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BRIEF COMMUNICATION

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Algal origin of sponge sterane biomarkers negates the oldest evidence for animals in the rock record

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The earliest fossils of animal-like organisms occur in Ediacaran rocks that are approximately 571 million years old. Yet 24-isopropylcholestanes and other C_{30} fossil sterol molecules have been suggested to reflect an important ecological role of demosponges as the first abundant animals by the end of the Cryogenian period (>635 million years ago). Here, we demonstrate that C_{30} 24-isopropylcholestane is not diagnostic for sponges and probably formed in Neoproterozoic sediments through the geological methylation of C_{29} sterols of chlorophyte algae, the dominant eukaryotes at that time. These findings reconcile biomarker evidence with the geological record and revert the oldest evidence for animals back into the latest Ediacaran.

Animals rapidly diversified and became ecologically dominant at the beginning of the Cambrian period (541–485 million years ago (Ma)). Yet, the first animals must have arisen much earlier, in the Neoproterozoic era (1,000–541 Ma), although the exact timing remains contentious. The oldest uncontroversial animals in the fossil record are found in deposits from 558–552 Ma among the biota of the Ediacaran period[1–3], as confirmed by fossil lipid signatures recovered from *Dickinsonia* fossils[4]. With some confidence, animals can be traced back as far as the earliest Ediacaran biota communities at 571 Ma (ref. [5]). However, most molecular clocks place the appearance of animals within the range of 900–635 Ma, during the Cryogenian or Tonian periods[6–10]. There is therefore a gap of up to 340 million years between the oldest animal fossils and molecular clock estimates that may be due to either the scarcity of the fossil record[11] or because of a systematic offset in molecular clocks that are normally constrained by fossil occurrences[12].

The discrepancy between molecular clocks and the fossil record is seemingly reduced by molecular fossils (biomarkers). Biomarkers are remains of biological compounds that are stable over geological timescales. Whereas most biomarkers are representative of broad phylogenetic groups, selected compounds can be diagnostic for more specific biological sources[13,14]. Sterols with 30 carbon atoms and unusual side chains, for example, are almost exclusively known from demosponges[15,16]. The fossil products of such sterols, C_{30} steranes, are particularly abundant in rocks from the Neoproterozoic and lower Palaeozoic eras and are commonly attributed to demosponge sources[15–19]. Although sterol precursors of C_{30} steranes with an *n*-propyl substitution at C-24 (24-npc, Fig. 1a) and—in trace amounts—isopropyl substitution (24-ipc, Fig. 1a) are also found in pelagophyte algae, their biosynthetic capability to produce these molecules possibly arose in the Palaeozoic era (541–419 Ma), leaving sponges as the most plausible source organisms of C_{30} steranes in older sedimentary rocks[20]. C_{30} sterols with a 26-methylstigmastane (26-mes, Fig. 1a) skeleton were likewise proposed as diagnostic sponge markers[15,17]. All three C_{30} steranes have been found in sediments dating to the beginning of the Ediacaran period (635–541 Ma), and even in one Cryogenian sample (>635 Ma), thus apparently extending the geological record of sponges ~100 million years beyond the oldest diagnostic body fossils (Fig. 1b)[15,17,18]. The uniform distribution of C_{30} steranes in Ediacaran sediments from diverse environments would indicate that demosponges were extraordinarily abundant and widespread across marine ecosystems[15,17,18].

However, several observations have challenged the assignment of these C_{30} steranes to a demosponge origin[21–24]. According to the fossil record, crown-group demosponges started to diversify only in the Cambrian[22]. C_{30} sterols are usually only minor constituents in demosponges that otherwise produce many more unusual sterol structures[15], which have never been identified in the rock record[21]. Furthermore, C_{30} steranes are ubiquitously present in Ediacaran and lower Palaeozoic sedimentary rocks, including those that formed in anoxic basins[25–27], whereas sponges, although tolerant of low levels of oxygen, are unable to survive in completely anoxic waters[28].

