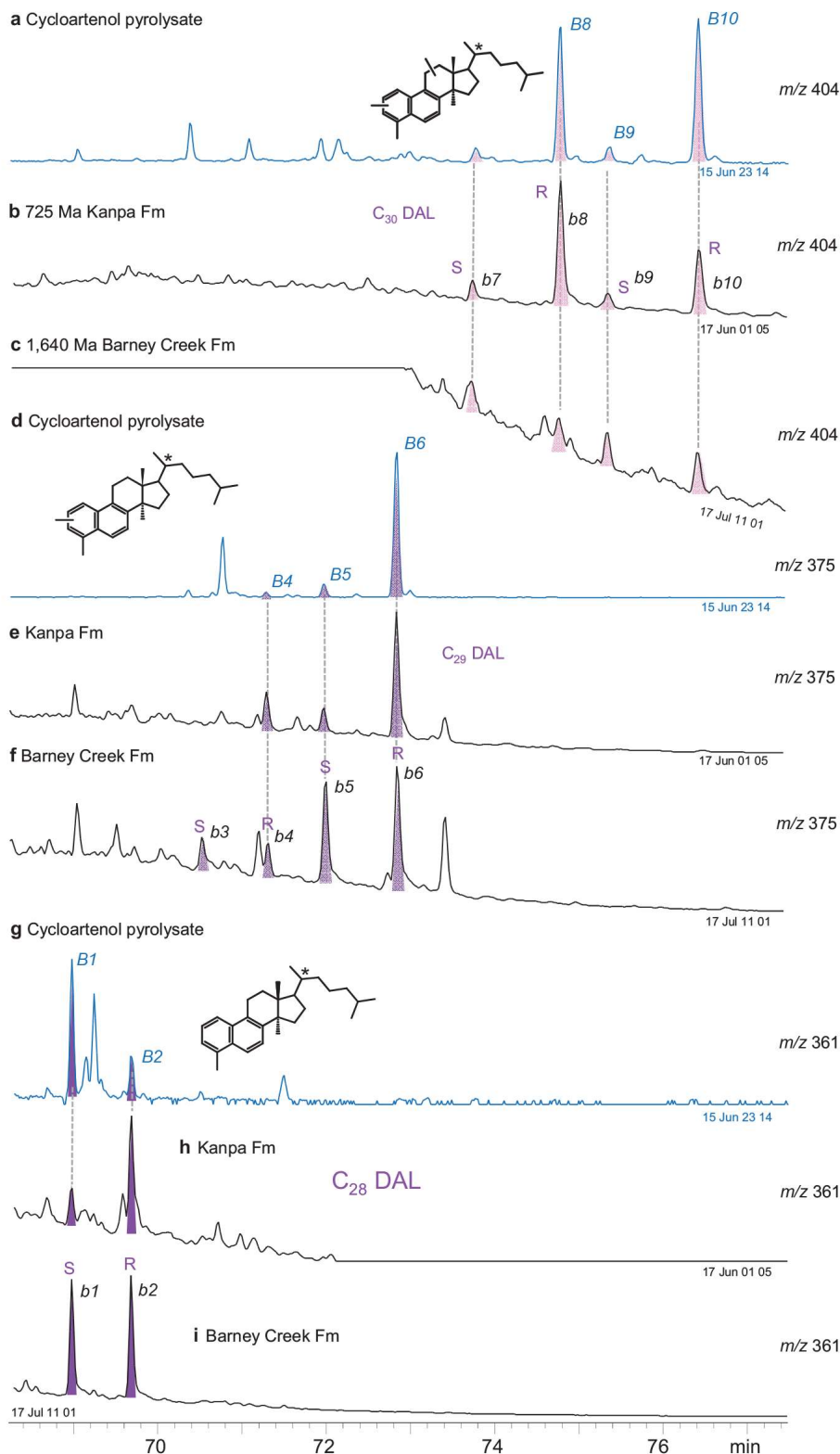
Extended Data Fig. 2 | Mass spectra and elution behaviour of cyclosterane and its cleavage products in a severely biodegraded migrabitumen (12Z083, drill core MY4, 103.3 m). a, M/z 412 to 300 partial ion chromatograms of cyclosterane and successive side-chain cleavage products. 'x' indicate absence or low concentration of pseudohomologs indicative of side-chain branching positions. R is the pentacyclic core of cyclosterane, '*' denotes a chiral centre. **b,** Total ion current (TIC) of the saturated hydrocarbon fraction of the biodegraded oil, highlighting the biodegradation resistance of the two cyclosterane isomers $k1$ and $k2$. '~' marks truncated signal of internal standard. **c,** Mass spectra of the cycloartane side-chain cleavage products. See text for explanation of red and blue dashed lines. **d,** Juxtaposition of the mass spectra of cyclosterane isomer $k2$ (upper panel) and cycloartane from the NIST 95 library (lower panel) (the mass spectrum of $k1$ is not shown as it is nearly identical to $k2$). **e,** Suggested major MS fragmentation of hypothetical cyclosterane structure **I** and cycloartane.



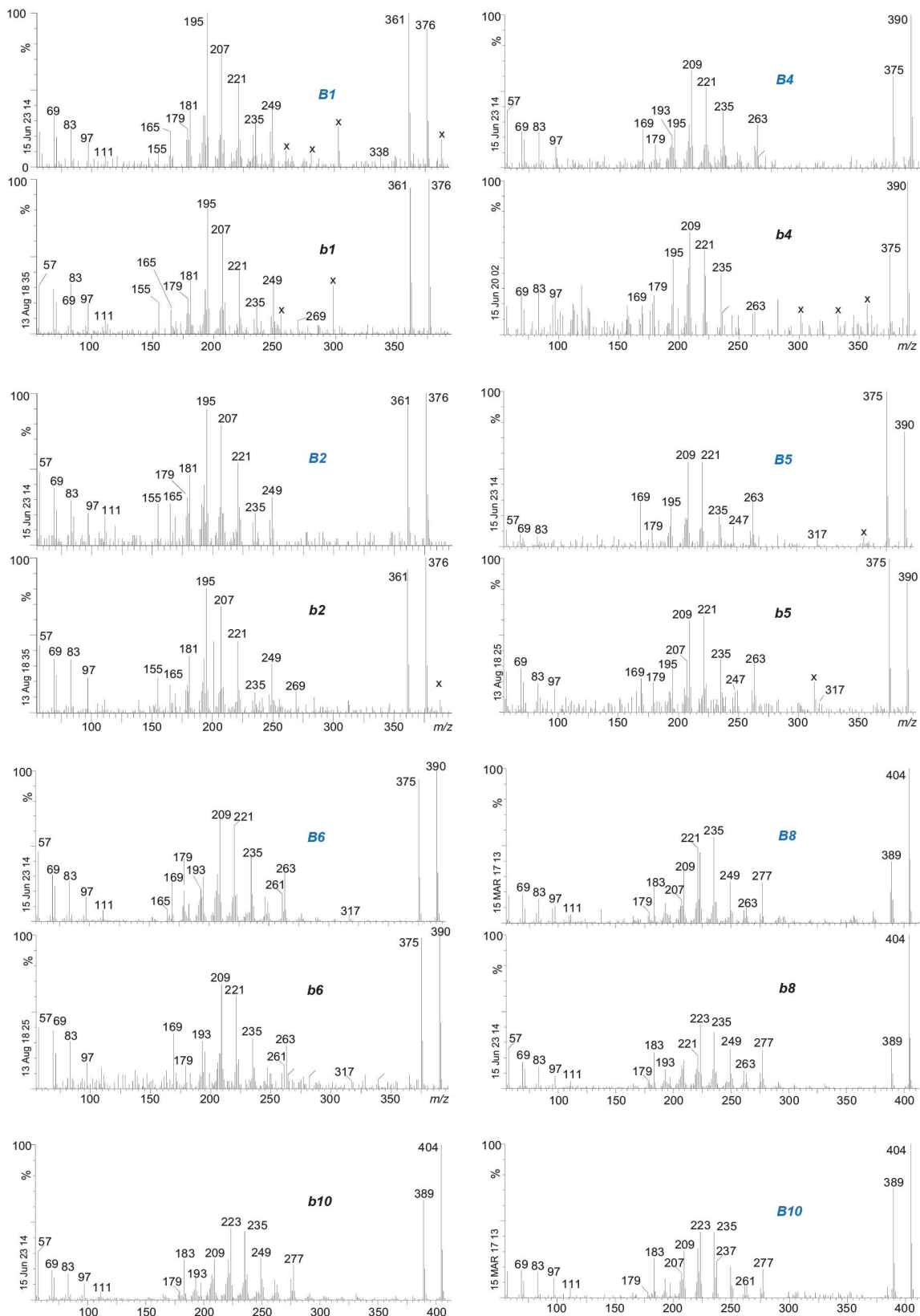
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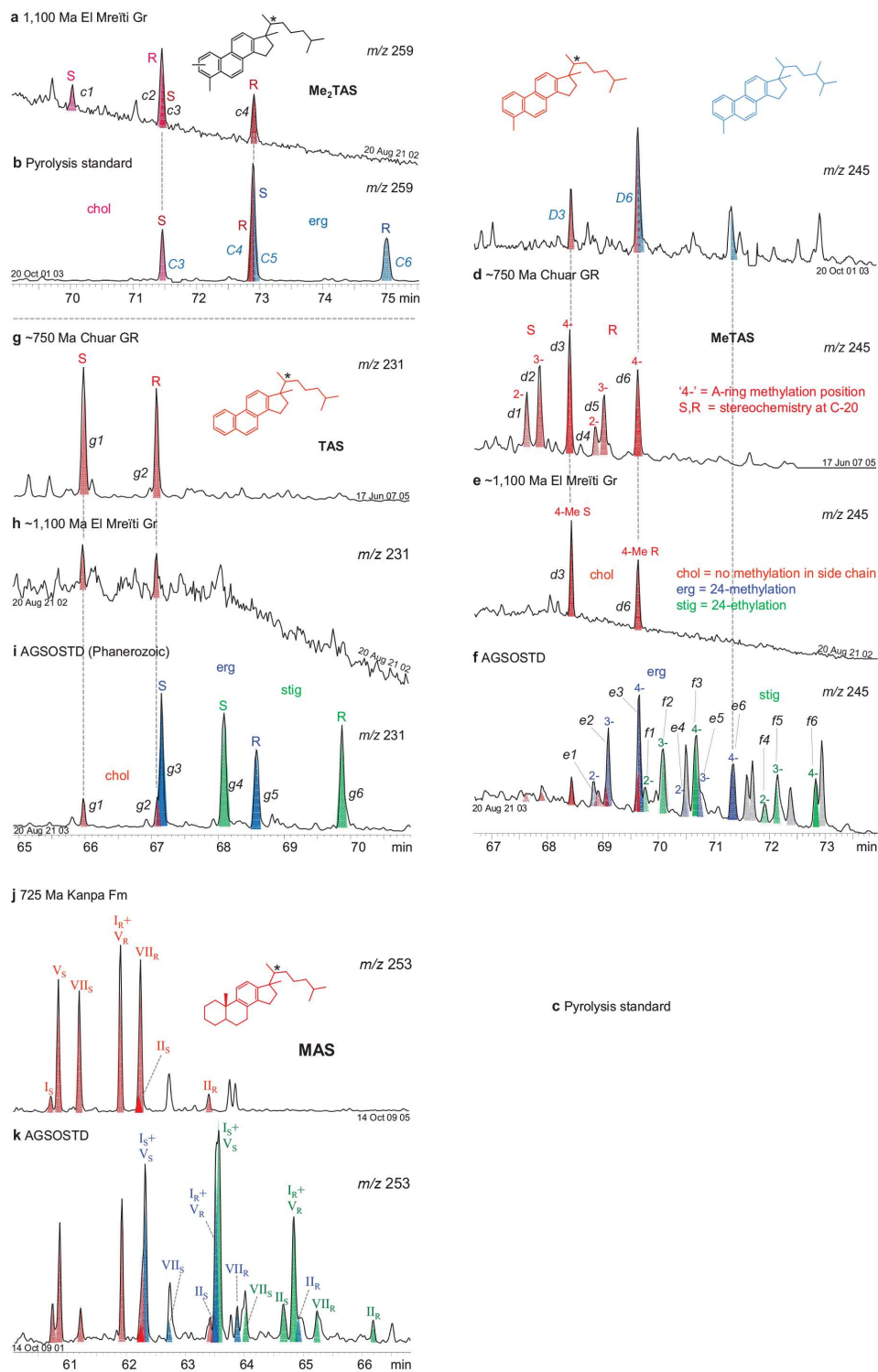
Extended Data Fig. 4 | Mass chromatograms and spectra of monoaromatic lanosteroids (MAL). **a**, M/z 379 mass chromatogram identifying the 20S and 20R isomers of C_{29} MAL in sample B03163 from the 1,640 Ma Barney Creek Fm (black) and coinjection experiment with an authentic MAL standard on a DB-5MS capillary column (red). **b**, Authentic C_{29} MAL standard. **c**, C_{28} MAL of sample 14B211 from the 725 Ma Kanpa Fm showing an immature isomer distribution ($S \ll R$). **d**, C_{28} MAL generated through pyrolysis of lanosterol (sample BEX20150624). **e**, C_{28} MAL of sample B03163 from the 1,640 Ma Barney Creek Fm showing a mature isomer distribution ($S \approx R$). seco-hop = monoaromatic 8,14-secohopanoids (see Extended Data Fig. 8a). **f**, Mass spectra of signals labelled in (a) to (e). **a1** to **a4** are chromatographic signal identifiers for geological bitumens, and **A1** to **A4** identifiers for corresponding authentic standards and pyrolysis products. Ancient bitumen chromatograms are in black, standard chromatograms in blue.



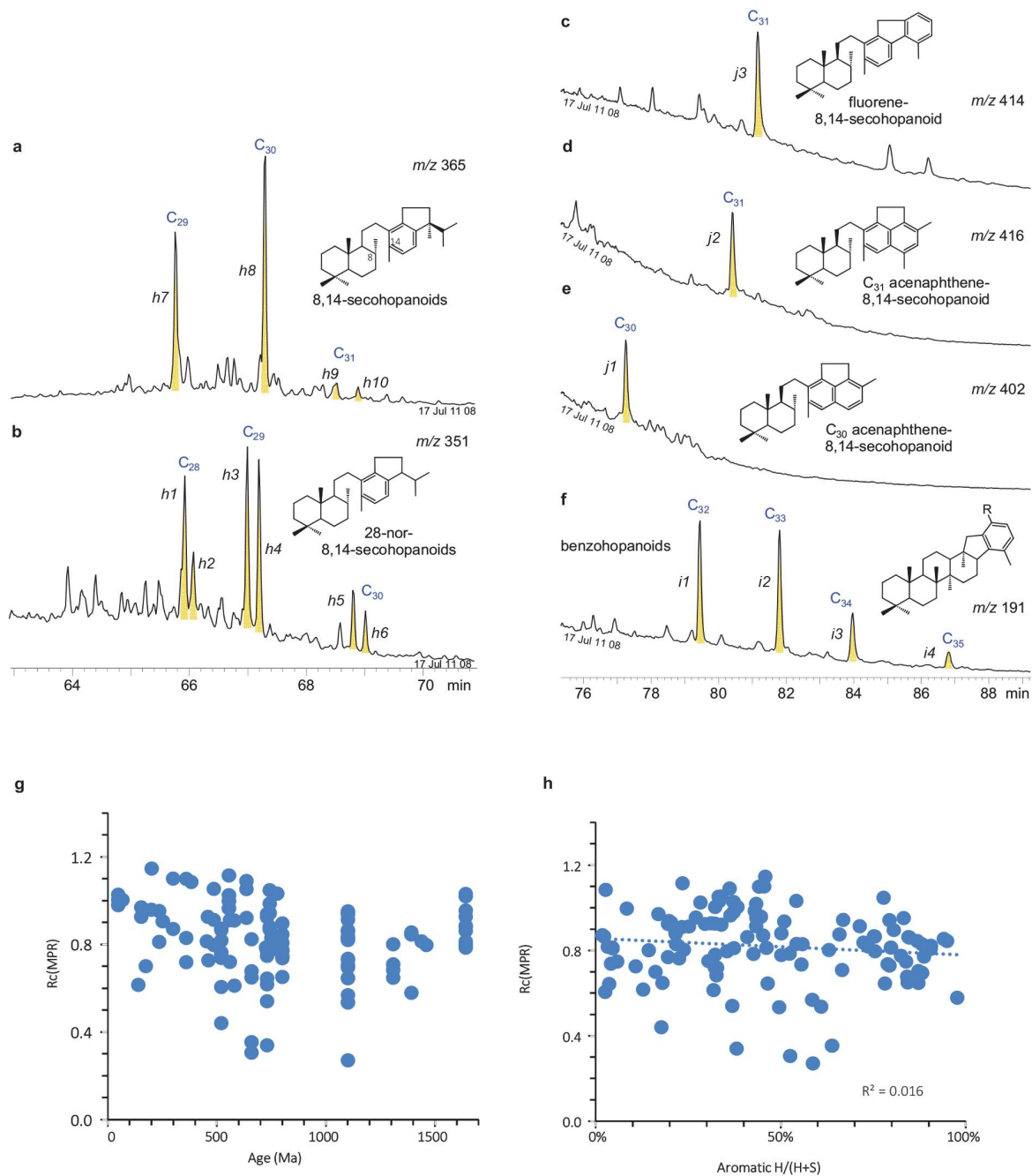
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Extended Data Fig. 6 | Mass spectra of DAL given in Extended Data Figure 5. *b1* to *b10* are chromatographic signal identifiers for geological bitumens, and *B1* to *B10* identifiers for pyrolysis products of cycloartenol. Vertical labels '13 Aug 18 35' are unique identifiers for individual GC-MS experiments. Signals marked 'x' are from coeluting compounds.

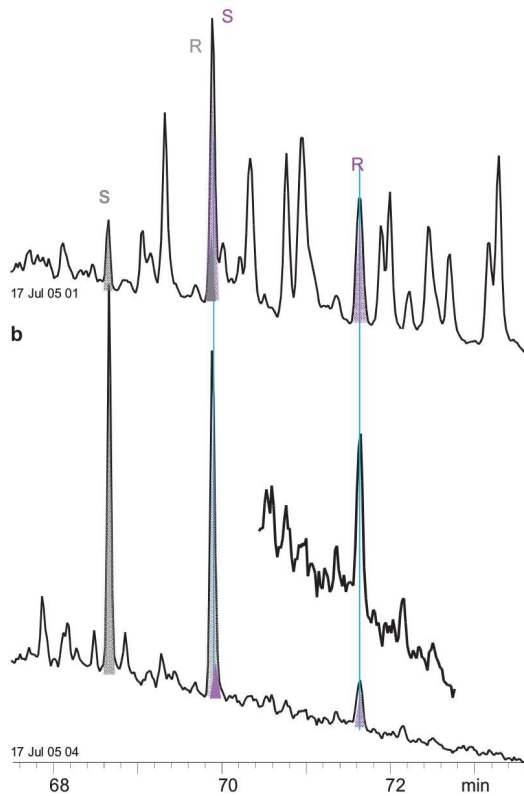


Extended Data Fig. 7 | Mass chromatograms of triaromatic steroids (TAS). **a**, M/z 259 mass chromatogram of TAS with two methylations in the ring system (Me₂TAS) in sample 13N14 from the 1,100 Ma El Mreiti Group, and **b**, in the pyrolysate of an enrichment mixture of cycloartenol (CA) and 24-methylene cycloartenol (MCA). **c**, M/z 245 chromatogram of A-ring methylated TAS (MeTAS) of the pyrolyzed CA and MCA mixture, **d**, of sample 10J093 of the ~750 Ma Chuar Group, **e** the El Mreiti Gr (as above), and **f**, the Phanerozoic-based AGSOSTD oil reference standard. TAS dinosteroids in grey. **g**, M/z 231 mass chromatogram of TAS without methylations in the ring system of the Chuar Gr, **h**, the El Mreiti Gr and **i**, the AGSOSTD (samples as above). **j**, M/z 253 chromatogram of monoaromatic steroids (MAS) of sample 14B212 of the ~725 Ma Kanpa Fm and **k**, the AGSOSTD. Colour coding as in panel (e). MAS compound identification and roman numeral nomenclature follows Figure 13.107 and 13.108 in ref 69.

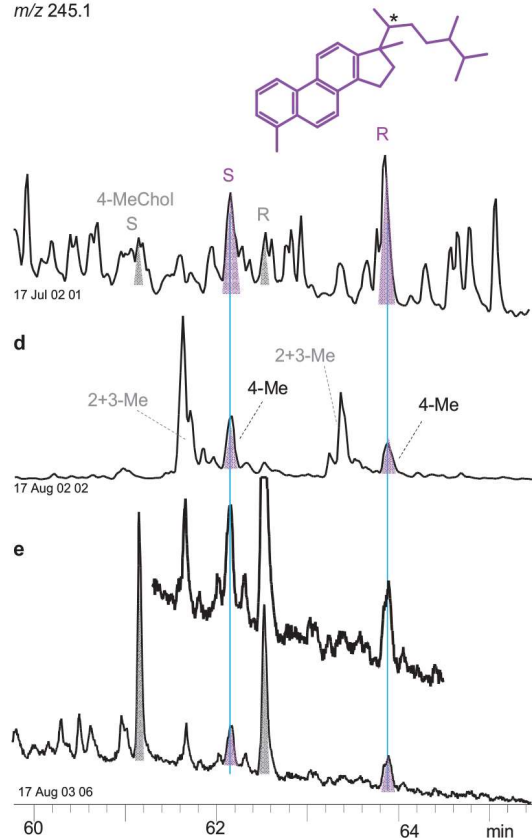


Extended Data Fig. 8 | Mass chromatograms of aromatic hopanoids in the 1,640 Ma Barney Creek Fm (12B117), and thermal maturity evaluation of the sample set. Mass chromatograms of **a**, m/z 365 identifying regular aromatic 8,14-secohopanoids, **b**, m/z 351 regular 28-nor-8,14-secohopanoids, **c**, m/z 414 8,14-secohopanoids with a fluorene moiety, **d**, m/z 416 and **e**, m/z 402 8,14-secohopanoids with an acenaphthene moiety, and **f**, m/z 191 benzohopanoids. **g**, **h**, Evaluation of possible bias in the aromatic steroid and hopanoid record caused by thermal maturity based on cross plots of thermal maturity indicator $R_c(\text{MPR})$ against (g) sample age, and (h) the relative abundance of aromatic hopanoids and steroids ($H/(H+S)$). $R_c(\text{MPR})$ = computed vitrinite reflectance (R_c) based on the Methyl Phenanthrene Ratio (MPR, see Supplementary Table 1).

a DB-5MS column
 m/z 245.1



c VZ-200 column
 m/z 245.1



Extended Data Fig. 9 | Confirmation of the elution position of S and R 4,24-dimethyl triaromatic cholesteroloids in SIR m/z 245 traces on DB-5MS (left) and VZ-200 (right) gas chromatographic columns. a, c, The AGSOSTD standard, b, e, the ~1,300 Ma Velkerri Formation, Roper Group (sample 230692, drill core Altree-2, 410.55 m). Note that the 20S-isomer of the 4,24-dimethyl triaromatic cholesteroloid coelutes with the 20R-isomer of 4-methyl triaromatic cholesteroloid on the DB-5MS capillary column, but is resolved on VZ-200. d, A-ring methylated 24-methyl triaromatic cholesteroloid standard formed by pyrolysis of ergosterol (Supplementary Methods). 4,24-dimethyl triaromatic cholesteroloids were also generated through pyrolysis of the C_{31} ursterol 24-methylene cycloartenol (Extended Data Fig. 7c).

Extended Data Table 1 : Information on Cryogenian to Palaeoproterozoic geological samples

