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

2  
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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<sup>1,2</sup>. 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 years<sup>3</sup>. 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 Bloch<sup>4</sup>. 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<sup>3,5-7</sup>. 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 ancestors<sup>7</sup>, 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 record<sup>8</sup>.

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<sup>1,8,9</sup>. The oldest fossils that can be assigned to the eukaryotic crown  
53 with confidence are the 1,050 Ma multicellular rhodophyte (red) alga *Bangiomorpha*<sup>10</sup> and 1,000  
54 Ma chlorophyte (green) alga *Proterocladus*<sup>11</sup> (Fig. 1c, specimens c,d). However, body fossils of  
55 the crown only diversified and became abundant around 900 Ma with the emergence of fungi<sup>12</sup>  
56 followed by possible rhizarians and testate amoebozoans<sup>13</sup> (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 biosynthesis<sup>14</sup>. 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<sup>15,16</sup>. 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 skeleton<sup>17</sup>. The trajectory of the  
71 relative abundances of the three saturated crown-steranes in the geological record reflects trends  
72 of eukaryote evolution<sup>18</sup>. For example, increasing abundances of ergostane from the Mesozoic to  
73 Caenozoic reflect the rise of secondary endosymbiotic algae in the oceans<sup>18</sup>, and the predominance  
74 of stigmastane in the Ediacaran and Paleozoic suggests that chlorophyte algae were the prevalent  
75 eukaryotic phototrophs<sup>18,19</sup>. 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 bias<sup>20</sup>, prevalence of anaerobic eukaryotes that do not  
84 produce sterols<sup>21-23</sup>, or ecosystems pervasively dominated by prokaryotes<sup>2,22,24-27</sup>. 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)<sup>26</sup>.  
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’<sup>4</sup>. 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<sup>28,29</sup>, 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 observed<sup>30</sup> 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 $\alpha$ ,14 $\beta$  stereochemistry.  
125 However, the absence of 13 $\alpha$ ,14 $\beta$ -euphanes and presence of 13 $\beta$ ,14 $\alpha$ -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 artefact<sup>20</sup> 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<sup>15,16,19</sup>, 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% ergosteroids<sup>15</sup>.



158 The data thus substantiate previous suggestions<sup>16,31,32</sup> 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<sup>15,19</sup>. 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 biosynthesis<sup>33</sup>. 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 sterols<sup>34</sup>.

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<sup>34,35</sup>, 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 Myxococcales<sup>33</sup>, 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, respectively<sup>35</sup>. 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 habitats<sup>35</sup>, 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 Methylococcales<sup>30</sup>. However, the carbon isotopic  
189 signature of triaromatic steroids from the 1,640 Ma Barney Creek Fm ( $\delta^{13}\text{C} = -19$  to  $-22\text{‰}$ )<sup>36</sup> is  
190 inconsistent with a methanotrophic origin and suggests heterotrophic<sup>36</sup> 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 system<sup>37</sup> with active curvature control,

203 a capability aided by sterols in extant Eukarya<sup>38</sup>. Moreover, large but simple fossils interpreted as  
204 possibly eukaryotic, such as leiosphaerids, are widespread in mid-Proterozoic sediments<sup>37</sup> 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 steroids 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<sup>33,39</sup> 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 extracts<sup>35</sup>. By contrast, SMT was already present in  
212 LECA<sup>17,33,40</sup>. 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<sup>3,5,6</sup> and rapidly diverged into major branches<sup>7</sup>. 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 algae<sup>10</sup>, and

225 protists capable of endocytosis, phagocytosis and locomotion<sup>41</sup>. 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 ref<sup>23</sup>). 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 rehydration<sup>42</sup>. 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<sup>29,43</sup>. 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<sup>29,43</sup>,  
242 may play a role in membrane repair during UV-B exposure<sup>43</sup> and promotes asymmetric cell  
243 growth<sup>29</sup>. Potentially important but more speculative is the role of down-stream modified sterols  
244 in the protection of cell membranes against attack by membranolytic toxins<sup>44-46</sup> and for the  
245 response of cells to changing O<sub>2</sub> levels<sup>43,47</sup>. 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<sup>10,11</sup> (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<sup>48,49</sup> and surface waters may have become anoxic during the dark hours or experienced  
261 incursions of sulphidic waters, toxic to crown-group Eukarya<sup>24</sup>. 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<sup>50,51</sup>.

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<sup>48,52</sup>. 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 aftermath<sup>53</sup>.

### 277 **Konrad Bloch's Ursterols**

278 In the essay 'Evolutionary Perfection of a Small Molecule'<sup>4</sup>, 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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425

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 Me<sub>2</sub>TAS' 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<sup>15,54</sup>. 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 protocols<sup>55-58</sup>. 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 surfaces<sup>55</sup>, 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<sup>55,56,58,59</sup>. 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<sup>56,59</sup> (Buehler Isomet™ 1000; Illinois, U.S.A) or, for fissile  
489 mudstones and shales, surface ablation using a modified KG-1 Mini-Sonic tumbler<sup>55</sup> (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-d<sub>10</sub>, Chiron) to the aromatic fraction. For metastable  
503 reaction monitoring (MRM) experiments, 25 to 50 ng of D4 (d<sub>4</sub>-C<sub>29</sub>-ααα-ethylcholestane; 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.  $^1\text{H}$  and  $^{13}\text{C}$ -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  $\mu\text{m}$  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  $\text{min}^{-1}$  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  $\text{FeCl}_2$  build-up in the MS ion source  
520 through use of halogenated solvents<sup>60</sup>. The GC oven was programmed from 60°C (held for 4 min)  
521 to 315°C at 4°C  $\text{min}^{-1}$ , 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  $\mu\text{m}$  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  $\sim 742$  Ma<sup>61</sup>. Image courtesy S. Porter, Fig. 15 (panel 3) in ref<sup>61</sup>. (b) *Bonniea dacruchares*, a testate  
577 amoebozoan with an age of  $\sim 742$  Ma<sup>61</sup>. Image courtesy S. Porter, Fig. 9 (panel 9) in ref<sup>61</sup>. (c)  
578 *Bangiomorpha pubescens*, a likely bangiacean rhodophyte alga<sup>62</sup> with an age of  $1,047 \pm 13/-17$   
579 Ma<sup>63</sup>. Image courtesy N. Butterfield, Fig. 9A in ref<sup>64</sup>. (d) *Proterocladus antiquus*, a likely  
580 multicellular, benthic, siphonocladalean chlorophyte alga with an age of  $1,056$  to  $947.8$  Ma<sup>11</sup>.  
581 Image courtesy Q. Tang, Fig. 2g in ref<sup>11</sup>. (e) *Ourasphaira giraldae*, a likely fungus with an age of  
582  $1,010$  to  $890$  Ma<sup>12</sup>. Image courtesy C. Loron, Fig. 1b in ref<sup>12</sup>. (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 ref<sup>65</sup>. (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 ref<sup>65</sup>. (h) *Satka favosa*, a microfossil with diagnostic eukaryotic features from the  $\sim 1,300$  Ma Roper  
587 Group<sup>9</sup>. Image courtesy E. Javaux, Fig. 5(9) in ref<sup>9</sup>. (i) *Valeria lophostriata*, a microfossil with  
588 diagnostic eukaryotic features from the  $\sim 1,300$  Ma Roper Group<sup>9</sup>. Image courtesy E. Javaux, Fig.  
589 7(2) in ref<sup>9</sup>. (j) *Tappania plana*, a microfossil with diagnostic eukaryotic features from the  $\sim 1,600$   
590 Ma Ruyang Formation<sup>1</sup>. Image courtesy Y. Leiming, Fig. 4A in ref<sup>1</sup>. (k) *Shuiyousphaeridium*  
591 *macroreticulatum*, a microfossil with diagnostic eukaryotic features from the  $1,600$  Ma Ruyang  
592 Formation<sup>1</sup>. Image courtesy Y. Leiming, Fig. 4B in ref<sup>1</sup>. [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.

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653 Shuizhang Zhang.

654

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 RuO<sub>4</sub> oxidation experiments and assisted with compound identification.  
660 J.J.B. conceived the project and compiled data and figures.

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662 **Competing interests statement**

663 The authors declare no competing interests.

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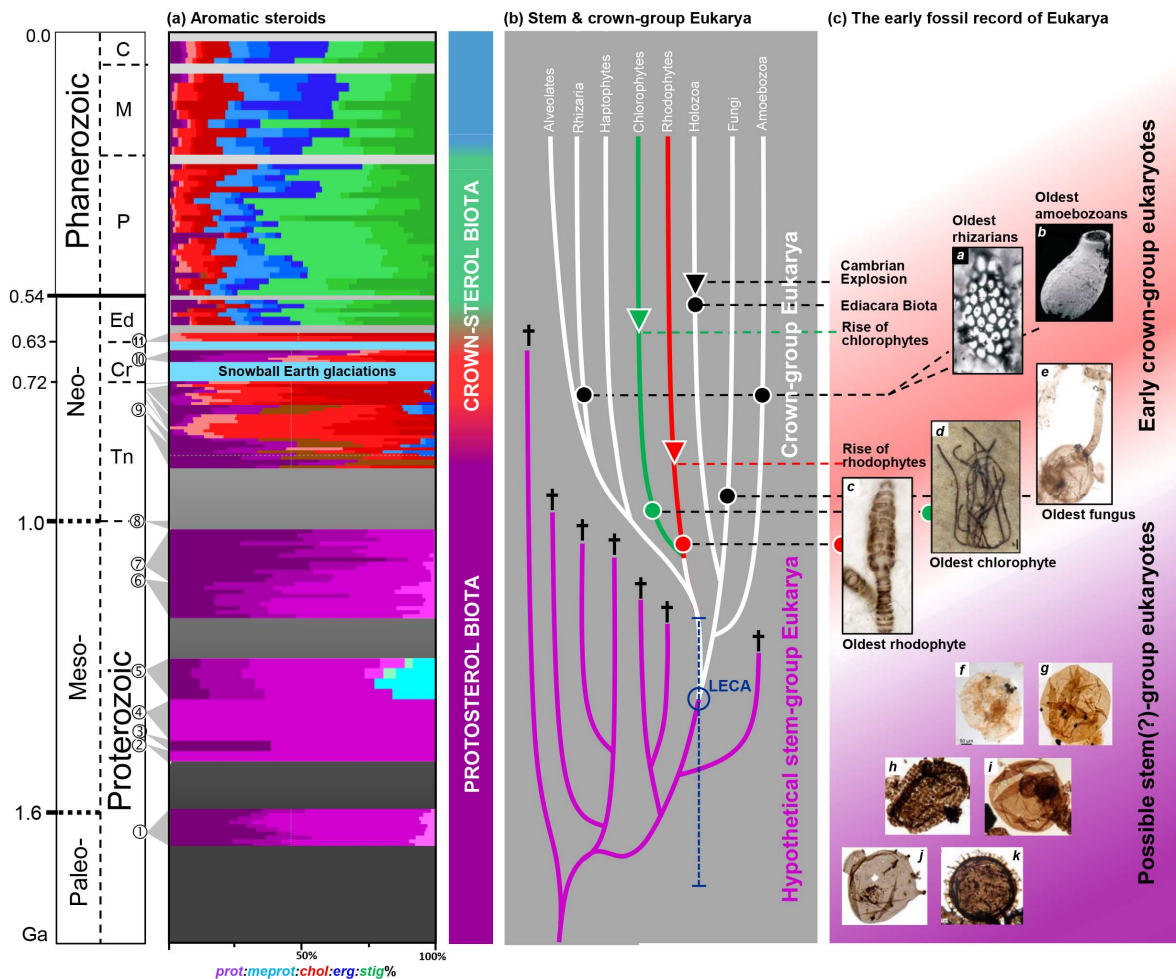
665 **Additional Information**

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.