An alternative interpretation was recently suggested for the origin of C_{30} steranes in the rock record[21]. The authors converted sterols of unicellular Rhizaria into geological sterane equivalents by means of hydrogenation, and suggested that precursors to all three C_{30} steranes are present in small amounts in these protists, in relative proportions that often match the deep-time biomarker record[21]. Rhizarians can be highly abundant in the upper ocean, potentially explaining the abundance of C_{30} steranes in anoxic basins[21]. However, all Rhizaria screened until now have only contained traces of the C_{30} sponge steranes 26-mes and 24-ipc at concentrations seemingly too low to account for the Proterozoic biomarker record[19].

Rhizarians and other heterotrophic protists were probably common among Neoproterozoic and early Palaeozoic planktonic communities[29], but there is one group of eukaryotes that would have constituted an even more notable proportion of deposited marine biomass, regardless of local environmental conditions; namely, eukaryotic algae. Suggestions that C_{30} steranes might be derived from algae have been voiced previously[22,24,30], but were hypothetical and not supported by sterol distributions in extant species[31].

The dominant sterane in Ediacaran and lower Palaeozoic sediments is stigmastane with 29 carbon atoms, which can represent up to 90% of C_{27}–C_{29} steranes[32–34]. Excluding phaeophyceaen algae,

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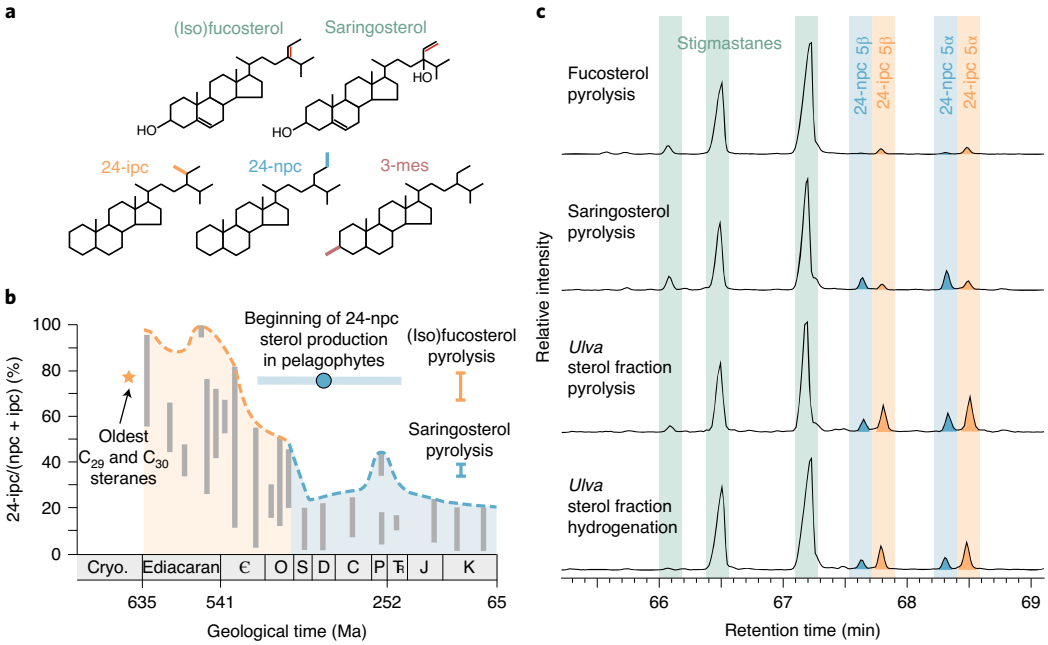