Sample ID	Age [Ma]	Basin or region	Geological unit	Drill Core	Depth [m]	Sample lithology and characteristics
Ediacaran						
Araras Group, Brazil – Terconi Outcrop (~635 Ma)						
LvM 00311	635	Araras Group	Mirassol d'Oeste Fm	Outcrop	11.7	Grey Dolomite
LvM 00313	635	Araras Group	Guia Fm.	Outcrop	16.4	Dark Carbonate
LvM 00315	635	Araras Group	Guia Fm.	Outcrop	31.2	Dark Carbonate
Cryogenian (635 – 717 Ma)						
Amadeus Basin – Central Australia						
Aralka Formation (659 Ma)						
11J005 i	659	Amadeus Basin	Aralka Formation	BR05	242.20	Laminated grey mudstone
13J005 i	659	Amadeus Basin	Aralka Formation	BR05	454.00	Dark grey siltstone
12J019 i2	659	Amadeus Basin	Aralka Formation	BR05	471.50	Black laminated shale
13J0902 i	659	Amadeus Basin	Aralka Formation	BR05	476.20	Medium grey laminated shale
13J0903 i	659	Amadeus Basin	Aralka Formation	BR05	480.45	Medium grey laminated shale
Tonian (717 – 1000 Ma)						
Wallara Formation (aka Finke Beds) (>750 Ma)						
11J021 i	>750	Amadeus Basin	Wallara Formation	BR05	573.06	Laminated grey carbonate
13J717 i	>750	Amadeus Basin	Wallara Formation	BR05	585.2	Brown micritic mudstone with cross cutting molar tooth structures
Johnnys Creek Formation, Bitter Springs Group (<820 Ma)						
11J038 i	<820	Amadeus Basin	Johnnys Creek Formation	BR05	762.18	Laminated grey micritic limestone
13J718 i	<820	Amadeus Basin	Johnnys Creek Formation	BR05	808.2	Laminated dolomitic mudstone with molar tooth structures
MG006 i	<820	Amadeus Basin	Johnnys Creek Formation	BR05	813.6	Dark brown microbial mat in a light grey carbonate matrix
11J024 i	<820	Amadeus Basin	Johnnys Creek Formation	BR05	816.26	Dark brown laminated carbonate with light grey to white microbial mat
MG013	<821	Amadeus Basin	Johnnys Creek Formation	BR05	931.33	Laminated dolomitic mudstone with stromatolitic features
MG007 i	<820	Amadeus Basin	Johnnys Creek Formation	BR05	924.62	Microbialite with micrite/sparite lamination
11J050 i	<820	Amadeus Basin	Johnnys Creek Formation	BR05	942.74	Laminated dark grey carbonate
13J036 i	<820	Amadeus Basin	Johnnys Creek Formation	BR05	956.25	Laminated dark grey carbonate
13J107 i	<820	Amadeus Basin	Johnnys Creek Formation	BR05	961.91	Laminated grey carbonate
Loves Creek Formation, Bitter Springs Group (<820 Ma)						
11J063 i	<820	Amadeus Basin	Loves Creek Formation	BR05	1224.69	Grey dolomitic cross-cut by thin white molar tooth structures
Officer Basin – Western Australia						
Stepptoe Formation, Buldya Group (717-725 Ma)						
14B214 i	717-25	Officer Basin	Stepptoe Formation	Empress 1A	509.14	Light and dark grey laminated mudstone
14B213 i	717-25	Officer Basin	Stepptoe Formation	Empress 1A	588.21	Black laminated mudstone with evaporite nodules
Kanpa Formation, Buldya Group (725 ± 11 Ma)						
14B212 i	725 ± 11	Officer Basin	Kanpa Formation	Empress 1A	629.6	Light grey dolomite, with soft sediment deformation
14B211 i	725 ± 11	Officer Basin	Kanpa Formation	Empress 1A	737.39	Dark grey to dark brown mudstone
14B21 i	725 ± 11	Officer Basin	Kanpa Formation	Empress 1A	757	Light grey siltstone with black mudstone laminae
14B209 i	725 ± 11	Officer Basin	Kanpa Formation	Empress 1A	764.64	Dark grey finely laminated mudstone
14B208a i	725 ± 11	Officer Basin	Kanpa Formation	Empress 1A	768.25	Stromatolite-like structure with wrinkled laminae, base and top
14B208b i	725 ± 11	Officer Basin	Kanpa Formation	Empress 1A	768.25	Medium grey laminated mudstone
14B206 i	725 ± 11	Officer Basin	Kanpa Formation	Empress 1A	829.42	Dark grey to black mudstone
14B204 i	725 ± 11	Officer Basin	Kanpa Formation	Empress 1A	830.35	Medium grey extremely soft mudstone, some wavy bedding
14B203 i	725 ± 11	Officer Basin	Kanpa Formation	Empress 1A	839.23	Mudstone
Hussar Formation, Buldya Group (777-725 Ma)						
14B202 i	777-725	Officer Basin	Hussar Formation	Empress 1A	1072.85	Finely laminated (stromatolitic?) light grey dolomite with anhydrite nodules
14B201-b i	777-725	Officer Basin	Hussar Formation	Empress 1A	1074.15	Light and dark grey planar laminae, with anhydrite nodule
14B201-a i	777-725	Officer Basin	Hussar Formation	Empress 1A	1074.15	Light and dark grey planar laminae, with anhydrite nodule
Grand Canyon - Arizona - Outcrop at Sixtymile Canyon and Nankowep Butte (depths are meters above base)						
Walcott Member, Kwagunt Formation, Chuar Group (729 ± 0.9 Ma)						
10J090	729 ± 0.9	Grand Canyon	Walcott Member	outcrop	1609	Dolomite nodule
10J093	729 ± 0.9	Grand Canyon	Walcott Member	outcrop	1544	Black shale
10J092	729 ± 0.9	Grand Canyon	Walcott Member	outcrop	1494	Massive black shale
10J091	729 ± 0.9	Grand Canyon	Walcott Member	outcrop	1480	Black shale
10J089	729 ± 0.9	Grand Canyon	Walcott Member	outcrop	1365	Black shale
B04026 - i	729 ± 0.9	Grand Canyon	Walcott Member	outcrop	1343	Black carbonaceous siltstone
B04029 - i	729 ± 0.9	Grand Canyon	Walcott Member	outcrop	1340	Black carbonaceous sandstone
Lake Vättern Basin - Sweden - Outcrop at Girabacken locality						
Visingsö Group (> 740 Ma)						
VG-20-03-i	~750	Lake Vättern Basin	Visingsö Group	outcrop		Black mudstone
Mesoproterozoic (1000 – 1600 Ma)						
Sette– Daban fold belt, Siberian Craton, Russia						
08m001 (LK-4)	1000	Sette-Daban fold belt	Lakhanda Group, Neryuen Fm	outcrop		black-grey, sometimes discontinuously laminated shale, associated sediments yield mat-forming filamentous microfossils
08m009 (LK-81)	1000	Sette-Daban fold belt	Ui Group, Kandyk Fm	outcrop		variegated green-grey shale assoc. with ripples & mudcracks
Taoudeni Basin, El Mreiti Group, Mauritania						
11N048	1100	Taoudeni Basin	Tourist Fm	S2	139.6	Shale with grey and brown sub-mm laminae, carbonate lenses, mass-movement
13N06	1100	Taoudeni Basin	Tourist Fm	S2	140.25	Shale with grey and brown wrinkly sub-mm laminae
11N052	1100	Taoudeni Basin	Tourist Fm	S2	140.55	Shale with brownish and grey wrinkly sub-mm laminae
12N020	1100	Taoudeni Basin	Tourist Fm	S2	141.9	Shale with grey and black sub-mm laminae
11N049	1100	Taoudeni Basin	Tourist Fm	S2	142.2	Shale with brownish and grey wrinkly sub-mm laminae
11N050	1100	Taoudeni Basin	Tourist Fm	S2	151.95	Shale with brown and grey sub-mm laminae
11N054	1100	Taoudeni Basin	En Nesoar Fm	S2	185.4	Shale with dark grey and black sub-mm laminae
11N045b	1100	Taoudeni Basin	En Nesoar Fm	S2	187.3	Shale with grey and black sub-mm laminae
13N13	1100	Taoudeni Basin	En Nesoar Fm	S2	188.1	Dark-grey laminated calcitic shale/siltstone, oxic facies based on iron spec data
13N14	1100	Taoudeni Basin	En Nesoar Fm	S2	188.6	Calcareous green-grey shale, anoxic facies based on iron speciation data
11N055	1100	Taoudeni Basin	En Nesoar Fm	S2	200.01	Shale with dark and medium grey sub-mm laminae
11N051	1100	Taoudeni Basin	En Nesoar Fm	S2	203.1	Shale with very dark and black sub-mm laminae, ~2mm wide pyrite layer
11N056	1100	Taoudeni Basin	En Nesoar Fm	S2	206.08	Shale with dark and light grey sub-mm laminae, sign of mass-movement
Keweenaw Rift, Michigan Basin, USA						
13N36	1100	Keweenaw Rift	Nonesuch Fm	PI-2	90.10	shale with sub-mm dark and mid-grey laminae interlayered with carbonates
13N41	1100	Keweenaw Rift	Nonesuch Fm	PI-2	226.20	Calcareous shale with light to dark grey slightly wrinkly layers, salty crust on surface
13N31	1100	Keweenaw Rift	Nonesuch Fm	D06	391.90	Shale with black and dark grey sub mm laminae
13N37	1100	Keweenaw Rift	Nonesuch Fm	D06	417.50	Shale with mid and light grey sub mm laminae
13N34	1100	Keweenaw Rift	Nonesuch Fm	WPB-3	204.70	Light grey shale interlayered with mid-grey silty clay and carbonate
B04005	1100	Keweenaw Rift	Nonesuch Fm	WPB-3	237.74	black shale with sub-mm laminae
13N45	1100	Keweenaw Rift	Nonesuch Fm	WPB-3	266.40	Shale with dark to light grey sub-mm laminae
13N42	1100	Keweenaw Rift	Nonesuch Fm	WPB-4	63.30	Shale with mid and light grey sub mm laminae
B04007	1100	Keweenaw Rift	Nonesuch Fm	WPB-4	140.67	Black shale with sub-mm Laminae
McArthur Basin, Northern Australia						
11N043	1313 ± 47	McArthur Basin	Kyalla Fm	Jamison-1	1649.81	Black shale
GA 20150106	1308 ± 41	McArthur Basin	Velkerri Fm	Altree-2	410.55	Dark brown to dark grey planar laminated siltstone
GA 20150115	1308 ± 41	McArthur Basin	Velkerri Fm	Altree-2	452.64	Dark grey to black siltstone, flaser-like cross-bedding
GA 20150118	1308 ± 41	McArthur Basin	Velkerri Fm	Altree-2	471.24	Dark grey laminated siltstone, silts are surrounded by 2 cm thick sand layers (couplets) with oil shows
GA 20150129	1308 ± 41	McArthur Basin	Velkerri Fm	Altree-2	527.37	Alternating layers of laminated light and dark grey siltstone surrounded by 2 cm thick sand layers (couplets)
Man 1	1308 ± 41	McArthur Basin	Velkerri Fm	McManus1	1150.00	finely laminated black shale
North China Craton						
14B311	1392	North China Craton	Xiamaling Fm	Core 1 [1]	53.48	Black shale (courtesy S. Zhang, Petrochina)
14B316	1392	North China Craton	Xiamaling Fm	Core 3 [1]	222.35	Black silty mudstone (courtesy S. Zhang, Petrochina)
14B313	1392	North China Craton	Xiamaling Fm	Core 2 [1]	285.90	Black shale (courtesy S. Zhang, Petrochina)
17B100	1392	North China Craton	Xiamaling Fm	YJ2	73.60	Black Shale (courtesy S. Zhang, Petrochina)
17B101	1439	North China Craton	Tieling Fm	YJ2	117.40	Black Shale (courtesy S. Zhang, Petrochina)
17B103	1460	North China Craton	Hongshuizhuang Fm	YJ2	463.40	Black silty mudstone (courtesy S. Zhang, Petrochina)
Palaeoproterozoic (1600 – 2500 Ma)						
McArthur Basin, Northern Australia						
B03162c	1640	McArthur Basin	Barney Creek Fm	GR-7	45.35	dolomitic siltstonewith dark laminae
B03167	1640	McArthur Basin	Barney Creek Fm	GR-7	71.65	Dark dolomitic siltstone
B03168	1640	McArthur Basin	Barney Creek Fm	GR-7	82.95	Dark dolomitic siltstone
B03169	1640	McArthur Basin	Barney Creek Fm	GR-7	90.30	Dark dolomitic siltstone
B03175	1640	McArthur Basin	Barney Creek Fm	GR-7	162.85	Dark dolomitic siltstone
B03178	1640	McArthur Basin	Barney Creek Fm	GR-7	199.08	Dark dolomitic siltstone overlain by breccia
12B117	1640	McArthur Basin	Barney Creek Fm	LV09	382.22	dark and light grey laminated dolomite
12B118	1640	McArthur Basin	Barney Creek Fm	LV09	423.10	finely laminated mudstone
12Z167	1640	McArthur Basin	Barney Creek Fm	LV09	455.52	finely laminated mudstone
12B119	1640	McArthur Basin	Barney Creek Fm	LV09	492.98	dark and light grey finely laminated dolomite

1 **Lost world of complex life and the late rise of the eukaryotic crown**

SUPPLEMENTARY INFORMATION

2 Jochen J. Brocks^{1*}, Benjamin J. Nettersheim^{1,2*}, Pierre Adam³, Philippe Schaeffer³, Amber J. M.
3 Jarrett^{1,4}, Nur Güneli¹, Tharika Liyanage¹, Lennart M. van Maldegem¹, Christian Hallmann⁵,
4 Janet M. Hope¹

5

6 ¹ Research School of Earth Sciences, The Australian National University, Canberra, ACT 2601, Australia.

7 ² MARUM – Center for Marine Environmental Sciences and Faculty of Geosciences, University of Bremen, 28359
8 Bremen, Germany

9 ³ Université de Strasbourg, CNRS, Institut de Chimie de Strasbourg UMR 7177, F-67000 Strasbourg, France

10 ⁴ Northern Territory Geological Survey, GPO Box 4550, Darwin NT 0801, Australia

11 ⁵ GFZ German Research Center for Geosciences, 14473 Potsdam, Germany

12 *These authors contributed equally to this work. e-mail: jochen.brocks@anu.edu.au; bnettersheim@marum.de

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16 **Supplementary Methods and Supplementary Discussion**

17 **1. Geology and samples**

18 Extended Data Table 1 and Supplementary Table 3 summarize information about the bitumens
19 (organic rock extracts) and oils used to assemble the saturated and aromatic steroid and hopanoid
20 records in Figure 1 and 3. Ediacaran and Phanerozoic biomarker data are included to provide a
21 baseline to evaluate the older Proterozoic record. The geological and environmental context for
22 Ediacaran and younger samples is available in the literature and not further described here.
23 Cryogenian and Tonian bitumens come from a transect across palaeocontinent Rodina, including
24 the Amadeus Basin in central Australia, the Officer Basin in Western Australia, the Lake Vättern
25 Basin in Sweden, and from the Grand Canyon, USA. Geological units include the 659 million
26 years old (Ma) Aralka Formation, > 750 Ma Wallara Formation, < 820 Ma Johnnys Creek and
27 Loves Creek formations (both from the Bitter Springs Group), the 717–725 Ma Steptoe, 725 ± 11
28 Ma Kanpa and 777–725 Hussar formations (all from the Buldya Group), the 729 ± 0.9 Ma⁷⁰
29 Walcott Member of the Kwagunt Formation, the Chuar Group, and the ≤ 886 and 740 Ma Visingsö
30 Group⁷¹. Age constraints for these formations and their geological and environmental context are
31 summarized in the Supplementary Discussion of Brocks et al. (2017)¹⁵. The geology of Meso- and
32 Palaeoproterozoic units are outlined below.

33

34 ***1.1. Late Mesoproterozoic El Mreïti Group, Taoudeni Basin, Mauritania (~1,100 Ma)***

35 The epicratonic Taoudeni Basin covers a total area > 1,750,000 km² on the West African Craton,
36 extending from Mauritania to northern Mali and Western Algeria^{65,72}. Up to 1,300 m of gently
37 dipping (< 0.5°) Mesoproterozoic to Palaeozoic sediments unconformably overlie an
38 Archean/Palaeoproterozoic basement and are covered by thin Mesozoic/Caenozoic strata. The
39 formations analysed in this study belong to the Hodh, the first of four Megasequences, resting
40 directly on the basement. In the north-central part of the basin, the Hodh comprises, from oldest to
41 youngest, the Douik, El Mreïti and Cheikhia groups.

42 The El Mreïti Group is subdivided into the Khatt, En Nesoar, Tourist, Aguelte el Mabha and
43 Gouamîr formations. Re-Os geochronology on black shales yielded an age of 1107 ± 12 Ma for
44 the Tourist and 1109 ± 22 Ma for the En Nesoar Formation⁷³. The sediments of the El Mreïti
45 Group are undeformed and unmetamorphosed and comprise marine stromatolitic carbonates and
46 shales. In the north-west of the basin, the sediments were deposited in an open, relatively shallow
47 epeiric sea⁷⁴, characterized by sea-level fluctuations in a general shallowing-upward trend⁷⁵⁻⁷⁷.

48 The eleven samples analysed in this study comprise black shales from the En Nesoar and Tourist
49 formations from drill core S2 that intersects the El Mreïti Group in the north-western part of the
50 basin⁷⁸. Five finely laminated black shales of the En Nesoar Formation have total organic carbon
51 (TOC) contents of ~2 to 15%. In drill core S2, the black shales occur as decimetre-thick layers
52 interbedded with dominant grey, green and brown shales and clayey siltstones with occasional
53 ripples, gutter casts and wavy bedding^{78,79}. These sediments were deposited in a relatively shallow,
54 subtidal environment during a transgressive phase⁷⁷. The Tourist Formation was deposited during
55 maximal flooding of the basin beneath fair-weather wave base. The lower Tourist Formation
56 comprises coniform stromatolites that thrived within the photic zone. The stromatolitic facies is
57 overlain by thin-beds of light- to dark-grey clayey dolomite and limestone interbedded with
58 decimetre to meter-thick layers of extremely carbon-rich black shales^{77,78}. The six black shales
59 analysed in this study (Extended Data Table 1) are finely planar- or wavy-laminated and have TOC
60 contents of 10 to 32%.

61 The black shales of both formations formed in quiet sub-wave base environments that were
62 possibly protected by off-shore stromatolite reefs⁷⁷ and, based on stratigraphic context, presumably
63 not very deep. The black shales intersected by drill hole S2 were deposited beneath (transitory)
64 ferruginous and occasionally sulphidic waters⁷⁸ and comprise micrometre-thin, plane to wrinkly,
65 continuous organic laminae with occasional framboidal pyrite and pyritized filamentous sheaths⁷⁸.
66 The organic laminae are interpreted as benthic microbial mats that either represent heterotrophic
67 and chemosynthetic microbial communities thriving beneath anoxic ferruginous to euxinic waters
68 or communities of anoxygenic and possibly oxygenic phototrophic bacteria^{78,2} persisting beneath

69 (transitory) anoxic waters. Fluorescence microscopy on thin-sections also revealed irregularly
70 shaped, discrete or bedding-parallel accumulations of organic particles interpreted as planktonic
71 debris².

72 Micropalaeontological work on the El Mreïti Group, including sediments from drill core S2,
73 yielded an exquisitely preserved assemblage of organic-walled microfossils⁶⁵. The assemblage
74 included 8 taxa of ornamented and 3 taxa of process-bearing vesicles interpreted as unambiguously
75 eukaryotic, 9 possibly eukaryotic taxa, 6 probable prokaryotes (diverse leiospheroids), and 22
76 other prokaryotic or eukaryotic taxa. The eleven, generally rare taxa classified as ‘diagnostic
77 eukaryotic’ were absent from all samples of the black shale facies, but six of nine taxa classified
78 as ‘possibly eukaryotic’ were present and partly abundant in this facies⁷⁸. In the basin, the
79 abundance of fossils with diagnostic eukaryotic features decreases from shallow to deeper
80 environments, and this was interpreted as the possible ecological preference of these taxa, although
81 preservational bias cannot be ruled out⁷⁸.

82 The Tourist Formation in drill core S2 did not contain any diagnostic eukaryotic taxa but yielded
83 numerous leiospheroids classified as ‘possibly eukaryotic’. Such leiospheroids were present in all
84 samples⁷⁸ that yielded protosteroids (Extended Data Table 1, Supplementary Table 1). Likewise,
85 all samples of the En Nesoar Formation that yielded protosteroids contained abundant
86 leiospheroids⁷⁸.

87 The En Nesoar Formation of drill core S2 also yielded the diagnostic eukaryotic fossils *Simia*
88 *annulare*, *Jacutianema solubila*, *Pterospermopsimorpha insolita*, *Trachyhystrichosphaera*
89 *aimika*, *Trachyhystrichosphaera botula*, *Valeria lophostriata* and *Vidaloppala*⁷⁸. Such fossils with
90 typical eukaryotic features were also found in two samples from the En Nesoar Formation that
91 yielded protosteroids but lacked crown-steroids: 13N13 contained *Jacutianema solubila*,
92 *Trachyhystrichosphaera aimika* and *Simia annulare*; and 13N14 yielded *Simia annulare*,
93 *Trachyhystrichosphaera aimika*, *Trachyhystrichosphaera botula*, and *Vidaloppala*⁷⁸. These
94 observations strengthen the hypothesis that the possibly eukaryotic and clearly eukaryotic fossils
95 of the El Mreïti Group have a stem-group origin.

96

97 **1.2. Late Mesoproterozoic Nonesuch Formation, Keweenaw Rift, USA (~1,100 Ma)**

98 The Nonesuch Formation is part of the Oronto Group, in the upper Keweenaw Peninsula, and is
99 recorded in several cores drilled around White Pine, Michigan, USA. The Oronto Group sits within
100 the failed Midcontinent Rift System. The grey to black shales (50 to 200 m) of the Nonesuch
101 formation overlie the Copper Harbor Conglomerate and are overlain by the Freda Sandstone. The
102 age of the Nonesuch Fm was determined by Re/Os dating to be 1078 (±24) Ma⁸⁰.

103 The nine samples of the Nonesuch investigated in this study were collected from drill cores PI-2,
104 DO-6, WPB-3 and WPB-4, all drilled near the White Pine Mine, Michigan, USA (Extended Data
105 Table 1). Seven samples contain sub-mm dark grey to black laminae, one sample is a light grey
106 shale interlayered with grey silty shale and carbonate (#13N34), and one sample is a calcific shale
107 with wrinkly grey laminae (#13N41).

108 The Oronto Group was originally described as a transgressive-regressive alluvial fan-lacustrine-
109 fluvial system⁸¹. The Nonesuch Formation contains several indicators that the basin was
110 shallowing upwards, including microbial induced sedimentary structures⁸², raindrop impressions,
111 desiccation polygons⁸³ and mud cracks⁸⁴. A stromatolitic horizon near the top of the Copper
112 Harbor Conglomerate was interpreted as the onset of lacustrine deposition⁸⁵. A lacustrine
113 interpretation for the depositional environment is supported by mineralogy and total iron over
114 aluminium (FeT/Al) systematics, with values reflective of authigenic enrichments of FeT/Al
115 representative of iron flux similar to modern palaeo-lake settings within a volcanic province⁸⁶.
116 Furthermore, the presence of pigmented hematite titanite, leucoxene and authigenic titanium-oxide
117 grains indicate the depositional basin witnessed in-place oxidation, supporting the hypothesis of
118 an oxygenated water column during deposition. However, others suggest the basin was influenced
119 by marine waters during deposition of the Nonesuch Fm causing brackish conditions. This
120 interpretation is based on an enrichment of redox-sensitive elements U, Mo, Co, Zn and V, sulfur
121 isotopes and slightly elevated Sr/Ba ratio⁸⁷.

122 Over the past decade, the Nonesuch lagerstätte has gathered a lot of interests as the organic rich
123 shales contain a wide range of unicellular and simple multicellular microfossils^{88,89}, providing a
124 window into eukaryotic diversification during the Late Mesoproterozoic. The formation yields a
125 large variety of simple, presumably prokaryotic sphaeromorphs and filaments, but also eukaryotic
126 microfossils such as *Valeria lophostriata*, linear multicellular clusters comparable with
127 *Gloeodiniopsis*, and the multicellular *Proterocladus* that is now recognized as a possible
128 chlorophyte^{11,83}.

129

130 ***1.3. Mid-Mesoproterozoic Roper Group, McArthur Basin, Australia (~1,300 Ma)***

131 The geology of the Roper Group was previously summarized⁹⁰⁻⁹³. The Roper Group is a
132 component of the Wilton Package and was deposited in the McArthur Basin of northern Australia
133 over an area of > 180,000 km². It consists of a 1 to 3 km thick package of siliciclastic rocks
134 characterised by alternating mudrock and cross-bedded sandstones. They are thickest in the
135 Beetaloo Sub-basin and gradually decreases in thickness away from the depocentre. Based on two
136 competing models, the Roper Group was either deposited in a shallow-marine to shelf environment
137 on an epicontinental platform or on an intracratonic ramp.

138 The Velkerri Fm, a shoaling sequence deposited between two sandstone units in the middle of the
139 Roper Group, dominantly comprises deep-water, basinal facies with abundant black shale and
140 organic-rich siltstones, and is sub-divided into the Kalala, Amungee and Wyworrie members⁹⁴.

141 The age of the Velkerri Formation is constrained using maximum and minimum depositional ages
142 as well as direct shale measurements. The maximum deposition age is constrained by detrital
143 zircon U-Pb ages of 1386 ± 13 Ma and 1385 ± 116 Ma in the underlying Bessie Creek Sandstone
144 ^{93,95} and a detrital zircon age of 1308 ± 41 Ma in the Wyworrie Member⁹⁵. The minimum age of
145 the Velkerri Formation is constrained by the intrusive Derim Derim Dolerite that has a TIMS U-
146 Pb baddeleyite age of 1312.9 ± 0.7 Ma⁹⁶. The Amungee Member of the Velkerri Fm yielded Re-
147 Os ages of 1361 ± 21 Ma (C shale) and 1417 ± 29 Ma (A shale)⁹⁷.

148 In the present study, four thermally immature²⁷ dark grey to black siltstones from the upper
149 Velkerri Fm from drill core Atree-2 yielded indigenous protosteroids. Atree-2 was collared in the
150 Beetaloo Sub-basin, the main depocentre of the Roper Seaway. During deposition of the siltstones
151 and shales of the upper Velkerri Fm in Atree-2, the water column was presumably suboxic to
152 anoxic and ferruginous at depth but not sulphidic⁹². Moreover, anoxic waters did not, or rarely,
153 rise into the euphotic zone of the water column^{22,27}.

154 The Roper Group is well known for well preserved assemblages of early eukaryotic fossils (Fig.
155 1c)⁹⁸. Diagnostic eukaryotic species such as *Satka favosa*, *Satka squamifera* and *Valeria*
156 *lophostriata* occur largely in marginal marine to inner shelf facies, while the process and
157 protrusion-bearing eukaryote *Tappania plana* is restricted to deeper, distal shelf facies. In basinal
158 facies, such as those of the Velkerri Fm, fossils with diagnostic eukaryotic characteristics are rare,
159 although possibly eukaryotic leiospheroids occur⁹⁸. Moreover, the probable eukaryote
160 *Blastanosphaera kokkoda* is abundant in basinal shales of the Mainoru Fm (a deep-water facies
161 towards the base of the Roper Group), forming local monospecific assemblages. Importantly, rare
162 specimens of *Dictyosphaera macroreticulata*, a large acritarch with convincing eukaryotic
163 features, is restricted to basinal facies in the Velkerri Fm⁹.

164

165 **1.4. Early Mesoproterozoic formations of the North China Craton (1,392 to 1,460 Ma)**

166 The Yanliao Basin in the North China Craton was formed during the rifting of the Nuna
167 (Columbia) Supercontinent and contains a ~10 km thick Mesoproterozoic and Neoproterozoic
168 sedimentary sequence. The black shales and silty mudstones in the present study come from the
169 Mesoproterozoic Jixian and Huailai groups, deposited between 1,685 and 1,320 Ma⁹⁹). The lower
170 Jixian Group is separated—listed from oldest to youngest—into the Gaoyuzhuang, Yangzhuang,
171 Wumishan, Honghuizhuang and Tieling formations. The overlying Huailai Group only contains

172 the Xiamaling Fm. In this study we collected six samples from four drill cores covering the
173 Xiamaling, Tieling and Hongshuizhuang formations (Extended Data Table 1).

174 The age of the Hongshuizhuang Fm is constrained by the ~1,485 Ma underlying Wumishan Fm¹⁰⁰
175 and the ~1,439 Ma overlying Tieling Fm (with an average age of 1,460 Ma given in Extended Data
176 Table 1). The ~1,439 Ma age of the Tieling Fm is based on U/Pb analyses on zircons recovered
177 from a tuff layer in the middle part of the formation^{100,101}. The Tieling and overlying Xiamaling
178 formations are separated by a ~14 million year unconformity linked to the Qinyu uplift (1,432–
179 1,418 Ma⁹⁹). A tuffaceous layer in Member 3 of the Xiamaling Fm is dated at ~1,392 Ma^{100,102}
180 while the upper limit of the top of the Xiamaling Fm is indicated to be ~1,350 Ma¹⁰³.

181 The Hongshuizhuang Fm consists of dolomitic carbonate interlayered with black to dark grey
182 mudstones. Redox elemental analyses (Mo, Cr, U) revealed ferruginous to intermittently euxinic
183 bottom waters during the deposition of the formation¹⁰⁴. The overlying Tieling Fm starts with silty
184 dolostone and shale deposits with occasional manganese dolostone in the basal Daizhuangzhi. The
185 deposits of the overlying Laohuding member comprise limestone deposits with increasing
186 stromatolitic features up-section, suggesting a shallowing basin¹⁰⁵.

187 The Xiamaling Fm displays a transgressive-regressive system with the lowest member (Member
188 1) recording ferruginous sandstone, sandy mudstones, and mudstones. Member 2 mainly contains
189 green and dark grey mudstones with marly concretions. Member 3 is dominated by black shale,
190 while the highest member (Member 4) consists of dark mudstones interlayered with stromatolitic
191 carbonate units¹⁰⁶. (Note that ref⁶⁷ divides the Xiamaling Fm into 6 units with a different
192 numbering system). The organic rich shale horizons of the Xiamaling Fm, with TOC values up to
193 20%⁶⁷, were presumably deposited in a restricted basin with varying degrees of connection to the
194 open ocean¹⁰⁷. During the deposition of the organic rich horizons, the basin was severely restricted,
195 while the geochemical characteristics of surrounding shale and silty mudstone units are interpreted
196 as a marine system with persistent euxinic waters^{106,107}. Others suggest that the Xiamaling Fm was
197 predominantly deposited under deep oxygenated waters^{67,108}, although the interpretation of the
198 exact redox conditions remains debated¹⁰⁹.

199 The fossil assemblages of the Xiamaling contain a large variety of palynomorphs, both of bacterial
200 and eukaryotic origin. The majority of microfossils are found in Member 1 of the Xiamaling
201 Formation preserved within dark grey and greyish-green shale, and silty shale interbedded with
202 thin-bedded iron-rich siltstone horizons¹¹⁰. Preserved microfossils of possibly to certainly
203 eukaryotic origin in the Xiamaling Fm include *Leiosphaeridia*, *Pterospermospimorpha insolita*,
204 *Simia annulare*, *Satka favosa*, *Dictyosphaera macroreticulata*, *Germinosphaera bispinosa* and
205 *Valeria lophostriata*¹¹⁰.

206

207 ***1.5. Late Palaeoproterozoic Barney Creek Formation (BCF), McArthur Basin, Australia***
208 ***(1,640 Ma)***

209 The McArthur Basin is a > 180,000 km² multiphase basin in northern Australia. Late
210 Paleoproterozoic sediments of the Glyde Package were deposited across the McArthur Basin
211 during north-south trending extension and active subsidence of the Northern Australian Craton
212 (NAC). As such, the basin is characterised by paleohighs and structurally controlled sub-basins
213 bounded by NNE-striking faults and south-dipping growth faults¹¹¹⁻¹¹³.

214 The geology of the Barney Creek Fm was summarized by Jackson et al. (1987)⁹⁰ and Ahmad et al.
215 (2013)¹¹⁴ and a recent facies analysis and sequence stratigraphy was provided by Kunzmann et al.
216 (2019)¹¹¹. The Barney Creek Fm, as part of the Umbolooga Subgroup of the McArthur Group, is
217 a regionally extensive but poorly outcropping dolomitic siltstone-dominated sedimentary rock
218 unit. The Barney Creek Fm comprises mainly dolomitic, carbonaceous and pyritic siltstones and
219 shales, locally with abundant tuff beds, breccias and graded units^{111,114}. Three tuff beds from the
220 lower Barney Creek Fm (HYC Pyritic Shale Member) yielded U-Pb ages of 1,638 ± 7 Ma, 1,639
221 ± 3 and 1,640 ± 3 Ma¹¹⁵. Thickness of the Barney Creek Fm varies from ~10 m on paleohighs, to
222 ~900 m in local subbasins such as Glyde River (drill core GRNT-79-7 = GR7), or ~160 m at the
223 Leviathan anomaly (drill core LV09001 = LV09) to the east of the Emu Fault. Major non-
224 carbonate components of the Barney Creek Fm were reported as microcrystalline K-feldspar,
225 terrigenous silt and clay, as wells as locally abundant sulphides, carbonaceous and bituminous
226 material^{114,116}.

