Demosponge steroid biomarker
26-methylstigmastane provides evidence for Neoproterozoic animals

J. Alex Zumberge1, Gordon D. Love2*, Paco Cárdenas2, Erik A. Sperling3, Sunithi Gunasekera2, Megan Rohrssen4, Emmanuelle Grosjean5, John P. Grotzinger6 and Roger E. Summons7

Sterane biomarkers preserved in ancient sedimentary rocks hold promise for tracking the diversification and ecological expansion of eukaryotes. The earliest proposed animal biomarkers from demosponges (Demospongiae) are recorded in a sequence around 100 Myr long of Neoproterozoic–Cambrian marine sedimentary strata from the Huqf Supergroup, South Oman Salt Basin. This C30 sterane biomarker, informally known as 24-isopropylcholestone (24-ipc), possesses the same carbon skeleton as sterols found in some modern-day demosponges. However, this evidence is controversial because 24-ipc is not exclusive to demosponges since 24-ipc sterols are found in trace amounts in some pelagophyte algae. Here, we report a new fossil sterane biomarker that co-occurs with 24-ipc in a suite of late Neoproterozoic–Cambrian sedimentary rocks and oils, which possesses a rare hydrocarbon skeleton that is uniquely found within extant demosponge taxa. This sterane is informally designated as 26-methylstigmastane (26-mes), reflecting the very unusual methylation at the terminus of the steroid side chain. It is the first animal-specific sterane marker detected in the geological record that can be unambiguously linked to precursor sterols only reported from extant demosponges. These new findings strongly suggest that demosponges, and hence multicellular animals, were prominent in some late Neoproterozoic marine environments at least extending back to the Cryogenian period.

The transition from unicellular protists to multicellular animals constitutes one of the most intriguing and enigmatic events in the evolutionary history of life, largely due to the absence of unambiguous physical fossils for the earliest fauna. The Neoproterozoic rise of eukaryotes1, including demosponges2, in marine environments can be discerned from lipid biomarker records preserved in ancient sedimentary rocks that have experienced a mild thermal history. Molecular phylogenies commonly show that sponges (Porifera) are the sister group of other animals3 and molecular evidence for Neoproterozoic animal life was first proposed based on the occurrence of unusual C29 demosponge-derived steranes informally known as 24-isopropylocholestone (24-ipc) steranes in sedimentary rocks and oils of that age4–9. These steranes are the hydrocarbon remains of 24-isopropylocholesterols and structurally related sterols10. The record of 24-ipc steranes commences in Cryogenian-aged sediments in South Oman (around 717–635 million years ago (Ma)11–13) and then occurs continuously through the Ediacaran–Cambrian formations of the Huqf Supergroup of the South Oman Salt Basin. Notably, these steroids also occur as covariantly bound constituents fixed within the immobile kerogen phase of the same rocks, which is an important confirmation that these are not younger contaminant compounds that migrated into the rocks14.

Demosponges are the only known extant taxon that can biosynthesize 24-ipc precursors as their major sterols. High relative abundances of 24-ipc steranes have now been reported in many other late Neoproterozoic–early Cambrian rocks and oils15–18. These 24-ipc occurrences—if interpreted correctly—reflect an early presence of Porifera and provide a conservative minimum time estimate for the origin of animal multicellularity and the sponge body plan. Others have hypothesized that the 24-ipc steranes could be derived from unicellular animal ancestors or have an algal origin since the parent sterols have been reported in trace amounts in some pelagophyte algae19. The claim that poribacterial sponge symbionts from the candidate phylum Poribacteria can make 24-ipc steroids10 has since been shown to be erroneous due to a genome assembly error12,13.