Fig. 1 | Distribution of C_{30} steranes in the rock record and in the pyrolysis/hydrogenation products from algal sterols. a, Molecular structures of isofucosterol, fucosterol, saringosterol and C_{30} steranes. **b**, Relative proportion of 24-ipc and 24-npc through time. A 24-ipc/(npc + ipc) ratio > 33% was suggested to indicate the presence of demosponges[18]. The orange star marks the only reported Cryogenian (Cryo.) sample that contains indigenous steranes[18]. The dashed line highlights the maximum reported 24-ipc/(ipc + npc) ratio through geological time, the colour indicates whether the maximum ratio is greater (orange) or smaller (blue) than 50%. The grey vertical lines show the range of reported 24-ipc/(ipc + npc) values[4,15–18,25,47]. The blue horizontal line marks a 95% confidence interval for the potential beginning of 24-npc input from pelagophyte algae[20] (modified after refs. [21,47]). The orange and blue bars on the right show the ranges of 24-ipc/(npc + ipc) ratio values produced by pyrolysis of (iso)fucosterol and saringosterol. **c**, MRM chromatograms (*m/z* 414 → 217) showing C_{30} steranes produced by pyrolysis or hydrogenation of (iso)fucosterol and saringosterol (Supplementary Table 3). The green lines highlight, from left to right, ααα20S, βαα20R and ααα20R stigmastanes (C_{29} steranes), which are ghost peaks on this transition and only presented to show the relative elution time and isomer distributions of C_{29} and C_{30} steranes. Note that the relative peak height between C_{29} and C_{30} steranes is not proportional.

which evolved after the Ediacaran[35], the only plausible organisms with such an extreme C_{29} predominance are green algae (Chlorophyta), some of which have a near-100% C_{29} predominance among sterols (Supplementary Table 1)[36,37]. Although sterols of green algae are well studied, C_{30} sterols have never been observed among them. However, it is well established that processes in sediments can generate molecular alteration products, including the removal and addition of methyl groups[38–40]. Based on the observation that C_{29} sterols were so abundant, it is possible that C_{30} steranes were generated within sediments through methylation of algal C_{29} sterols.

In specific types of sterols, the presence of functional groups, such as double bonds, might provide a site for increased reactivity, potentially mediating methylation of the side chain during diagenesis. This process commonly occurs at ring A of sterols and results in the wide occurrence of 2- and 3-methyl steranes in oils and bitumens (Fig. 1a, 3-mes structure)[38,39]. In principle, double bonds in the side chain of algal C_{29} sterols might similarly facilitate methylation of the ethyl moiety of the side chain (Fig. 1a, red double bonds). Both microscopic and macroscopic green algae that have the strongest C_{29} predominance (consistent with the strong C_{29} predominance in Ediacaran–Cambrian rocks and oils) contain side-chain functionalized C_{29} sterols, such as isofucosterol (Fig. 1a), as their primary sterol (up to 100%; Supplementary Tables 1,2). In theory, double bonds in the side chain of isofucosterol might facilitate alkylation at the 24-ethyl moiety, thus producing 24-ipc and/or 24-npc.

To test whether 24-ipc and 24-npc may form by side-chain alkylation of algal C_{29} sterols, we subjected chlorophyte lipid extracts to pyrolysis, a method that is classically used to mimic transformations of biological molecules within sediments[40–43]. We pyrolysed algal extracts containing varying amounts of isofucosterol, its *trans*-isomer fucosterol, and their most common autoxidation product, saringosterol, which carries an additional hydroxyl group at the C-24 position alongside an unsaturation between C-28 and C-29 (Fig. 1a, Δ^{28}). Our experiments showed that 24-ipc and 24-npc (up to 1.6% of resulting steranes) can indeed form by abiogenic side-chain methylation of C_{29} sterol precursors. Fucosterol and isofucosterol with a double bond at $\Delta^{24(28)}$ were predominantly methylated at C-28, generating 24-ipc, and saringosterol with a $\Delta^{28(29)}$ double bond largely yielded 24-npc (Fig. 1c and Supplementary Table 3). The pyrolysis of isolated isofucosterol and fucosterol thus created more 24-ipc than 24-npc (24-ipc/(24-ipc + 24-npc) = 70–77%), and saringosterol yielded 24-ipc/(24-ipc + 24-npc) = 34–39%. The results of our pyrolysis experiments thus cover the large range of C_{30} sterane distributions observed in Ediacaran and early Palaeozoic deposits (Fig. 1b). As the fresh algal extracts did not contain any biological C_{30} sterols, the C_{30} steranes must have been generated during pyrolysis through methylation of the C_{29} precursors. Likewise, we posit that C_{30} steranes in Ediacaran to early Palaeozoic deposits largely represent diagenetic methylation products of algal C_{29} sterols that possess functionalized side chains.