227 Deposition is inferred as having occurred mostly under subtidal to slope conditions with local
228 shoaling to shallow subtidal environments on paleohighs. The Barney Creek Fm comprises three
229 members, the W-Fold shale, HYC pyritic Shale and Cooley Dolostone (only along fault scarps)
230 that are overlain by the undifferentiated upper part of the formation. Jointly with the conformably
231 overlying Reward Dolostone, the Barney Creek Fm comprises two 3rd-order transgressive-
232 regressive stratigraphic sequences. A maximum flooding surface is located in the base metal
233 prospective HYC Pyritic Shale Member that constitutes the most pyritic and organic matter-rich
234 interval deposited during basin deepening, while another maximum flooding surface is located in
235 the upper undifferentiated Barney Creek Fm¹¹¹. Early interpretations of depositional environments
236 ranged from shallow marine to lacustrine or lagoonal¹¹¹, but the HYC Pyritic Shale Member is
237 generally regarded to have formed under deep subtidal conditions^{117,118}. Nevertheless, similarities
238 in carotenoid biomarker signatures between the undifferentiated Barney Creek Fm³⁰ and the saline
239 lacustrine Eocene Green River Fm, were used to infer lacustrine depositional conditions¹¹⁹.
240 However, redox stratification, generally low sulphate concentrations in Palaeoproterozoic marine
241 basins¹²⁰ and the intracratonic setting of the BCF could also explain similarities in carotenoid

242 biomarker patterns. Fe-speciation indicates dominantly anoxic ferruginous redox conditions^{121,122},
243 even though sulfidic excursion may have been common with possible temporary injection of oxic
244 waters¹²².

245 Abundant fossil pigments of green (Chlorobiaceae) and purple (Chromatiaceae) sulfur bacteria
246 indicate a shallow chemocline and an important ecological role of anoxygenic phototrophic
247 primary producers, while indigenous algal steranes were not discovered^{30,122}. Despite the lack of
248 saturated steranes, the aromatic fractions of Barney Creek Fm rock extracts were previously found
249 to contain high abundances of triaromatic steroids with an enigmatic dominance (> 90%) of
250 steroids methylated at the C-4 position³⁰. As a dominance of 4-methylated steroids without side-
251 chain alkylation was mainly known from certain methanotrophic bacteria, these were tentatively
252 proposed as the most likely source organisms, although the lack of saturated sterane equivalents
253 hampered reconstructions of biological precursors and source organisms in previous studies. Most
254 putative microfossils described from the Barney Creek Fm appear to be bacterial, although some
255 ambiguous, larger and more complex structures have been interpreted as putative eukaryotic
256 fossils¹²³.

257 After the underlying, 1,730 Ma Wollongorang Fm¹²⁴, the Barney Creek Fm contains the probably
258 oldest-known, clearly indigenous biomarkers¹²⁵ and may constitute the oldest active petroleum
259 system¹²⁶. Samples analysed in the present study come from the thermally least mature sections in
260 the basin, the upper 200 m of drill core GR7 and drill core LV09. The thermal maturity falls into
261 the early oil window-range, with Tmax ranging from ~435 to ≤440°C in the upper 200 m of
262 GR7¹²⁷, whereas biomarkers show an even better thermal preservation throughout core LV09
263 where Tmax values range from 423 to 440°C¹²⁸. One additional sample (12Z083, 103.3 m depth)
264 used to evaluate the identity of cyclosterane, contains strongly biodegraded migrabitumen filling
265 a calcite vug in the Coxco Dolomite of drill core MY4 collared in the Myrtle area in the southern
266 McArthur Basin, but this bitumen was likely sourced from the Barney Creek Fm and was
267 subsequently biodegraded in the permeable carbonate reservoir.

268 **2. Supplementary Methods**

269 ***2.1. Generation of protosterol, ursterol and euphol pyrolysis products***

270 ***2.1.1. Pyrolysis of cycloartenol and lanosterol***

271 Identification standards for saturated and aromatic hydrocarbon derivatives of protosterols were
272 generated by pyrolysis of commercially available cycloartenol (by GC-MS full scan: 95%
273 cycloartenol, 5% 24-methylene cycloartenol, traces of cycloartenol isomers, no detectable 4- or
274 14-demethylated products, no detectable crown sterols; aber GmbH & Co. KG, cat AB 165530)
275 and lanosteroid mixture (by GC-MS full scan: isomers of lanosterol, lanostenol and lanostadienols,

276 impurities of lanosteroid oxidation products, no detectable 4- or 14-demethylated products, no
277 detectable crown sterols; abcr GmbH & Co. KG, cat AB 137660). Similar to activated carbon
278 pyrolysis procedures described in the literature^{129,130}, ~10 mg of activated carbon and ~1 mg of
279 standard were inserted into a ~3 mm i.d. glass tube and sealed under vacuum. The tubes were
280 heated at 300°C or 330°C for 18 h and after cooling to room temperature, scored and opened.
281 Pyrolysis products and active carbon were transferred with DCM into a glass wool plugged silica
282 pipette and extracted with ~4 mL *n*-hexane and DCM. After removal of the solvent under a stream
283 of purified nitrogen gas, products were re-dissolved in *n*-hexane for GC-MS analyses. Half of the
284 pyrolysates were hydrogenated by stirring for 1 h in the presence of PtO₂ catalyst under a
285 continuous flow of H₂ gas, before capping and stirring for another 3 h.

286 2.1.2. *Pyrolysis of a cycloartenol/24-methylenecycloartenol mixture*

287 To obtain elution positions and mass spectra, 24-methylated protosteroids were generated by
288 pyrolysis of rice bran powder (Lotus Foods, Australia) containing subequal amounts of
289 cycloartenol and 24-methylenecycloartenol (among other sterols). 150 mg of rice bran powder
290 were placed into baked glass tubes (200 mm long, 6.3 mm OD and 4.1 mm ID) sealed at one end.
291 A glass fibre plug was placed on top of the bran to prevent dispersion of the powder. To remove
292 air, the tubes were evacuated and purged with nitrogen gas three times and then sealed under
293 vacuum. The 160 mm long pyrolysis tubes were inserted into a custom-built heating block so that
294 the upper 70 mm remained at room temperature outside the heating zone to allow condensation of
295 water and pyrolysis products. The heating block was set to an initial temperature of 300°C and
296 then rapidly ramped to 500°C. Tubes were removed after 5 to 15 minutes, cooled, scored and
297 opened. Pyrolysis products were extracted with dichloromethane (1 mL, 3 times). After removal
298 of the solvent under a stream of purified nitrogen gas, the pyrolysis products were taken up in *n*-
299 hexane (2 mL) and hydrogenated with H₂ gas on ~20 mg PtO₂/C under constant stirring for 12
300 hours. *n*-Alkanes were removed from the rice bran pyrolysate by filtration over silicalite and
301 elution with pentane¹³¹.

302 2.1.3. *Pyrolysis of euphenol (24(25)-dihydroeuphol)*

303 Euphanes were generated by pyrolysis of ~1 mg euphenol (24(25)-dihydroeuphol, purity > 95%
304 by GC), obtained from the natural products reference collection of late Prof Ourisson, University
305 of Strasbourg, France, following the protocol in Section 2.1.2. Saturated+unsaturated
306 hydrocarbons were separated from aromatic and polar pyrolysis products over silica gel using *n*-
307 hexane as eluent, followed by hydrogenation of the saturated+unsaturated hydrocarbon fraction
308 on PtO₂/C as described above. Pyrolysis products were characterized by GC-MS in full scan and
309 MRM mode on a DB5-MS column (Extended Data Fig. 3b, c).

310 **2.2. Synthesis of authentic C₂₉ monoaromatic lanosteroid (MAL)**

311 The monoaromatic lanosteroid was synthesized from lanost-8-ene following an established
312 method¹³² for the synthesis of a monoaromatic fernene derivative starting from fern-9(11)-ene.
313 Lanost-8-ene was first heated with elemental sulfur (200°C, 5 h). The crude mixture obtained after
314 removal of the elemental sulfur using activated copper was sequentially treated with Raney nickel
315 and hydrogenated over Pd/C under H₂ atmosphere. The monoaromatic lanosteroid was purified
316 from the crude mixture by column chromatography on a silica gel column using *n*-hexane as eluent.

317 GC-MS (EI, 70 eV): *m/z* (relative intensity) 394 (15%), 379 (100), 267 (17), 253 (12), 239 (20),
318 225 (33), 213 (55). 199 (19), 169 (11), 155 (11), 83 (7).

319
320 The structure of the synthesized compound (Extended Data Fig. 1, 20R MAL) was confirmed by
321 mass spectrometry and NMR studies comprising 1D (¹H, ¹³C, DEPT) and 2D homo (¹H-¹H COSY)
322 and heteronuclear (¹H-¹³C-HSQC, and ¹H-¹³C-HMBC) experiments. NMR analyses were
323 performed on a Bruker Avance I – 500 MHz spectrometer operating at an observation frequency
324 of 500 MHz (¹H) and 125 MHz (¹³C) (¹H, ¹³C, DEPT, ¹H-¹³C-HSQC, and ¹H-¹³C-HMBC) and on
325 a Bruker Avance III – 600 MHz spectrometer at an observation frequency of 600 MHz (¹H) (¹H,
326 ¹H-¹H COSY). The chemical shifts (Supplementary Table 4) are reported in ppm relative to
327 tetramethylsilane with the solvent used as internal standard (CD₂Cl₂: δ¹H 5.32 ppm; δ¹³C 53.5
328 ppm).

329
330 **Supplementary Table 4.** ¹H and ¹³C-NMR chemical shifts of synthetic monoaromatic lanosteroid
331 (20R MAL) (CD₂Cl₂).

Position	δ _C	δ _H	δ _H	Position	δ _C	δ _H	δ _H
1	27.4	2.51	2.51	16	28.3	2.10	1.45
2	19.5	1.81	1.81	17	50.6	1.65	-
3	38.7	1.61	1.61	18	-	-	-
4	33.6	-	-	19	15.6	0.62	-
5	142.3	-	-	20	36.4	1.45	-
6	123.4	7.12	-	21	18.6	0.96	-
7	122.2	6.83	-	22	36.4	1.40	1.05
8	144.2	-	-	23	24.1 ^c	1.40	1.19
9	133.1	-	-	24	39.5	1.16	1.16
10	133.8	-	-	25	28.0	1.51	-
11	24.0 ^c	2.62	2.62	26	22.6 ^a	0.880 ^b	-
12	30.9	1.98	1.98	27	22.3 ^a	0.876 ^b	-
13	43.9	-	-	28	31.8	1.26	-
14	50.1	-	-	29	31.8	1.26	-
15	32.1	1.82	1.70	30	27.9	1.02	-

332 a, b, c: can be exchanged

333

334 **2.3. Elucidation of the possibility of double bonds and cyclopropyl moieties in cyclosterane**

335 Aliquots of the saturate fraction of the biodegraded oil from drill core MY4 (section 2.6.1) were
336 subjected to experiments in order to evaluate the possible presence of a double bond or cyclopropyl
337 moiety in cyclosterane (*k1* and *k2*, Extended Data Fig. 2b). In the first experiment, an aliquot of
338 the bitumen was hydrogenated by stirring for 3 h in the presence of PtO₂ catalyst and a constant
339 stream of H₂ gas, followed by extraction of products with 1 mL *n*-hexane and 3 mL DCM.
340 Subsequently, the hydrogenation products on ~7 mg active carbon were transferred into a glass
341 tube that was evacuated and sealed, followed by pyrolysis at 300°C for 18h. After extracting the
342 active carbon twice with 2 mL *n*-hexane and 2 mL DCM, the pyrolysis products were hydrogenated
343 by stirring for 30 min in the presence of PtO₂ catalyst and a constant stream of H₂ gas. In the
344 second experiment, an aliquot of bitumen was subjected to acidic conditions for 30 minutes by
345 stirring it in 2 mL DCM acidified with 10 μL concentrated HCl dissolved in 50 μL methanol,
346 followed by removal of the solvent under a stream of N₂ gas. The products were taken up in *n*-
347 hexane and hydrogenated first for 4 h with H₂ gas on PtO₂, and then on Pt/C for 21 h. In the third
348 experiment, an aliquot of bitumen was hydrogenated under acetic acid conditions under a constant
349 stream of H₂ gas by stirring for 3 h in 50 μL acetic acid and 2 mL DCM in the presence of PtO₂
350 catalyst, before the vial was capped and stirred for another 69 hours. The same experiment was
351 also conducted on cycloartenol. All hydrogenation products were extracted by ultrasonication (5
352 min) in DCM, followed by extraction with 1.5 mL *n*-hexane and 1.5 mL DCM. In a fourth
353 experiment, HCl gas was reacted with cycloartenol, cycloartane (BOC Sciences, NY, USA, CAS#
354 511-64-8) and an aliquot of the saturate fraction of the MY4 biodegraded bitumen. HCl gas was
355 let through a capillary into a 4 mL vial filled with the organic substrates dissolved in 2 mL DCM.
356 After being flushed several times with HCl gas, the reactant vial was capped and stirred overnight.
357 To remove HCl, the DCM was washed with water (4 times) and dried over NaSO₄.

358 **2.4. RuO₄ degradation of a fraction containing *k1* and *k2***

359 A small fraction containing *k1* and *k2* (neat) was treated with a few drops of a solution of RuO₄
360 freshly prepared according to Piatak et al. (1969)¹³³, but replacing acetone by CCl₄. After 10 min,
361 a mixture of CCl₄ and isopropanol (1 ml) was added in order to destroy the excess of reagent. The
362 solvents were removed under a flow of argon, the crude mixture was filtered on a short silica gel
363 column using dichloromethane as eluent and analysed using GC-MS.

364 **2.5. Mass spectrometer parameters for the detection of (proto)steroids and hopanoids**

365 Metastable Reaction Monitoring (MRM) precursor → product transitions for the measurement of
366 saturated steranes and hopanes is given in Supplementary Table 5 (total cycle time 1362.1 ms),
367 MRM precursor → product transitions for the measurement of saturated protosteranes and
368 ursteranes in Supplementary Table 6 (total cycle time 962.9 ms), and Selected Ion Recording (SIR)

369 mass to charge (m/z) ratios for the measurement of aromatic protosteroids, steroids and hopanoids
370 in Supplementary Table 7 (total cycle time 1280.1 ms).

371

372 **Supplementary Table 5.** MRM parameters for saturated steranes and hopanes.

Transition	Precursor	Product	Dwell [ms]	Delay [ms]
1	358.35	217.20	10.0	40.0
2	358.35	217.20	25.0	40.0
3	414.42	231.21	25.0	21.6
4	372.38	217.20	20.0	21.4
5	386.39	217.20	20.0	22.8
6	404.41	221.22	15.0	20.7
7	400.41	217.20	20.0	22.0
8	414.42	217.20	25.0	22.5
9	426.42	205.19	20.0	29.8
10	370.36	191.18	20.0	20.0
11	440.44	205.19	20.0	22.1
12	384.37	191.18	25.0	20.3
13	454.45	205.19	20.0	21.7
14	398.39	191.18	20.0	20.6
15	468.47	205.19	25.0	21.3
16	412.41	191.18	20.0	20.9
17	482.48	205.19	100.0	21.0
18	426.42	191.18	20.0	21.1
19	496.50	205.19	150.0	20.7
20	440.44	191.18	20.0	21.3
21	454.45	191.18	20.0	21.9
22	398.39	177.16	20.0	21.2
23	468.47	191.18	20.0	20.6
24	482.48	191.18	20.0	21.7

373

374 **Supplementary Table 6.** MRM parameters for protosteranes and ursteranes.

Transition	Precursor	Product	Dwell [ms]	Delay [ms]
1	412.40	288.29	10.0	40.0
2	412.41	288.29	40.0	20.0
3	426.40	288.29	40.0	23.1
4	412.41	274.27	60.0	26.1
5	414.40	259.25	40.0	30.3
6	400.40	245.24	30.0	26.4
7	414.40	245.25	30.0	22.8
8	386.38	231.21	30.0	23.8
9	400.40	231.21	30.0	22.8
10	414.42	231.21	30.0	22.6
11	372.37	217.20	10.0	21.4
12	386.39	217.20	10.0	22.8
13	404.41	221.22	10.0	20.7
14	400.40	217.20	10.0	22.0
15	414.41	217.20	30.0	22.5
16	370.40	191.20	10.0	50.0

375 **Supplementary Table 7.** SIR parameters for aromatic (proto)steroids and hopanoids

Transition	<i>m/z</i>	Dwell [ms]	Delay [ms]
1	416.34	20.0	40.0
2	416.34	20.0	20.0
3	414.33	20.0	20.7
4	404.34	20.0	23.3
5	402.33	20.0	20.7
6	393.35	20.0	23.0
7	379.34	20.0	24.8
8	375.31	20.0	21.4
9	365.32	20.0	23.5
10	361.29	20.0	21.4
11	351.31	20.0	23.5
12	347.27	20.0	21.5
13	340.33	20.0	22.5
14	267.21	20.0	140.0
15	367.21	20.0	20.0
16	359.15	20.0	23.3
17	253.20	20.0	22.5
18	245.13	20.0	23.4
19	231.12	20.0	26.1
20	217.20	20.0	20.0
21	212.14	10.0	40.0
22	191.18	10.0	40.0
23	191.18	20.0	20.0

376

377

378 **2.6. Characterization of saturated protosteranes**

379 The GC elution behaviour, mass spectra and structures of saturated protosterol derivatives were
380 investigated on thermally well preserved bitumens from the Barney Creek Fm from the upper ~200
381 m of drill core GR-7 (Extended Data Table 1, and additional samples B03163, GR7, 47.55 m;
382 12Z083, MY4, 103.3 m) and on strongly biodegraded migrabitumen filling a calcite vug in the
383 Coxco Dolomite of drill core MY4 collared in the Myrtle area in the McArthur Basin (12Z083).
384 In drill core MY4, the Coxco Dolomite directly underlies the BCF, and the solid migrabitumen
385 filling the vug was likely sourced from the BCF or deeper units.

386 **2.6.1. Cyclosterane (I)**

387 Extended Data Figure 2b shows the total ion chromatogram of the saturated fraction of the
388 migrabitumen filling the calcite vug in drill core MY4. The bitumen is strongly biodegraded with
389 a very prominent unresolved complex mixture (UCM) and absence of *n*-alkanes and acyclic
390 isoprenoids pristane and phytane. Hopanes only occur in traces. The aromatic fraction (not shown)
391 is also dominated by a UCM. Triaromatic steroids (TAS), particularly abundant in most BCF

392 bitumens, were below detection limits. TAS are generally among the most biodegradation-resistant
393 compounds, and their absence places the migrabitumen into biodegradation category 10, the
394 highest rank¹³⁴. Despite severe biodegradation, the saturated fraction shows two dominant, well-
395 resolved chromatographic signals (*k1* and *k2*) with nearly identical mass spectra containing
396 molecular ion $[M^{+\circ}]$ at m/z 412 and base ion m/z 274 (Extended Data Fig. 2d). *k1* and *k2* were
397 subsequently also detected in lower relative concentrations in all thermally well preserved BCF
398 bitumens, suggesting that the compounds were enriched from the original migrated oil through
399 biodegradation and that they possess unusual structural features placing them among the most
400 biodegradation resistant hydrocarbons known to date. The molecular mass of 412 Da points to C₃₀
401 compounds with five double bond equivalents (DBE), i.e. with a total of five rings or double bonds
402 (compare also with peaks P in ref¹²⁵).

403 Due to the unusual enrichment of the unknown compounds in the biodegraded migrabitumen, it
404 was possible to detect a pseudo-homologous series of side-chain cleavage products *k3* to *k11* with
405 22 to 29 carbon atoms, corresponding to the sequential loss of up to 8 carbon atoms from the
406 hydrocarbon core (Extended Data Fig. 2a). However, two members of the pseudo-homologous
407 series with molecular ions $[M^{+\circ}]$ at m/z 384 (C₂₈) and 314 (C₂₃) are close to detection limits (marked
408 'x'), pointing to two branching positions in the side chain. In total, the pseudo-homologous series
409 reveals a 6-methylhept-2-yl side chain, the typical C₈ side chain of sterols such as cholesterol,
410 lanosterol or cycloartenol. Moreover, all pseudohomologs from *k1* to *k8* show two isomers with
411 nearly identical mass spectra, while *k10* and *k11* are single peaks. This likely indicates that the
412 signal pairs represent S and R isomers with a chiral centre in the side chain marked '*' in Extended
413 Data Figure 2a, another characteristic of protosterol side chains.

414 Extended Data Figure 2c, d shows the mass spectra of chromatographic signals *k1* to *k11*. The
415 dashed lines follow particular fragments through the pseudo-homologous series, whereby constant
416 mass throughout the pseudo-homologous series (red) indicates that the fragment belongs to the
417 'left-hand side' of the compounds (i.e. the polycyclic core), whereas successive shifts of m/z 14
418 (blue) indicate fragments belonging to the right-hand side (side-chain including fragments). The
419 main side-chain including fragments of the inferred parent compounds *k1* and *k2* ($[M^{+\circ}]$ 412) are
420 m/z 397, 288, 274, 259 and 205. By contrast, the m/z 299 fragment is constant in *k1* to *k10* and is
421 represented by m/z 300 in the last member of the series *k11*. The gain of one mass unit in *k11*
422 indicates that no further methyl group is lost, i.e. substitution with hydrogen instead of an alkyl-
423 group. This confirms that the m/z 299 fragment corresponds to a polycyclic core with 22 carbon
424 atoms and five DBE attached to a typical saturated C₈ sterol side chain as, for instance, found in
425 cycloartane, the hydrocarbon equivalent of cycloartenol. A comparison of the spectra of *k1* and *k2*
426 with the NIST MS database yielded a 63% agreement with cycloartane (Extended Data Fig. 2d),
427 albeit with several slightly shifted masses and different relative intensities. *k1* also elutes ~1.5

428 minutes earlier than commercially available cycloartane (CAS# 511-64-8, BOC Sciences, NY,
429 USA) under the described chromatographic conditions (Methods). The similarity of the mass
430 spectra suggests that *k1* and *k2* are closely related structural isomers of cycloartane.

431 In comparison to typical steranes that possess a core with four rings, compounds *k1* to *k11* possess
432 an additional DBE in the ring system. The fifth DBE may represent a double bond (as in
433 lanosterol), a cyclopropyl ring (as in cycloartenol) or a bridge across an existing ring (as found in
434 some geological oleanane-type triterpenoids¹³⁵). Non-aromatic double bonds are not particularly
435 stable in sedimentary environments and commonly do not persist over geologic periods of time in
436 systems as mature as the BCF. Moreover, unsaturated hydrocarbons are commonly more prone to
437 biodegradation than saturated counterparts, contradicting the resistance of *k1* to *k11* to
438 biodegradation. However, to test for the presence of a sterically highly protected double bond, the
439 biodegraded migrabitumen was subjected to prolonged catalytic hydrogenation (SI Methods). We
440 found that hydrogenation on platinum catalyst for several hours is commonly sufficient to even
441 hydrogenate many aromatic hydrocarbons. However, *k1* and *k2* survived such catalytic treatment
442 even under the harshest conditions, hydrogenation on PtO₂ and Pt/C for more than 20 h and under
443 acidification with concentrated HCl (Section 2.3). The fact that *k1* and *k2* survived this treatment
444 argues against the presence of a double bond. The potential presence of a sterically hindered double
445 bond was further tested under yet harsher conditions using RuO₄ (Section 2.4). *k1* and *k2* survived
446 this treatment, strongly arguing against the presence of a double bond, which would otherwise
447 have been oxidized. The combined experiments thus identify *k1* to *k11* as saturated pentacyclic
448 hydrocarbons.

449 Cyclopropyl rings are not stable under geological conditions and have, to our knowledge, never
450 been observed in mature sedimentary rocks. However, to test for the presence of a cyclopropyl
451 moiety, the biodegraded oil was subjected to strong heating, acidified PtO₂ hydrogenation and
452 treatment with HCl gas (Section 2.3). Pyrolysis of cycloartenol at 300°C for 18 hours in an
453 evacuated silica tube resulted in quantitative conversion, indicating that cyclopropyl-rings are
454 efficiently opened under these conditions. However, *k1* and *k2* survived this treatment. In a second
455 experiment, cycloartenol and *k1* and *k2* in the biodegraded bitumen were subjected to 70 hours of
456 acidified (concentrated acetic acid in DCM) PtO₂-catalyzed hydrogenation. Under this treatment,
457 the cyclopropyl moiety of cycloartenol was quantitatively opened and the final product was
458 lanostanol, whereas *k1* and *k2* again survived the treatment. In a further test, HCl gas was reacted
459 with cycloartenol, cycloartane and *k1* and *k2* in the biodegraded oil. The reaction of cycloartenol
460 with HCl gas quantitatively opened the three-ring, leading to the formation of compounds with
461 lanostenone-like mass spectra. The experiment was repeated using commercially available
462 cycloartane, resulting in quantitative conversion to lanostenes, thus confirming that HCl gas

463 treatment also opens cyclopropyl moieties of otherwise unfunctionalized hydrocarbons. However,
464 *k1* and *k2* in the biodegraded oil again survived the same HCl gas treatment.

465 The combined experiments suggest that *k1* and *k2* do not possess a double bond or cyclopropyl
466 ring, and the only remaining plausible option for the fifth DBE is a bridge across one of the four
467 rings, a bicyclo-structure. This assignment is supported by the observation of bicyclo-structures in
468 oleanane-type triterpanes that presumably formed through geological rearrangement processes¹³⁵.
469 We thus suggest the informal name ‘**cyclosterane**’ for *k1* and *k2*. The bridge structure may explain
470 the unusual resistance of these compounds to biodegradation.

471 The position of the bridge in cyclosterane remains unknown, but the mass spectra of *k1* to *k11*
472 (Extended Data Fig. 2c, d) are most consistent with a bridge across ring C, such as structure **I**
473 (Extended Data Fig. 1, 2b). The mass spectrum of cyclosterane is similar to cycloartane, suggesting
474 that the compounds are structurally related and that the differences in spectra may provide clues
475 to structural distinctions. Notable differences in the mass spectra of *k1* and *k2* versus cycloartane
476 are shifts from *m/z* 121 in cycloartane to *m/z* 123 in cyclosterane, from *m/z* 135 to *m/z* 137 and
477 from *m/z* 203 to *m/z* 205 (Extended Data Fig. 2d). The *m/z* 121 fragment of cycloartane represents
478 a C₉H₁₃ left-hand fragment with 3 DBE that encompasses the A-ring and is generated by cleavage
479 through the B-ring as well as the cyclopropyl ring (Extended Data Fig. 2e). *M/z* 121 is thus
480 indicative of the attachment of the cyclopropyl ring to the A and B rings. By contrast, the *m/z* 123
481 fragment of *k1* to *k11* represents a C₉H₁₅ left-hand fragment with 2 DBE that encompasses the A-
482 ring and is generated by cleavage through the B-ring, but indicating that there is no bridge across
483 ring A or a ring or bridge attached to any of the carbon atoms of ring A. According to the
484 degradation series in Extended Data Figure 2d, the *m/z* 205 fragment of cyclosterane represents a
485 C₁₅H₂₅ right-hand fragment with 3 DBE that includes the D-ring and is generated by cleavage
486 through the C-ring and one additional DBE that tentatively points to a bridge across ring C that is
487 attached to ring D, for example structure **I** (although other bridge configurations and substitution
488 patterns are possible). Extended Data Figure 2e shows possible MS fragmentation patterns that we
489 would expect from hypothetical structure **I**, although other structural isomers may also explain the
490 data.

491 In mid-Proterozoic bitumens, lanostane and cyclosterane are the only detected saturated
492 hydrocarbon products of sterols. However, cyclosterane was also detected in low percentage levels
493 (relative to total steranes) in virtually all Phanerozoic bitumens and oils that were investigated
494 using MRM 412 → 274 transitions (Supplementary Table 6) and yielded conventional steranes.
495 Cyclosterane is thus the diagenetic product of a biological compound that persisted through
496 geological time but is far less abundant than crown-steranes. Based on the inferred structure of
497 cyclosterane, likely precursors are sterols with the cycloartane skeleton such as cycloartenol.