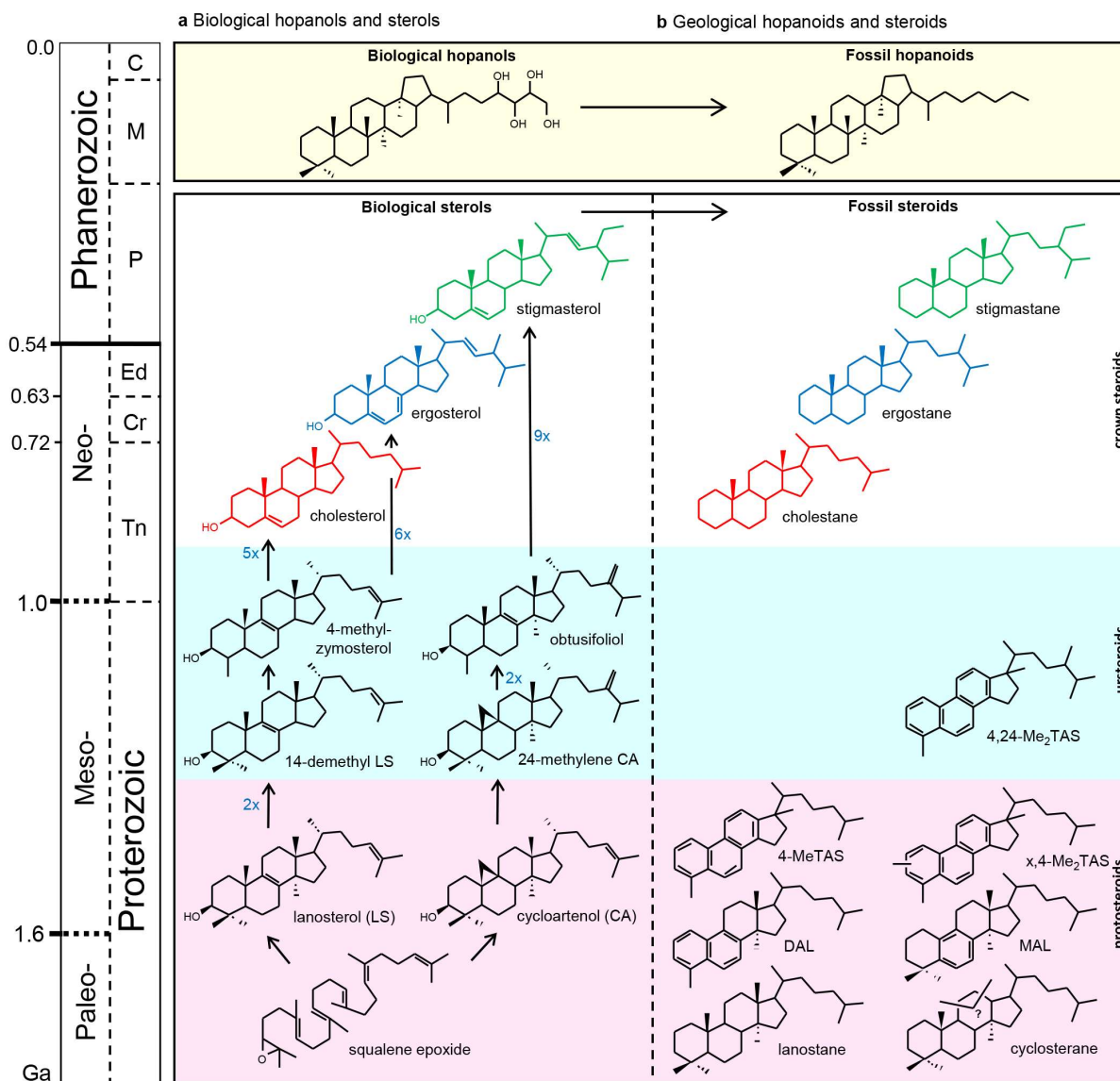
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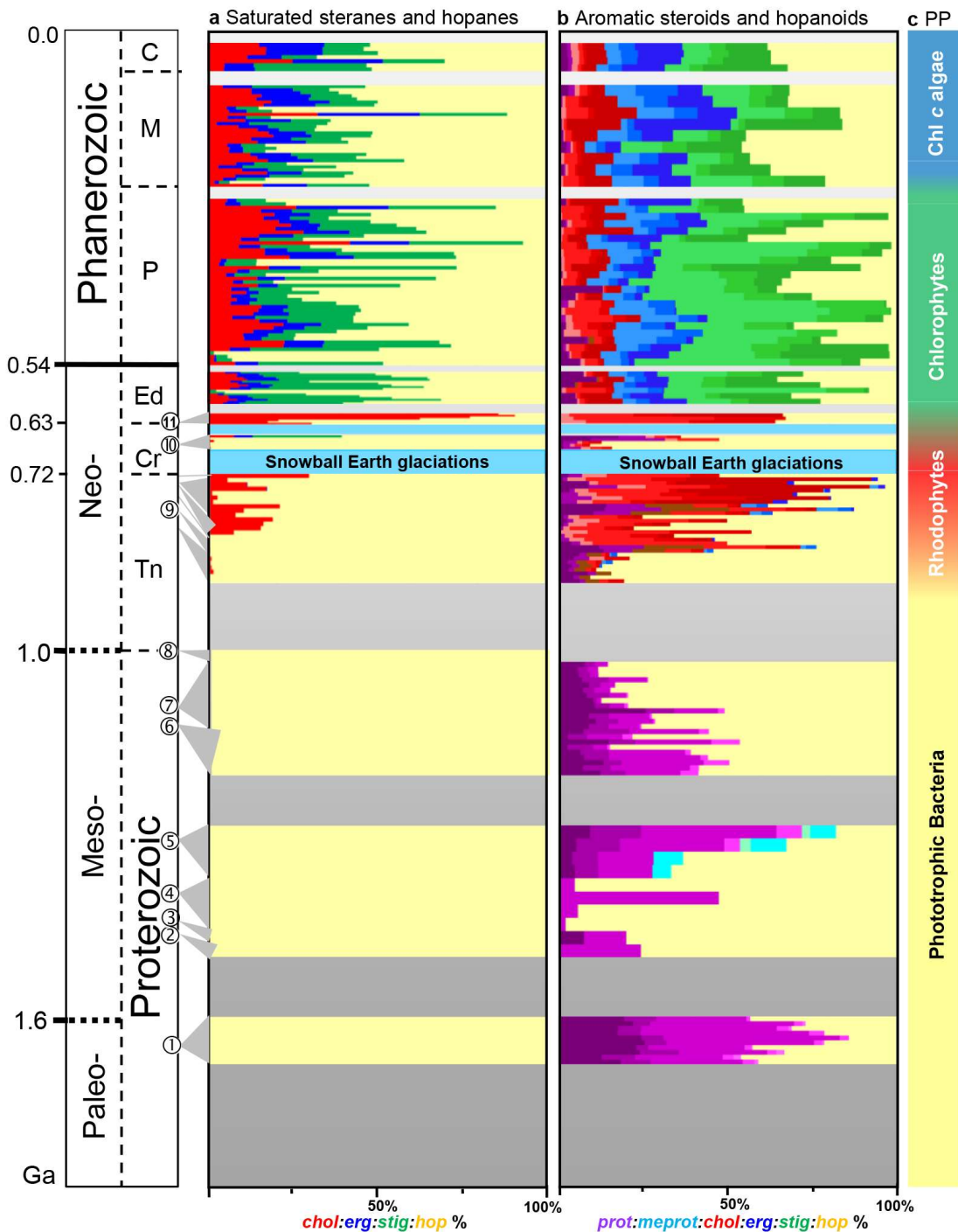


**Fig. 1 | Geological time chart (in billion years, Ga) comparing the molecular fossil, microfossil and phylogenetic records of early eukaryote evolution. a,** Relative abundances of aromatic protosteroids (purple and cyan tones) and crown-steroids (reds, blues and greens), highlighting the transition 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 = cholesterol, 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 Cenozoic 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-Me<sub>2</sub>TAS’ 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<sup>66,67</sup>.

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-Me<sub>3</sub>TAS' 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 $\beta$ (H),9 $\alpha$ (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 $\beta$ ,13 $\alpha$ ,14 $\beta$ ,17 $\alpha$ -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 C<sub>27</sub> 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 isomers<sup>68</sup>. Note that  
705 8 $\beta$ (H),9 $\alpha$ (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 C<sub>29</sub> 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 C<sub>29</sub> MAL standard. **c**,  
712 C<sub>28</sub> MAL of sample 14B211 from the 725 Ma Kanpa Fm showing an immature isomer distribution  
713 (S  $\ll$  R). **d**, C<sub>28</sub> MAL generated through pyrolysis of lanosterol (sample BEX20150624). **e**, C<sub>28</sub>

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.

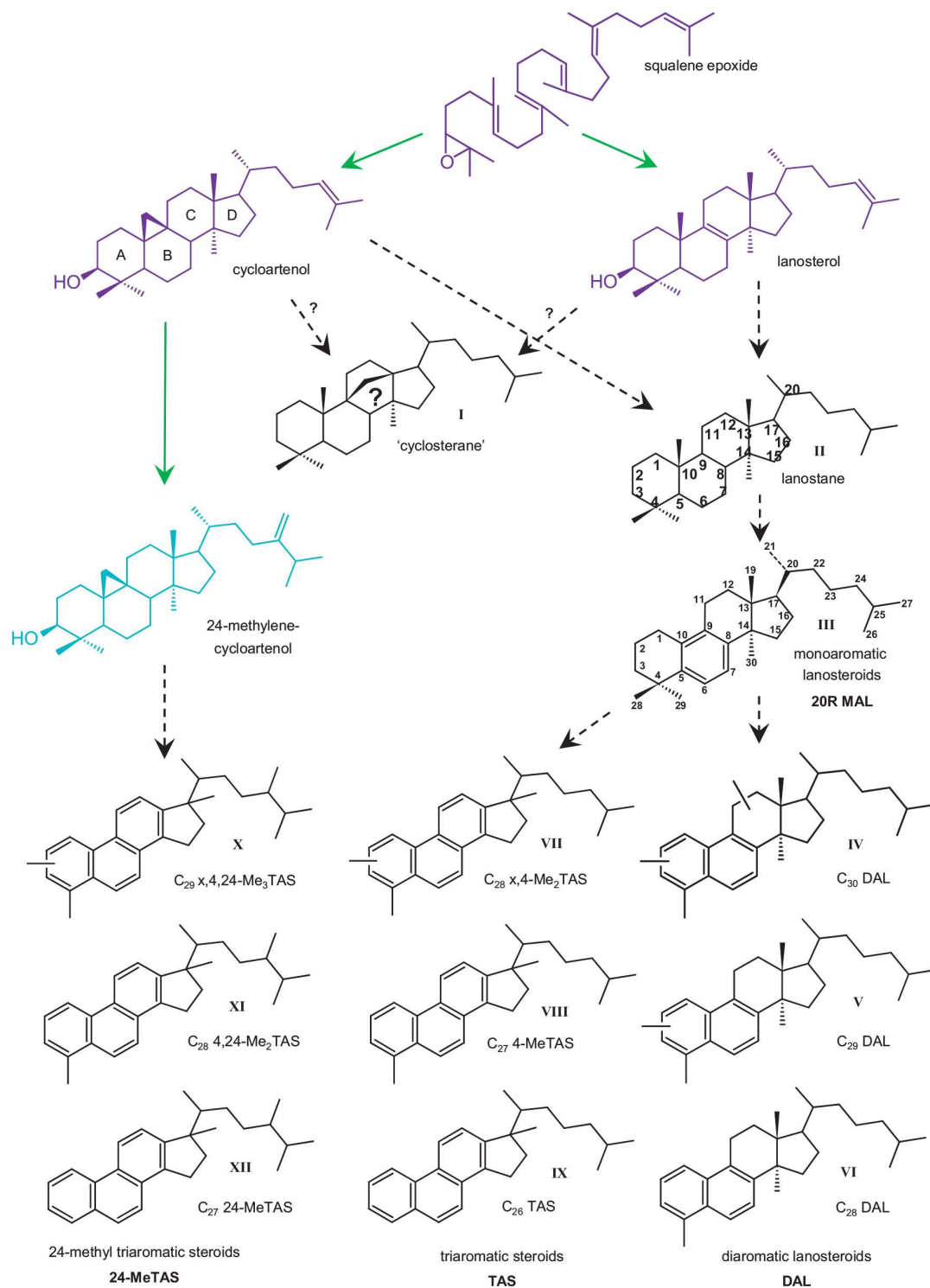
730 **Extended Data Fig. 7 | Mass chromatograms of triaromatic steroids (TAS). a,  $M/z$  259** mass  
731 chromatogram of TAS with two methylations in the ring system ( $Me_2TAS$ ) in sample 13N14 from  
732 the 1,100 Ma El Mreïti Group, and **b**, in the pyrolysate of an enrichment mixture of cycloartenol  
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 ref<sup>69</sup>.

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).