We also performed catalytic hydrogenation experiments on algal extracts containing isofucosterol and saringosterol. These experiments produced low amounts of C_{30} steranes (0.005–0.17% of total steranes), with 24-ipc/24-npc ratios similar to those produced by pyrolysis (Fig. 1 and Supplementary Table 3), suggesting that the small amounts of C_{30} steranes previously reported from either

pyrolysis or hydrogenation of algae[44], rhizarians[21] and sponges[15,18] may also derive from C_{29} sterol precursors.

Our results show that 24-ipc and 24-npc can be generated from algal C_{29} sterol in experiments that simulate diagenetic processes. Moreover, a previous study[45] demonstrated that the other two proposed sponge biomarkers, 26-methylcholestane and 26-methylstigmastane, also form during pyrolysis and that this could have occurred in Neoproterozoic sediments. The diagenetic methylation of algal sterols explains unusual characteristics of the Neoproterozoic sterane record, such as the parallel emergence and distribution of C_{29} and C_{30} steranes in Earth history[18] (Fig. 1b) and the unchanging presence of alleged sponge indicators in diverse aquatic ecosystems, including anoxic basins[15,18,25–27].

The pyrolysis experiments further suggest that the predominance of 24-ipc among C_{30} steranes, typical for the Ediacaran and early Palaeozoic, reflects specific unsaturation patterns of the dominant algal sterols, such as high proportions of isofucosterol. Together, the results of this study and of ref. [45] imply that sterols are readily methylated during diagenesis at positions carrying a double bond or other functional group, that the process is abiogenic, and that any interpretation of minor steranes in the rock record needs to consider both biogenic and diagenetic origins. As a consequence, the 'sponge biomarkers', although in some cases possibly of direct biogenic origin, are no longer diagnostic for early animals. The rock record thus becomes consistent with an early Cambrian diversification of crown-group demosponges as deduced from body fossils[22]. In the absence of diagnostic sponge markers, the oldest evidence for animals in the rock record comes from the Ediacaran biota fossils and their associated biomarker signatures[1–5], and the exact timing of the appearance of animals still remains to be uncovered.

Methods

Materials. All solvents used in the study were 99.9% grade (UltimAR; Mallinckrodt Chemicals). Glassware, glass fibre filters and aluminium foil used in the laboratory were cleaned by combustion at 300 °C for 9 hours, and metal appliances were washed with methanol and dichloromethane (DCM) before use.

Both macroscopic and microscopic green algae include species with isofucosterol predominance, and we chose macroalgae for our experiments because of the convenience of their purification and extraction. Collected macroalgae were identified visually. Green macroalgae, *Ulva lactuca*, which were used for the pyrolysis and hydrogenation experiments, and *Chaetomorpha* sp., only used for screening of sterol composition, were collected on the south-east coast of Australia (Batemans Bay area, New South Wales), washed with deionized water to clean the algae from any epiphytes (Millipore Elix 3 UV), and dried at room temperature (20–25 °C). *Sargassum fusiforme* and *Undaria pinnatifida*, the brown macroalgae used for the pyrolysis and hydrogenation experiments, were bought dried (Spiral Foods, Australia).