498 Cycloartenol occurs in low percentage levels as a biosynthetic intermediate in a large proportion
499 of extant eukaryotes, including all plants and algae (Supplementary Text). While cyclosterane was
500 not observed in our pyrolysis experiments on cycloartenol and cycloartane, low-temperature
501 diagenetic opening of the cyclopropyl-ring may plausibly lead to the stabilization of the resulting
502 carbocation or radical by intramolecular formation of a carbon bridge. The fact that lower
503 temperatures are sufficient, and possibly even required, for bridge formation in geological
504 triterpenoids is illustrated by the presence of methylene-bridge containing oleanane derivatives in
505 notable quantities in immature (R_o 0.36–0.41%) sedimentary samples¹³⁵. In this case, the bridged
506 oleanane compound most likely derives from a specific triterpenic precursor functionalized at the
507 C-27 methyl group. Formation of the additional ring can be explained by early diagenetic processes
508 likely to occur under low temperature conditions and leading first to a hexacyclic functionalized
509 oleanoid that is further diagenetically reduced into an oleanane saturated hydrocarbon with a
510 methylene-bridge. Similarly, it can also be envisaged that the cyclosterane skeleton is formed from
511 functionalized cycloartenol-related triterpenes at the earliest stage of diagenesis. This process
512 would first lead to a functionalized compound with the cyclosterane skeleton which is further
513 reduced into the geological cyclosterane hydrocarbons. Since cyclosteranes are not necessarily
514 formed by diagenetic rearrangement of a triterpenoid saturated hydrocarbon already having five
515 rings, the possibility that the cyclosterane skeleton resulted from early diagenetic transformation
516 of lanosterol-related precursors rather than from cycloartenol cannot be excluded. In this process,
517 the double bond of lanosterol would aid formation of the additional ring.

518 Synthesis of authentic standards, purification and NMR experiments (if sufficient quantities of
519 cyclosterane-containing oils or bitumens can be obtained in the future) and/or diagenesis
520 experiments on cycloartenol and related compounds are needed to confirm the exact structure and
521 biological precursors of cyclosterane **I**.

522 2.6.2. *Lanostane (II)*

523 In 1989, Chen and colleagues¹³⁶ described the first geologic record of lanostane **II** in Eocene
524 sample Y2 (2036 m) from the Biyang Basin, China. They confirmed the structure through a co-
525 elution experiment with authentic $8\beta(H),9\alpha(H)$ -lanostane (95% purity, Chiron). An aliquot of
526 original sample Y2 (courtesy J.H. Chen, Geoscience Australia) was used to evaluate the presence
527 of lanostane in the BCF. A co-injection experiment of Y2 on a 60 m DB-5 MS capillary column
528 with a BCF sample (B03178, GR7, 199.08 m depth) and comparison of mass spectra confirmed
529 the presence of $8\beta(H),9\alpha(H)$ -lanostane in the BCF (Extended Data Fig. 3a, signal *l4*). However,
530 many BCF bitumens show three additional chromatographic signals *l1* to *l3* with mass spectra
531 similar to *l4* (Fig. 9-26 in Nettersheim, 2017¹²²), suggesting existence of further stereo or structural
532 isomers (Extended Data Fig. 3b). To evaluate the origin of these compounds, we performed

533 artificial maturation experiments on lanosterol and cycloartenol through pyrolysis at 300°C on
534 active carbon followed by hydrogenation with H₂ gas on PtO₂. These experiments yielded a wide
535 array of compounds with the molecular mass of lanostane (414 Dalton) and mass spectra similar
536 to lanostane (Fig. 9-30 in Nettersheim, 2017¹²²), including signals corresponding to the elution
537 positions of *11* to *14* (Extended Data Fig. 3b). The pyrolysis experiments show that lanosterol as
538 well as cycloartenol are suitable biogenic precursors of 8β(H),9α(H)-lanostane *14* and potentially
539 some of the other BCF isomers with lanostane-type MS fragmentation patterns *11* to *13*, although
540 the exact identity and origin of these additional compounds in the BCF has not yet been
541 unambiguously established.

542 *2.7. Characterization of aromatic protosteroids and ursteroids*

543 Previous studies reporting aromatic steroids nearly exclusively focused on derivatives of crown-
544 sterols such as cholesterol, ergosterol, stigmasterol and dinosterol. However, in contrast
545 to the Phanerozoic where such compounds are ubiquitous and abundant, these crown-sterol
546 derivatives are below detection limits in mid-Proterozoic bitumens, with two exceptions. Firstly,
547 mid-Proterozoic bitumens that contain abundant protosterol degradation products also commonly
548 yield traces of C₂₆ triaromatic steroid **IX**. These compounds may form as aromatization products
549 of C₂₇ crown-sterols. However, as the corresponding saturated cholestanes and monoaromatic
550 cholesterol always remain below detection limits, it is likely that **IX** in mid-Proterozoic
551 sediments formed by aromatization and demethylation of protosteroid precursors (Extended Data
552 Fig. 1). Secondly, solvent extracts from 1,300 Ma old sediments of the Roper Group yielded traces
553 of triaromatic ergosteroid **XII**. However, as with **IX**, the corresponding saturated and
554 monoaromatic ergosteroids remain below detection limits. Moreover, the 4-methylated
555 counterpart **XI** is more abundant than **XII**, suggesting that the precursor was a 4,24-dimethylated
556 ursterol such as 24-methylenecycloartenol or a 4- and/or 14-demethylated downstream products
557 (see Section 2.7.2.1).

558 In this study, we identified a large variety of additional aromatic steroids that are likely derived
559 from ursterols and/or protosterols. They were identified based on their elution behaviour and mass
560 spectra in comparison to the literature, authentic standards, reference oils and/or laboratory
561 pyrolysis products of protosterols.

562 *2.7.1. Triaromatic steroids (TAS)*

563 In this study we identified triaromatic steroids without ring methylation (**IX**), with one methyl
564 group at ring-A (almost exclusively at position C-4, **VIII**, among the most abundant compounds
565 in some mid-Proterozoic bitumens) and TAS with two methyl-groups attached to the aromatic core
566 (**VII**). Electron impact (EI⁺) mass spectra of TAS are strongly dominated by a single mass

567 fragment comprising the aromatized polycyclic ring system and are best identified and quantified
568 using base ions m/z 231, 245 and 259 respectively.

569 2.7.2. *TAS without methyl groups at the ring system (m/z 231)*

570 TAS in m/z 231 mass chromatograms were identified through comparison with the well-
571 characterized Phanerozoic-based AGSOSTD oil reference standard⁴⁶ (Extended Data Fig. 7i).
572 Extended Data Figure 7g shows abundant S and R isomers of **IX** in the ~750 Ma Chuar Group
573 where they presumably derive from C₂₇ crown-sterols as they co-occur with abundant saturated
574 cholestane and MAS cholesteroids⁴⁶. By contrast, in the mid-Proterozoic they only occur in traces,
575 as highlighted for the 1,100 Ma El Mreïti Gr in Extended Data Figure 7h, and are interpreted as
576 demethylation products of 4-methyl TAS **VIII**, which are abundant in such samples (Extended
577 Data Fig. 7e). TAS cholesteroids **IX** were also generated in traces in protosterol pyrolysis
578 experiments (not shown). In the mid-Proterozoic, 24-methylated TAS **XII** were exclusively
579 detected in traces in the 1,300 Ma Roper Group (Supplementary Table 2).

580 2.7.2.1. *A-ring methylated TAS (m/z 245)*

581 A-ring methylated TAS were identified in m/z 245 mass chromatograms through comparison with
582 AGSOSTD⁴⁶. In the AGSOSTD, the distribution of cholesterol-, ergosteroid- and stigmasteroid-
583 type TAS with methylation at positions C-2, C-3 or C-4 of the aromatic ring system, as well as
584 4,23,24-trimethylated TAS (dinosteroids) is shown in Extended Data Fig. 7f.

585 The ~750 Ma Chuar Group contains abundant 2-, 3- and 4-methylated TAS cholesteroids, but no
586 detectable ergosteroids or stigmasteroids (Extended Data Fig. 7d), a pattern that mirrors the
587 saturated and monoaromatic steroid fractions and is typical for the Tonian period^{15,46}. These
588 Tonian TAS are interpreted as A-ring methylation products of C₂₇ crown-sterols. By contrast, mid-
589 Proterozoic bitumens only contain the 4-methylated TAS isomer **VIII**, exemplified by the 1,100
590 Ma El Mreïti Gr in Extended Data Figure 7e. The only exception is some bitumens of the 1,640
591 Ma Barney Creek Fm where traces of 2- and 3-methylated isomers were also detected
592 (Supplementary Table 2), interpreted as isomerization products of the 4-methyl isomers, which are
593 one to two orders of magnitude more abundant (Supplementary Table 2). Pyrolysis experiments
594 on lanosterol and cycloartenol confirm that 4-methylated TAS **VIII** (but not 2- or 3-methylated
595 TAS) are generated by thermal degradation of protosterols (red-coloured signals in Extended Data
596 Fig. 7c).

597 In the mid-Proterozoic, 4,24-dimethylated TAS **XI** were detected in the 1,300 Ma Roper Group
598 (Supplementary Table 2). The identity of these compounds was verified by comparing elution
599 positions in m/z 245 mass chromatograms on two GC-columns with different polarities (DB-5MS

600 and VZ-200) relative to AGSOSTD (Extended Data Fig. 9). On the DB-5MS column, the 20R
601 isomer of 4-methylated TAS cholesterol **VIII** coelutes with the 20S isomer of the 4,24-
602 dimethylated TAS ergosteroid **XI**, making confirmation of the presence of the 20S/R isomer pair
603 uncertain (Extended Data Fig. 9a, b). However, on the VZ-200 column, all isomers are
604 chromatographically fully separated, confirming the presence of these compounds. The identity of
605 **XI** is further supported by the unusual and broad chromatographic peak shape that is presumably
606 caused by the presence of closely eluting 24S and R isomers. In the absence of 2- and 3-methylated
607 isomers and of 24-methylated ergosteroids in the saturated and monoaromatic steroid fractions, **XI**
608 are interpreted as the degradation products of 24-methylated ursterols such as 24-
609 methylenecycloartenol, obtusifoliol or 4-methylfecosterol. Pyrolysis of 24-methylenecycloartenol
610 confirms that 4,24-dimethylated TAS **XI** (but not 2,24- or 3,24-dimethylated isomers) can be
611 generated during degradation of 24-methylated ursterols (blue-coloured signals in Extended Data
612 Fig. 7c).

613 2.7.2.2. *TAS with double-methylated ring-system (m/z 259)*

614 Virtually all mid-Proterozoic bitumens in the data set (Supplementary Table 1) that yielded 4-
615 methylated TAS **VIII** of the *m/z* 245 series also yielded four signals (*c1* to *c4*, Extended Data Fig.
616 7a) in *m/z* 259 ion chromatograms, indicative of additional methylation in the aromatic ring
617 system. (Signals *c2* and *c3* coelute on DB5-MS chromatographic columns but are separated on
618 DB1-MS columns). TAS of the *m/z* 259 series are highly abundant in mid-Proterozoic bitumens,
619 on average 25% of the sum of all TAS. However, relative concentrations drop to 15% in the Tonian
620 period and to less than 1% in the Ediacaran and Phanerozoic (Supplementary Table 1).

621 The identity of compounds *c1* to *c4* as protosteroid aromatization products was confirmed in
622 laboratory pyrolysis experiments on a cycloartenol + 24-methylene cycloartenol enrichment
623 mixture (Extended Data Fig. 7b) as well as on pure cycloartenol and lanosterol. The mass spectra
624 of the pyrolysis products *C3* and *C4* showed close similarity to the mass spectra of *c2/c3* and *c4*
625 in Barney Creek Fm bitumens, revealing the dominant base ion *m/z* 259 as well as a minor
626 molecular ion [M^{+o}] at *m/z* 372 and minor fragments *m/z* 244 and 229, corroborating the presence
627 of two methyl groups in the triaromatic ring system (Fig. 10-10 in Nettersheim, 2017¹²²). By
628 comparison with signals of the *m/z* 231 and *m/z* 245 series, and by comparison of bitumens with
629 different thermal maturities, *c1* and *c3* are likely 20S isomers while *c2* and *c4* possess the
630 biological 20R configuration. The precise location of the two methyl groups in *c1* to *c4* remains
631 unknown, but it is plausible that all isomers retain one methyl group at C-4. We thus suggest the
632 tentative structure **VII** (Extended Data Fig. 1)

633

634 2.7.3. *Diaromatic lanosteroids (DAL)*

635 Diaromatic steroids are rarely reported in the literature. Among the few reports are a series of 14-
636 methyl steroids with aromatic A- and B-rings that constitute the major hydrocarbon constituents
637 in biodegraded oil seeps in Pakistan, tentatively attributed to 14-methyl sterol precursors in green
638 algae¹³⁷. A second report tentatively describes A/B-ring diaromatic steroids methylated at C-4, C-
639 13 and C-14 from sediments of Lake Caçó (Brazil). As the lake sediments were rich in aromatized
640 higher plant-derived triterpenoids, the diaromatic lanosteroids were tentatively attributed to higher
641 plant sources. The mass spectral interpretation of these structures was further substantiated by the
642 presence of a methyl ether, a ketone and an alcohol derivative of lanosteroids¹³⁸. Here, we describe
643 the detection of diaromatic lanosteroids in Proterozoic sediments based on mass spectral
644 characteristics and comparison to protosterol pyrolysates.

645 2.7.3.1. *C₂₈ diaromatic lanosteroids (C₂₈ DAL)*

646 In bitumens of the 1,640 Ma Barney Creek Formation, two molecules (*b1* and *b2*) with a molecular
647 mass of 376 Da and base ion *m/z* 361 are among the most abundant aromatic compounds (Extended
648 Data Fig. 5i). The chromatographic signals *b1* and *b2* are commonly well resolved in total ion
649 chromatograms where they elute between the two characteristic 4-MeTAS peaks *d3* and *d6*.
650 Signals *b1* and *b2* exhibit the same main mass spectral fragments (*m/z* 376, 361, 195, 207, 221,
651 181, 249, 235) as the 4,13,14-trimethylated A/B-ring diaromatic lanosteroids previously identified
652 in Lake Caçó (compare Extended Data Fig. 6 with Fig. 4 in ref¹³⁸). In the present work, the
653 lanosteroid structure was confirmed by pyrolysis experiments on cycloartenol, which yielded
654 chromatographic signals *B1* and *B2* with identical mass spectra and elution positions to *b1* and *b2*
655 (Extended Data Fig. 5g-i, 6). Therefore, *b1* and *b2* are tentatively identified as DAL structure **VI**.
656 The high abundance of these diaromatic steroids supports the 13,14-methylation pattern as these
657 methyl groups would hamper progressive aromatization of the C-ring¹³⁷, which is also observed
658 for B-ring aromatized isoarborinol and fernane-derivatives¹³⁹. It is therefore probably no
659 coincidence that the sparse reports of diaromatic steroids are restricted to 13,14-dimethylated
660 derivatives^{137,138}, as the slow pace of B-ring aromatization in crown-sterols combined with the
661 considerable pace of A-ring and C-ring aromatization¹⁴⁰ usually results in the formation of either
662 mono- or triaromatic steroid derivatives.

663 2.7.3.2. *C₂₉ diaromatic lanosteroids (C₂₉ DAL)*

664 The aromatic fraction of most mid-Proterozoic bitumens also contained a series of later eluting
665 compounds (*b3* to *b6*) with a molecular mass of 390 Da and base ion *m/z* 375 (Extended Data Fig.
666 5e, f and 6). These compounds possess mass spectra with major fragments at *m/z* 390, 375, 209,

667 221, 195, 179, 263 and 235. In analogy to the very similar fragmentation patterns of the C₂₈ DAL
668 series, but with main fragments shifted by 14 Da to higher masses (e.g. 209 instead of 195), these
669 compounds likely constitute a series of four C₂₉ DAL with additional methylation in the ring
670 system. Cycloartenol pyrolysis yielded chromatographic signals *B4* to *B6* with matching elution
671 positions and mass spectra to *b4* to *b6* (Extended Data Fig. 5d and 6), confirming a diaromatic
672 lanosteroid structure (**V**).

673 2.7.3.3. C₃₀ diaromatic lanosteroids (C₃₀ DAL)

674 Several mid-Proterozoic bitumens yielded a series of yet later eluting compounds *b7* to *b10* with
675 molecular masses of 404 Da and mass spectra pointing to a C₃₀ homolog of the C₂₈ and C₂₉ DAL
676 series with an additional methylation in the ring system (Extended Data Fig. 5a-c and 6).
677 Cycloartenol pyrolysis yielded signals *B7* to *B10* with identical mass spectra and elution positions
678 to *b7* to *b10* (Extended Data Fig. 5a and 6), confirming a lanosteroid structure (**IV**). The positions
679 of the methyl groups remain to be investigated.

680 2.7.4. B-ring monoaromatic lanosteroids (MAL)

681 Previously, B-ring monoaromatic lanosteroids were only known from Oligocene evaporites in
682 France¹⁴¹. In the present study, the identity of C₂₉ B-ring monoaromatic lanosteroids was
683 confirmed by co-injection with an authentic standard.

684 2.7.4.1. C₂₉ B-ring monoaromatic lanosteroids (C₂₉ MAL)

685 Almost all Proterozoic bitumens analysed in this study yielded two mass chromatographic peaks
686 *a3* and *a4* with a molecular mass of 394 Da and base ion *m/z* 379 (Extended Data Figure 4).
687 Compounds *a3* and *a4* have mass spectral fragmentation patterns similar to C₂₉ DAL signals *b4* to
688 *b6* (cmp. Extended Data Fig. 4f and 6), but all main fragments are shifted four mass units to heavier
689 values indicative of one instead of two aromatic rings. The identity of *a4* was confirmed by
690 comparison of mass spectra and coelution experiments of 1,640 Ma Barney Creek Formation
691 sample B03163 (GR7, 47.55 m depth) with an authentic standard (section 2.2) represented by
692 signal *A4* (20R C₂₉ MAL) in Extended Data Figure 4a, b, f. Compound *a3* with identical mass
693 spectrum was identified as the 20S isomer of *a4*, a hypothesis supported by the co-occurrence of
694 this compound in small amounts with the 20R C₂₉ MAL standard (signal *A3* in Extended Data Fig.
695 4b; compound with same mass spectrum and retention time as *a3*). While protosterol pyrolysates
696 yielded several peaks with very similar mass spectra, compounds with the exact elution positions
697 of *a3* and *a4* were not generated in detectable quantities.

698 2.7.4.2. C₂₈ B-ring monoaromatic lanosteroids (C₂₈ MAL)

699 Some samples also yielded two chromatographic signals *a1* and *a2* in *m/z* 365 chromatograms
700 eluting between aromatic C₂₉ and C₃₀ secohopanoids (Extended Data Fig. 4e). The mass spectra of
701 the M⁺ 380 compounds are very similar to those of the C₂₉ MAL with main fragments shifted 14
702 Da towards lighter masses, suggestive of methyl-loss from the polycyclic core (Extended Data Fig.
703 4f). Elution positions (Extended Data Fig. 4d) also match those of compounds produced during
704 the laboratory maturation of lanosterol (*A1* and *A2* in Extended Data Fig. 4f) and cycloartenol.
705 However, due to poor signal to noise ratios, it remains unclear whether the mass spectra are
706 identical (compare *a2* and *A2* in Extended Data Fig. 4f). Authentic standards are required for
707 unambiguous structural identification.

708 **2.7.4.3. Characterization of aromatic hopanoids and benzohopanoids**

709 In addition to the commonly analysed C₃₂-C₃₅ benzohopanoids that encompass side-chain
710 cyclisation and aromatization, we included the aromatic hopanoid derivatives C₃₀ and C₃₁
711 acenaphthene-8,14-secohopanoids, fluorene-8,14-secohopanoids, 8,14-secohopanoids and their
712 28-nor derivatives to calculate aromatic hopanoid abundances (Extended Data Fig. 8).

713 C₃₂-C₃₅ benzohopanoids *i1-i4* (Extended Data Fig. 8f) were identified relative to compounds in
714 AGSOSTD reference oil (Fig. 8-14 in Nettersheim, 2017¹²²) and mass spectral characteristics¹⁴².
715 Eluting between the C₃₂ and C₃₃ benzohopanoids under our chromatographic conditions, the
716 chromatographic signal *j3* (Extended Data Fig. 8c) is more abundant and clearly resolvable in the
717 TIC of many BCF samples. It yields a molecular ion [M⁺] at *m/z* 414 and was identified as a C₃₁
718 8,14-secohopanoid with a fluorene moiety based on comparison with published mass spectral
719 characteristics¹⁴³ (see also Fig. 8-15 in Nettersheim, 2017¹²²). Eluting before *j3*, the
720 chromatographic signal *j2* (Extended Data Fig. 8d)—following Carillo-Hernández et al.
721 (2001)¹⁴⁴—was tentatively identified as a C₃₁ acenaphthene-8,14-secohopanoid based on its mass
722 spectral characteristics of dominant [M⁺] at *m/z* 416 and a similarly abundant *m/z* 209 fragment
723 attributed to cleavage through the former C ring that is facilitated by the 8,14-seco (cleavage)
724 structure. Similarly, chromatographic signal *j1*, eluting before C₃₂ benzohopanoid *i1* (Extended
725 Data Fig. 8e) was tentatively identified as the corresponding C₃₀ acenaphthene-8,14-secohopanoid
726 based on dominant [M⁺] at *m/z* 402 and *m/z* 195 fragments¹⁴⁴. Chromatographic signals *h7* to *h10*
727 in *m/z* 365 ion chromatograms (Extended Data Fig. 8a) were identified as a series of regular
728 aromatic C₂₉-C₃₁ 8,14-secohopanoids by comparison with published elution patterns and mass
729 spectra¹⁴⁵ (see also Fig. 8-19 and 8-20 in Nettersheim, 2017¹²²). These compounds are
730 characterized by a *m/z* 365 base ion comprising the polycyclic core after side-chain cleavage, a
731 *m/z* 159 fragment comprising the part of the polycyclic core that contains the aromatic moiety after
732 cleavage of the C-11 to C-12 bond in the 8,14-cleaved (seco) former C-ring; as well as the
733 equivalent fragment after 11,12-cleavage that retains the side chain (i.e. *m/z* 187 in the C₂₉

734 derivative h7). A similar series is found in m/z 351 (Extended Data Fig. 8b) and m/z 145 ion
735 chromatograms, comprising double peaks $h1-h6$ with $[M^{+o}]$ of 380, 394 and 408 corresponding to
736 aromatic C₂₈-C₃₀ 8,14-secohopanoids that lost one of the methyl groups at the aromatic or
737 pentacyclic ring¹⁴⁶.

738 **2.8. Assignment of steroids to stem or crown sources for Figures 1a and 3b**

739 As described in Methods, Figure 1a and 3b summarize the evolution of the relative abundances of
740 aromatic (proto)steroids and hopanoids from the late Palaeoproterozoic to the Phanerozoic,
741 assigning colours to different compound classes. Supplementary Table 1 provides a key to the
742 chromatographic signals that contribute to each compound class or colour. Shown in shades of
743 purple to pink are aromatic protosteroids interpreted as degradation products of lanosterol and/or
744 cycloartenol, and in cyan and light blue-green aromatic derivatives of 24-methylated protosterols
745 such as cyclolaudenol or 24-methyl lanosterol, and in shades of red, blue and green the aromatic
746 derivatives of cholesterol, ergosteroids and stigmateroids respectively. Several groups of
747 aromatic steroids in the data set (Supplementary Table 1) may be the diagenetic products of stem
748 as well as crown-sterol precursors: triaromatic cholesterol and ergosteroids without ring-system
749 methylation (TAS 231 Chol and TAS 245 Erg, where TAS stands for ‘triaromatic steroid’, and
750 231 denotes the base ion m/z 231 used to quantify the compounds), and 2-, 3- and 4-methylated
751 TAS Chol and Erg (TAS 245 MeChol and MeErg). For instance, TAS 231 Chol may be the
752 aromatisation product of cholesterol but may also form through aromatization and demethylation
753 of cycloartenol. TAS 245 4-MeChol may be generated through diagenetic methylation of
754 cholesterol precursors but also through demethylation of cycloartenol precursors. However, it is
755 possible to distinguish the dominant precursors by investigating the absence or presence of other
756 protosterol products. Supplementary Table 1 assigns TAS 245 4-MeChol to a protosterol source
757 (entry 3 in SI Table 1) if the corresponding saturated bitumen fraction lacks cholestanes AND the
758 aromatic fraction contains no, or almost no, 2- and 3-methylated TAS AND the aromatic fraction
759 lacks monoaromatic cholesterol (MAS). Moreover, bitumens of the 1,640 Ma Barney Creek Fm
760 contain traces (usually close to detection limits) of 2- and 3-methylated TAS, and these are
761 interpreted as rearrangement products of 4-methylated protosteroid precursors (entry 5 in SI Table
762 1). Conversely, if indigenous cholestanes are present in the saturated fraction OR 2- and 3-
763 methylated TAS are present in more than traces, then SI Table 1 assigns methylated TAS to a
764 crown-sterol source (entries 7 and 9 in SI Table 1). The same criteria apply to TAS 231 Erg and
765 TAS 245 4-MeErg.

766 Several samples in the age range 800 to 520 Ma possess a mixed signal where major proportions
767 of TAS 245 Chol appear to have a protosterol as well as C₂₇ sterol origin. This is typified by the
768 presence of indigenous cholesterol in the saturated fraction in combination with a very high

769 proportion of TAS 4-methylated cholesteroids over 2- and 3-methylated isomers. In these cases, it
770 is currently not possible to quantify the proportion of stem- and crown- contributions. However,
771 to acknowledge this effect, SI Table 1 arbitrarily assigns TAS 245 4MeChol to a 'mixed' source
772 (entry 6 in SI Table 1) if all off the following is true: (a) cholestane is present in the saturated
773 fraction, (b) [TAS 245 4MeChol] > 0.5 * [TAS 231 Chol], and (c) [TAS 245 4MeChol] > [TAS
774 245 2+3MeChol]. These mixed sources of 4-methylated TAS are plotted in a red-brown hue,
775 optically between the purple tones of stem sources and red tones of crown-sterol sources.

776 3. Evaluation of euphol/tirucallol series precursors for Proterozoic tetracyclic terpenoids

777 The structures of tetracyclic triterpenoids of the euphol/tirucallol series resemble those from
778 protosterols but have a different stereochemistry at positions C-13, C-14 and C-17¹⁴⁷. As these
779 compounds are not true protosterols, it is crucial to test whether they are a potential source of
780 tetracyclic terpenoids found in Proterozoic sedimentary rocks.

781 Tetracyclic triterpenoids of the euphol/tirucallol series are found in the latex of some higher plants
782 such as *Euphorbia* (spurges)¹⁴⁷, and in traces in bacteria as accidental side products of hopanoid
783 biosynthesis^{148,149}. Higher plants can evidently be ruled out as sources for such compounds in the
784 Precambrian, and the very high concentrations of MAL, DAL and TAS defy an origin as accidental
785 side products. However, it cannot be ruled out that unknown or extinct microorganisms may have
786 produced such compounds as major natural products in the past.

787 The stereochemistry of protosterol and euphol/tirucallol series compounds is determined during
788 enzymatic folding of squalene epoxide. The biosynthesis of lanosterol and cycloartenol involves
789 the intermediate formation of the protosteryl cation that has a chair-boat-chair conformation,
790 leading to products with a 13 β (Me),14 α (Me) configuration. By contrast, euphol
791 (3 β ,13 α ,14 β ,17 α ,20R-lanost-8,24-dien-3-ol) and tirucallol (the 20S isomer of euphol) are
792 examples of triterpenes biosynthesized by cyclization of squalene epoxide in a chair-chair-chair
793 conformation, with a dammarenyl cation as intermediate, leading to products with a
794 13 β (Me),14 α (Me) configuration¹⁵⁰. Thus, euphols and tirucallols are strictly not sterols.