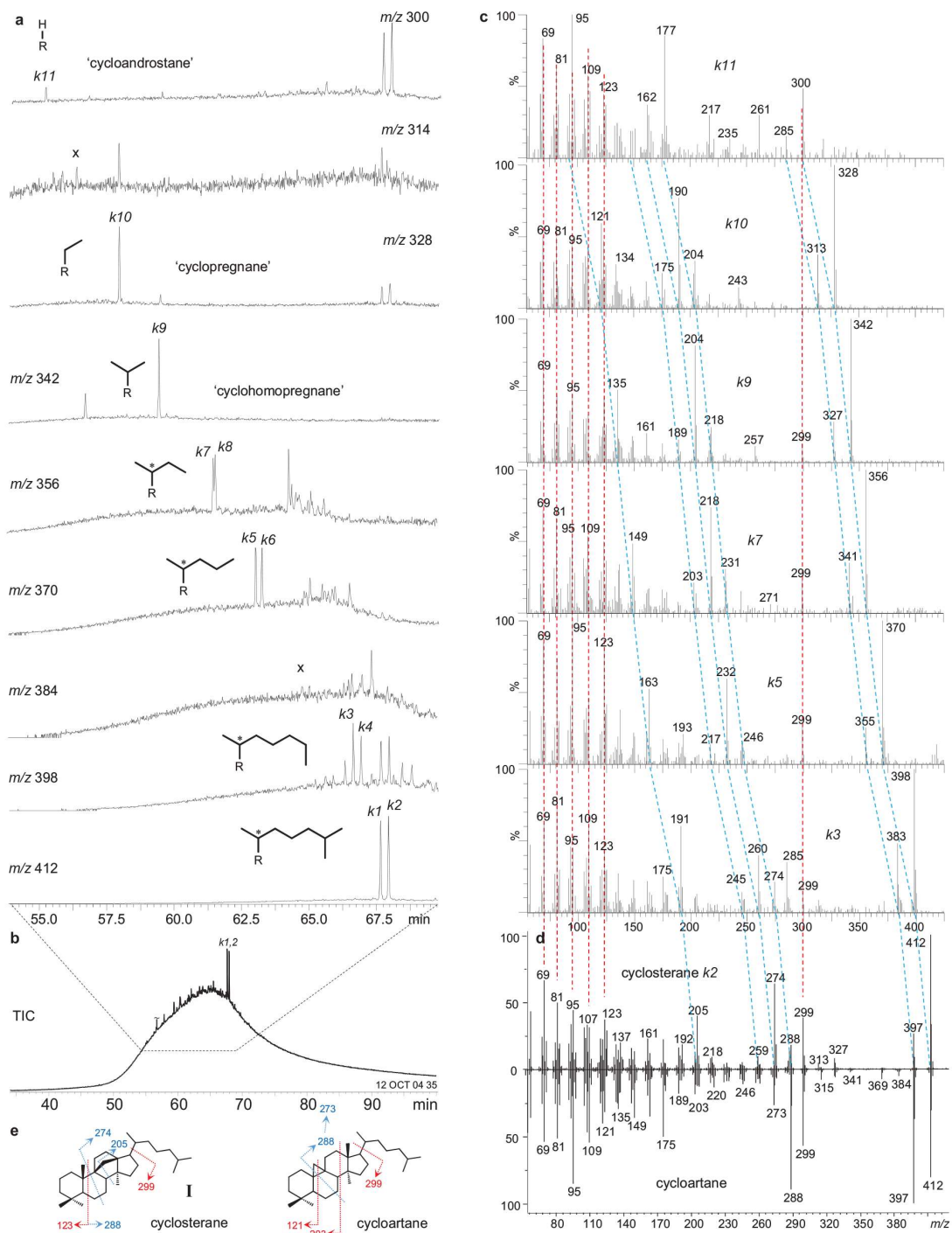
750 **Extended Data Fig. 9 | Confirmation of the elution position of S and R 4,24-dimethyl**  
751 **triaromatic cholesterol in SIR  $m/z$  245 traces on DB-5MS (left) and VZ-200 (right) gas**  
752 **chromatographic columns.** **a, c**, The AGSOSTD standard, **b, e**, the ~1,300 Ma Velkerri  
753 Formation, 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 C<sub>31</sub> ursterol 24-methylene cycloartenol (Extended Data Fig. 7c).

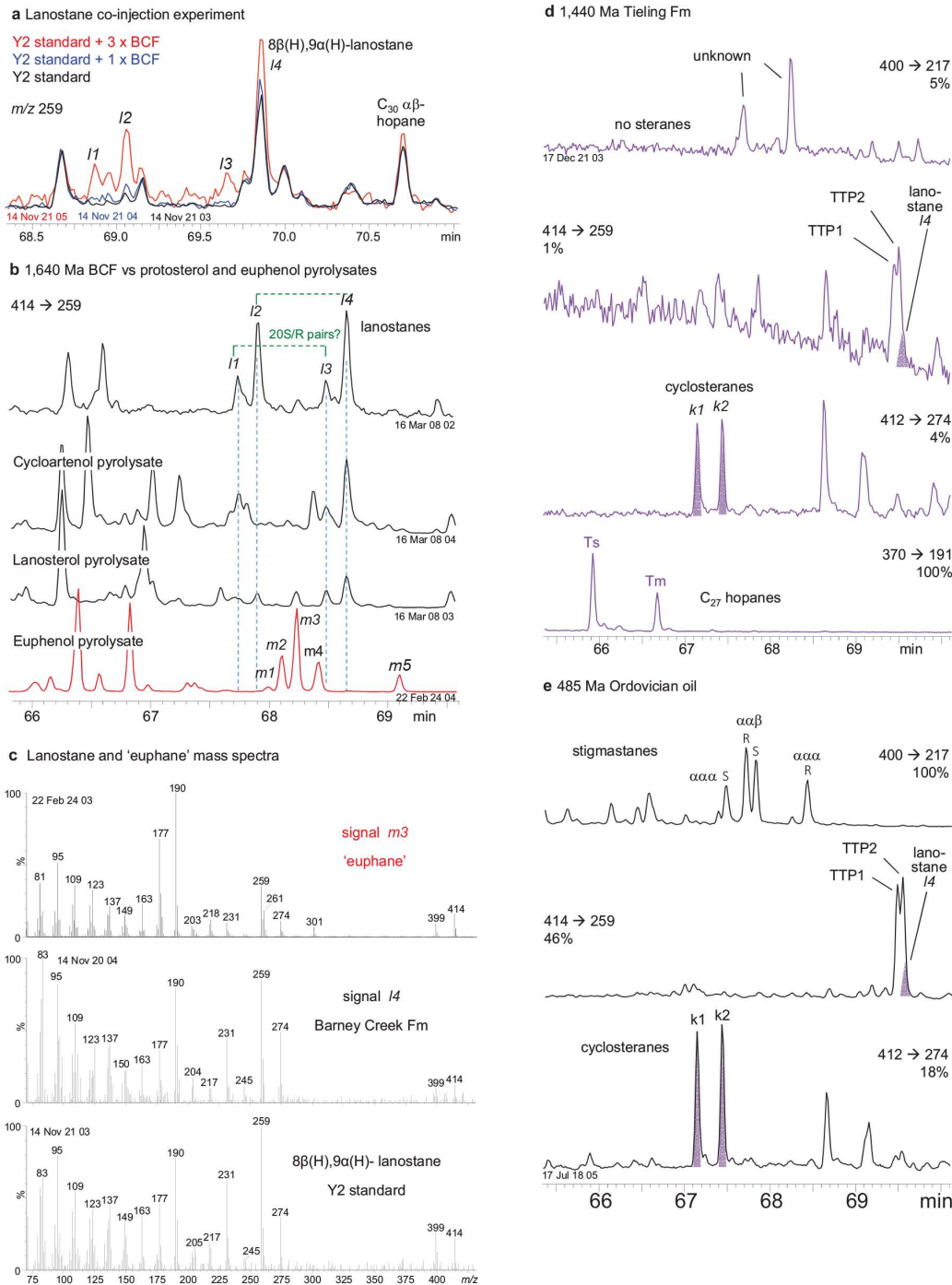


**Extended Data Fig. 1 | Diagenetic and pyrolysis products of ur- and protosterols.** Chemical structures in purple and cyan are biogenic precursors. Structures in black are fossil lipids detected in mid-Proterozoic sedimentary rocks and generated in pyrolysis experiments of the respective biolipids. Green arrows signify biosynthetic reactions, black dashed arrows point to products of diagenesis (and laboratory pyrolysis). Note that the dashed arrows do not imply direct product precursor relationships as diagenetic reactions commonly involve numerous intermediates and complex reaction networks. All structures in black, apart from X, were found in mid-Proterozoic bitumens. TAS = triaromatic steroids, MAL = monoaromatic lanosteroids, DAL = diaromatic lanosteroids. 'x,4,24-Me<sub>3</sub>TAS' signifies a TAS methylated at C-4, C-24 and at an unknown position x.