Extraction and purification of sterols. All algae were extracted by means of ultrasonic agitation in solvents (methanol:DCM, 3:2 for 15 min, twice; and DCM:*n*-hexane, 2:3 for 15 min). The extract was fractionated into non-polar and polar fractions using microcolumn chromatography over annealed (300 °C, 12 hours) and dry-packed silica gel (Silica Gel 60, 230–600 mesh, EM Science). Non-polar molecules were eluted with 1.5 ml of *n*-hexane:DCM (1:1) and the polar fraction with 1 ml DCM:methanol (1:1). The polar fractions were saponified in 1 ml of 5% KOH methanol:water (4:1) solution at 50 °C overnight, 1 ml of water was added and a neutral fraction was extracted by vortexing for 1 min with 1 ml of DCM:*n*-hexane (1:4, three times). The neutral fraction, which we refer to as the 'sterol fraction', consisted predominantly of sterols as well as some phytol, the only other notable compound present. Sterols were further converted to the trimethylsilyl (TMS) ether derivatives for gas chromatography–mass spectrometry (GC–MS) analysis.

To separate isofucosterol and fucosterol from saringosterol in the sterol fractions of *S. fusiforme* and *U. pinnatifida*, a part of the TMS-derivatized neutral fraction was again fractionated into non-polar and polar fractions using silica gel microcolumn chromatography. As the hydroxyl group in the ring system was derivatized with TMS, isofucosterol and fucosterol eluted in the non-polar fraction. The additional hydroxyl group in the side chain of saringosterol, however, was not derivatized with TMS[44]. Therefore, saringosterol eluted in the polar fraction, which was also slightly contaminated with a small proportion of isofucosterol and fucosterol (Supplementary Table 4). To reconvert the TMS-ether group back to the hydroxyl group in all fractions, samples were saponified once again as described above, and after 1 ml of water was added, the solutions were acidified with two drops of 36% hydrochloric acid before extraction with 1 ml of DCM:*n*-hexane (1:4, three times).

Pyrolysis and hydrogenation of sterols. For pyrolysis, total neutral fractions, as well as purified (iso)fucosterol and saringosterol fractions, were dried under a gentle stream of nitrogen, redissolved in DCM and transferred to glass pyrolysis tubes (6 mm external diameter). After the DCM evaporated, the tubes were flushed with nitrogen gas, evacuated using a vacuum line, and flame sealed. The sealed tubes were then heated to 310 °C for 16 hours. After cooling, the tubes were opened and washed with DCM to collect the pyrolysate, which was fractionated into non-polar and polar fractions, as described above. The non-polar fraction was dissolved in *n*-hexane and hydrogenated in a 4 ml vial by bubbling a gentle stream of hydrogen gas for 20 min while stirring, with ~0.2 mg of platinum (IV) oxide on charcoal added as a catalyst. Then the hydrogen gas was turned off, the vials were closed and stirring was continued for 16 hours. The samples were separated from the catalyst by means of ultrasonic agitation in solvents (*n*-hexane (twice) and *n*-hexane:DCM (1:1, once)) followed each time by centrifugation. Samples were cleaned from any remaining catalyst by eluting with an excess of *n*-hexane over a small chromatographic column filled with activated silica gel.

Additionally, to separate the effects of pyrolysis from subsequent hydrogenation on the non-polar fractions of pyrolysates, we performed hydrogenation-control experiments: three samples of *U. lactuca* neutral fractions were directly hydrogenated (that is, without prior pyrolysis) following the same procedure as for the non-polar fractions of pyrolysates.

For hydrogenation-only experiments (without pyrolysis), the neutral fraction of *U. lactuca* was dissolved in *n*-hexane and hydrogenated in a 4 ml vial by bubbling a gentle stream of hydrogen gas for 5 hours while stirring, with ~2 mg of platinum (IV) oxide on charcoal added as a catalyst, and subsequently separated from the catalyst as described above. Hydrogenated samples were fractionated into non-polar and polar fractions as described above, and the non-polar fraction was used for analysis.