795 The recognition of diagenetic products of the euphol/tirucallol series among protosteroids is not
796 trivial. TAS retain their original stereochemistry at C-17 and MAL and DAL at C-13, C-14 and C-
797 17 (Extended Data Fig. 1). However, on achiral GC columns, these compounds cannot be
798 distinguished from their enantiomers (i.e. stereochemical mirror images) that may derive from the
799 euphol/tirucallol series. A similar situation was encountered in the case of enantiomeric
800 monoaromatic derivatives of isoarborinol and fernenol (or fernene), which could only be separated
801 using chiral phase HPLC and measurement of optical activities¹³². However, distinguishing TAS,
802 MAL and DAL from their enantiomers without authentic GC injection standards and suitable

803 chiral GC columns is not feasible at present. By contrast, the saturated hydrocarbon products of
804 protosterols and euphols/tirucallols should possess unique GC elution times and mass spectra. To
805 test this, we subjected lanosterol and euphenol (3 β ,13 α ,14 β ,17 α ,20R-lanost-8-en-3-ol) to pyrolysis
806 followed by hydrogenation on PtO₂ (see Section 2.1.3) and comparison of the products with the
807 saturated hydrocarbon fraction of the Proterozoic BCF using GC-MS.

808 Lanosterol pyrolysis yielded compounds *l1* to *l4* in the BCF, including unambiguously identified
809 8 β (H),9 α (H)-lanostane *l4*. Euphenol pyrolysis yielded five compounds *m1* to *m5* in the broad
810 elution region of lanostanes, but none corresponds to a compound present in the BCF (Extended
811 Data Fig. 3b). Moreover, while the mass spectra of ‘euphanes’ *m1* to *m5* are nearly identical, they
812 differ notably from those of lanostanes (Extended Data Fig. 3c). Major differences include the
813 much higher relative intensity of *m/z* 177 and 190 fragment ions, the presence of notable *m/z* 161
814 and 301 fragments, and much lower *m/z* 83, 231, 259 and 274 ions. Compounds with such mass
815 spectral characteristics were not detected in the BCF. We thus conclude that euphane isomers
816 generated by pyrolysis of euphenol are not detectable in the BCF, while lanostanes are
817 unambiguously identified. By extension, compounds labelled MAL, DAL and TAS most likely
818 belong to the lanosterol/cycloartenol series and are not the corresponding enantiomers of the
819 euphol/tirucallol series.

820 **4. Maturity dependencies of aromatic steroid parameters**

821 Systematic changes of biomarker distributions over geological time, such as the relative
822 abundances of hopanoids and steroids, might be influenced by thermal maturity trends. The
823 average relative abundance of thermally more labile compounds may decrease with age,
824 particularly towards the Palaeo- and Mesoproterozoic where the number of thermally well
825 preserved sedimentary successions decreases. To test for such biases, we determined the Methyl
826 Phenanthrene Ratio¹⁵¹ (MPR = 2-MP/1-MP; MP = methylphenanthrene) for samples in the data
827 base and computed the corresponding vitrinite reflectance equivalent $R_c(\text{MPR}) =$
828 $1.1 \cdot \log_{10}(\text{MPR}) + 0.95$. $R_c(\text{MPR})$ was chosen as maturity parameter as it is minimally influenced
829 by kinetic and source effects¹⁵² and was available for the majority of samples (Table S2).

830 Extended Data Figure 8g plots $R_c(\text{MPR})$ against geological age, revealing that there is no
831 systematic trend. The average R_c for the Phanerozoic is $0.87 \pm 0.16\%$ ($n = 33$), for the
832 Neoproterozoic $0.79 \pm 0.18\%$ ($n = 39$) and for the Meso- and Palaeoproterozoic $0.78 \pm 0.15\%$ (n
833 $= 38$). Furthermore, Extended Data Figure 8h shows that the relative abundance of aromatic
834 hopanoids and steroids shows no systematic trend with calculated vitrinite reflectance ($r^2 = 0.016$,
835 $n = 110$). We also did not find a trend between thermal maturity and the abundance of aromatic
836 stem-steroids relative to all aromatic steroids and hopanoids ($r^2 = 0.052$), the ratio of mono- to

837 triaromatic steroids (i.e. MAS in m/z 253 over TAS in m/z 231, $r^2 = 0.003$), and the percentage of
838 triaromatic stigmasteroids among all triaromatic steroids in the m/z 231 trace ($r^2 = 0.001$) (not
839 shown). We thus conclude that the trends presented in Figures 1 and 3 are not significantly affected
840 by thermal maturity.

841 **5. Phanerozoic and Recent Protosteroids**

842 The following synopsis provides a summary of the most relevant information of Appendix A,
843 which provides an encyclopaedic review on protosterol synthesis genes in bacteria, protosterol
844 production in extant organisms, and protosteroids detected in modern environments and ancient
845 sediments.

- 846 1. All major groups of extant eukaryotes produce sterols and LECA had the biosynthetic
847 capacity to produce crown-sterols. Crown-sterols can be found in virtually all modern
848 sedimentary environments. Likewise, fossil crown-steroids are found in virtually all
849 Tonian to Phanerozoic bitumens and oils that are diagenetically and thermally well
850 preserved.
- 851 2. In our dataset, fossil protosteroids are present in all bitumens and oils that yield crown-
852 steroids, without exception. However, in the Phanerozoic the proportion of protosteroids is
853 usually low. Aromatic protosteroids account for $3.8 \pm 4.0\%$ ($n = 36$) of the total aromatic
854 steroid pool, and the approximate average percentage of saturated protosteranes among
855 total steranes is $0.9 \pm 1.4\%$ ($n = 15$, see Section 10.1). These low relative abundances may
856 largely represent eukaryotic biosynthetic intermediates (Section 8).
- 857 3. Based on metagenomic surveys, sterol producing bacteria exist in a wide variety of modern
858 environments (Section 8.2.7). Yet, the bacterial contribution of protosterols to such
859 sedimentary environments remains unexplored (Section 9), with a few notable exceptions.
860 Strongly elevated levels of sterols of likely bacterial origin have only been found at
861 hydrothermal vents, methane seeps and beneath the surface of some cyanobacterial mats
862 (Section 9).
- 863 4. There are only few modern and Phanerozoic examples of common sedimentary
864 environments with strongly elevated protosterol levels (Section 9). In some instances, such
865 elevated abundances are likely derived from higher plants (Section 8.1.2).
- 866 5. Stem-group eukaryotes likely acquired the ability to produce protosterols before 1,700 Ma
867 (Section 8.2). These organisms must have started to contribute sterols to the sedimentary
868 record by that time at the latest. Likewise, at least one lineage of bacteria likely possessed
869 the ability to produce sterols by 1,700 Ma.
- 870 6. The first two genes for sterol biosynthesis, SQMO and OSC, have been detected in at least
871 twelve phyla of bacteria, and actual sterol production has been demonstrated for three of

872 them. However, all bacterial clusters that possess SQMO and OSC, except
873 Methylococcales and Myxococcales, have relatively shallow clade depths, suggesting that
874 most acquired the genes via horizontal gene transfer (HGT) relatively recently. It is
875 currently not possible to state with certainty that any of the currently know groups of sterol
876 producing bacteria existed in the early mid-Proterozoic or, in fact, predated LECA (Section
877 8.2).

878 7. The suggestion that stem-group eukaryotes acquired cholesterol biosynthesis genes as a
879 full syntenic set from the Myxococcales suborder Cystobacterineae³³ is not supported by
880 biomarker data, and is not supported by molecular clocks that point to a Phanerozoic origin
881 of these bacteria. The direction and timing of any gene transfer between Eukarya and
882 Myxococcales remains unresolved (Section 8.2.3).

883 6. Biological origins of mid-Proterozoic steroids

884 6.1. The biological origin of side-chain methylated ursteroids

885 The 1,300 Ma Roper Group contains indigenous triaromatic steroids that are methylated at Ring-
886 A at position C-4 and in the side chain at position C-24. The precursor lipid may have been the C₃₁
887 sterol 24-methylenecycloartenol, which is the second intermediate in the biosynthetic pathway to
888 phytosterols¹⁷. However, more advanced downstream sterols demethylated at C-14 and/or
889 demethylated once at C-4 are plausible precursors as well. Saturated steranes were below detection
890 limits in the Roper Group bitumens, so the exact precursor carbon skeletons cannot be confirmed.
891 The 24-methylated ursteroids occur in high relative abundances of 12 to 24% of total steroids (18
892 \pm 4%, n = 4), suggesting that they are biogenic and not diagenetic methylation products.
893 Functionalized sterol side chains may generally become methylated during diagenesis, and this
894 has also been observed in pyrolysis experiments^{32,153}. However, these diagenetic methylation
895 products form in relatively low abundances (usually low single digit percentage levels), and
896 abiogenic methylation has thus far only been observed at penultimate and terminal carbon
897 positions in the sterol side chain. For example, pyrolysis of the C₂₇ sterol desmosterol, which
898 possesses an unsaturation at C-24, resulted in products alkylated dominantly at the terminal C-26
899 position. Likewise, sedimentary rocks of Tonian and Cryogenian age contain 26-methyl
900 cholestanes (cryostanes) at a relative abundance of $1.7 \pm 3.1\%$ (n = 47, range is 0 to 7.8% plus an
901 outlier at 15%)^{15,46} that are demonstrably the product of diagenetic C₂₇ sterol methylation³². By
902 contrast, among the 47 Tonian and Cryogenian samples, only two contained detectable 24-methyl
903 cholestanes (ergostanes) at relative abundances of 0.9 and 1.4%, and these may be biogenic. These
904 values suggest that diagenetic 24-methylation does not occur or is uncommon even in sediments
905 where diagenetic 26-methylation is notable. Moreover, outside the Roper Group, not one mid-

906 Proterozoic bitumen yielded 24-methylated steroids, highlighting that diagenetic 24-methylation,
907 if it occurs at all, is not a common sedimentary process. Thus, there is currently no indication that
908 24-methylated steroids can form through abiogenic processes, particularly at the high relative
909 abundances observed in the Roper Group.

910 Sterol methyltransferase (SMT), the gene responsible for the alkylation of sterols in the side chain
911 at C-24, is found in eukaryotes and some bacteria. The C-24 methylated sterols of the Roper
912 Group may thus, in principle, have a bacterial and/or stem-group eukaryotic origin. Although none
913 of the bacteria screened for sterols to date yielded side-chain alkylated sterols³⁵, SMT genes of
914 bacteria that live in symbiosis with sponges, when expressed *in vitro*, methylate C₂₇ sterols to form
915 24-methylated or even 24-ethylated cholesteroloids^{154,155}. Some SMTs even yield 24-
916 isopropenylcholesterol and related C₃₀ sterols that had been regarded as diagnostic for sponges.
917 Similar functionalities were found for bacterial SMTs identified in metagenomes of marine,
918 freshwater and other environments. These experiments demonstrate that bacterial SMTs are
919 functional. The study also found bacterial SMTs that occur in sterol biosynthesis gene clusters,
920 suggesting that some bacteria have the capacity to produce side-chain alkylated sterols *de novo*¹⁵⁵.
921 Biosynthesis of side-chain methylated sterols may thus not be a strictly diagnostic eukaryotic trait.
922 Future analyses have to determine whether and which groups of SMT-bearing bacteria produce
923 sterols *de novo* or, conversely, alkylate sterols taken from the environment (as shown for
924 Chlamydiae)¹⁵⁵. It will also be crucial to assess whether sterol alkylation by bacteria has a
925 detectable impact on sedimentary sterol profiles.

926 For the interpretation of the 1,300 Ma old 24-methylated ursteroids, the most important question
927 is the antiquity of SMT genes in bacteria. While this question cannot be answered with certainty
928 at present, SMT trees provided in refs^{33,39,155} suggest that bacterial SMTs have relatively shallow
929 clade depths. Moreover, in the trees provided by refs^{39,155} all analysed bacterial SMTs branch
930 within extant Eukarya, suggesting that they were acquired after the emergence of LECA. While
931 these observations do not exclude bacterial side-chain methylation by 1,300 Ma, it makes a stem-
932 group eukaryotic origin for these ursteroids more likely. Generally, 24-alkylation is a common
933 characteristic of Eukarya^{35,156}. It occurs across all major eukaryotic groups and it was probably
934 already present in LECA¹⁷. The few eukaryotic clades that do not alkylate the side chain, such as
935 most eumetazoans, lost the ability later. The presence of 24-methylation in LECA demonstrates
936 that SMT was acquired in the eukaryotic stem. Thus, the 24-methylated ursteroids extracted from
937 1,300 Ma old sedimentary rocks are consistent with a eukaryotic origin and, in combination with
938 a lack of crown-steroids in these sediments, with a stem-group eukaryotic origin.

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941 **6.2. Anaerobic stem and crown-group eukaryotes**

942 Porter et al. (2018)²¹ suggested that anaerobic eukaryotes may have been common in the mid-
943 Proterozoic interval. As anaerobic eukaryotes cannot produce sterols, this hypothesis elegantly
944 reconciled the existence of diagnostic eukaryotic fossils with the paucity of crown-group steranes.
945 However, the new protosteroid record demonstrates that steroids were in fact abundant throughout
946 the interval. Thus, although anaerobic stem and crown-group eukaryotes likely existed, they are
947 not needed to explain the record.

948 **6.3. Microaerophilic crown-group eukaryotes**

949 It is conceivable that mid-Proterozoic protosteroids derive from crown-group eukaryotes that
950 secondarily reverted to a microaerophilic lifestyle, conserving scarce oxygen resources by
951 truncating the sterol biosynthetic pathway after the first step. Yet, there is no independent fossil
952 evidence for the existence of microaerophilic crown-group eukaryotes in the mid-Proterozoic, nor
953 is there any evidence that microaerophilic crown-group eukaryotes revert to protosterol
954 biosynthesis. Extant eukaryotes living under extreme oxygen deficiency either take up sterols from
955 their environment, produce sterol surrogates such as tetrahymanol or entirely lack polycyclic
956 membrane modifiers¹⁵⁷⁻¹⁵⁹. Moreover, crown-group eukaryotes only require molecular oxygen at
957 very low levels to complete the full sterol biosynthetic pathway¹⁶⁰, and crown-sterol biosynthesis
958 may have been possible in the mixed zone of most open water habitats, in cyanobacterial mats and
959 ventilated deep basins⁶⁷ throughout the mid-Proterozoic interval. Thus, considering the paucity of
960 downstream modified steranes in the mid-Proterozoic biomarker record, a notable contribution of
961 protosterols by crown-group eukaryotes is an unlikely scenario.

962 **7. The nature of mid-Proterozoic eukaryotic fossils in light of the steroid record**

963 The mid-Proterozoic record of microfossils that bear diagnostic eukaryotic characteristics is
964 sparse. The oldest fossils confidently assigned to crown-group Eukarya emerge about one billion
965 years ago, the 1,050 Ma multicellular rhodophyte alga *Bangiomorpha*¹⁰ and ~1,000 Ma
966 chlorophyte alga *Proterocladus*¹¹. Other fossils with diagnostic eukaryotic features, such as
967 processes, protrusions and cell-wall ornamentation, occur throughout the Mesoproterozoic and
968 date back to ~1,650 Ma (Fig. 1c). However, these fossils lack characteristics of extant clades, and
969 it remains unknown whether they belong to the eukaryotic stem or crown^{1,8,9}. By contrast, fossil
970 steroids extracted from 1,640 to 1,000 Ma old sedimentary rocks have a likely stem-group
971 eukaryotic origin, while clearly indigenous and generally accepted traces of crown-steroids do not
972 emerge until ~800 Ma (Fig. 3; note that there is no data for the critical interval < 1,000 to > 800
973 Ma).

974 To interpret these combined fossil and biomarker records, it is important to consider the different
975 taphonomic conditions responsible for microfossil and molecular fossil preservation. Biomarkers
976 are preserved in sedimentary environments where a fraction of organic matter escapes
977 mineralization and is protected by reducing conditions. This commonly occurs in sediments
978 deposited in low energy environments characterized by small mineral grains such as clay particles
979 that protect organic matter through surface adsorption and limited diffusion of oxygen and other
980 oxidants. Biomarkers are thus not commonly preserved in coarse sedimentary rocks such as
981 sandstones, or in environments with persistently well-oxygenated bottom waters such as many
982 near-shore environments, although they may be found in oxygenated, tide-influenced
983 environments where microbial mats limit oxygen exchange between sediment and water
984 column¹⁶¹. Moreover, organic matter from shallow oxygenated waters (and in the post-Silurian
985 also from terrestrial environments) may be transported to, and preserved in, deeper depositional
986 settings. So, while a part of the biomarker signal likely represents contributions from well
987 oxygenated, high-energy environments, the record is clearly biased towards a limited number of
988 specific depositional environments favouring organic preservation. However, in those
989 environments where biomarkers are well preserved, they afford semi-quantitative information
990 about relative abundances of broad taxonomic groups.

991 In contrast to biomarkers, mid-Proterozoic microfossils have very different modes of preservation,
992 are found in a far wider range of habitats, and may provide morphological information to the
993 species level. However, they lack information about ecological importance and abundance. Soft
994 bodied, single cellular organisms lacking preservable structures such as resistant cell wall material
995 or mineral tests, will only leave a fossil record under exceptional circumstances. Such organisms
996 may entirely evade detection in the body fossil record. Therefore, based on body fossils, we may
997 remain oblivious of the most abundant and ecologically dominant organisms of the mid-
998 Proterozoic interval. It is possible that we do not possess a single fossil of an organism that
999 contributed detectable abundances of protosteroids to sediments. Conversely, organisms that only
1000 constitute a small percentage of total biomass but have outstanding potential to fossilize, will be
1001 overrepresented in the body fossil record, possibly by orders of magnitude. Thus, organisms
1002 leaving a copious fossil record in specific sediments may never have been abundant and may fail
1003 to contribute detectable quantities of biomarkers.

1004 In summary, while molecular fossils are biased towards oxygen-starved environments, they can
1005 provide information about the relative abundances of broad organism groups. Body fossils are
1006 taxonomically far more specific, are found in a wider range of depositional environments, but
1007 rarely provide data on ecological importance. Thus, while the two records are complementary, it
1008 is currently not possible to link individual mid-Proterozoic microfossils to specific biomarkers and
1009 vice versa. While the lack of crown-steroids and the immense abundance of protosteroids in mid-

1010 Proterozoic sediments suggests that most fossils are stem-group representatives, it is not possible
1011 to make statements about individual specimens. The 1,050 Ma old red alga *Bangiomorpha* and
1012 chlorophyte alga *Proterocladus* are cases in point, and other fossils may also belong to the crown-
1013 group.

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APPENDIX

1021 **APPENDIX A**

1022 This Appendix provides a literature review and collection of information on protosterol synthesis
1023 genes in bacteria, protosterol production in extant organisms, and protosteroids detected in modern
1024 environments and ancient sediments. The Appendix is intended to be encyclopaedic and does not
1025 have to be read in any specific order. A synopsis of the most relevant information is provided in
1026 Section 5.

1027 **8. Protosterols in extant organisms**

1028 The abundance of protosteroids is generally low in Phanerozoic bitumens and oils relative to other
1029 steroids, but individual samples display strongly elevated proportions. In Supplementary Table 1,
1030 aromatic protosteroids in the Phanerozoic account for $3.8 \pm 4.0\%$ ($n = 36$), and the approximate
1031 average percentage of protosteranes among total steranes is $0.9 \pm 1.4\%$ ($n = 15$, see Section 10.1).
1032 Examples for elevated abundances include a Cretaceous lacustrine oil (GA224) with 7.1%
1033 aromatic protosteroids, a Jurassic coal-resinitic oil (GA19999642) containing 6.8%, Ordovician
1034 Tarim oil (HD_1 #5458) with 20.5%, and bitumen from a Cambrian marine limestone (15B602)
1035 at 13.4%. The following section discusses potential biogenic sources that may explain typical
1036 background abundances but also elevated concentrations of protosteroids in modern and
1037 Phanerozoic depositional environments.

1038 **8.1. Eukaryotic sources**

1039 **8.1.1. Protosterols as biosynthetic intermediates**

1040 Lanosterol or cycloartenol are the first polycyclic biosynthetic intermediates in the biosynthesis of
1041 sterols in virtually all living eukaryotes and thus occur at least in low abundances in all species
1042 that produce sterols *de novo*. Some species contain major concentrations of protosterols, albeit
1043 largely for unknown reasons, and these exceptions are reviewed in Sections 8.1.2 and 8.1.3.

1044 Information on the abundance of protosterols as biosynthetic intermediates is surprisingly sparse.
1045 In early work, Goad and Goodwin (1967)¹⁶² purified sterols from larch leaves (*Larix decidua*) and
1046 found a mass content of ~1% cycloartenol and 13% methylenecycloartenol relative to total sterol
1047 content (computed from data provided in the text). A sterol survey¹⁶³ on basal, flagellated fungi
1048 found a lanosterol proportion of 2 to 7% of total sterols (average $3.6 \pm 2.2\%$, $n = 5$; data excludes
1049 two samples where the lanosterol content was not reported and one outlier where lanosterol was
1050 the major sterol, see Section 8.1.3). Organisms of the phylum Hyphochytriomycota are heterokont
1051 protists with rhizoidal or hypha-like vegetative system that, in the past, were grouped with fungi.
1052 Two analysed species had a lanosterol content of 2% relative to total sterols¹⁶³. In a detailed sterol

1053 survey¹⁶⁴ of 106 diatom species, 104 did not contain detectable concentrations of protosterols
1054 (detection limit presumably < 0.5% of total sterols). The authors concluded that diatoms are
1055 generally unlikely to be major sources of lanosterol or cycloartenol in natural environments.
1056 However, in two species, protosterols were exceptionally high, and this is further discussed in
1057 Section 8.1.3. Among higher plants, the *Arabidopsis* wildtype contains 3% cycloartenol and 4%
1058 24-methylenecycloartanol¹⁶⁵. In summary, while the data are sparse, the abundance of protosterols
1059 as biosynthetic intermediates in eukaryotes presumably falls into the low single digit percentage
1060 range relative to total sterols.

1061 8.1.2. *Elevated protosterol abundances in higher plants*

1062 Many higher plants contain terpenoids that may, for instance, function as a defence against
1063 herbivores and pathogens. In some plants, compounds with protosteroid skeletons are part of this
1064 terpenoid pool, which can result in significantly elevated protosterol levels in plant organic matter
1065 relative to crown-sterols. Since protosterols are commonly isolated together with triterpenoids
1066 rather than crown-sterols, most studies only report their abundances relative to other triterpenoids
1067 such as oleanoids. Here we briefly review some of the reported occurrence of protosterols in a
1068 variety of higher plants, and where possible provide information about the abundance of
1069 protosterols relative to ursterols and crown-sterols.

1070 Vegetable oils may contain substantial quantities of protosterols¹⁶⁶. The triterpene alcohol fraction
1071 of 19 vegetable oils were strongly dominated by cycloartenol and 24-methylene cycloartenol.
1072 Based on data in refs^{166,167}, calculated protosterol ratios (protosterols/[protosterols + crown-
1073 sterols]*100) for the oils ranged between ~1% and 37%, with an average of ~15%. For commercial
1074 Shea butter, the non-glyceride saponifiable fraction (6 wt% of Shea butter; compared to 1 wt%
1075 free triterpene alcohols and sterols) yielded 1 wt% parkeol and 1.2 wt% 24-methylene-lanost-
1076 9(11)en-3-ol, compared to a crown-sterol content of 1.5% α -spinasterol and 1.5% stigmastenol,
1077 yielding a protosterol ratio of ~42%¹⁶⁸. In addition to the more common cycloartenol or
1078 cyclolaudenol, some plants also contain protosterol derivatives with various types of functional
1079 groups¹⁶⁹.

1080 The bark of some trees is particularly rich in cycloartanol-derivatives. A petroleum extract (1% of
1081 wood weight) of birch wood (*Betula verrucosa*) contained 2% cycloartenol, 4% 24-methylene
1082 cycloartenol, 5% 4-methylstigmastadienol and 6% β -sitosterol derivatives in addition to 4% non-
1083 esterified β -sitosterol¹⁷⁰, corresponding to a protosterol ratio of ~37% ([cycloartenol + methylene
1084 cycloartenol]/ [cycloartenol + methylene cycloartenol + β -sitosterol]*100). Abietospiran, a
1085 cycloartanol-derived ether with poly-functionalized side chain is highly abundant in the bark of
1086 white fir (*Abies alba*), comprising 0.14 g/g bark¹⁷¹. Similar cycloartanol-derivatives were also

1087 isolated in bark of other firs (e.g. *Abies grandis*¹⁷²). The stem and branches of the angiosperm
1088 *Neolitsea aciculata* (Magnoliids) contain not only cycloartenol and parkeol, but also a large variety
1089 of 24-methylated and 24-ethylated lano- and cycloartanol-type triterpenoids and even some with
1090 dimethylation at C-24 and trimethylation at C-25. The triterpene alcohol fraction comprises near-
1091 exclusively protosterol-derivatives, except for 12.4% lupeol and 6.4% unidentified compounds.
1092 The 4-methyl sterol fraction comprises 24-methyl and 24-ethyl 31-norlanosteroids as well as
1093 citrostadienol, a 24-ethylated dinorlanosteroid lacking one of the C-4 and the C-14 methyl
1094 groups¹⁷³.

1095 Latex may also contain high absolute abundances and proportions of protosterols and their
1096 derivatives. The tetracyclic and pentacyclic latex constituents of five different varieties of
1097 *Euphorbia pulcherrima* contain 2.0 to 3.2% sitosterol and 0.4 to 1.2% cycloartenol by dry
1098 weight¹⁷⁴, corresponding to protosterol ratios (cycloartenol/[cycloartenol + sitosterol]*100) of ~12
1099 to 37% (average = 26%; n =5). The latex of *Syngonium auritum* contains ~1.85 mg sterols/mL,
1100 and cycloartenol constitutes ~32% of this sterol fraction. Likewise, *Xanthosoma sagittifolium*
1101 contains 6.2 mg sterols/mL latex, including ~27% cycloartenol, and *Xanthosoma violaceum*
1102 contains 8.4 mg sterols/mL latex with ~23% cycloartenol¹⁷⁵. Since the other major sterols were
1103 not identified in the study (and might comprise additional protosterol derivatives), the cycloartenol
1104 ratios of 23 to 32% can be considered as minimum protosterol ratios for the latex of these three
1105 New World Araceae species.

1106 Some grasses (Gramineae or Poaceae) also contain substantial amounts of protosteroids. For
1107 example, non-saponifiable fractions of 2-3 weeks-old maize shoot extracts contained ~7.3 mg 4,4-
1108 dimethyl and 187.6 mg 4-desmethyl sterols per gram of shoots¹⁷⁶. The 4-desmethyl sterols were
1109 found to contain ~75% β -sitosterol, ~14% stigmasterol, ~9% campesterol and ~2% cholesterol,
1110 while the 4,4-dimethyl sterol fraction was reported to contain ~65% cycloartenol, ~13% 24-
1111 methylenecycloartenol and ~22% were comprised of two unidentified compounds¹⁷⁶. Based on the
1112 $\geq 78\%$ cycloartenol + methylene cycloartenol content of the 4,4-dimethyl-fraction and the ~100%
1113 crown-sterol content of the 4-desmethyl fraction, we calculate a minimum protosterol ratio of
1114 ~2.9%. Similarly, pea leaves have also been shown to contain cycloartenol and minor 24-
1115 methylene cycloartenol. Assuming that the 4,4-dimethyl triterpene fraction comprises ~90%
1116 protosterols (cf. Fig. 5a in ref¹⁷⁷), from the 4,4-dimethyl (1.5 mg) and 4-desmethyl sterol fraction
1117 contents (71.6 mg)¹⁷⁷, we estimated a protosterol ratio of ~1.9%.

1118 Plant waxes may also contain substantial amounts of protosteroids. They were reported for
1119 example from the epicuticular wax of spurges (*Euphorbia*)¹⁷⁸, maple leaves¹⁷⁹ and tomatoes¹⁸⁰. In
1120 *Euphorbia esula*, cyclolaudenol was found to comprise between 5 and 31% (average = 21.2%) of
1121 total triterpenoids in five *E. esula* biotypes, corresponding to 0.5 to 2.5% (average = 1.35%) of the

1122 total wax (calculated from data reported in ref¹⁷⁸). While sterol contents were unfortunately not
1123 reported, the high proportion of protosterols of total wax content, in combination with the
1124 observation that plant waxes often form a prominent component of many sedimentary biomarker
1125 signatures, we infer that plant waxes may contribute a substantial proportion of the protosterol
1126 inventory of near-shore post-Silurian strata.