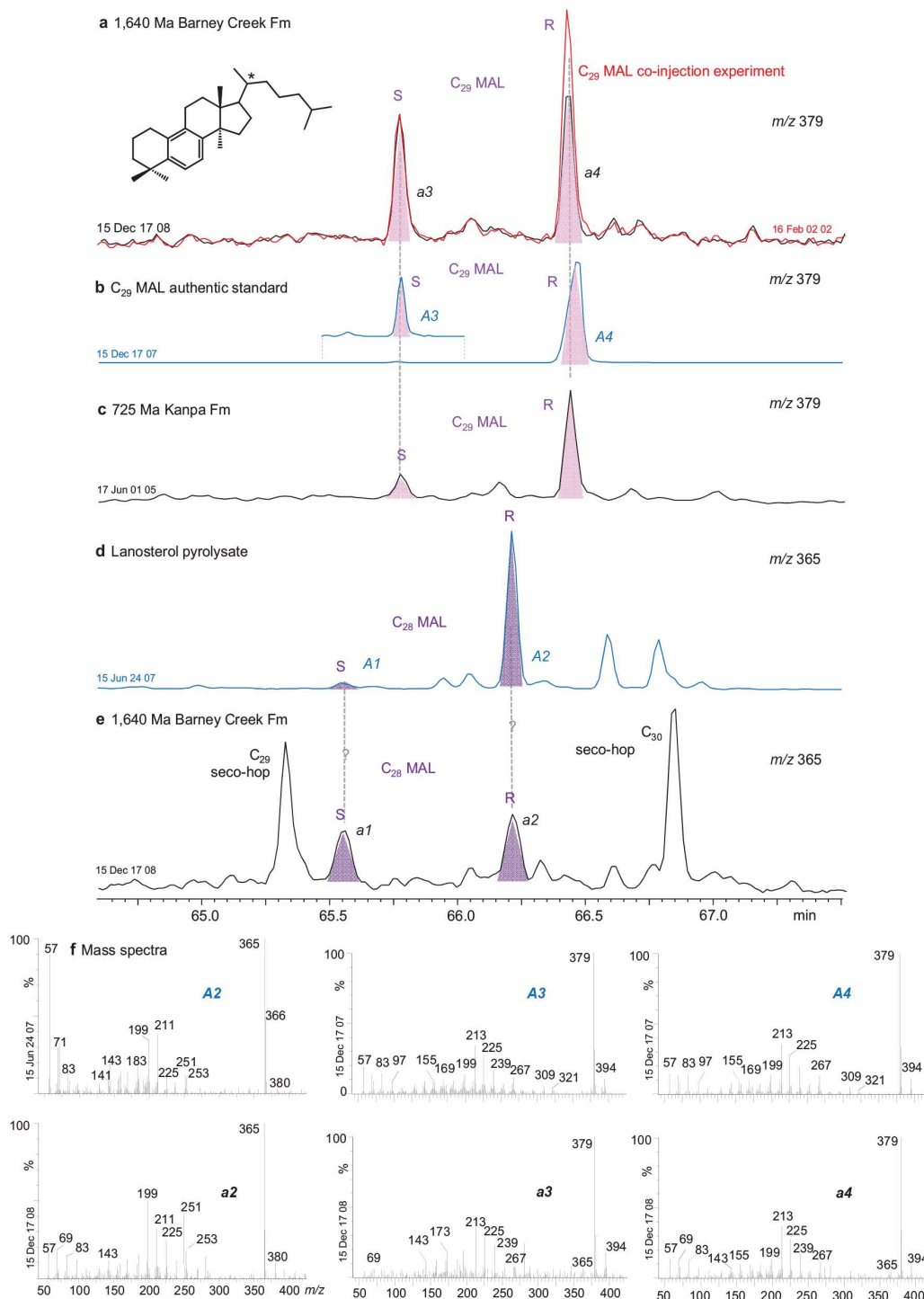




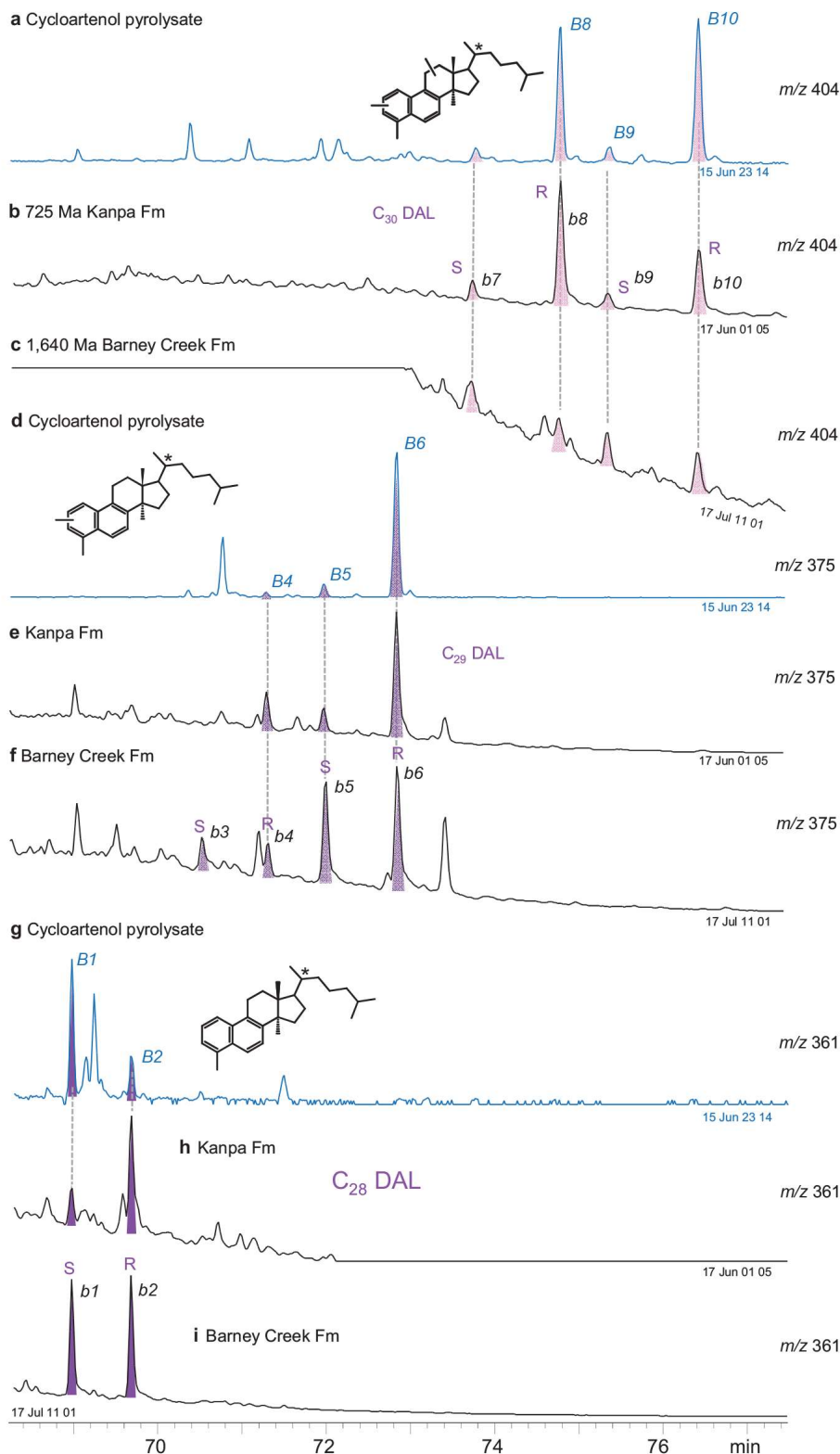
**Extended Data Fig. 2 | Mass spectra and elution behaviour of cyclosterane and its cleavage products in a severely biodegraded migrabitumen (12Z083, drill core MY4, 103.3 m). a,**  $M/z$  412 to 300 partial ion chromatograms of cyclosterane and successive side-chain cleavage products. 'x' indicate absence 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.



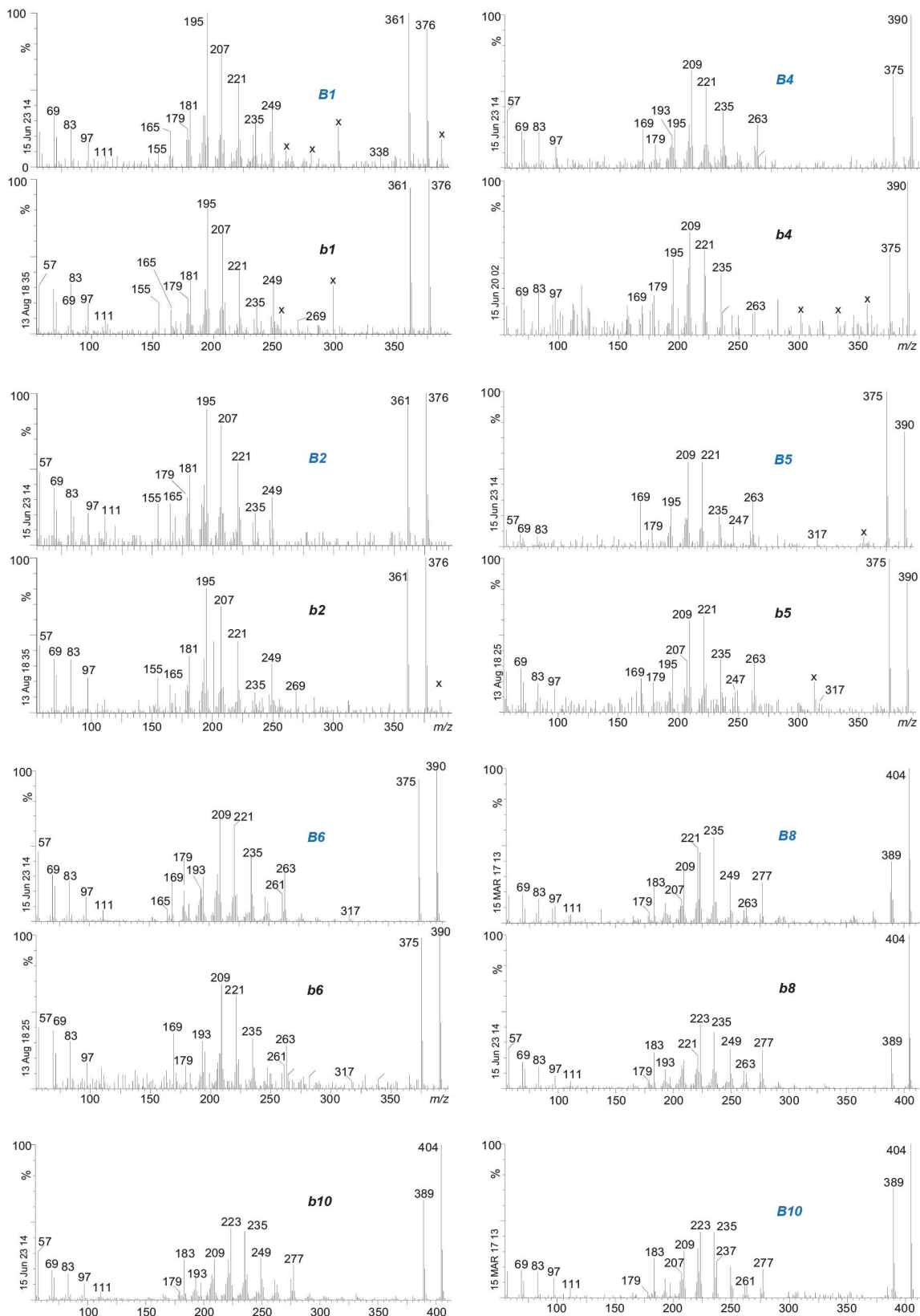
**Extended Data Fig. 3 | Mass chromatograms and spectra of protosteranes.** **a**,  $M/z$  259 partial mass chromatograms of a co-injection experiment of Eocene bitumen Y2 containing  $8\beta(H),9\alpha(H)$ -lanostane *I4* (see text) and 1,640 Ma Barney Creek Fm (B03178). **b**, MRM 414  $\rightarrow$  259 showing the elution positions of lanostane isomers *I1* to *I4*, and the results of pyrolysis experiments on cycloartenol, lanosterol and euphenol ( $3\beta,13\alpha,14\beta,17\alpha$ -lanost-8-en-3-ol). **c**, Mass spectra of signal m3 of euphenol pyrolysis, *I4* in BCF sample B03178 and lanostane in the Y2 standard. **d**, **e**, MRM chromatograms highlighting the relative elution positions of stigmastanes, lanostane, cyclosteranes and  $C_{27}$  hopanes in (d) the 1,440 Ma Tieling Fm (sample 17B101) and (e) an Ordovician oil from Australia (GA#299). TTP1 and 2 are Tetracyclic Terpene isomers<sup>68</sup>. Note that  $8\beta(H),9\alpha(H)$ -lanostane *I4* co-elutes with TTP2, but that its presence can be recognized by an elevated TTP2 peak or a shoulder trailing the peak. The chromatograms are identified by MRM precursor  $\rightarrow$  product transitions and relative signal heights in % relative to the highest signal.



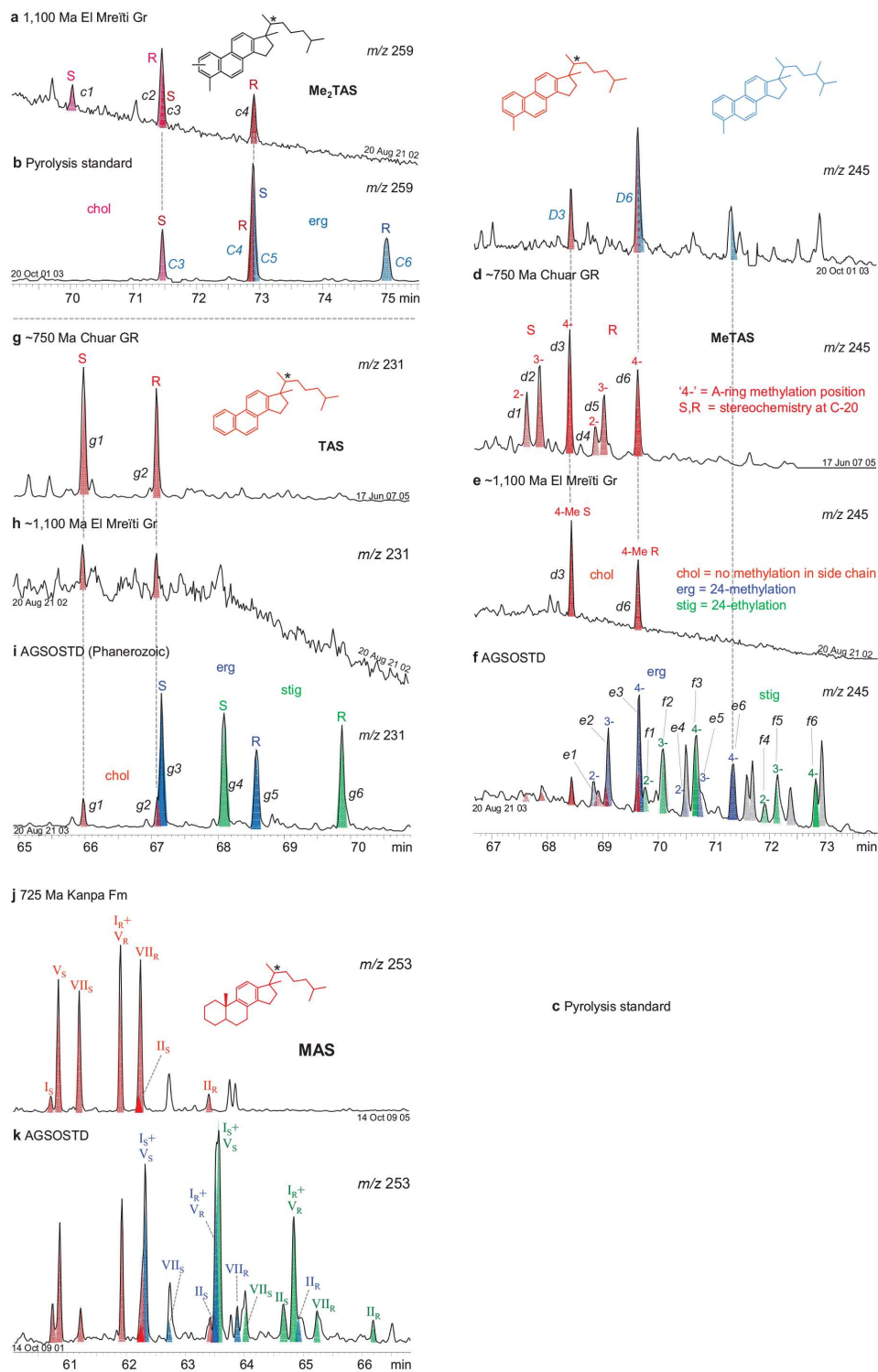
**Extended Data Fig. 4 | Mass chromatograms and spectra of monoaromatic lanosteroids (MAL).** **a**,  $m/z$  379 mass chromatogram identifying the 20S and 20R isomers of  $C_{29}$  MAL in sample B03163 from the 1,640 Ma Barney Creek Fm (black) and coinjection experiment with an authentic MAL standard 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.