Gas chromatography–mass spectrometry. GC–MS analyses of biomarker extracts were carried out on an Agilent 6890 gas chromatograph coupled to a Micromass Autospec Premier double sector mass spectrometer (Waters Corporation) at the Australian National University. The gas chromatograph was equipped with a 60 m DB-5 MS capillary column (0.25 mm inner diameter, 0.25 µm film thickness, Agilent), and helium was used as the carrier gas at a constant flow of 1 ml min^{-1}. Samples were injected in splitless mode into a Gerstel PTV injector at 60 °C (held for 0.1 min) and heated at 260 °C min^{-1} to 300 °C. For full-scan and metastable reaction monitoring (MRM) analyses, the GC oven was programmed from 60 °C (held for 4 min) to 315 °C at 4 °C min^{-1}, with a total run time of 100 min. All samples were injected in *n*-hexane to avoid deterioration of chromatographic signals by $FeCl_2$ build-up in the MS ion source through the use of halogenated solvents[46]. Molecules were identified based on their retention time and mass spectra (Extended Data Fig. 1) in comparison to laboratory standards. Relative proportions of steranes were quantified in MRM mode in $M^+ \rightarrow 217$ transitions. To ensure accurate integration, sterol TMS-ethers from algae were quantified in full-scan mode using the m/z 129 chromatographic trace. Peak areas are uncorrected for differences in GC–MS response.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data that support the paper are presented in the main text of the manuscript and in the Supplementary Information.

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Author contributions

I.B. conceived the study, performed the analyses and wrote the first draft. J.M.H. and J.K.V. helped with compound identification. I.B. and B.J.N. interpreted the data. I.B., J.M.H., B.J.N., J.K.V., C.H. and J.J.B. prepared the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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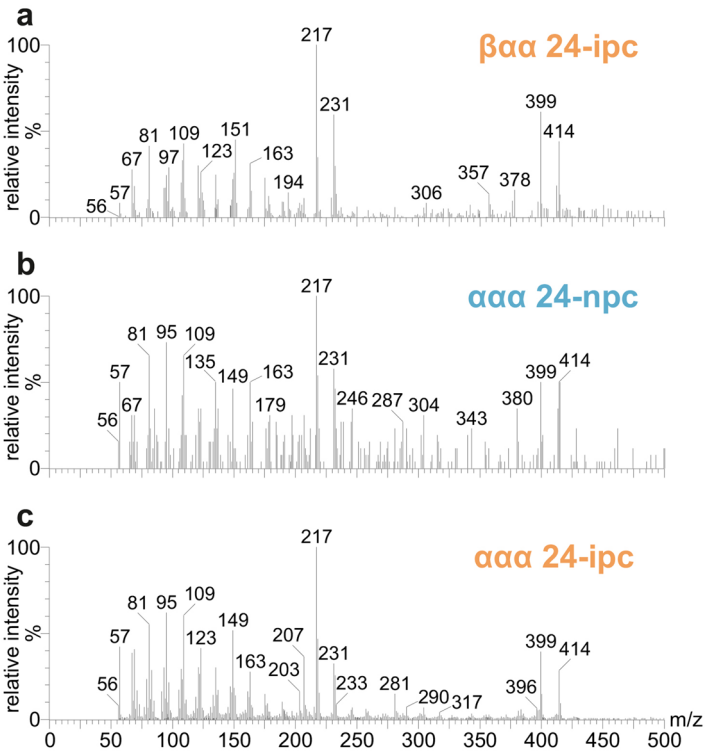

Extended Data Fig. 1 | Mass spectra of steranes produced via pyrolysis of (iso)fucosterol and saringosterol. All mass spectra presented in this figure were obtained from one of the *S. fusiforme* pyrolysates; it was not possible to obtain a pure mass spectrum for βαα 24-npc due to coelution with other compounds.

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Study description	Pyrolysis of sterols in laboratory
Research sample	Sterols of Ulva lactuca, Chaetomorpha sp., Sargassum fusiforme, and Undaria pinnatifida
Sampling strategy	No sample-size calculation was performed
Data collection	Experiments were performed at the Australian National University
Timing and spatial scale	No time-dependent measurements were made
Data exclusions	No data were excluded
Reproducibility	All attempts to repeat the experiment were successful.
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