1127 Bryophytes, non-vascular plants such as mosses and liverworts, often contain notable amounts of
1128 cycloartane-type triterpenoids¹⁸¹. *Sphagnum* peat mosses are particularly noteworthy as they
1129 dominate many bogs and might thus be important protosteroid sources in peats, lignite coals and
1130 lake deposits. In the green part (capitulum) of the rusty peat moss *Sphagnum fuscum*, cycloartenol
1131 and methylene cycloartenol mainly occurred in esterified form and constituted ~0.02 wt% of the
1132 entire biomass of the capitulum¹⁸². In the 21-24 cm segment below the green top of a *S. fuscum*
1133 shoot, where first signs of decay at the microscopic level were observed, protosterols constituted
1134 ~0.004 wt% of the dry weight of the plant material, yielding a calculated global (free and esterified)
1135 protosterol ratio of ~15% (calculated from data in ref¹⁸²). Protosteroids also occur in a variety of
1136 other bryophytes. A review of the chemical constituents of bryophytes¹⁸¹ lists (in their Table IIIa)
1137 15 different species of bryophytes from three different classes (Sphagnopsida, Polytrichopsida and
1138 Bryopsida) for which cycloartenol-, cyclolaudenol, cycloeucalenol and/or obtusifoliol-type
1139 triterpenoids have been reported. In the moss *Pseudoscleropodium purum* (Bryopsida),
1140 cyclolaudenol comprises 0.008% (dry wt.) and 31-norcyclolaudenol 0.006% compared to 0.015%
1141 campesterol, 0.01% stigmasterol and 0.015% sitosterol¹⁸³, corresponding to a protosterol ratio of
1142 ~26%. This shows that some of the earliest land plants may already have contributed notable
1143 amounts of protosteroids to the Phanerozoic rock record.

1144 8.1.3. *Elevated protosterol abundances in other eukaryotes*

1145 Sterol production is an energy and oxygen-intensive process. The conversion of the isoprenoid
1146 precursor squalene to a protosterol requires only one O₂ molecule to introduce the characteristic
1147 3-hydroxylation of sterols via the epoxidation of squalene prior to enzymatic folding. The
1148 subsequent enzymatic down-stream modifications that result in the formation of typical crown-
1149 sterol such as ergosterol, cholesterol or sitosterol requires an additional 10 (cholesterol, sitosterol)
1150 to 11 (ergosterol) O₂ molecules¹⁵⁶. It is thus plausible that eukaryotes with a very limited energy
1151 or oxygen supply may abbreviate the pathway to accumulate protosterols as major sterols unless
1152 this is detrimental to membrane and metabolic function. It is also plausible that some eukaryotes
1153 other than higher plants (section 8.1.2) accumulate protosterols as a deterrent against grazers or
1154 predators.

1155 Reports of elevated protosterol abundances in eukaryotes (other than higher plants) are scarce,
1156 although this may be strongly biased because protosterols are not routinely screened in lipid
1157 surveys, whereas they typically elute in triterpenoid fractions included in many plant studies. An
1158 exception among algae are diatoms. The diatom *Haslea sp.* was shown to biosynthesize 20%
1159 lanosterol and 5% 4-norlanosterol as part of its sterol mix¹⁶⁴. Similarly, the diatom *Stauroneis*
1160 *simulans* contained 11% 24-methylcycloartenol, 2% lanosterol and 82% 24-methyl-14-
1161 norcycloartenol. The function of the elevated protosterol content in these species is unknown.
1162 However, elevated protosterol abundances in diatoms are generally an exception as protosterols
1163 were below detection limits (presumably < 0.5%) in a further 104 species. The authors concluded
1164 that diatoms are generally unlikely to be major sources of protosterols in natural environments¹⁶⁴.
1165 Yet, blooms of specific diatom species with high protosterol abundances may well have
1166 contributed to elevated protosteroid occurrences in Phanerozoic sediments.

1167 A relatively high protosterol content was observed in the excavate protist *Naegleria gruberi* that
1168 accumulates cycloartenol in substantial amounts (500 µg/g dry weight)¹⁸⁴. Based on the reported
1169 high abundance of 4-methyl sterols (3-4 mg/g dry weight) and the reportedly similar abundances
1170 of 4-desmethyl sterols, we determine a protosterol ratio of ~6-8%.

1171 In animals, lanosterol-derivatives are highly enriched in lanolin (wool wax). The unsaponifiable
1172 fraction of wool wax consists of ~30% steroids (mostly cholesterol, but also cholestanol and 7-
1173 ketocholesterol) and 25% triterpenes (predominantly lanosterol, smaller amounts of lanostatrienol,
1174 and the respective dihydro derivatives)¹⁸⁵, corresponding to a protosterol ratio of ~46%
1175 (percentage of protosterols of total identified sterols).

1176 Among fungi, *Catenaria anguillulae*, a member of the Chytridiomycota and an endoparasite of
1177 nematodes, yields lanosterol (45%) as the main sterol¹⁶³. While two more of the ten fungi
1178 (*Chytridium confervae* and *Rhizophlyctis rosea*) analysed by ref¹⁶³ also accumulated appreciable
1179 amounts of lanosterol (7% of total sterols), the other fungi in that study only contained minor
1180 amounts (typically ~2%). The function of elevated lanosterol levels in above fungi is unknown,
1181 but there is evidence that fungi can down-regulate crown-sterol biosynthesis under conditions of
1182 O₂ limitation and intermittently accumulate 24-methylene lanosterol, albeit presumably only as a
1183 signalling molecule, not as a critical membrane component¹⁸⁶.

1184 **8.2. Bacterial sources**

1185 **8.2.1. Notes on gene and enzyme nomenclature**

1186 For the nomenclature of genes and enzymes, different combinations of capital, small, italic and
1187 roman fonts are recommended to distinguish enzymes and genes, and also genes of bacteria,

1188 eukaryotes and humans. In the context of the following discussion, these distinctions are irrelevant,
1189 and we follow Hoshino and Gaucher (2021)³³ using capitalized roman letters for all genes and
1190 enzymes, e.g. SQMO and OSC.

1191 8.2.2. *The phylogeny of sterol producing Bacteria*

1192 Based on genomic and metagenomic surveys, knowledge about the distribution of sterol
1193 biosynthesis genes in the domain Bacteria has increased markedly in the past years^{17,33-35,187,188}.
1194 Most studies focused on SQMO, squalene monooxygenase, catalysing the oxygenation of squalene
1195 to oxidosqualene, and OSC, oxidosqualene cyclase, catalysing the cyclization of oxidosqualene to
1196 protosterols. A comprehensive survey of all genes involved in sterol biosynthesis was provided by
1197 Hoshino and Gaucher (2021)³³.

1198 In a pioneering combined genomic and lipid analysis, Pearson et al. (2003)¹⁸⁷ detected SQMO and
1199 OSC in the planctomycete bacterium *Gemmata obscuriglobus* and subsequently identified the
1200 protosterol lanosterol and its double-bond isomer parkeol in saponified cell extracts. Subsequent
1201 genome surveys^{17,33-35,188} provided a comprehensive overview of the presence of sterol
1202 biosynthesis genes in bacterial genomes that were available in the public domain at that time. Wei
1203 et al. (2016)³⁵ performed a combined genomic and lipid analysis and searched for homologs of
1204 OSC through BLASTP in all bacterial genomes (31,237) available in 2016 in the JGI database.
1205 The search yielded 34 bacterial OSC homologs in five different phyla, including the bacterial
1206 groups where sterols had been detected in earlier lipid studies^{187,189,190} plus several new
1207 occurrences. Similarly, in a search for SQMO and OSC protein sequences in the National Center
1208 for Biotechnology Information (NCBI) protein database, Gold et al. (2017)³⁴ found 27 bacterial
1209 taxa across 6 phyla, 9 classes and 9 orders, confirming the findings of Wei et al. (2016)³⁵. In an
1210 update on sterol biosynthesis genes in the NCBI data base, Hoshino and Gaucher (2021)³³ detected
1211 OSC in 94 bacterial taxa across twelve phyla (based on their Table S1).

1212 The combined studies detected the genetic potential for protosterol biosynthesis in the following
1213 major bacterial groups:

- 1214 ○ Phylum **Bacteroidetes**: Genera *Fluviivola* (Crocinitomicaceae), *Eudoraea*
1215 (Flavobacteriaceae) and an unnamed species in Cytophagales
- 1216 ○ Phylum **Actinobacteria**: Eight named and six unnamed species, including the genera
1217 *Streptomyces*, *Streptosporangium*, *Nocardia*, *Lentzea*, *Nonomuraea* and *Actinomadura*
- 1218 ○ Phylum **Cyanobacteria**: Six genera from three different orders (Oscillatoriales,
1219 Synechococcales, Nostocales)
- 1220 ○ Phylum **Planctomycetes**: two species of the genus *Gemmata*
- 1221 ○ Phylum **Verrucomicrobia**: Eleven unnamed species

- 1222 ○ Phylum **Chloroflexi**: three unnamed species
- 1223 ○ Phylum **Nitrospirae**: two species of the genus *Nitrospira*
- 1224 ○ Phylum **Rokubacteria**: three unnamed species
- 1225 ○ Phylum **Dadabacteria**: one unnamed species
- 1226 ○ Phylum **Gemmatimonadetes**: two unnamed species
- 1227 ○ Phylum **Acidobacteria**: one unnamed species in the Holophagales
- 1228 ○ Class **Alphaproteobacteria**: One unnamed species each in Rhodobacterales and
- 1229 Rhodospirillales, and the methanotrophs *Methyloceanibacter caenitepidi* and
- 1230 *Methylobacterium methanicus* belonging to two different families in the order
- 1231 Hyphomicrobiales (=Rhizobiales)
- 1232 ○ Class **Deltaproteobacteria**: Twelve genera of the order Myxococcales in the suborders
- 1233 Cystobacterineae (5), Nannocystineae (3) and Sorangiineae(4), ten more unnamed
- 1234 Myxococcales isolates, and one isolate not further characterized.
- 1235 ○ Class **Gammaproteobacteria**: Nine genera of aerobic methanotrophic bacteria in the
- 1236 order Methylococcales (four Type 1a and five Type 1b), the genus *Glaciecola* in
- 1237 Alteromonadales, two unnamed species in Chromatiales, and seven isolates not further
- 1238 characterized

1239 Wei et al. (2016)³⁵ also detected 176 OSC sequences in 2707 environmental metagenomes, of
 1240 which at least 37 clustered among bacterial OSC. Many of the detected genes clustered closely
 1241 with known bacterial OSC, while others may belong to additional classes and phyla. However, all
 1242 OSC from the metagenomic data set clustered within the known major Groups 1 and 2a-d of Wei
 1243 et al. (2016)³⁵, and addition of metagenomic data only caused relatively minor changes to the
 1244 topology of their OSC phylogenetic tree.

1245 The OSC phylogenetic trees presented in different studies have the same major characteristics and
 1246 some relatively minor differences. The OSC tree of Desmond and Gribaldo (2009)¹⁷ only included
 1247 four bacterial species. In that study, *Plesiocystis* (Myxococcales), *Methylococcus* (Type 1b
 1248 Methylococcales) and *Gemmata* (Planctomycetes) formed an outgroup to eukaryotic OSC while a
 1249 second genus within Myxococcales (*Stigmatella*) branched within Eukarya, invoking a relatively
 1250 recent horizontal gene transfer (HGT). In the OSC tree of Villanueva et al. (2014)¹⁸⁸, which is
 1251 based on twelve bacterial OSC sequences, *Stigmatella* falls outside eukaryotic OSC, but *Fluviicola*
 1252 (Bacterioidetes) and two further genera of Methylococcales of Type 1a (*Methylobacter*,
 1253 *Methylomicrobium*) branch within Eukarya, distributing the Methylococcales between two distinct
 1254 major clusters. The larger data set of Wei et al. (2016)³⁵ largely echoes the topology of Villanueva
 1255 et al. (2014)¹⁸⁸, with a major cluster ‘Group 1’ outside Eukarya that includes the Myxococcales
 1256 suborders Nannocystineae and Sorangiineae (‘Myxococcales Group I’), the three cyanobacterial

1257 orders, *Gemmata* and two genera of Type 1b Methylococcales. Three smaller clusters, Groups 2a-
1258 c, branch close to Eukarya, including the Myxococcales suborder Cystobacterineae
1259 (‘Myxococcales Group I’). Group 2d falls within Eukarya, including three genera of Type 1a
1260 Methylococcales, *Fluviicola* and a representative of phylum Verrucomicrobia. Notable is the
1261 distribution of different genera of Myxococcales, Methylococcales and Bacterioidetes across major
1262 different groupings. The OSC tree topologies of Gold et al. (2017)³⁴ and Hoshino and Gaucher
1263 (2021)³³ (their Fig. S13) show the same general features, with Bacterioidetes and Type 1a
1264 Methylococcales branching within Eukarya, and similar splits of Myxococcales, Methylococcales
1265 and Bacterioidetes across different clusters.

1266 SQMO and OSC phylogenetic trees offer several insights. Firstly, the topologies of the SQMO
1267 and OSC trees are complex and do not follow rDNA based evolutionary patterns of bacteria.
1268 Secondly, apart from Myxococcales and Methylococcales, the occurrence of OSC is
1269 phylogenetically shallow, largely occurring in individual genera and species within larger
1270 groupings that otherwise lack SQMO and OSC. For instance, in Cyanobacteria, OSC only occurs
1271 in six genera and these are distributed across three different orders. As the number of published
1272 cyanobacterial genomes and metagenomes is large, it is unlikely that OSC is widespread within
1273 the phylum but remains undetected. In the words of Gold et al. (2017)³⁴, “the shallow clade depth
1274 and sparse phylogenetic distribution of bacterial genes—combined with the relatively long
1275 branches leading to eukaryal representatives—are most consistent with these genes being
1276 transferred one or more times from stem eukaryotes to bacteria”. We agree with this assessment,
1277 but note that the data is currently not robust enough to determine the sources, direction and timing
1278 of HGT events in most cases. This is exemplified by the most comprehensive study of Hoshino
1279 and Gaucher (2021)³³, discussed Section 8.2.3.

1280 8.2.3. *Myxococcales as the possible origin of sterol biosynthesis*

1281 Hoshino and Gaucher (2021)³³ performed a comprehensive analysis of all genes involved in sterol
1282 biosynthesis, including ‘Stage 1’ enzymes involved in the biosynthesis of squalene, and ‘Stage 2’
1283 enzymes that generate and modify the polycyclic core of sterols, in particular demethylation at
1284 positions C-4 and C14, plus further downstream enzymes involved in the modification of
1285 unsaturations and side-chain alkylation. The authors found that Stage 1 genes in Myxococcales
1286 (and one species of Dadabacteria) were more similar to the eukaryotic pathway than to other
1287 bacteria. Moreover, they found that Stage 2 genes involved in the removal of methyl groups from
1288 the protosterol core to generate C₂₇ cholesteroloids are present in the two Myxococcales suborders
1289 Nannocystineae and Sorangiineae (Myxococcales Group I), but not in suborder Cystobacterineae
1290 (Myxococcales Group II) or any other group of bacteria. The core Stage 2 genes in bacteria were

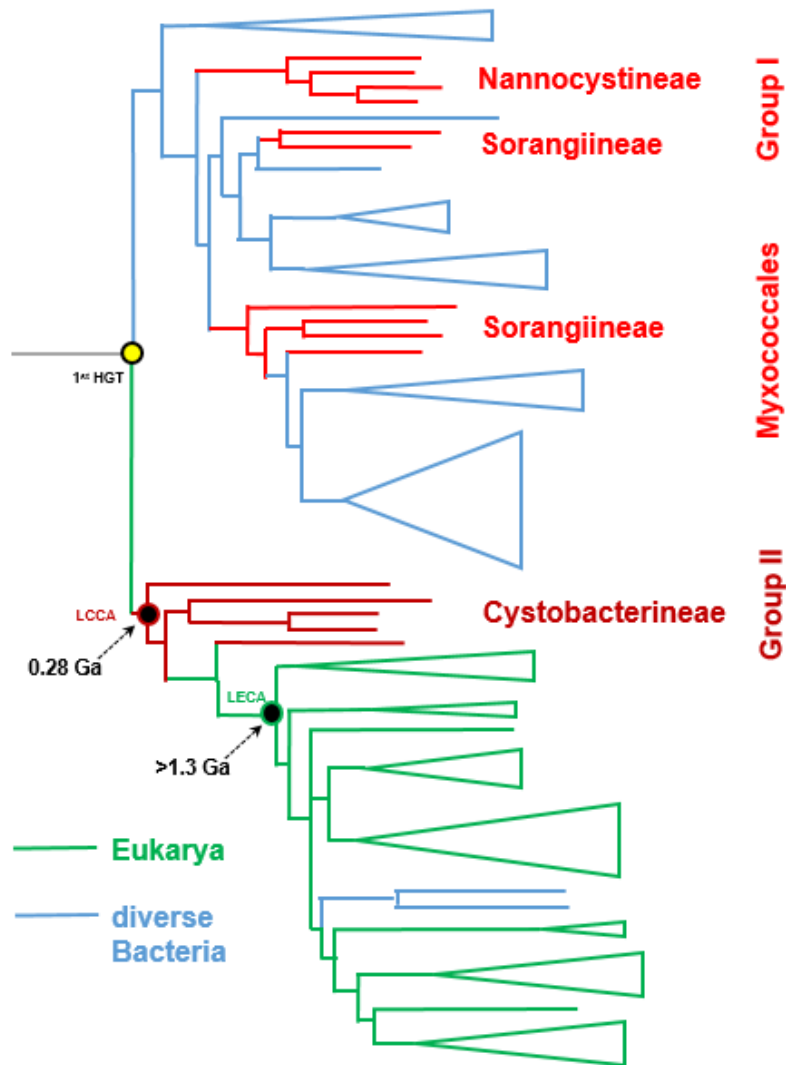
1291 mostly in a syntenic relationship with each other, i.e. they were physically co-localized with other
1292 sterol biosynthesis genes.

1293 To create a possibly more robust phylogenetic tree of sterol biosynthesis genes, Hoshino and
1294 Gaucher (2021)³³ used concatenated sequences of Stage 2 genes involved in the biosynthesis of
1295 protosterols (SQMO, OSC), the opening of the three-ring of cycloartenol (CPI1) and the
1296 subsequent removal of methyl groups from C-14 (CYP51, ERG24) and C-4 (ERG25, ERG26 and
1297 ERG27-analog). A simplified version of the tree is reproduced in Supplementary Figure 1. In the
1298 concatenated tree, but also in the individual gene trees, crown-group Eukarya always branches
1299 within Bacteria. Moreover, in the OSC, SQMO and concatenated tree, Eukarya branch within
1300 Myxococcales Group II (suborder Cystobacterineae) (Supplementary Fig. 1). The branching
1301 pattern of Myxococcales in these trees broadly follows phylogeny, i.e. the sterol genes often (but
1302 not always) fall into the correct suborders. Based on these observations, Hoshino and Gaucher
1303 (2021)³³ suggested that sterol biosynthesis, including demethylation of the sterol core, evolved
1304 within Bacteria and was transferred from Myxococcales Group II (suborder Cystobacterineae) to
1305 stem-group eukaryotes. Moreover, sterol biosynthesis was probably acquired by eukaryotes from
1306 Myxococcales Group II as a full syntenic set. As Myxococcales Group II does not possess any
1307 genes for downstream modification, the authors suggest that all genes apart from SQMO and OSC
1308 were later lost from these Myxococcales.

1309 The model of Hoshino and Gaucher (2021)³³ makes strong predictions about the timing of this
1310 HGT event. As LECA presumably already possessed all eight genes of the concatenated set, the
1311 transfer must have occurred broadly before ~1,300 Ma ago. However, the authors further suggest
1312 that sterols were likely needed for endocytosis and the acquisition of the mitochondrion,
1313 presumably moving the HGT event considerably further back in time. Based on molecular clock
1314 estimates of Gold et al. (2017)³⁴, the maximum probability for the horizontal transfer of the eight
1315 Stage 2 sterol biosynthesis genes from Myxococcales Group II to eukaryotes would then be ~2,300
1316 Ma, with a minimum age of ~1,700 Ma.

1317 The timing of these events and the direction of gene transfer critically depend on correct tree
1318 topology. However, the phylogeny generated by the concatenation of the eight Stage 2 sterol
1319 biosynthesis genes (Supplementary Fig. 1), as well as the individual gene trees, are presumably
1320 inconsistent with the evolution of Myxococcales. In the concatenated gene tree, the
1321 Cystobacterineae cluster includes the species *Archangium gephyra*, *Cystobacter fuscus*,
1322 *Corallocooccus coralloides*, *Stigmatella aurantiaca* and an unnamed species. The four named
1323 species are terrestrial chitin degraders that acquired chitinase of the GH18 family via HGT from
1324 Bacilli (Firmicutes), which in turn received it via HGT from crown-group eukaryotic fungi¹⁹¹.
1325 Based on well calibrated molecular clocks, Gruen (2019)¹⁹¹ estimated the date of the HGT of

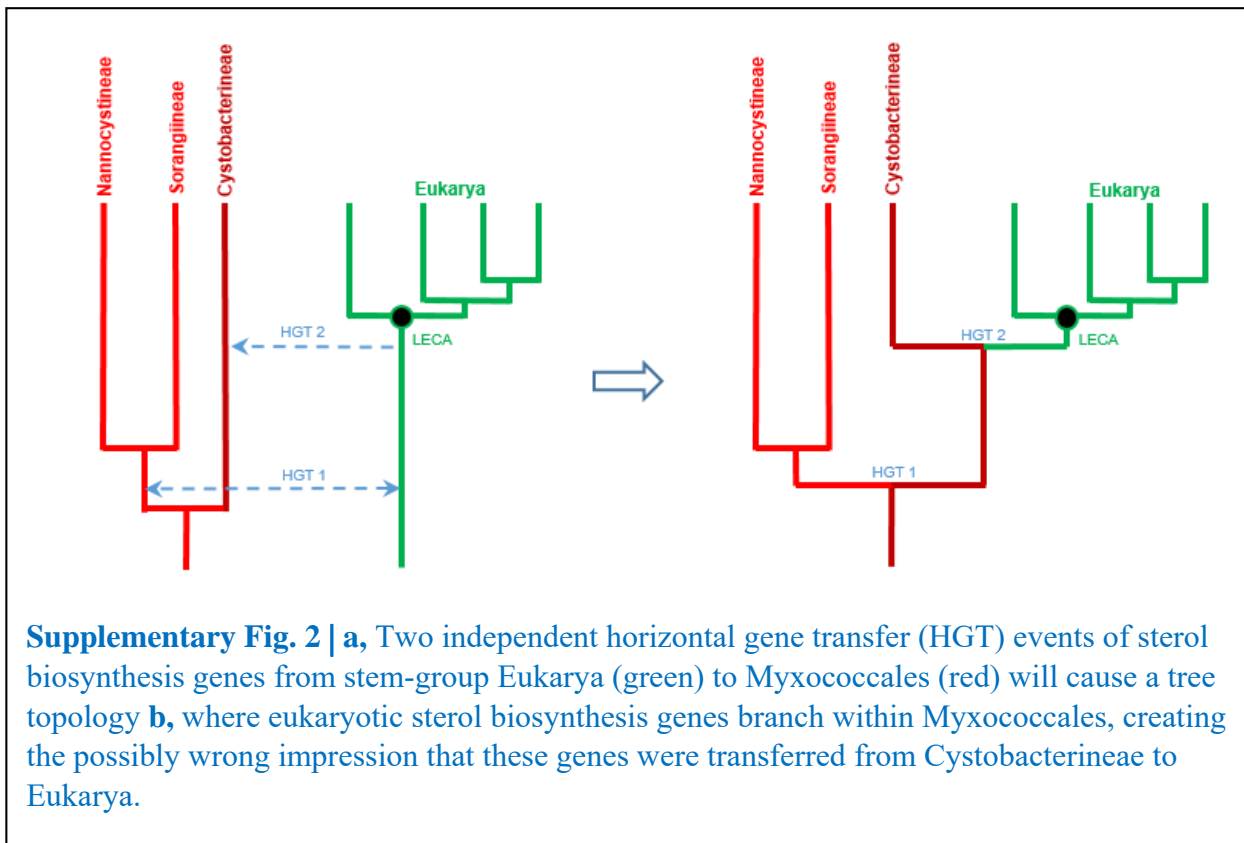
1326 chitinase from fungi to Bacilli at 408 Ma (323 to 498 Ma, 95% confidence interval) and from
1327 Bacilli to Cystobacterineae at 275 Ma ago (174 to 389 Ma, 95% confidence interval) (see age
1328 annotation in Supplementary Fig. 1). Based on these age estimates, Cystobacterineae is a very
1329 young bacterial clade, and the origin of chitinase in these organisms may postdate LECA by over
1330 one billion years. Even if these age estimates were widely wrong, an ancestor of the group
1331 *Melittangium* + *Archangium* + *Cystobacter* + *Corallococcus* + *Stigmatella*, which comprises most
1332 of the clade depth of named Cystobacterineae species, did receive a fungal gene in a single HGT
1333 event¹⁹¹. Fungi are descendants of LECA. Thus, it is not possible that an ancestor of LECA
1334 acquired sterol biosynthesis from these Cystobacterineae. This means that the gene trees of
1335 Hoshino and Gaucher (2021)³³ apparently failed to produce the correct topology around the
1336 Eukarya-Cystobacterineae branching point.



Supplementary Fig. 1 | Phylogenetic tree modified from Figure 3 in Hoshino and Gaucher (2021)³³. The tree is based on concatenated sequences of SQMO, OSC, CYP51, ERG24, ERG25, ERG26, ERG27-analog and CPI1 (i.e. genes generating and modifying the polycyclic core of sterols). Eukaryotes (green) branch within Bacteria among Cystobacterineae (dark red), a suborder of Myxococcales (red). The two nodes (black filled circles) are annotated with molecular clock ages for the last eukaryotic common ancestor (LECA) and the common ancestor of the Cystobacterineae species *Archangium gephyra*, *Cystobacter fuscus*, *Coralloccoccus coralloides*, *Stigmatella aurantiaca* (LCCA). For discussion of contradictory ages see text. The yellow node indicates the first hypothetical horizontal gene transfer (HGT) between stem-group Eukarya and Bacteria.

1337 While the correct evolutionary relationship of sterol biosynthesis genes between Myxococcales
 1338 and Eukarya remains unresolved, it is clear that crown-group eukaryotes should not branch within

1339 Myxococcales Group II and, thus, sterol biosynthesis genes of Myxococcales Group II are either
 1340 a close outgroup to crown-group Eukarya or branch within crown-group Eukarya. Both scenarios
 1341 significantly reduce the probability that stem-group eukaryotes received a full syntenic set of sterol
 1342 biosynthesis genes from Myxococcales. We thus suggest that the gene trees are best explained by
 1343 two HGT events, as shown in Supplementary Figure 2: The first HGT event is the transfer of sterol
 1344 biosynthesis genes (possibly in a syntenic set) between stem-group Eukarya and Myxococcales
 1345 Group I (Nannocystineae + Sorangiineae). However, this transfer may have occurred in either
 1346 direction. Second, a more recent HGT event of SQMO and OSC from Eukarya to Myxococcales
 1347 Group II (Cystobacterineae). The second transfer may have occurred as recently as the
 1348 Phanerozoic from crown-group eukaryotes to Cystobacterineae, or in the Proterozoic from late
 1349 stem-group eukaryotes to Cystobacterineae (Supplementary Fig. 2a). Such a second gene transfer



1350 will create a tree topology where Eukarya branch within Myxococcales, creating the possibly
 1351 wrong impression that sterol biosynthesis genes were transferred from Cystobacterineae to
 1352 Eukarya. (Supplementary Fig. 2b). This alternative scenario also explains the absence of
 1353 downstream Stage 2 genes in Myxococcales Group II without requiring the loss of these genes
 1354 from Cystobacterineae.

1355 Are two sterol gene transfers likely between eukaryotes and Myxococcales? Generally, HGT of
1356 sterol biosynthesis genes has occurred many times based on the large number of isolated bacterial
1357 groups that have acquired these genes independently, although these genes may have been largely
1358 transferred among bacterial clades. However, several groups of bacteria also possess sterol
1359 biosynthesis genes derived from crown-group eukaryotes (e.g. SQMO and OSC in Bacteroidetes,
1360 Methylococcales, and Alteromonadales within the Deltaproteobacteria), demonstrating that the
1361 transfer crown-group Eukarya → Bacteria has occurred. It is not unlikely that similar transfers
1362 occurred from the long stem of Eukarya to bacterial groups, and this may account for some of the
1363 occurrences of SQMO and OSC in bacteria that branch outside crown-group Eukarya. Many
1364 Myxococcales feed on insoluble eukaryotic substrates such as starch, cellulose, and fungal and
1365 arthropod chitin. The close physical association with eukaryotic biomass may increase the
1366 probability of gene transfer.