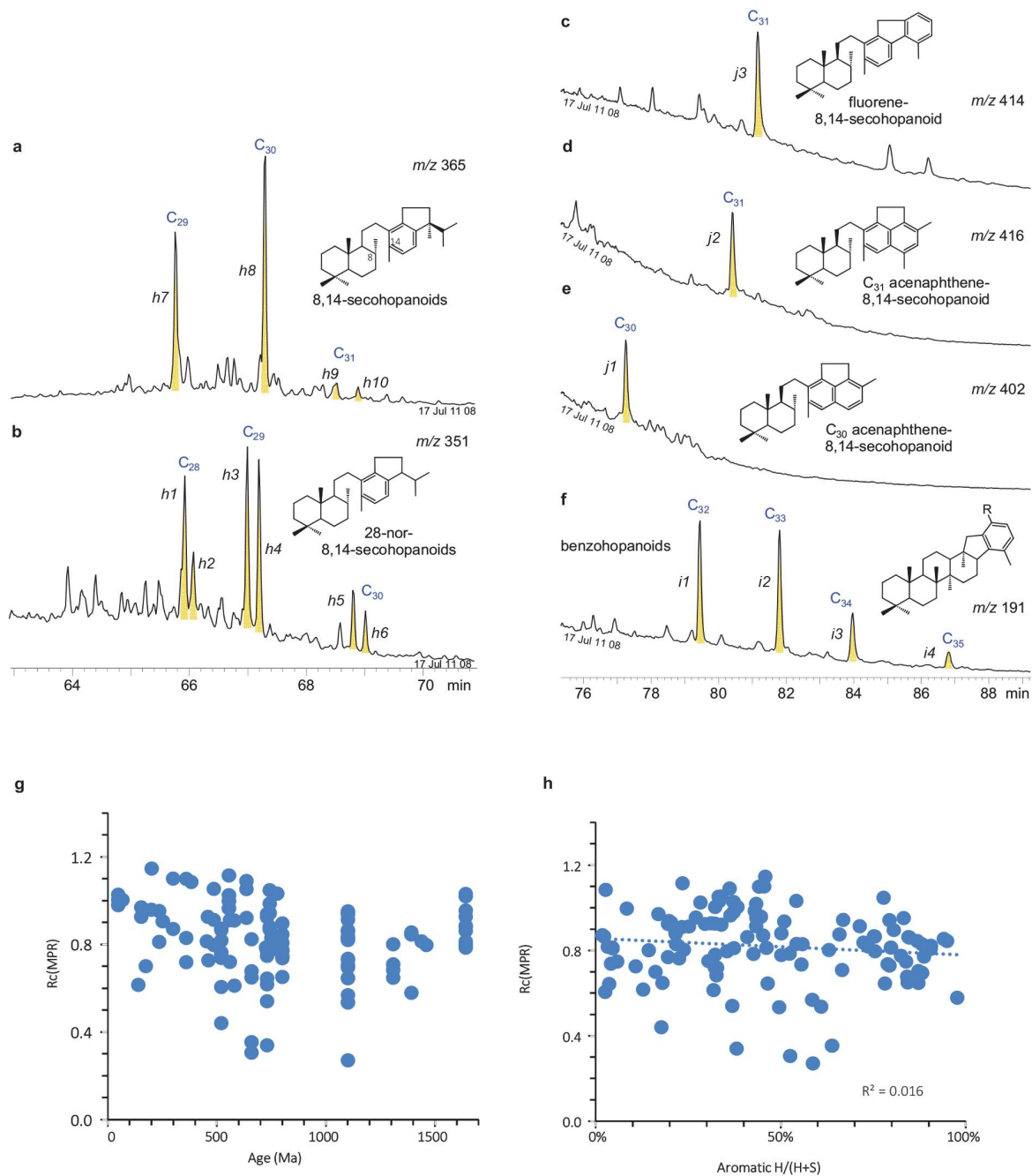
**Extended Data Fig. 5 | Mass chromatograms of diaromatic lanosteroids (DAL).** **a, d, g,** *M/z* 404, 379 and 361 mass chromatograms identifying isomers of C<sub>28</sub>, C<sub>29</sub> and C<sub>30</sub> DAL generated through pyrolysis of cycloartenol. **b, e, h,** C<sub>28</sub>, C<sub>29</sub>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



**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.

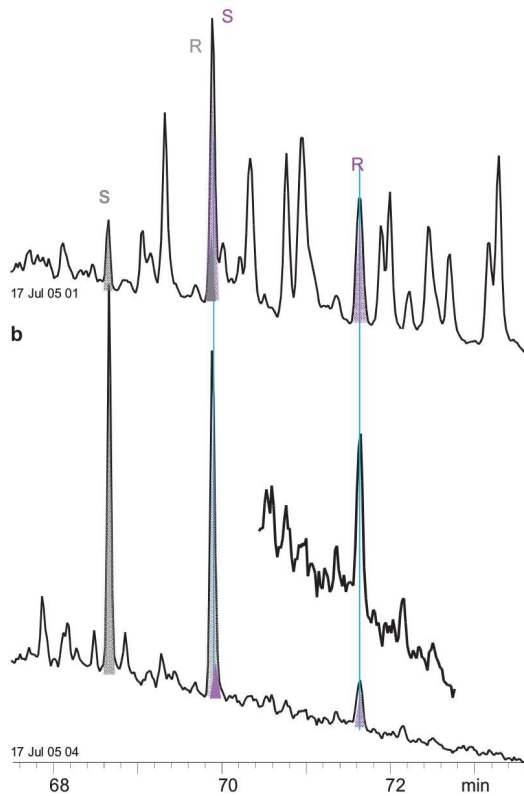


**Extended Data Fig. 7 | Mass chromatograms of triaromatic steroids (TAS).** **a**,  $M/z$  259 mass chromatogram of TAS with two methylations in the ring system ( $Me_2TAS$ ) in sample 13N14 from the 1,100 Ma El Mreiti Group, and **b**, in the pyrolysate of an enrichment mixture of cycloartenol (CA) 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.

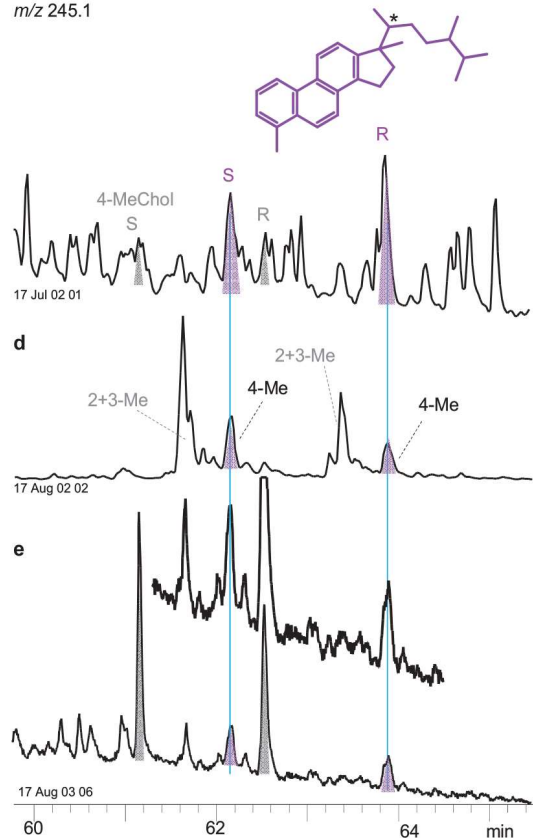


**Extended Data Fig. 8 | Mass chromatograms of aromatic hopanoids in the 1,640 Ma Barney Creek Fm (12B117), and thermal maturity evaluation of the sample set.** Mass chromatograms of **a**,  $m/z$  365 identifying regular aromatic 8,14-secohopanoids, **b**,  $m/z$  351 regular 28-nor-8,14-secohopanoids, **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  $Rc(MPR)$  against (g) sample age, and (h) the relative abundance of aromatic hopanoids and steroids (H/(H+S)).  $Rc(MPR)$  = computed vitrinite reflectance ( $Rc$ ) 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
<b>Ediacaran</b>						
<b>Araras Group, Brazil – Terconi Outcrop (~635 Ma)</b>						
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
<b>Cryogenian (635 – 717 Ma)</b>						
<b>Amadeus Basin – Central Australia</b>						
<b>Aralka Formation (659 Ma)</b>						
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
<b>Tonian (717 – 1000 Ma)</b>						
<b>Wallara Formation (aka Finke Beds) (&gt;750 Ma)</b>						
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
<b>Johnnys Creek Formation, Bitter Springs Group (&lt;820 Ma)</b>						
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
<b>Loves Creek Formation, Bitter Springs Group (&lt;820 Ma)</b>						
11J063 i	<820	Amadeus Basin	Loves Creek Formation	BR05	1224.69	Grey dolomitic cross-cut by thin white molar tooth structures
<b>Officer Basin – Western Australia</b>						
<b>Step toe Formation, Buldya Group (717-725 Ma)</b>						
14B214 i	717-25	Officer Basin	Step toe Formation	Empress 1A	509.14	Light and dark grey laminated mudstone
14B213 i	717-25	Officer Basin	Step toe Formation	Empress 1A	588.21	Black laminated mudstone with evaporite nodules
<b>Kanpa Formation, Buldya Group (725 ± 11 Ma)</b>						
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
<b>Hussar Formation, Buldya Group (777-725 Ma)</b>						
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
<b>Grand Canyon - Arizona - Outcrop at Sixtymile Canyon and Nankowep Butte (depths are meters above base)</b>						
<b>Walcott Member, Kwagunt Formation, Chuar Group (729 ± 0.9 Ma)</b>						
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
<b>Lake Vättern Basin - Sweden - Outcrop at Girabacken locality</b>						
<b>Visingsö Group (&gt; 740 Ma)</b>						
VG-20-03-i	~750	Lake Vättern Basin	Visingsö Group	outcrop		Black mudstone
<b>Mesoproterozoic (1000 – 1600 Ma)</b>						
<b>Sette– Daban fold belt, Siberian Craton, Russia</b>						
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
<b>Taoudeni Basin, El Mreiti Group, Mauritania</b>						
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
<b>Keweenaw Rift, Michigan Basin, USA</b>						
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
<b>McArthur Basin, Northern Australia</b>						
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
<b>North China Craton</b>						
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)
<b>Palaeoproterozoic (1600 – 2500 Ma)</b>						
<b>McArthur Basin, Northern Australia</b>						
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

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14

15

## 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 Ma<sup>70</sup>  
29 Walcott Member of the Kwagunt Formation, the Chuar Group, and the ≤ 886 and 740 Ma Visingsö  
30 Group<sup>71</sup>. Age constraints for these formations and their geological and environmental context are  
31 summarized in the Supplementary Discussion of Brocks et al. (2017)<sup>15</sup>. 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 km<sup>2</sup> on the West African Craton,  
36 extending from Mauritania to northern Mali and Western Algeria<sup>65,72</sup>. 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 Formation<sup>73</sup>. 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 sea<sup>74</sup>, characterized by sea-level fluctuations in a general shallowing-upward trend<sup>75-77</sup>.

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 basin<sup>78</sup>. 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<sup>78,79</sup>. These sediments were deposited in a relatively shallow,  
54 subtidal environment during a transgressive phase<sup>77</sup>. 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<sup>77,78</sup>. 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 reefs<sup>77</sup> 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 waters<sup>78</sup> and comprise micrometre-thin, plane to wrinkly,  
65 continuous organic laminae with occasional framboidal pyrite and pyritized filamentous sheaths<sup>78</sup>.  
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<sup>78,2</sup> 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 debris<sup>2</sup>.

72 Micropalaeontological work on the El Mreïti Group, including sediments from drill core S2,  
73 yielded an exquisitely preserved assemblage of organic-walled microfossils<sup>65</sup>. 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 facies<sup>78</sup>. 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 out<sup>78</sup>.

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 samples<sup>78</sup> 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 leiospheroids<sup>78</sup>.

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*<sup>78</sup>. 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*<sup>78</sup>. 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) Ma<sup>80</sup>.

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 system<sup>81</sup>. The Nonesuch Formation contains several indicators that the basin was  
110 shallowing upwards, including microbial induced sedimentary structures<sup>82</sup>, raindrop impressions,  
111 desiccation polygons<sup>83</sup> and mud cracks<sup>84</sup>. A stromatolitic horizon near the top of the Copper  
112 Harbor Conglomerate was interpreted as the onset of lacustrine deposition<sup>85</sup>. 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 province<sup>86</sup>.  
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 ratio<sup>87</sup>.

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<sup>88,89</sup>, 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<sup>11,83</sup>.

129

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

131 The geology of the Roper Group was previously summarized<sup>90-93</sup>. 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 km<sup>2</sup>. 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 members<sup>94</sup>.

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  $1386 \pm 13$  Ma and  $1385 \pm 116$  Ma in the underlying Bessie Creek Sandstone  
144 <sup>93,95</sup> and a detrital zircon age of  $1308 \pm 41$  Ma in the Wyworrie Member<sup>95</sup>. 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  $1312.9 \pm 0.7$  Ma<sup>96</sup>. The Amungee Member of the Velkerri Fm yielded Re-  
147 Os ages of  $1361 \pm 21$  Ma (C shale) and  $1417 \pm 29$  Ma (A shale)<sup>97</sup>.

148 In the present study, four thermally immature<sup>27</sup> 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 sulphidic<sup>92</sup>. Moreover, anoxic waters did not, or rarely,  
153 rise into the euphotic zone of the water column<sup>22,27</sup>.

154 The Roper Group is well known for well preserved assemblages of early eukaryotic fossils (Fig.  
155 1c)<sup>98</sup>. 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 occur<sup>98</sup>. 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 Fm<sup>9</sup>.

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 Ma<sup>99</sup>). 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 Fm<sup>100</sup>  
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<sup>100,101</sup>. 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 Ma<sup>99</sup>). A tuffaceous layer in Member 3 of the Xiamaling Fm is dated at ~1,392 Ma<sup>100,102</sup>  
180 while the upper limit of the top of the Xiamaling Fm is indicated to be ~1,350 Ma<sup>103</sup>.

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 formation<sup>104</sup>. 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 basin<sup>105</sup>.

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 units<sup>106</sup>. (Note that ref<sup>67</sup> 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%<sup>67</sup>, were presumably deposited in a restricted basin with varying degrees of connection to the  
194 open ocean<sup>107</sup>. 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<sup>106,107</sup>. Others suggest that the Xiamaling Fm was  
197 predominantly deposited under deep oxygenated waters<sup>67,108</sup>, although the interpretation of the  
198 exact redox conditions remains debated<sup>109</sup>.

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 horizons<sup>110</sup>. 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*<sup>110</sup>.

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 km<sup>2</sup> 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 faults<sup>111-113</sup>.

214 The geology of the Barney Creek Fm was summarized by Jackson et al. (1987)<sup>90</sup> and Ahmad et al.  
215 (2013)<sup>114</sup> and a recent facies analysis and sequence stratigraphy was provided by Kunzmann et al.  
216 (2019)<sup>111</sup>. 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<sup>111,114</sup>. 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 Ma<sup>115</sup>. 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<sup>114,116</sup>.

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 3<sup>rd</sup>-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 Fm<sup>111</sup>. Early interpretations of depositional environments  
236 ranged from shallow marine to lacustrine or lagoonal<sup>111</sup>, but the HYC Pyritic Shale Member is  
237 generally regarded to have formed under deep subtidal conditions<sup>117,118</sup>. Nevertheless, similarities  
238 in carotenoid biomarker signatures between the undifferentiated Barney Creek Fm<sup>30</sup> and the saline  
239 lacustrine Eocene Green River Fm, were used to infer lacustrine depositional conditions<sup>119</sup>.  
240 However, redox stratification, generally low sulphate concentrations in Palaeoproterozoic marine  
241 basins<sup>120</sup> and the intracratonic setting of the BCF could also explain similarities in carotenoid



242 biomarker patterns. Fe-speciation indicates dominantly anoxic ferruginous redox conditions<sup>121,122</sup>,  
243 even though sulfidic excursion may have been common with possible temporary injection of oxic  
244 waters<sup>122</sup>.

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<sup>30,122</sup>. 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 position<sup>30</sup>. 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 fossils<sup>123</sup>.

257 After the underlying, 1,730 Ma Wollgorang Fm<sup>124</sup>, the Barney Creek Fm contains the probably  
258 oldest-known, clearly indigenous biomarkers<sup>125</sup> and may constitute the oldest active petroleum  
259 system<sup>126</sup>. 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 GR7<sup>127</sup>, whereas biomarkers show an even better thermal preservation throughout core LV09  
263 where Tmax values range from 423 to 440°C<sup>128</sup>. 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<sup>129,130</sup>, ~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 PtO<sub>2</sub> catalyst under a  
285 continuous flow of H<sub>2</sub> 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 H<sub>2</sub> gas on ~20 mg PtO<sub>2</sub>/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 pentane<sup>131</sup>.

#### 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 PtO<sub>2</sub>/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 C<sub>29</sub> monoaromatic lanosteroid (MAL)**

311 The monoaromatic lanosteroid was synthesized from lanost-8-ene following an established  
 312 method<sup>132</sup> 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 H<sub>2</sub> 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 (<sup>1</sup>H, <sup>13</sup>C, DEPT) and 2D homo (<sup>1</sup>H-<sup>1</sup>H COSY)  
 322 and heteronuclear (<sup>1</sup>H-<sup>13</sup>C-HSQC, and <sup>1</sup>H-<sup>13</sup>C-HMBC) experiments. NMR analyses were  
 323 performed on a Bruker Avance I – 500 MHz spectrometer operating at an observation frequency  
 324 of 500 MHz (<sup>1</sup>H) and 125 MHz (<sup>13</sup>C) (<sup>1</sup>H, <sup>13</sup>C, DEPT, <sup>1</sup>H-<sup>13</sup>C-HSQC, and <sup>1</sup>H-<sup>13</sup>C-HMBC) and on  
 325 a Bruker Avance III – 600 MHz spectrometer at an observation frequency of 600 MHz (<sup>1</sup>H) (<sup>1</sup>H,  
 326 <sup>1</sup>H-<sup>1</sup>H COSY). The chemical shifts (Supplementary Table 4) are reported in ppm relative to  
 327 tetramethylsilane with the solvent used as internal standard (CD<sub>2</sub>Cl<sub>2</sub>: δ<sup>1</sup>H 5.32 ppm; δ<sup>13</sup>C 53.5  
 328 ppm).

329  
 330 **Supplementary Table 4.** <sup>1</sup>H and <sup>13</sup>C-NMR chemical shifts of synthetic monoaromatic lanosteroid  
 331 (20R MAL) (CD<sub>2</sub>Cl<sub>2</sub>).

Position	δ <sub>C</sub>	δ <sub>H</sub>	δ <sub>H</sub>	Position	δ <sub>C</sub>	δ <sub>H</sub>	δ <sub>H</sub>
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 <sup>c</sup>	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 <sup>c</sup>	2.62	2.62	26	22.6 <sup>a</sup>	0.880 <sup>b</sup>	-
12	30.9	1.98	1.98	27	22.3 <sup>a</sup>	0.876 <sup>b</sup>	-
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 PtO<sub>2</sub> catalyst and a constant  
339 stream of H<sub>2</sub> 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 PtO<sub>2</sub> catalyst and a constant stream of H<sub>2</sub> 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 N<sub>2</sub> gas. The products were taken up in *n*-  
347 hexane and hydrogenated first for 4 h with H<sub>2</sub> gas on PtO<sub>2</sub>, 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 H<sub>2</sub> gas by stirring for 3 h in 50 μL acetic acid and 2 mL DCM in the presence of PtO<sub>2</sub>  
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 NaSO<sub>4</sub>.

#### 358 **2.4. RuO<sub>4</sub> 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 RuO<sub>4</sub>  
360 freshly prepared according to Piatak et al. (1969)<sup>133</sup>, but replacing acetone by CCl<sub>4</sub>. After 10 min,  
361 a mixture of CCl<sub>4</sub> 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 rank<sup>134</sup>. 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 C<sub>30</sub>  
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 ref<sup>125</sup>).

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 (C<sub>28</sub>) and 314 (C<sub>23</sub>) 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 C<sub>8</sub> 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 C<sub>8</sub> 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 triterpenoids<sup>135</sup>). 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 PtO<sub>2</sub> 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 RuO<sub>4</sub> (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 PtO<sub>2</sub> 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) PtO<sub>2</sub>-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 processes<sup>135</sup>.  
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 C<sub>9</sub>H<sub>13</sub> 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 C<sub>9</sub>H<sub>15</sub> 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 C<sub>15</sub>H<sub>25</sub> 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 samples<sup>135</sup>. 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 colleagues<sup>136</sup> 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, 2017<sup>122</sup>), 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 H<sub>2</sub> gas on PtO<sub>2</sub>. 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, 2017<sup>122</sup>), 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 C<sub>26</sub> triaromatic steroid **IX**. These compounds may form as aromatization products  
549 of C<sub>27</sub> 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<sup>+</sup>) 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 standard<sup>46</sup> (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 C<sub>27</sub> crown-sterols as they co-occur with abundant saturated  
574 cholestane and MAS cholesteroids<sup>46</sup>. 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 AGSOSTD<sup>46</sup>. 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<sup>15,46</sup>. These  
588 Tonian TAS are interpreted as A-ring methylation products of C<sub>27</sub> 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, 2017<sup>122</sup>). 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 algae<sup>137</sup>. 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 lanosteroids<sup>138</sup>. 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. *C<sub>28</sub> diaromatic lanosteroids (C<sub>28</sub> 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 ref<sup>138</sup>). 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-ring<sup>137</sup>, which is also observed  
658 for B-ring aromatized isoarborinol and fernane-derivatives<sup>139</sup>. It is therefore probably no  
659 coincidence that the sparse reports of diaromatic steroids are restricted to 13,14-dimethylated  
660 derivatives<sup>137,138</sup>, as the slow pace of B-ring aromatization in crown-sterols combined with the  
661 considerable pace of A-ring and C-ring aromatization<sup>140</sup> usually results in the formation of either  
662 mono- or triaromatic steroid derivatives.

663                   2.7.3.2. *C<sub>29</sub> diaromatic lanosteroids (C<sub>29</sub> 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 C<sub>28</sub> 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 C<sub>29</sub> 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. C<sub>30</sub> diaromatic lanosteroids (C<sub>30</sub> 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 C<sub>30</sub> homolog of the C<sub>28</sub> and C<sub>29</sub> 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 France<sup>141</sup>. In the present study, the identity of C<sub>29</sub> B-ring monoaromatic lanosteroids was  
683 confirmed by co-injection with an authentic standard.

##### 684 2.7.4.1. C<sub>29</sub> B-ring monoaromatic lanosteroids (C<sub>29</sub> 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 C<sub>29</sub> 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 C<sub>29</sub> 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 C<sub>29</sub> 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. C<sub>28</sub> B-ring monoaromatic lanosteroids (C<sub>28</sub> MAL)

699 Some samples also yielded two chromatographic signals *a1* and *a2* in *m/z* 365 chromatograms  
700 eluting between aromatic C<sub>29</sub> and C<sub>30</sub> secohopanoids (Extended Data Fig. 4e). The mass spectra of  
701 the M<sup>+</sup> 380 compounds are very similar to those of the C<sub>29</sub> 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 C<sub>32</sub>-C<sub>35</sub> benzohopanoids that encompass side-chain  
710 cyclisation and aromatization, we included the aromatic hopanoid derivatives C<sub>30</sub> and C<sub>31</sub>  
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 C<sub>32</sub>-C<sub>35</sub> benzohopanoids *i1-i4* (Extended Data Fig. 8f) were identified relative to compounds in  
714 AGSOSTD reference oil (Fig. 8-14 in Nettersheim, 2017<sup>122</sup>) and mass spectral characteristics<sup>142</sup>.  
715 Eluting between the C<sub>32</sub> and C<sub>33</sub> 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<sup>+</sup>] at *m/z* 414 and was identified as a C<sub>31</sub>  
718 8,14-secohopanoid with a fluorene moiety based on comparison with published mass spectral  
719 characteristics<sup>143</sup> (see also Fig. 8-15 in Nettersheim, 2017<sup>122</sup>). Eluting before *j3*, the  
720 chromatographic signal *j2* (Extended Data Fig. 8d)—following Carillo-Hernández et al.  
721 (2001)<sup>144</sup>—was tentatively identified as a C<sub>31</sub> acenaphthene-8,14-secohopanoid based on its mass  
722 spectral characteristics of dominant [M<sup>+</sup>] 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 C<sub>32</sub> benzohopanoid *i1* (Extended  
725 Data Fig. 8e) was tentatively identified as the corresponding C<sub>30</sub> acenaphthene-8,14-secohopanoid  
726 based on dominant [M<sup>+</sup>] at *m/z* 402 and *m/z* 195 fragments<sup>144</sup>. 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 C<sub>29</sub>-C<sub>31</sub> 8,14-secohopanoids by comparison with published elution patterns and mass  
729 spectra<sup>145</sup> (see also Fig. 8-19 and 8-20 in Nettersheim, 2017<sup>122</sup>). 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 C<sub>29</sub>



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  $[M^{+\circ}]$  of 380, 394 and 408 corresponding to  
736 aromatic C<sub>28</sub>-C<sub>30</sub> 8,14-secohopanoids that lost one of the methyl groups at the aromatic or  
737 pentacyclic ring<sup>146</sup>.