1367 Hoshino and Gaucher (2021)³³ suggest that the synteny of sterol synthesis genes in Myxococcales
1368 Group I speaks for a simultaneous lateral transfer of all Stage 2 sterol biosynthesis genes as a set
1369 to stem-group Eukarya. If correct, then Myxococcales as well as the first sterol synthesizing
1370 eukaryotes had the ability to make C₂₇ cholesteroloids as early as 2,300 Ma ago, and by 1,700 Ma
1371 ago at the latest³⁴. This implies that Myxococcales and eukaryotes were capable of C₂₇ sterol
1372 production through the entire mid-Proterozoic interval. Yet, despite the abundance of
1373 protosteroids, not even traces of indigenous, saturated C₂₇ steranes have been detected in
1374 sedimentary rocks during that time interval. In our opinion it is implausible that cholesteroloid
1375 biosynthesis existed in eukaryotes and bacteria by 2,300 Ma ago but remained undetectable in the
1376 rock record until 800 Ma ago. It is more likely that a very ancient transfer of an entire Stage 2
1377 syntenic set of sterol biosynthesis genes between Eukarya and Myxococcales did not occur and
1378 that downstream modification of the protosterol core evolved later.

1379 In Summary, Hoshino and Gaucher (2021)³³ provided the most comprehensive and thorough
1380 analyses of the phylogenetic distribution and evolution of sterol biosynthesis in bacteria and
1381 eukaryotes to date. However, the conclusion that stem-group eukaryotes acquired sterol
1382 biosynthesis from Myxococcales Group 2 is poorly supported due to the very young age of that
1383 group. Future studies need to evaluate whether Myxococcales Group II branches within Eukarya
1384 or just outside Eukarya, and whether this group acquired its sterol biosynthetic genes from
1385 eukaryotes via HGT or via vertical inheritance from stem-group Myxococcales. The most difficult
1386 unresolved question is whether Myxococcales Group I obtained sterol biosynthesis from stem-
1387 group Eukarya or transferred it to Eukarya, and whether syntenic relationships were inherited via
1388 HGT or were achieved independently.

1389

1390 8.2.4. *The geological age of sterol producing bacteria*

1391 To interpret protosteroids in the mid-Proterozoic biomarker record, it is important to assess which
1392 groups of extant bacteria had the capacity to produce protosterols between 1,640 and 1,000 Ma
1393 ago. With the exception of Myxococcales and Methylococcales, known bacterial SQMO and OSC
1394 have very shallow clade depths, and some even branch within crown-group Eukarya³³⁻³⁵. Thus,
1395 sterol production in most bacteria likely has quite recent origins via HGT from eukaryotes and
1396 among bacteria. Yet, it is not possible at present to determine precisely when various lineages of
1397 bacteria acquired sterol biosynthesis. The scarcity of calibration points in the bacterial fossil
1398 record, and hundred million to billion-year error bars associated with molecular clocks, make it
1399 difficult in most cases to determine which groups of extant sterol producers existed in the mid-
1400 Proterozoic interval. However, existing data provides some estimates for the two deepest
1401 branching lineages, Myxococcales and Methylococcales.

1402 8.2.4.1. *Myxococcales ('slime bacteria', Deltaproteobacteria)*

1403 The age of the last common ancestor of Myxococcales was roughly estimated at ~900 Ma, and the
1404 divergence of Myxococcales from other bacteria at ~1,000 Ma¹⁹². These estimates are broadly
1405 consistent with more refined molecular clock calculations based on the horizontal transfer of a
1406 fungal chitinase gene to Myxococcales calibrated with well dated fossils of fungi¹⁹¹. The clock
1407 places the base of suborder Cystobacterineae at 275 Ma (with 95% confidence interval from 174
1408 to 389 Ma). Although we are not aware of molecular clock estimates for the ages of suborders
1409 Nannocystineae and Sorangiineae, a comparison of the data of Gruen et al. (2019)¹⁹¹ with
1410 published phylogenetic trees¹⁹³⁻¹⁹⁵ suggest that an origin of Myxococcales of around 1,000 to 1,200
1411 Ma is reasonable. If these estimates are broadly correct, then it is unlikely that crown-group
1412 representatives of the order Myxococcales go back to 1,640 Ma, the age of the oldest known
1413 protosteroids.

1414 8.2.4.2. *Methylococcales (aerobic methanotrophs, Gammaproteobacteria)*

1415 The literature offers widely diverging views on the age of Methylococcales. Based on one study¹⁹⁶,
1416 Methylococcales and Thiotrichaceae (sulfide oxidizing bacteria, SOB) radiate near the base of the
1417 Gammaproteobacteria-Betaproteobacteria division (but also see ref¹⁹⁷ where the radiation of
1418 gammaproteobacterial orders is spread across nearly 1 billion years). Using molecular clocks
1419 calibrated on SOB symbionts in bivalves, the pioneering work of Canfield and Teske (1996)¹⁹⁸
1420 suggested that the radiation of Thiotrichaceae occurred in the Neoproterozoic 760 ± 320 to $620 \pm$
1421 280 Ma ago (and with an unlikely absolute age maximum of 1,400 Ma). Based on this data, the
1422 order Methylococcales may have a similar age and did not likely exist early in the mid-Proterozoic

1423 interval. By contrast, a second molecular clock study places the origin of Methylococcales at
1424 ~1,850 to 2,200 Ma¹⁹⁷. The authors applied the carotenoid biomarker okenane as a calibration
1425 point for Chromatiaceae, which is a sister group of Methylococcales. Okenane occurs in the 1,640
1426 Ma Barney Creek Formation together with protosteroids and is regarded as a marker for purple
1427 sulfur bacteria^{30,199}. However, the calibration age of 1,640 Ma was used as the minimum age for
1428 the last common ancestor of all Chromatiaceae while it would have been more correct to assign it
1429 to total-group Chromatiaceae, which would move the age for Methylococcales a few hundred
1430 million years to the present. In any case, if okenane is correctly interpreted as a biomarker for
1431 purple sulfur bacteria, then total-group Methylococcales should also have existed by 1,640 Ma. It
1432 is thus crucial to determine when sterol biosynthesis evolved *within* Methylococcales.

1433 SQMO and OSC are observed in nine named genera of the order Methylococcales that fall in two
1434 different groupings. *Methylocaldum*, *Methylococcus*, *Methylospira*, *Methyloterricola* and
1435 *Methyloumidiphilus* belong to Group 1 of Wei et al. (2016)³⁵ and Hoshino and Gaucher (2021)³³,
1436 which branches outside crown-group eukaryotic sterol biosynthesis genes. These genera belong to
1437 the phylogenetic cluster of so-called Type 1b methanotrophs as defined by Knief (2015)²⁰⁰.
1438 Conversely, *Methylobacter*, *Methylomicrobium*, *Methylosarcina* and *Methyloprofundus* belong to
1439 a phylogenetic cluster within a second branch of Methylococcales, the Type 1a methanotrophs.
1440 Interestingly, this cluster possesses distinct protosterol biosynthesis genes that were obtained from
1441 crown-group eukaryotes via HGT³³⁻³⁵. As the cluster of sterol-producing Type 1a methanotrophs
1442 demonstrably obtained SQMO and OSC from crown-group eukaryotes, it is not unlikely that Type
1443 1b methanotrophs obtained them from stem-group eukaryotes or from bacteria such as the
1444 Myxococcales.

1445 It is difficult to ascertain when Type 1b methanotrophs acquired sterol biosynthesis genes. If we
1446 apply the most extreme molecular clock estimate where Methylococcales emerge at 2,200 Ma¹⁹⁷,
1447 then the last common ancestor of Type 1b may well have existed by 1,640 Ma. However, based
1448 on other estimates discussed above, methanotrophs likely acquired sterol biosynthesis later and are
1449 not a likely source of protosterols by 1,640 Ma.

1450 Irrespective of the age of origin of sterol biosynthesis in methanotrophic bacteria, one additional
1451 observation speaks against a major contribution of steroids from Methylococcales to the mid-
1452 Proterozoic biomarker pool: all extant sterol-producing Type 1a and 1b Methylococcales, and thus
1453 presumably their respective last common ancestors, synthesize 4-methyl and 4,4-dimethyl
1454 cholesteroloids as their major sterols. Yet, the corresponding saturated hydrocarbon biomarkers, 4-
1455 methylcholestane and 4,4-dimethylcholestane, have not been detected in mid-Proterozoic
1456 sediments of any age despite the fact that 4,4,14-trimethylated cholesteroloids, including lanostane,
1457 are abundant. This observation suggests that crown-group representatives of Type 1a and 1b

1458 Methylococcales were not a major component of the Protosterol Biota irrespective of their age of
1459 origin. This also means that an earlier interpretation that attributed triaromatic steroids of the 1,640
1460 Ma Barney Creek Formation to methanotrophic bacteria³⁰ is no longer supported. However, the
1461 structure of these steroids is, in principle, still consistent with a stem-group methylococcalean
1462 origin.

1463 Additional strong evidence against crown- or stem-group Methylococcales sources comes from
1464 the carbon isotopic composition of individual biomarker from the Barney Creek Fm. Methane
1465 generation in mid-Proterozoic marine sediments was likely generally higher than today,
1466 potentially providing substrate for substantial activity of methane oxidizing bacteria³⁰. As
1467 methane consumed by Methylococcales is usually isotopically strongly depleted in ¹³C, their
1468 lipids possess diagnostically low $\delta^{13}\text{C}$ values ($\delta^{13}\text{C} < -60\text{‰}$). This is incompatible with $\delta^{13}\text{C}$
1469 values of 4-methylated triaromatic steroids from the Barney Creek Fm that are isotopically
1470 strongly enriched ($\delta^{13}\text{C} = -19$ to -22‰) relative to bulk biomass ($\delta^{13}\text{C}_{\text{kerogen}} = -30.4$ to -32.1‰)
1471 and cyanobacterial lipids ($\delta^{13}\text{C}_{\beta\text{-carotane}} = -37.3 \pm 0.9\text{‰}$)³⁶. The carbon isotopic values of the
1472 steroids are consistent with a heterotrophic³⁶ or phototrophic source, but exclude
1473 Methylococcales as major sterol producers.

1474 **8.2.4.3. Summary**

1475 Based on current age estimates, none of the crown-groups of sterol-producing bacteria are likely
1476 sources of protosteroids going as far back as 1,640 Ma. Bacterial steroid contributions to mid-
1477 Proterozoic sediments are thus more likely derived from unknown or extinct bacterial lineages or
1478 from stem-group representatives of Myxococcales and Type 1b Methylococcales. However, this
1479 assessment is tentative as molecular clock estimates and phylogenetic tree topologies may change
1480 with emerging sequence data.

1481 **8.2.5. Did bacteria or eukaryotes produce sterols in the Palaeoproterozoic at all?**

1482 Based on molecular clocks, Gold et al. (2017)³⁴ estimate that the maximum marginal probability
1483 for the divergence of bacterial and eukaryotic protosterol biosynthesis genes is ~2,300 Ma, and the
1484 minimum age ~1,700 Ma. The authors cautiously and correctly did not claim that this age range
1485 corresponds to a HGT event of SQMO and OSC between bacteria and eukaryotes. This is because
1486 the computed divergence age might correspond to one of two alternate scenarios: (1) the age may
1487 reflect the bifurcation of two stem-group eukaryotic lineages, one of which later transferred SQMO
1488 and OSC to a bacterial group and then went extinct, while the second lineage gave rise to LECA.
1489 (2) The age may reflect the bifurcation of two bacterial lineages, one of which later transferred
1490 SQMO and OSC to stem-group eukaryotes and the other to an extant bacterial lineages (e.g.

1491 Myxococcales), and then both went extinct. Scenario (1) is not unlikely given the generally high
1492 frequency of HGT from eukaryotes to bacteria, the fact that all stem lineages went extinct (except
1493 the one giving rise to LECA), and the knowledge that transfers of SQMO and OSC from
1494 eukaryotes to bacteria do in fact occur^{34,35}.

1495 There are additional hypotheses about sterol origins that are currently intractable. For instance,
1496 stem-group eukaryotes and extant bacterial lineages may have received protosterol biosynthesis
1497 from an extinct bacterial group, or from a bacterial group that has since lost these genes or remains
1498 to be discovered.

1499 8.2.6. *The sterols produced by bacteria*

1500 For many bacterial species that possess SQMO and OSC, it remains unknown whether these genes
1501 are expressed and, if so, which sterols are produced. In particular, sterol biosynthesis has not been
1502 confirmed through lipid analysis in any representative of Cyanobacteria, Actinobacteria and
1503 Verrucomicrobia³⁵. However, for 18 species, sterol biosynthesis has been confirmed through lipid
1504 surveys. *Gemmata obscuriglobus* (Planctomycetes) produces lanosterol and its double-bond
1505 isomer parkeol¹⁸⁷. The sterols are essential to growth and function as membrane molecules²⁰¹.
1506 *Fluviicola taffensis* (Bacterioidetes) and the aerobic methanotroph *Methyloceanibacter caenitepidi*
1507 (Alphaproteobacteria) exclusively produce cycloartenol, while aerobic methanotrophs of the
1508 Methylococcales (Gammaproteobacteria) have the capacity to demethylate protosterols in the C-
1509 14 position and remove one methyl group from the C-4 position. The final sterol products are 4-
1510 methyl and 4,4-dimethyl cholesteroloids. Methylococcales do not appear to accumulate notable
1511 amounts of C₃₀ protosterols^{35,189,202}. Myxococcales (Deltaproteobacteria) of the suborders
1512 Nannocystineae and Sorangiineae (part of Group 1 and branching deeply outside Eukarya³⁵) have
1513 the distinguishing capacity to remove all methyl groups from positions C-4 and C-14 to produce
1514 the C₂₇ sterol zymosterol as the main product. *Enhygromyxa salina* (Nannocystineae)
1515 biosynthesizes cholesterol that is bound to a lipid or protein moiety but does not occur as a free
1516 membrane component²⁰³. By contrast, representatives of the suborder Cystobacterineae, which
1517 form a separate cluster in the OSC tree closer to eukaryotes, either did not contain detectable sterols
1518 or produce cycloartenol and/or lanosterol^{35,190}. An unusual case is *Eudoraea adriatica*
1519 (Bacteroidetes) that only yielded traces of lanosterol while the main products of the OSC-like
1520 cyclase were two pentacyclic terpenols with an arborane skeleton, eudoraenol and adriaticol²⁰⁴.

1521 8.2.7. *Environments inhabited by sterol producing Bacteria*

1522 Bacteria with genes for protosterol biosynthesis are found in a wide variety of habitats. OSC
1523 protein sequences were found in metagenomes from soil, in marine and freshwater environments,
1524 estuarine microbial mats, hydrothermal vent fluids, wastewater and in the deep subsurface. Most

1525 sterol producing Myxococcales were acquired from soil, but at least two come from marine
1526 environments. Sterol producing Methylococcales were enriched from sewage, marine pelagic
1527 environments, hot springs, freshwater lake sediments, and soda lake sediments. The
1528 alphaproteobacterial methanotroph *Methyloceanibacter caenitepidi* (Rhizobiales) was isolated
1529 from a marine hydrothermal vent, *Eudoraea adriatica* came from a coastal sediment, and
1530 *Fluviicola taffensis* from river sediment³⁵. *Gemmata obscuriglobus* was isolated from the littoral
1531 zone of a freshwater lake. Based on genomic data, no particular environment stands out as a
1532 preferred habitat for bacterial sterol producers.

1533 8.2.8. *Summary*

1534 Phylogenetic studies on sterol biosynthesis provide six notable insights: (1) SQMO and OSC of
1535 extant bacteria and crown-group eukaryotes most likely diverged ~2,300 Ma ago and probably
1536 before 1,700 Ma ago³⁴. (2) After this first major divergence of SQMO and OSC, at least one
1537 transfer of these genes occurred between stem-group eukaryotes and bacteria, but the direction of
1538 transfer remains unknown (see ref³³ for a contrasting view). (3) At least one transfer of SQMO and
1539 OSC occurred from crown-group eukaryotes to bacteria, and these eukaryotic genes are now found
1540 in representatives of Methylococcales, Alteromonadales and Bacteriodetes. (4) The distribution of
1541 sterol biosynthesis genes in bacteria is sporadic and complex and presumably affected by a
1542 combination of frequent HGT events and gene losses, some vertical inheritance (chiefly in
1543 Myxococcales and Methylococcales) and minor convergent evolution²⁰⁵ (see also existence of
1544 SQMO and alternative-SQMO in eukaryotes²⁰⁶). (5) The only bacterial clusters where sterol
1545 biosynthesis has very deep roots is Myxococcales, but it is currently not possible to state with
1546 certainty when this group emerged and whether it predates LECA.

1547 8.3. *Comparison of sterol abundances in eukaryotic and bacterial cells*

1548 To assess whether bacteria can make a significant contribution to the sterol content of sediments
1549 and sedimentary rocks, we need to compare the absolute sterol content of bacterial and eukaryotic
1550 cells. All sterol contents in the following discussion are reported in percent of dry weight.

1551 The sterol content of plant matter can range from very low to very high depending on wood and
1552 carbohydrate contents and presence of sterol-rich oils. Lipid surveys measured sterol contents of
1553 0.02-0.4% in vegetables, 0.05-0.29% for fruit²⁰⁷, 0.04-0.22% in berries, 0.02-0.1% in the kernels
1554 of grain crops, 0.1-0.3% in seeds of oil crops, and 0.1-0.2% in legumes²⁰⁸. These values are
1555 comparable to a 0.1% sterol content in the polypore mushroom *Polystictus versicolor*¹⁸⁹. The sterol
1556 content of algae is generally higher than of land plants with an average content of $0.31 \pm 0.18\%$
1557 (range 0.06-0.65%) in 18 species of macroalgae²⁰⁹ and an average of $0.51 \pm 0.71\%$ (range 0.02-
1558 2.6%) in ten species of microalgae²¹⁰. Yet higher is the average sterol content of single celled

1559 protists and fungi. The free living amoeba *Acanthamoeba polyphaga* contains 0.4-0.5% sterols²¹¹
1560 and the fungal mould *Aspergillus nidulans* yielded 0.6-0.9%¹⁸⁹. A sterol survey¹⁶³ on basal
1561 flagellated fungi found high sterol contents of 0.3-3.6% (average $1.7 \pm 1.1\%$, n = 8). Similarly
1562 high sterol contents of 1.4% and 2.2% were found in two heterokonts of the phylum
1563 Hyphochytriomycota, protists which possess a fungi-like rhizoidal or hypha-like vegetative
1564 system¹⁶³.

1565 Bloch (1983)²⁸ suggested that the few bacteria that appear to produce sterols only contain traces.
1566 Although there is still only limited reliable information on the abundance of sterols in bacteria, we
1567 now know that sterol concentrations per dry weight are not significantly different from eukaryotes.
1568 Reports of very low concentration are most likely based on culture contamination¹⁵⁶ such as traces
1569 of cholesterol (0.0035%) in *Streptomyces olivaceus*²¹², 0.01% of a mixture of six sterols (including
1570 ergosterol) in *Azotobacter chroococcum*²¹³ and traces in some other bacteria²¹⁴. Reliable data exists
1571 for *Gemmata obscuriglobus* with 2.0%¹⁸⁷, *Nannocystis exedens* with 0.4%²¹⁵ and *Methylococcus*
1572 *capsulatus* with 0.22%¹⁸⁹. Extremely low concentrations of 0.00002% lanosta-8,22,24-triene were
1573 detected in *Methylobacterium organophilum* (Alphaproteobacteria, Rhizobiales)²¹⁶. This latter
1574 report seems credible as this unusual sterol is not a likely contaminant and the related bacterium
1575 *Methyloceanibacter caenitepidi* is known to produce sterols. Yet, it cannot be excluded that the
1576 sterol was contributed by other, minor methanotrophic bacteria present in the medium, so the
1577 existence of such low sterol concentrations needs to be re-evaluated in pure culture.

1578 Based on this limited data, the sterol content of bacteria per dry weight is in the same range as in
1579 eukaryotes, supporting the hypothesis that sterols can be a notable component of bacterial
1580 membranes. Bacterial sterols may thus comprise a significant fraction of sedimentary lipids in
1581 those environments where sterol-producing bacteria are abundant (Section 9).

1582 **9. Modern environments and sediments with elevated protosterol levels**

1583 All organisms that produce sterols also contain protosterols as biosynthetic intermediates,
1584 commonly at low percentage levels. Thus, all modern environments and sediments that contain
1585 sterols should yield protosterols. Yet, there are very few studies that report these compounds,
1586 presumably because the small chromatographic signals are overlooked or not reported. By
1587 contrast, protosterol abundances far above typical biosynthetic background levels are usually
1588 conspicuous in chromatograms and difficult to overlook. Yet, despite the vast number of sterol
1589 surveys of recent environments and sediments, elevated protosterol abundances are very rarely
1590 observed, and the few instances in the literature invariably point to uncommon and unusual
1591 environments such as methane seeps and microbial mats. It thus appears that protosterols rarely

1592 exceed background levels in almost all modern marine, lacustrine and terrestrial environments.
1593 Known exceptions are discussed below.

1594 ***9.1. Cycloartenol in the Lost City hydrothermal system***

1595 Lost City is an ocean-floor hydrothermal field located at a depth of 750 to 900 meters at the mid-
1596 Atlantic ridge. At Lost City, hot reducing fluids rich in hydrogen and methane and with
1597 temperatures of < 40 to 90°C, emanate from large carbonate chimneys. A lipid survey²¹⁷ at Lost
1598 City detected cycloartenol in concentrations approaching 500 ng per gram of dry rock.
1599 Cycloartenol was the most abundant sterol in some samples. In one case, cycloartenol occurred at
1600 an abundance of 100 ng/g, while the only other sterol, cholesterol, merely yielded 40 ng/g rock.
1601 Moreover, in that sample, cycloartenol was enriched in ¹³C by more than 12‰ relative to
1602 cholesterol. Based on the high relative abundance and diverging carbon isotopes, the authors
1603 concluded that the protosterol at Lost City is a final product, not a biosynthetic intermediate²¹⁷.
1604 The authors excluded Myxococcales as a source of cycloartenol as these were not detected in
1605 rRNA gene surveys. The authors further concluded that “*the most probable source of abundant*
1606 *cycloartenol is one or more of the protists inhabiting the carbonate chimneys*”, noting that “*a*
1607 *diverse population of protists has been detected in Lost City carbonates. Ciliates are the most*
1608 *dominant, and other alveolates, fungi, heterokonts, radiolaria and other cercozoa, and*
1609 *heterolobosea have also been detected* (citing ref²¹⁸)”. However, the authors also cautioned that
1610 the sterol biosynthetic pathway of most protists remains unknown²¹⁷. As protosterol biosynthesis
1611 requires ten times less oxygen than crown-sterol biosynthesis, they further speculated that (quote)
1612 “*cycloartenol might be a favored sterol from an amphiaerobic eukaryote inhabiting vent fluids*
1613 *with only sporadic access to oxygenated seawater*“. Based on the carbon isotopic enrichment of
1614 the Lost City cycloartenol, the authors further suggest that it may derive from protists that inhabit
1615 the carbonate chimneys and feed on prokaryotes that are part of the methanogenic community. We
1616 agree with the assessment that the protosterol in Lost City may, in principle, derive from a crown-
1617 group eukaryote adapted to microaerobic conditions. However, more recent studies reveal
1618 additional groups of bacteria, apart from Myxobacteria, that produce cycloartenol as their final
1619 sterol product, including one representative of the phylum Bacteroidetes and, notably, the aerobic
1620 methanotroph *Methyloceanibacter* (Alphaproteobacteria) that inhabits marine sediments at
1621 hydrothermal vents³⁵. A bacterial source is thus likely.

1622 Crown-sterols were also surprisingly abundant at Lost City. Detected were cholesterol at
1623 concentrations of up to 580 ng per gram of dry carbonate, attributed to animals, C₂₈ and C₂₉ sterols
1624 likely derived from phytoplankton, and ergosterol ascribed to fungi that had been detected at the
1625 site. The abundance of crown-sterols in carbonates from a deep-water hydrothermal vent is
1626 notable.

1627 **9.2. Cycloartenol in benthic mats of the Black Sea**

1628 Protosterols were also detected in a study on a 1 to 5 cm thick microbial mat covering several m²
1629 of sea floor at 180 m depth in the Black Sea²¹⁹. The mat covered the interior of a 10 m wide caldera-
1630 like structure (pockmark), presumably the remnant of an old gas outburst. The rim of the pockmark
1631 consisted of carbonate crust generated by ANME (anaerobic methanotroph) activity. The
1632 microbial mat was floating on the seafloor, trapping methane rich fluids emanating from below.
1633 At a depth of 180 m, the mat was located just below the anoxic/oxic interface in the water column.
1634 ANME consortia were active in the mat, while aerobic methane oxidizing bacteria were detected
1635 at the top of the mat, possibly becoming active upon sporadic oxygen exposure. Alternatively, the
1636 aerobic methanotrophs are of planktonic origin from the overlying water column.

1637 Solvent extracts of the mat yielded cycloartanol, minor cycloartenol and 4,4-dimethyl-
1638 cholestenol²¹⁹. The authors refer to these sterols as “major” with up to 3.6 µg/g dry weight for
1639 cycloartanol, but no information is provided about the presence and abundance of other sterols,
1640 such as phytosterols, so it is difficult to place these concentrations into context. The carbon isotopic
1641 composition of cycloartanol ranged from -74.4‰ to -69.8‰, and 4,4-dimethylcholestenol
1642 from -80.9‰ to -64.3‰. However, in individual extracts, δ¹³C of the two sterols diverged
1643 considerably, with offsets ranging from +8.6‰ to -7.9‰. The carbon isotopic compositions
1644 suggest that the source organisms directly or indirectly consumed methane-derived carbon, and
1645 the isotopic offsets imply that the two sterols have different biogenic sources²¹⁹. However, the bulk
1646 exopolymeric substance (EPS) of the mat was also carbon isotopically strongly depleted (-73‰),
1647 suggesting that the bulk of the mat community is fuelled by methane.

1648 The authors of the study suggest that the 4,4-dimethylcholestenol is either derived from
1649 methylococcalean bacteria consuming methane at the top of the mat or from “a microbe thriving
1650 in the water column above”²¹⁹. We agree that Methylococcales are a plausible source but consider
1651 the suggested mat origin more likely than planktonic sources based on the high concentration of
1652 the sterol of up to 15 µg/g dry weight and based on the observations that this sterol is not commonly
1653 observed at such concentrations elsewhere in Black Sea sediments. For cycloartenol and
1654 cycloartanol, the authors suggested eukaryotic ciliates as a source, feeding on isotopically depleted
1655 biomass in the mat or in the water column above. They noted that ciliates are generally an
1656 important component of the Black Sea food chain and point to a study²²⁰ that reported cycloartenol
1657 in ciliates. However, based on the cited work, cycloartenol is only a minor sterol in ciliates and
1658 presumably of dietary origin^{220,221}. In fact, based on current knowledge, ciliates do not appear to
1659 produce or contain protosterols as major membrane lipids^{157,159}. Yet, we agree in principle that
1660 cycloartanol in Black Sea mats may derive from eukaryotes thriving under oxygen limiting
1661 conditions. This possibility is supported by PCR data revealing eukaryotic ribosomal genes in the

1662 mats²¹⁹, although no further information about the nature of these organisms is provided. However,
1663 it is now known that the methane consuming alphaproteobacterium *Methyloceanibacter* produces
1664 cycloartenol (although not cycloartanol)³⁵, and we suggest that these or related methanotrophs are
1665 the most plausible source of the protosterols in the Black Sea mat.

1666 ***9.3. 4-Methylated cholesteroloids in a methane-emitting mud volcano***

1667 A study of steroids and hopanoids in microbial mats associated with a methane-emitting mud
1668 volcano at the Norwegian margin of the Barents Sea yielded abundant 4-methylated cholesteroloids
1669 and traces of 4,4-dimethyl cholesteroloids that were carbon isotopically depleted by -68 to -77‰,
1670 pointing to organisms directly or indirectly consuming methane carbon²²². A 16S rRNA survey
1671 confirmed the presence of aerobic methanotrophic bacteria of the order Methylococcales
1672 (Gammaproteobacteria) that are known to produce 4-methylated cholesteroloids. However, the
1673 protosterols lanosterol and cycloartenol were not detected at the site, consistent with the
1674 observation that all known sterol-producing Methylococcales remove carbon C-14 from lanosterol
1675 to produce downstream sterols. The crown-sterols cholesterol and brassicasterol were detected in
1676 concentrations similar to the 4-methylated sterols. Their carbon isotopic composition was around
1677 -30‰, indicating a eukaryotic planktonic origin.