## 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 C<sub>27</sub> 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-17<sup>147</sup>. 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)<sup>147</sup>, and in traces in bacteria as accidental side products of hopanoid  
783 biosynthesis<sup>148,149</sup>. 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 $\beta$ (Me),14 $\alpha$ (Me) configuration. By contrast, euphol  
791 (3 $\beta$ ,13 $\alpha$ ,14 $\beta$ ,17 $\alpha$ ,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 $\beta$ (Me),14 $\alpha$ (Me) configuration<sup>150</sup>. 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 activities<sup>132</sup>. 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 $\beta$ ,13 $\alpha$ ,14 $\beta$ ,17 $\alpha$ ,20R-lanost-8-en-3-ol) to pyrolysis  
806 followed by hydrogenation on PtO<sub>2</sub> (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 $\beta$ (H),9 $\alpha$ (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 Ratio<sup>151</sup> (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 effects<sup>152</sup> 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 Cystobacterineae<sup>33</sup> 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 C<sub>31</sub>  
887 sterol 24-methylenecycloartenol, which is the second intermediate in the biosynthetic pathway to  
888 phytosterols<sup>17</sup>. 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<sup>32,153</sup>. 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 C<sub>27</sub> 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%)<sup>15,46</sup> that are demonstrably the product of diagenetic C<sub>27</sub> sterol methylation<sup>32</sup>. 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 sterols<sup>35</sup>, SMT genes of  
914 bacteria that live in symbiosis with sponges, when expressed *in vitro*, methylate C<sub>27</sub> sterols to form  
915 24-methylated or even 24-ethylated cholesteroloids<sup>154,155</sup>. Some SMTs even yield 24-  
916 isopropenylcholesterol and related C<sub>30</sub> 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*<sup>155</sup>.  
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)<sup>155</sup>. 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<sup>33,39,155</sup> suggest that bacterial SMTs have relatively shallow  
929 clade depths. Moreover, in the trees provided by refs<sup>39,155</sup> 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<sup>35,156</sup>. It occurs across all major eukaryotic groups and it was probably  
934 already present in LECA<sup>17</sup>. 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.

939

940

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

942 Porter et al. (2018)<sup>21</sup> 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 modifiers<sup>157-159</sup>. Moreover, crown-group eukaryotes only require molecular oxygen at  
957 very low levels to complete the full sterol biosynthetic pathway<sup>160</sup>, 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 basins<sup>67</sup> 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*<sup>10</sup> and ~1,000 Ma  
966 chlorophyte alga *Proterocladus*<sup>11</sup>. 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<sup>1,8,9</sup>. 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 column<sup>161</sup>. 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)<sup>162</sup> 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 survey<sup>163</sup> 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 sterols<sup>163</sup>. In a detailed sterol

1053 survey<sup>164</sup> 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-methylenecycloartanol<sup>165</sup>. 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 protosterols<sup>166</sup>. The triterpene alcohol fraction  
1071 of 19 vegetable oils were strongly dominated by cycloartenol and 24-methylene cycloartenol.  
1072 Based on data in refs<sup>166,167</sup>, 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%  $\alpha$ -spinasterol and 1.5% stigmastenol,  
1077 yielding a protosterol ratio of ~42%<sup>168</sup>. In addition to the more common cycloartenol or  
1078 cyclolaudenol, some plants also contain protosterol derivatives with various types of functional  
1079 groups<sup>169</sup>.

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%  $\beta$ -sitosterol derivatives in addition to 4% non-  
1083 esterified  $\beta$ -sitosterol<sup>170</sup>, corresponding to a protosterol ratio of ~37% ([cycloartenol + methylene  
1084 cycloartenol]/ [cycloartenol + methylene cycloartenol +  $\beta$ -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 bark<sup>171</sup>. Similar cycloartanol-derivatives were also

1087 isolated in bark of other firs (e.g. *Abies grandis*<sup>172</sup>). 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 groups<sup>173</sup>.

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 weight<sup>174</sup>, 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% cycloartenol<sup>175</sup>. 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 shoots<sup>176</sup>. The 4-desmethyl sterols were  
1109 found to contain ~75%  $\beta$ -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 compounds<sup>176</sup>. 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 ref<sup>177</sup>), from the 4,4-dimethyl (1.5 mg) and 4-desmethyl sterol fraction  
1117 contents (71.6 mg)<sup>177</sup>, 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*)<sup>178</sup>, maple leaves<sup>179</sup> and tomatoes<sup>180</sup>. 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 ref<sup>178</sup>). 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 triterpenoids<sup>181</sup>. *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 capitulum<sup>182</sup>. 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 ref<sup>182</sup>). Protosteroids also occur in a variety of  
1136 other bryophytes. A review of the chemical constituents of bryophytes<sup>181</sup> 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% sitosterol<sup>183</sup>, 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 O<sub>2</sub> 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) O<sub>2</sub> molecules<sup>156</sup>. 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 mix<sup>164</sup>. 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 environments<sup>164</sup>.  
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)<sup>184</sup>. 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)<sup>185</sup>, 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 sterol<sup>163</sup>. While two more of the ten fungi  
1178 (*Chytridium confervae* and *Rhizophlyctis rosea*) analysed by ref<sup>163</sup> 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 O<sub>2</sub> limitation and intermittently accumulate 24-methylene lanosterol, albeit presumably only as a  
1183 signalling molecule, not as a critical membrane component<sup>186</sup>.

## 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)<sup>33</sup> 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<sup>17,33-35,187,188</sup>.  
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)<sup>33</sup>.

1198 In a pioneering combined genomic and lipid analysis, Pearson et al. (2003)<sup>187</sup> 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<sup>17,33-35,188</sup> 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)<sup>35</sup> 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<sup>187,189,190</sup> 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)<sup>34</sup> found 27 bacterial  
1209 taxa across 6 phyla, 9 classes and 9 orders, confirming the findings of Wei et al. (2016)<sup>35</sup>. In an  
1210 update on sterol biosynthesis genes in the NCBI data base, Hoshino and Gaucher (2021)<sup>33</sup> 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)<sup>35</sup> 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)<sup>35</sup>, 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)<sup>17</sup> 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)<sup>188</sup>, 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)<sup>35</sup> largely echoes the topology of Villanueva  
 1255 et al. (2014)<sup>188</sup>, 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)<sup>34</sup> and Hoshino and Gaucher  
1263 (2021)<sup>33</sup> (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)<sup>34</sup>, “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)<sup>33</sup>, discussed Section 8.2.3.

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

1281 Hoshino and Gaucher (2021)<sup>33</sup> 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 C<sub>27</sub> 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

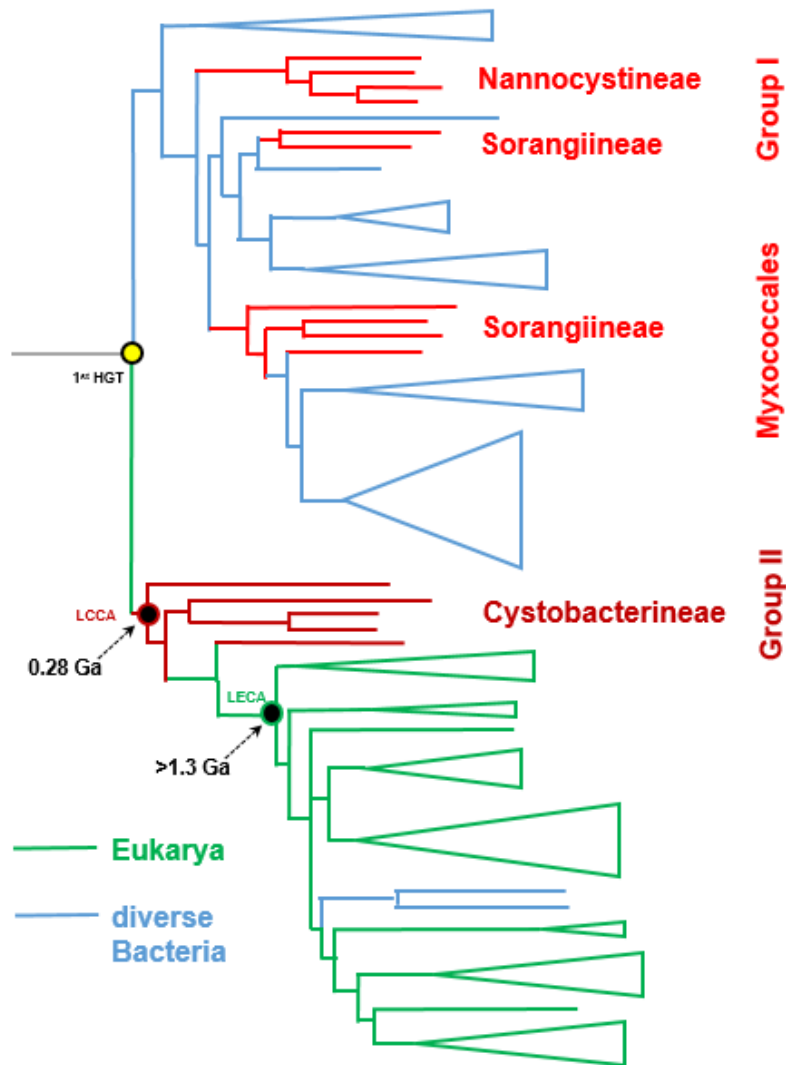
1291 mostly in a syntenic relationship with each other, i.e. they were physically co-localized with other  
1292 sterol biosynthesis genes.

1293 To create a possibly more robust phylogenetic tree of sterol biosynthesis genes, Hoshino and  
1294 Gaucher (2021)<sup>33</sup> 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)<sup>33</sup> 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)<sup>33</sup> 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)<sup>34</sup>, 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 fungi<sup>191</sup>.  
1325 Based on well calibrated molecular clocks, Gruen (2019)<sup>191</sup> estimated the date of the HGT of

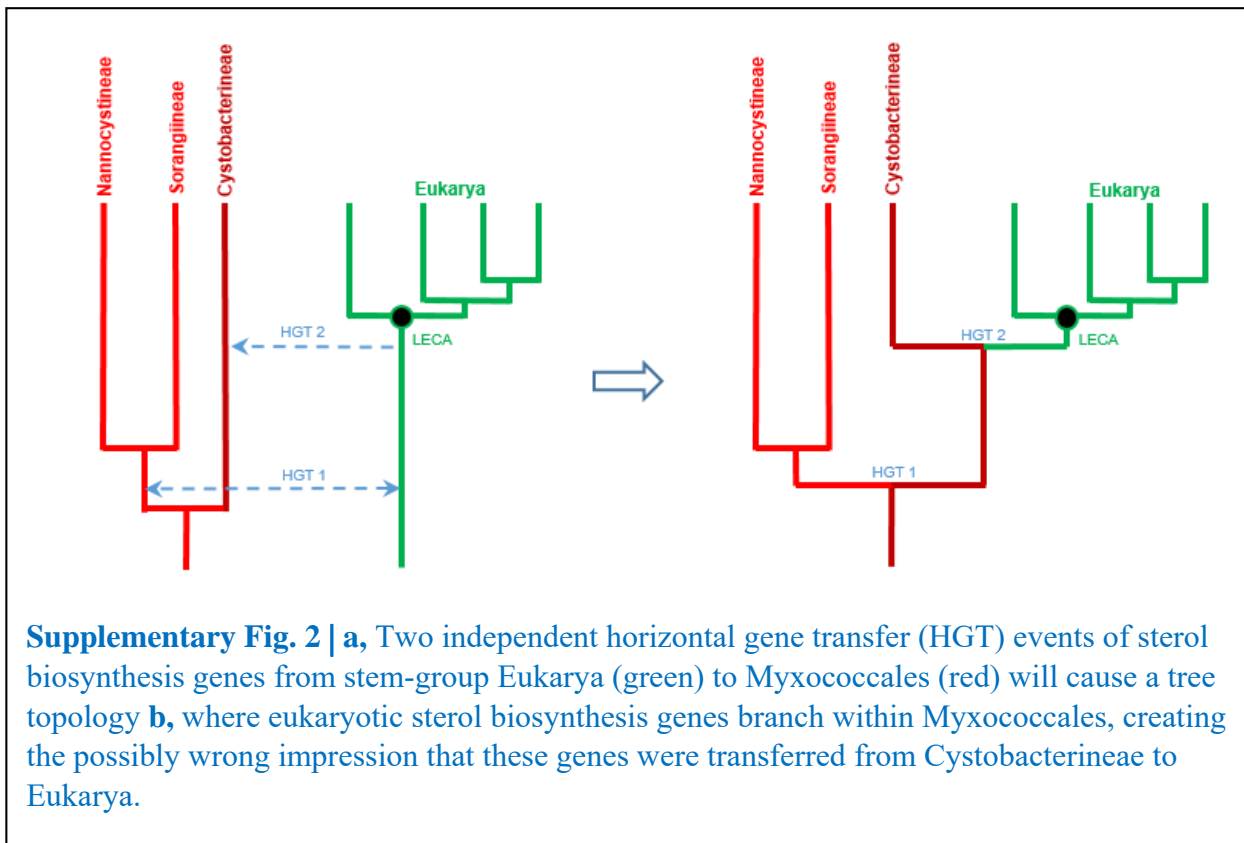
1326 chitinase from fungi to Bacilli at 408 Ma (323 to 498 Ma, 95% confidence interval) and from  
1327 Bacilli to Cystobacterineae at 275 Ma ago (174 to 389 Ma, 95% confidence interval) (see age  
1328 annotation in Supplementary Fig. 1). Based on these age estimates, Cystobacterineae is a very  
1329 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 event<sup>191</sup>. 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)<sup>33</sup> 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)<sup>33</sup>. 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)<sup>33</sup> 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 C<sub>27</sub> cholesteroloids as early as 2,300 Ma ago, and by 1,700 Ma  
1371 ago at the latest<sup>34</sup>. This implies that Myxococcales and eukaryotes were capable of C<sub>27</sub> sterol  
1372 production through the entire mid-Proterozoic interval. Yet, despite the abundance of  
1373 protosteroids, not even traces of indigenous, saturated C<sub>27</sub> 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)<sup>33</sup> 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 Eukarya<sup>33-35</sup>. 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 Ma<sup>192</sup>. 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 fungi<sup>191</sup>. 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)<sup>191</sup> with  
1410    published phylogenetic trees<sup>193-195</sup> 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 study<sup>196</sup>,  
1416    Methylococcales and Thiotrichaceae (sulfide oxidizing bacteria, SOB) radiate near the base of the  
1417    Gammaproteobacteria-Betaproteobacteria division (but also see ref<sup>197</sup> 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)<sup>198</sup>  
1420    suggested that the radiation of Thiotrichaceae occurred in the Neoproterozoic  $760 \pm 320$  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 Ma<sup>197</sup>. 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<sup>30,199</sup>. 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)<sup>35</sup> and Hoshino and Gaucher (2021)<sup>33</sup>,  
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)<sup>200</sup>.  
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 HGT<sup>33-35</sup>. 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 Ma<sup>197</sup>,  
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 bacteria<sup>30</sup> 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 bacteria<sup>30</sup>. As  
1467 methane consumed by Methylococcales is usually isotopically strongly depleted in <sup>13</sup>C, 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  $-22\text{‰}$ ) relative to bulk biomass ( $\delta^{13}\text{C}_{\text{kerogen}} = -30.4$  to  $-32.1\text{‰}$ )  
1471 and cyanobacterial lipids ( $\delta^{13}\text{C}_{\beta\text{-carotane}} = -37.3 \pm 0.9\text{‰}$ )<sup>36</sup>. The carbon isotopic values of the  
1472 steroids are consistent with a heterotrophic<sup>36</sup> 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)<sup>34</sup> 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<sup>34,35</sup>.

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 Verrucomicrobia<sup>35</sup>. However, for 18 species, sterol biosynthesis has been confirmed through lipid  
1504 surveys. *Gemmata obscuriglobus* (Planctomycetes) produces lanosterol and its double-bond  
1505 isomer parkeol<sup>187</sup>. The sterols are essential to growth and function as membrane molecules<sup>201</sup>.  
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 C<sub>30</sub> protosterols<sup>35,189,202</sup>. Myxococcales (Deltaproteobacteria) of the suborders  
1512 Nannocystineae and Sorangiineae (part of Group 1 and branching deeply outside Eukarya<sup>35</sup>) have  
1513 the distinguishing capacity to remove all methyl groups from positions C-4 and C-14 to produce  
1514 the C<sub>27</sub> 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 component<sup>203</sup>. 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<sup>35,190</sup>. 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 adriaticol<sup>204</sup>.

#### 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 sediment<sup>35</sup>. *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 ago<sup>34</sup>. (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 ref<sup>33</sup> 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 evolution<sup>205</sup> (see also existence of  
1544 SQMO and alternative-SQMO in eukaryotes<sup>206</sup>). (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 fruit<sup>207</sup>, 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 legumes<sup>208</sup>. These values are  
1555 comparable to a 0.1% sterol content in the polypore mushroom *Polystictus versicolor*<sup>189</sup>. 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 macroalgae<sup>209</sup> and an average of  $0.51 \pm 0.71\%$  (range 0.02-  
1558 2.6%) in ten species of microalgae<sup>210</sup>. 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% sterols<sup>211</sup>  
1560 and the fungal mould *Aspergillus nidulans* yielded 0.6-0.9%<sup>189</sup>. A sterol survey<sup>163</sup> 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 system<sup>163</sup>.

1565 Bloch (1983)<sup>28</sup> 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 contamination<sup>156</sup> such as traces  
1569 of cholesterol (0.0035%) in *Streptomyces olivaceus*<sup>212</sup>, 0.01% of a mixture of six sterols (including  
1570 ergosterol) in *Azotobacter chroococcum*<sup>213</sup> and traces in some other bacteria<sup>214</sup>. Reliable data exists  
1571 for *Gemmata obscuriglobus* with 2.0%<sup>187</sup>, *Nannocystis exedens* with 0.4%<sup>215</sup> and *Methylococcus*  
1572 *capsulatus* with 0.22%<sup>189</sup>. Extremely low concentrations of 0.00002% lanosta-8,22,24-triene were  
1573 detected in *Methylobacterium organophilum* (Alphaproteobacteria, Rhizobiales)<sup>216</sup>. 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 survey<sup>217</sup> 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 <sup>13</sup>C 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 intermediate<sup>217</sup>.  
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 ref<sup>218</sup>)*”. However, the authors also cautioned that  
1610 the sterol biosynthetic pathway of most protists remains unknown<sup>217</sup>. 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 vents<sup>35</sup>. 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, C<sub>28</sub> and C<sub>29</sub> 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 m<sup>2</sup>  
1629 of sea floor at 180 m depth in the Black Sea<sup>219</sup>. 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 cholestenol<sup>219</sup>. 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, δ<sup>13</sup>C 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 sources<sup>219</sup>. 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”<sup>219</sup>. 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 study<sup>220</sup> 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<sup>220,221</sup>. In fact, based on current knowledge, ciliates do not appear to  
1659 produce or contain protosterols as major membrane lipids<sup>157,159</sup>. 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 mats<sup>219</sup>, 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)<sup>35</sup>, 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 carbon<sup>222</sup>. 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 Eglinton (1984)<sup>223</sup> 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 ref<sup>184</sup>). 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 sterols<sup>224</sup>, 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 (C<sub>27</sub>, C<sub>28</sub> and C<sub>29</sub>) with minor contributions of cycloartenol and several 4-methyl  
1709 sterols<sup>225</sup>. 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 ref<sup>225</sup>). 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)<sup>226</sup> 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 C<sub>31</sub> 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 plants<sup>17</sup>, 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 sterols<sup>226</sup>, 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 mass<sup>227</sup>. 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 mat<sup>227</sup>. 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 chain<sup>33</sup>. Such organisms would have remained invisible  
1754 during the binocular microscopic inspection by McCaffrey et al. (1989)<sup>226</sup>.

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 study<sup>228</sup> 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 observed<sup>226</sup> 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 study<sup>222</sup> 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 study<sup>227</sup> purified sterols extracted from *Beggiatoa* mats from a hydrothermal  
1772 vent in the Guaymas Basin, reporting C<sub>27</sub> to C<sub>29</sub> sterols attributed to eukaryotes, but no  
1773 cyclolaudenol or protosterols. Likewise, a fourth study<sup>229</sup> 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 survey<sup>230</sup> 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 C<sub>29</sub>, C<sub>30</sub>, and C<sub>31</sub> steranes. The C<sub>30</sub> compound was identified as lanostane. The steranes were  
1779 strongly depleted in <sup>13</sup>C, suggesting a methanotrophic bacterial source. The C<sub>31</sub> compound is  
1780 described as a derivative of lanostane “methylated in ring A or ring B” (quote ref<sup>230</sup>), and can thus  
1781 not be a cyclolaudanoid or other side-chain methylated protosteroid. Based on a chromatogram  
1782 given in Figure 4 of ref<sup>230</sup>, the concentration of lanostane was substantially higher than of other  
1783 steranes (which are not visible in the chromatogram) and also higher than C<sub>30</sub> 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 sterols<sup>231</sup>. 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 sterols<sup>232</sup>. 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* mats<sup>226</sup>. 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 present<sup>233-242</sup>. Yet, many of these studies cite the work that had reported  
1798 cyclolaudenol in *Thioploca* mats<sup>226</sup>, 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)<sup>230</sup>

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*<sup>243</sup>. 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 Thiotrichales<sup>35</sup>. 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 Thiotrichales<sup>34</sup>. Likewise, the most recent and vastly expanded survey of sterol  
1814 biosynthesis genes did not yield any hits for Thiotrichales<sup>33</sup>

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 Peru<sup>226</sup>. 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 division<sup>196</sup>. Using molecular clocks calibrated on  
1820 SOB symbionts in bivalves, Canfield and Teske (1996)<sup>198</sup> estimated that this radiation of  
1821 Thiotrichaceae occurred in the Neoproterozoic  $760 \pm 320$  to  $620 \pm 280$  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 cholesterol, 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 study<sup>244</sup> 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-dimethylcholesterols were not detected,

1835 and minor abundances of 4-methyl cholesteroloids were attributed to dinoflagellates. This is  
1836 corroborated by a second survey<sup>245</sup> 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)<sup>246,247</sup>  
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 seasons<sup>246</sup> and, according to previous chemo-  
1862 taxonomic studies, grasses (Gramineae) colonized the savannas of Northern Brazil at the time of  
1863 deposition<sup>247</sup>. 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-rush<sup>246</sup>.

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<sup>248,249</sup>, 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<sup>248,249</sup>. Based on mass spectral interpretations, Jacob et al. (2007)<sup>246</sup> 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 ref<sup>246</sup>), 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)<sup>246</sup> 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<sup>246,247</sup>, 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<sup>246,247</sup> as protosterol methyl ethers do occur in several Gramineae species<sup>250</sup>.  
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)<sup>136</sup> 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 C<sub>30</sub> lanostane and  
1895 C<sub>31</sub> 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 ref<sup>251</sup>, lanostane is less abundant than 3 $\beta$ -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 lake<sup>251</sup>. 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 C<sub>30</sub> to C<sub>32</sub> lanostanes were also observed in Eocene lime-mudstones deposited under saline  
1908 stratified waters of the Bohai Bay basin, China<sup>252</sup>. 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  $\delta^{13}\text{C} = -80\text{‰}$ <sup>253</sup>. 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 C<sub>30</sub> lanostane and C<sub>31</sub> 24-methyl lanostane<sup>254</sup>. While hopanes and steranes were  
1917 abundant in all fractions of the oil, the lanostanes were exclusively detected in the sulfide  
1918 fraction<sup>254</sup>, 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 study<sup>254</sup> 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 study<sup>255</sup> 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 ref<sup>255</sup>, 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 C<sub>27</sub> to C<sub>29</sub> regular and diasteranes, lanostanes and 4-desmethyl  
1928 lanostanes). The origin of the lanostanes remains unknown.

1929 C<sub>30</sub> to C<sub>32</sub> 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 India<sup>256</sup>. Mass spectra were not provided for the two higher  
1932 homologs, so their structures remain unclear. The authors speculate that the C<sub>31</sub> and C<sub>32</sub>  
1933 compounds formed in the sediment through diagenetic methylation. However, given the apparent  
1934 high relative signal intensities of the C<sub>31</sub> and C<sub>32</sub> 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 lignite<sup>249</sup> and a sub-bituminous coal from the East Elbian coal basin<sup>248</sup>,  
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 Lauraceae<sup>257</sup>.

### 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 source<sup>258</sup>, 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)<sup>136</sup>  
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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