1678 ***9.4. Cycloartenol in cyanobacterial mats***

1679 Protosteroids and protosteroid derivatives have also been observed in modern cyanobacterial mats
1680 alongside crown-group sterols, occasionally in substantial abundances. In extracts of different
1681 microbial mat layers at Solar Lake, North-East Sinai, Edmund and Eglington (1984)²²³ observed a
1682 varied and diverse distribution of sterols up to a depth of 70 cm. Among the sterols were
1683 cycloartanol, oxidation products such as cycloartanone, and cycloartanol derivatives demethylated
1684 at C-4 once or twice. Cycloartanol and cycloartanone were found in all layers ranging from 2% at
1685 the surface, 0.8% at a depth of ~1 cm, and rising sharply to a maximum of 19% at 27 cm
1686 (percentages relative to total sterols). Cycloartanoids that lost one or two methyl groups at C-4
1687 were even more abundant with 11% at the surface and a maximum of 21% at 27 cm. The maximum
1688 abundance of all cycloartanoid derivatives combined was 37% at a depth of 27 cm beneath the
1689 surface of the mat.

1690 The biological origin of the protosteroids in Solar Lake mats remains unknown. In particular, the
1691 observation of cycloartanoids demethylated at C-4 once or even twice, leading to 4,14-
1692 dimethylated and 14-methylated sterols, is unusual as these sterols have not been recorded in
1693 bacteria and are also not typical biosynthetic intermediates of crown-sterol biosynthesis. While
1694 4,14-dimethylated sterols occur as biosynthetic intermediates between cycloartenol and
1695 phytosterols in plants, these compounds are also always methylated at C-24, which is missing in

1696 the Solar Lake sterols. However, the excavate protist *Naegleria gruberi* contains ~6-8%
1697 cycloartenol and ~7% 4,14-dimethylcholesterol derivatives relative to total sterols (computed from
1698 data in ref¹⁸⁴). Generally, the sterol content of eukaryotic protists is poorly explored, making it
1699 likely that *Naegleria* is not the only genus with such unusual sterols. Hence, the cycloartenol and
1700 the 4-desmethyl cycloartanoids in Solar Lake mats may well have a eukaryotic origin. It is also
1701 currently impossible to exclude the hypothesis that these sterols and sterones became enriched by
1702 heterotrophic processes, e.g. through selective degradation of crown-group sterols, and that the 4-
1703 demethylated cycloartanoids are diagenetic products without direct biogenic precursors.

1704 In cyanobacteria-dominated microbial mats from Abu Dhabi, the relative proportion of
1705 cycloartenol among sterols is yet higher at ~60% of total sterols²²⁴, but still one order of magnitude
1706 lower than hopanols. A similar study of lipids preserved in flat-laminated cyanobacterial mats from
1707 the Guerrero Negro saltworks found that the surface layer of the mat mainly contained crown-
1708 group sterols (C₂₇, C₂₈ and C₂₉) with minor contributions of cycloartenol and several 4-methyl
1709 sterols²²⁵. However, in an apparently anoxic layer at a depth of 0.4 to 1.0 cm, the concentration of
1710 protosterol derivatives “increased dramatically” (quote ref²²⁵). However, absolute and relative
1711 abundances of the protosterols were not reported and it remains unclear whether they are as
1712 abundant as in the mats from Abu Dhabi and Solar Lake. Furthermore, no comments were made
1713 about possible origins of the cycloartenol in the Guerrero Negro saltworks. Yet, contrary to the
1714 older studies, a genomic survey assessed the activity of eukaryotes in the mat. The survey reported
1715 15 species of eukaryotes, more than half of which were nematodes, one insect, one crustacean, a
1716 stramenopile alga and, intriguingly, a eukaryote cluster with no known kingdom level affiliation.
1717 Thus, bacterial as well eukaryotic sources are possible for the protosterols, and an enrichment of
1718 these compounds by selective heterotrophic processes cannot be ruled out.

1719 ***9.5. Cyclolaudenol in SOB mats beneath the Peru upwelling zone***

1720 McCaffrey et al. (1989)²²⁶ analysed the lipid composition of microbial mats covering surface
1721 sediments beneath dysaerobic (< 0.1 mL/L) bottom waters in the coastal upwelling zone off the
1722 coast of Peru at a depth of 73 to 100 meters. The mats were predominantly (80%) composed of
1723 thick, filamentous sulfide oxidizing bacteria (SOB) of the genus *Thioploca*
1724 (Gammaproteobacteria). For lipid analysis, *Thioploca* filaments from two mats collected 17 km
1725 apart were cleaned with distilled water and inspected with a binocular microscope for
1726 contaminants. The enriched *Thioploca* filaments yielded ~4% lipids by dry weight, mostly
1727 comprising fatty acids and 0.008% cyclolaudenol per dry weight total biomass, but no
1728 hydrocarbons or hopanoids. The mat extracts also included traces of unspecified sterols, but
1729 cyclolaudenol constituted 95% of total sterols. By contrast, sediment directly beneath a *Thioploca*
1730 mat only contained traces of cyclolaudenol (1.3 µg/g dry weight) but much higher concentrations

1731 of other sterols of presumably planktonic animal and algal origins (29 µg/g cholesterol; 22 µg/g
1732 dinosterol). This distribution suggests that the cyclolaudenol was produced within the mat and is
1733 not derived from planktonic sources or from the sediment below. The distribution also
1734 demonstrates that cyclolaudenol is the final sterol product and not a biosynthetic intermediate.

1735 Cyclolaudenol is a rare C₃₁ sterol, a cycloartanoid methylated at the side chain in position C-24
1736 (24-methylcycloart-25-enol). Its detection in a microaerobic mat is interesting as it possesses the
1737 same carbon skeleton as 24-methylenecycloartenol, the second sterol intermediate in the
1738 biosynthesis of phytosterols in land plants¹⁷, and it is a potential diagenetic precursor for the 4,24-
1739 dimethylated triaromatic steroids in the ~1,300 old Roper Group. It is thus important to understand
1740 whether cyclolaudenol is biosynthesized by *Thioploca*, whether SOB generally have the capacity
1741 to produce cycloartanoids, and whether SOB may be the source for 24-methylated steroids in the
1742 mid-Proterozoic.

1743 Due to the low sterol content of only 0.008% per dry weight of mat, it is not possible to assign
1744 cyclolaudenol to *Thioploca* with certainty. KOH treatment of the lipid extract and alkaline and
1745 acid hydrolyses of the solid residue did not release any further sterols²²⁶, confirming the low
1746 concentration. Cyclolaudenol in the *Thioploca* mats is thus ~30 to 250 times less abundant than
1747 confirmed sterols in bacteria (Section 8.3). SOB mats may host numerous other species including
1748 prokaryotes and microeukaryotes, and smaller bacteria growing on filament surfaces can
1749 theoretically make up 60% of the total lipid mass²²⁷. For example, 40 to 46% of the sterols detected
1750 in an SOB mat of the sister genus *Beggiatoa* were contributed by eukaryotes that consume biomass
1751 within the mat²²⁷. It is thus plausible that cyclolaudenol is not produced by *Thioploca* but derived
1752 from other prokaryotes or microeukaryotes inhabiting SOB filaments. Some bacteria possess the
1753 SMT gene required to methylate the side chain³³. Such organisms would have remained invisible
1754 during the binocular microscopic inspection by McCaffrey et al. (1989)²²⁶.

1755 While cyclolaudenol cannot be assigned to a specific organism at present, *Thioploca* cannot be
1756 ruled out as the source. Although the sterol content in dried *Thioploca* mats is very low, the mass
1757 of the *Thioploca* sheaths may suppresses the content considerably. We thus have to investigate
1758 whether cyclolaudenol has been observed in other SOB mats and examine genomic evidence for
1759 or against sterol biosynthesis in the group.

1760 There are several studies that provide lipid analyses of SOB mats, or of sediments associated with
1761 SOB mats, that explicitly report sterol contents, however cyclolaudenol or protosterols were not
1762 detected in any of these analyses. One study²²⁸ analysed surface sediments in the upwelling region
1763 off Namibia, Peru and Chile with dense populations of SOB *Thiomargarita*, *Beggiatoa*, and
1764 *Thioploca*. *Thioploca* was dominant in the Chilean sediments. Lipid analysis yielded typical,
1765 previously observed²²⁶ SOB fatty acid patterns. The study also included a detailed survey of

1766 polyterpenoids where diverse algal sterols and hopanoids were observed. Yet, cyclolaudenol was
1767 not detected in any of the SOB communities. A second study²²² provided a detailed study of sterols
1768 in a *Beggiatoa* mat at an active marine mud volcano at the Norwegian margin of the Barents Sea.
1769 The survey detected unusual 4-methylated cholesteroloids that were assigned to methylococcalean
1770 bacteria based on their carbon isotopic composition. However, cyclolaudenol or protosterols were
1771 not detected. A third study²²⁷ purified sterols extracted from *Beggiatoa* mats from a hydrothermal
1772 vent in the Guaymas Basin, reporting C₂₇ to C₂₉ sterols attributed to eukaryotes, but no
1773 cyclolaudenol or protosterols. Likewise, a fourth study²²⁹ analysed lipids from the *Beggiatoa alba*
1774 layer of an intertidal microbial mat in Qatar, detecting abundant algal sterols but no unusual sterols.
1775 A fifth lipid survey²³⁰ reported lipids from a well-preserved fossil *Beggiatoa*-like microbial mat
1776 consisting of filamentous fossils in Miocene methane-seep limestone from the Romagna Apennine
1777 (Pietralunga, Italy). They study reported an unusual pseudohomologous series of C-4 methylated
1778 C₂₉, C₃₀, and C₃₁ steranes. The C₃₀ compound was identified as lanostane. The steranes were
1779 strongly depleted in ¹³C, suggesting a methanotrophic bacterial source. The C₃₁ compound is
1780 described as a derivative of lanostane “methylated in ring A or ring B” (quote ref²³⁰), and can thus
1781 not be a cyclolaudanoid or other side-chain methylated protosteroid. Based on a chromatogram
1782 given in Figure 4 of ref²³⁰, the concentration of lanostane was substantially higher than of other
1783 steranes (which are not visible in the chromatogram) and also higher than C₃₀ hopene, the only
1784 hopanoid in the chromatogram.

1785 In a comprehensive lipid analysis of three species of *Bathymodiolus* mussels from the Pacific
1786 Antarctic Ridge hosting methane-oxidizing and/or SOB, 4-methyl cholesteroloids, 4,4-dimethyl
1787 cholesteroloids and lanosteroids were found in bivalves with methanotrophic symbionts, an origin
1788 that was confirmed by the carbon isotopic composition of the sterols²³¹. However, bivalves
1789 harbouring SOB symbionts, but lacking methanotrophic bacteria, explicitly lacked unusual sterols.
1790 Similarly, analysis of sterols of the bivalve *Solemya velum* harbouring SOB symbionts yielded
1791 abundant sterols but no cyclolaudenol or protosterols. Likewise, analysis of the lipids of the SOB
1792 *Thiomicrospira crunogena* failed to detect any sterols²³². Based on a statement in the
1793 acknowledgements, the authors of that study received advice on lipid analysis from the first author
1794 of the original study that had detected cyclolaudenol in *Thioploca* mats²²⁶. It is thus likely that
1795 cyclolaudenol was absent rather than overlooked. There are also numerous studies that investigated
1796 lipids in SOB mats but do not mention whether sterols were monitored or whether cyclolaudenol
1797 or protosterols were present²³³⁻²⁴². Yet, many of these studies cite the work that had reported
1798 cyclolaudenol in *Thioploca* mats²²⁶, so we surmise that at least some of these studies would have
1799 detected cyclolaudenol if it was present. Based on lipid analyses, it is thus clear that sterol
1800 biosynthesis is not widespread among SOB, updating the statement of Peckmann et al. (2004)²³⁰

1801 that “sterol biosynthesis has so far been corroborated neither for *Thioploca* nor for any other
1802 sulfide-oxidizing bacteria.”

1803 Biosynthesis of sterols by SOB is also not supported by genomic analyses. There are now hundreds
1804 of genomes available in the Joint Genome Institute Integrated Microbial Genomes database of the
1805 order Thiotrichales, and dozens for Thiotrichaceae, the family comprising SOB, including finished
1806 genomes of the genera *Beggiatoa*, *Thiomicrohabdus*, *Thiomicrospira*, and *Thioploca*, and draft
1807 genomes of *Achromatium*, *Thiomargarita* and *Thiothrix*. For the genus *Thioploca*, genomes are
1808 available for *T. ingraca* and *T. araucae*²⁴³. A surveys of homologs of OSC through BLASTP in all
1809 bacterial genomes (31,237) available at that time in the JGI database yielded 34 bacterial OSC
1810 homologs in five different phyla, but no Thiotrichales³⁵. Similarly, a search for SQMO and OSC
1811 protein sequences in the National Center for Biotechnology Information (NCBI) protein database
1812 found 27 bacterial taxa, across 6 phyla, 9 classes and 9 orders, but again did not observe any hits
1813 among Thiotrichales³⁴. Likewise, the most recent and vastly expanded survey of sterol
1814 biosynthesis genes did not yield any hits for Thiotrichales³³

1815 Based on the combined lipid and genomic data, we conclude that cyclolaudenol biosynthesis in
1816 SOB, if it occurs at all, would be restricted to a specific *Thioploca* strain found in the upwelling
1817 region of Peru²²⁶. Based on molecular clock estimates, this strain would have gained the required
1818 genes via horizontal gene transfer relatively recently. SOB (Thiotrichaceae) radiate near the base
1819 of the Gammaproteobacteria-Betaproteobacteria division¹⁹⁶. Using molecular clocks calibrated on
1820 SOB symbionts in bivalves, Canfield and Teske (1996)¹⁹⁸ estimated that this radiation of
1821 Thiotrichaceae occurred in the Neoproterozoic 760 ± 320 to 620 ± 280 Ma ago (and with an
1822 unlikely absolute ago maximum of 1,400 Ma). Even if these divergence estimates are wrong and
1823 true ages are substantially greater, any particular *Thioploca* strain must have acquired sterol
1824 biosynthesis relatively recently. It is thus unlikely that SOB contributed to the mid-Proterozoic
1825 steroid pool, and exceedingly unlikely that *Thioploca* is the source for 4,24-dimethylated steroids
1826 in the ~1,300 Ma Roper Group.

1827 **9.6. Lack of bacterial sterols in dysoxic water columns**

1828 In contrast to microaerobic benthic environments, we were not able to find reports on notable
1829 abundances of protosterols, or bacterial 4-methylated cholesteroids, within water bodies. Even in
1830 euxinic water bodies that may serve as closest analogues for Proterozoic basins, protosterols
1831 remain inconspicuous. For example, one study²⁴⁴ provided a detailed survey of sterols in the
1832 particulate organic matter of the water column and in bottom sediments of the Black Sea, with
1833 particular attention to the chemocline. While crown-sterols were highly abundant throughout the
1834 water column and in the sediment, protosterols and 4,4-dimethylcholesteroids were not detected,

1835 and minor abundances of 4-methyl cholesteroloids were attributed to dinoflagellates. This is
1836 corroborated by a second survey²⁴⁵ of lipids of aerobic methanotrophic bacteria in the chemocline
1837 of the Black Sea that failed to detect any unusual sterols.

1838 **10. The geological record of protosteroids**

1839 **10.1. Average protosteroid abundances through time (this study)**

1840 There are only few published studies reporting fossil protosteroids. As with modern protosterols,
1841 the likely reason is that these biomarkers are rarely conspicuous in total ion chromatograms and
1842 only detectable using targeted MS experiments. However, with appropriate elution standards,
1843 identification of these biomarkers is straightforward. In the present study, all thermally well
1844 preserved Palaeoproterozoic to Phanerozoic bitumens and oils yielded, without exception, fossil
1845 protosteroids. However, the proportion of protosteroids to crown-group steroids changes notably
1846 through geological time. Supplementary Table 1 shows that the average proportion of aromatic
1847 protosteroids relative to total aromatic steroids for the Palaeo- and Mesoproterozoic is $100 \pm 0\%$
1848 ($n = 42$; average \pm stdev), for the Tonian and Cryogenian $33 \pm 24\%$ ($n = 34$), for the Ediacaran 5.8
1849 $\pm 6.3\%$ ($n = 9$), and for the Phanerozoic $3.8 \pm 4.0\%$ ($n = 36$). We were not able to compute reliable
1850 corresponding values for saturated protosteranes because the major lanostane isomer coelutes with
1851 the tetracyclic triterpane ‘TPP’, and we also lack data for most samples of the Geoscience Australia
1852 Phanerozoic oil collection (Supplementary Table 3). However, the approximate percentages of
1853 cyclosterane over steranes for the Palaeo- to Mesoproterozoic is $100 \pm 0\%$ ($n = 34$; average \pm
1854 stdev), for the Tonian and Cryogenian $14 \pm 19\%$ ($n = 22$), for the Ediacaran $0.5 \pm 0.5\%$ ($n = 8$),
1855 and for the Phanerozoic $0.9 \pm 1.4\%$ ($n = 15$).

1856 **10.2. Fossil protosteroids in the literature**

1857 **10.2.1. Protosteroids in sub-Recent sediments of Lake Caçó**

1858 Lake Caçó is a small oligotrophic lake in north-eastern Brazil. Jacob et al. (2005, 2007)^{246,247}
1859 analysed the aromatic and polar triterpenoids extracted from (sub)recent sediments in a 6 m long
1860 Lake Caçó core. The sediments had been deposited during the last 20,000 years under different
1861 environmental conditions during wet and dry seasons²⁴⁶ and, according to previous chemo-
1862 taxonomic studies, grasses (Gramineae) colonized the savannas of Northern Brazil at the time of
1863 deposition²⁴⁷. In particular the upper 3 m of core are thought to be influenced by influx of material
1864 from the development of a belt of the sedge-plant spike-rush²⁴⁶.

1865 The lake sediment extracts yielded dominantly plant-derived aromatic biomarkers with only few
1866 saturate triterpanes such as onocerane and some hopanoids. Strikingly, aromatic triterpenoid

1867 fingerprints from two samples (1.6 m and 4.5 m depth) resembled those described for a lignite and
1868 sub-bituminous coal respectively^{248,249}, comprising a series of pentacyclic monoaromatic
1869 compounds with various structures and unsaturations, triaromatic pentacyclic compounds, and a
1870 series of supposedly diaromatic tetracyclic triterpene derivatives dominated by a m/z 195 peak—
1871 mass spectral characteristics also observed in unidentified triterpenoids reported in the lignite and
1872 coal^{248,249}. Based on mass spectral interpretations, Jacob et al. (2007)²⁴⁶ provided the first tentative
1873 identification of these biomarkers as diaromatic tetracyclic triterpenes with a lanostane/euphane
1874 structure. These compounds show the same mass spectra (M^+ shifted downwards by 2 Da for those
1875 isomers still containing a double bond in the side chain), as the diaromatic lanosteroids produced
1876 in our protosterol pyrolysis experiments, supporting the structural assignment of the authors. In
1877 total ion chromatogram (TIC), the lanosteroids are amongst the most abundant aromatic
1878 triterpenoids (Fig. 3 in ref²⁴⁶), attesting to exceptionally high protosteroid vs crown-steroid and
1879 hopanoid ratios in some of the plant/terrestrial-influenced lacustrine sediments.

1880 The aromatization in Lake Caçó is extremely early and may have occurred in the watershed prior
1881 to entering the lake waters and sediments. The lake sediments contain a variety of typical plant-
1882 derived triterpenoids such as oleanane-derivatives. Accordingly, Jacob et al. (2007)²⁴⁶ interpret the
1883 aromatic triterpenoids to largely derive from higher plants, and the authors also consider these as
1884 the most likely sources of lanosteroids. As grasses (Gramineae) are thought to be amongst the
1885 dominant sources of triterpenoids in Lake Caçó^{246,247}, grasses are also likely sources of the
1886 lanosteroids. This inference is consistent with the tentative identification of a lanosteroid methyl
1887 ether in Lake Caçó^{246,247} as protosterol methyl ethers do occur in several Gramineae species²⁵⁰.
1888 However, organic matter from trees, mosses or other plants, as well as washed-in plant waxes are
1889 also plausible sources for the elevated lanosteroid content (see Section 8.1.2).

1890 10.2.2. *Lanostanes from an Eocene saline lake*

1891 Chen et al. (1989)¹³⁶ were the first to discover lanostanes in the sedimentary record. They detected
1892 saturated lanostane, 24-methyl lanostane and 24-ethyl lanostane in Eocene sediments from a saline
1893 lake of the Biyang Basin, China. The lanostanes only occurred in higher salinity samples and were
1894 not detected in sediments deposited under lower salinities. The abundances of C₃₀ lanostane and
1895 C₃₁ 24-methylated lanostane were similar, while the ethylated homolog was about three times
1896 lower. Yet higher homologs were not detected. Information about the abundance of the lanostanes
1897 relative to other steranes was unfortunately not provided. However, based on an MRM 414 → 259
1898 chromatogram in Figure 7 of ref²⁵¹, lanostane is less abundant than 3 β -propylcholestane, a
1899 diagenetic propylation product that commonly only occurs in traces in bitumens and oils. It is thus
1900 probable that the abundance of lanostanes relative to other steranes was very low (sub percentage
1901 levels). These low-concentrated lanostanes may have a plant origin. The bitumens extracted from

1902 the Eocene lake sediments yielded odd-over-even predominant *n*-alkane patterns, the plant
1903 terpanoid oleanane and a dominance of stigmastane among other steranes, all pointing to influx of
1904 plant matter into the lake²⁵¹. Moreover, side-chain methylation and ethylation of protosterols is
1905 currently unknown among bacteria but common in plants, several of which producing side-chain
1906 methylated and ethylated protosteroids in high relative abundances (Section 8.1.2).

1907 C₃₀ to C₃₂ lanostanes were also observed in Eocene lime-mudstones deposited under saline
1908 stratified waters of the Bohai Bay basin, China²⁵². The bitumens contained highly abundant
1909 oleanane, so a higher plant source for the side-chain methylated and ethylated lanostanes is
1910 plausible here as well.

1911 10.2.3. *Other protosteroid occurrences*

1912 Solvent extracts of Miocene Pietralunga methane seep limestone yielded lanostane with a carbon
1913 isotopic composition $\delta^{13}\text{C} = -70\text{‰}$ and 4-desmethyl lanostane with $\delta^{13}\text{C} = -80\text{‰}$ ²⁵³. The authors
1914 attributed the steranes to methanotrophic bacteria (see also Section 9.5).

1915 A highly sulphidic Paleocene crude oil from Jiangnan Basin, China, yielded cyclic sulfide
1916 derivatives of C₃₀ lanostane and C₃₁ 24-methyl lanostane²⁵⁴. While hopanes and steranes were
1917 abundant in all fractions of the oil, the lanostanes were exclusively detected in the sulfide
1918 fraction²⁵⁴, pointing to a specific unsaturation pattern that promoted sulfurization, or to an unusual
1919 biogenic origin that exposed these sterols preferentially to a sulfur rich environment.
1920 Unfortunately, the study²⁵⁴ did not provide information about the abundance of the lanostanes
1921 relative to other steranes, so it is currently not possible to assess whether these compounds are
1922 low-concentrated biosynthetic intermediates or have a more unusual biogenic origin.

1923 Another study²⁵⁵ detected two lanostane isomers and four isomers of 4-desmethyl lanostane in
1924 limestones of the Lower Cambrian Sinyaya Formation on the eastern Siberian Platform. Based on
1925 peak heights taken from chromatograms in Figures 3 and 6 of ref²⁵⁵, we estimated that lanostane
1926 constituted ~9%, and 4-desmethyl lanostanes ~31%, of total steranes in the richest samples (where
1927 ‘total steranes’ refers to the sum of C₂₇ to C₂₉ regular and diasteranes, lanostanes and 4-desmethyl
1928 lanostanes). The origin of the lanostanes remains unknown.

1929 C₃₀ to C₃₂ lanostanes were also detected in migrated petroleum presumably sourced from the
1930 carbonate facies of the latest Ediacaran Bilara Fm, Marwar Supergroup from the shallow marine
1931 Bikaner-Nagaur Basin, north-west India²⁵⁶. Mass spectra were not provided for the two higher
1932 homologs, so their structures remain unclear. The authors speculate that the C₃₁ and C₃₂
1933 compounds formed in the sediment through diagenetic methylation. However, given the apparent
1934 high relative signal intensities of the C₃₁ and C₃₂ homologs provided in GCxGC-TOFMS spectra,

1935 this interpretation appears unlikely. No information was provided about the abundances of the
1936 lanostanes relative to other biomarkers.

1937 Triterpenoids with diaromatic lanosteroid (DAL) fragmentation patterns have been reported from
1938 a Tertiary angiospermous lignite²⁴⁹ and a sub-bituminous coal from the East Elbian coal basin²⁴⁸,
1939 and these occurrences can be plausibly attributed to plants. A plant origin is also likely for 24-
1940 methyl-28-norlanosteroids and 24-methyl-19,28-bisnorlanosteroids extracted from a 2 million
1941 year old fossil plant of the family Lauraceae²⁵⁷.

1942 **10.3. The origins of low and high protosteroid abundances in the Phanerozoic**

1943 Most Phanerozoic bitumens and oils contain low single-digit percentages of protosteroids relative
1944 to total steroids (Section 10.1). These background levels are presumable largely derived from
1945 biosynthetic intermediates found in crown-group eukaryotes plus an unknown contribution from
1946 bacteria. However, as mention in Section 8, some geological samples yield elevated abundances,
1947 such as Cretaceous lacustrine oil (GA224) with 7.1% aromatic protosteroids, a Jurassic coal-
1948 resinitic oil (GA19999642) containing 6.8%, Ordovician Tarim oil (HD_1 #5458) with 20.5%,
1949 bitumen from a Cambrian marine limestone from Australia (15B602) at 13.4% and from Siberia
1950 with 9% lanostane and ~31% 4-desmethyl lanostanes (Section 10.2.3). In samples with terrestrial
1951 influence (lacustrine oils, coals), higher plant origins are likely (Section 8.1.2), but for pre-
1952 Devonian organic matter, the origin of the elevated abundances is unclear. (The predominantly
1953 Ordovician Tarim oil also contains plant biomarkers, pointing to a mixed age source²⁵⁸, so the
1954 protosteroids in HD_1 #5458 could be plant derived).

1955 Biodegradation is a likely cause for some elevated protosteroid occurrences. Chen et al. (1989)¹³⁶
1956 observed lanostanes in biodegraded tar sands but failed to detect these compounds in the non-
1957 degraded Eocene source rocks. The authors suggested that lanostanes may be particularly
1958 biodegradation resistant, causing enrichment relative to regular steranes and hopanes in the
1959 degraded tars. This interpretation is supported by our observations on biodegraded migrated
1960 bitumens from the 1,640 Ma Barney Creek Formation. GC-MS analysis of solid bitumen recovered
1961 from a calcite vug within dolomite of drill core LV09 revealed severely biodegraded hydrocarbons
1962 consisting of a smooth unresolved complex mixture lacking *n*-alkanes, methyl alkanes and
1963 isoprenoids, and only containing traces of hopanes close to detection limits. Yet, the chromatogram
1964 also showed outstanding signals of cyclosteranes I (Extended Data Fig. 2), attesting to their
1965 extreme resistance to biodegradation. The resistance of cyclosterane to biodegradation is
1966 presumably caused by the inferred bicyclo/bridge structure and by the relatively large proportion
1967 of quaternary carbon atoms. For comparison, while cholestane possesses two quaternary carbon
1968 centres, lanostane has four and cyclosterane presumably five. Likewise, mono- and diaromatic

1969 lanosteroids possess a quaternary carbon atom at C-4 that is absent in aromatic crown-steroids,
1970 possibly increasing their resistance to biodegradation. Although the effects of biodegradation on
1971 protosteroids requires systematic evaluation, it is plausible that this effect may cause protosteroid
1972 enrichment.

1973 Although entirely speculative at present, the high number of quaternary carbon centres in
1974 functionalized protosterols may also increase their resistance to heterotrophic reworking in modern
1975 environments. Increased resistance of quaternary carbon centres to heterotrophic reworking may
1976 lead to a general enrichment of protosterols in sediments and soils relative to other sterols. Such
1977 an effect may generally contribute to an elevated average abundance of protosteroids in
1978 Phanerozoic bitumens and oils when compared to protosterol fractions in living eukaryotes
1979 (Section 8.1.1).

1980

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