Currently, two chromatographically resolvable series of ancient C29 steranes are known: 24-n-propylcholestone (24-npc) and 24-ipc. Demosponges are the most plausible Neoproterozoic–Cambrian source of 24-npc as well as 24-ipc because both are produced by extant demosponges20. Foraminifera are another possible source of 24-npc21. Pelagophyte algae probably account for the 24-npc steranes that are found in Devonian and younger marine sediments and their derived oils22. Various recent findings support a pre-Ediacaran origin of animals and sponges, and arguably reinforce the validity of the 24-ipc biomarker record, including: (1) steroid assays and genomic analyses of extant taxa23, which suggest that sponges were the most likely Neoproterozoic source biota for 24-ipc steranes; and (2) nuclear and mitochondrial gene molecular clock studies, which consistently support a pre-Ediacaran origin of animals and Neoproterozoic demosponges15–18. The discovery of other sponge biomarkers to augment the 24-ipc sterane record would greatly strengthen evidence for the presence of animals before the appearance of the Ediacara fauna, since the efficacy of the standalone
24-ipc sterane record for tracking early demosponges has been contested\textsuperscript{16,12}.

**Results and discussion**

Here, we report the presence of a new C\textsubscript{30} sterane designated 26-methylstigmastane (26-mes) in a suite of Neoproterozoic–Cambrian rocks and oils (Fig. 1 and Supplementary Tables 1–3). Furthermore, we attribute this biomarker to demosponges since these are the only known organisms among extant taxa to produce sterols with the same carbon skeleton (Supplementary Information). The abundance of 26-mes sterane biomarkers is of comparable magnitude to 24-ipc and 24-npc (Fig. 1), although the relative proportions of the three main C\textsubscript{30} sterane compounds can vary from sample to sample (Fig. 1 and Supplementary Tables 1 and 2). Summed C\textsubscript{30} steranes are typically 1–4% of the total C\textsubscript{27–30} sterane signal in South Oman rocks, although higher contents >5% can also be found. Our analyses confirm the presence of 26-mes along with 24-ipc steranes in the Neoproterozoic–Cambrian rock extracts and kerogen pyrolysates from South Oman reported previously\textsuperscript{2,8}, as well as in Ediacaran–Cambrian-sourced oils from Eastern Siberia\textsuperscript{1} and India\textsuperscript{1}, for which representative samples are shown in Fig. 1 (see also Supplementary Table 3). When 26-mes is detectable in Cryogenian to Cambrian age rocks and oils, it is found alongside both the 24-ipc and 24-npc sterane compounds. These three different sterane series constitute only a small subset of all the structural possibilities for C\textsubscript{30} sterane compounds, which are feasible from adding three additional carbons to a cholestane side chain, and they correspond with the three most commonly occurring sterane skeletons for C\textsubscript{30} sterols found in extant demosponges (Fig. 1 and Supplementary Tables 4 and 5). In contrast, 26-mes abundance is typically lower or absent for the small suite of Phanerozoic oils and rocks analysed thus far but can be detected, along with 24-ipc and 24-npc, in some samples but not in the procedural blanks (Supplementary Table 3).

To unequivocally confirm the assignment of the newly identified ancient sterane series as 26-mes, we compared the C\textsubscript{30} sterane distributions of Neoproterozoic rocks and oils with sterane products derived from steroids of modern sponges comprising demosponges, hexactinellids, homocladomorphs and calcisponges (Supplementary Tables 4 and 5). We applied catalytic hydroxyprolysis (HyPy)—a mild reductive technique employing high-pressure hydrogen—to transform sterols from sponge biomass into steranes with minimal structural and stereochemical disturbance\textsuperscript{21}. Only three compatible parent C\textsubscript{29} sterols, with an identical side chain skeleton to 26-mes, are currently known in extant taxa (Fig. 2), and these were the probable precursors to the sedimentary 26-mes described above (Supplementary Information). *Rhabdastrella globostellata* was used as a model sponge species for initial investigations since its sterols have been previously well characterized\textsuperscript{16,21} and it contains stellasterol as the major C\textsubscript{30} sterol constituent, which was verified for our specimens (Supplementary Fig. 1). We generated a simple C\textsubscript{30} sterane distribution as expected from the HyPy conversion of the *R. globostellata* sterols, dominated by 26-mes stereoisomers (Supplementary Fig. 2), and these products were used as a sterane standard to unequivocally test for presence or absence of the 26-mes biomarker in modern and ancient samples (Fig. 1). The identification of the fossil 26-mes sterane series was verified by observing co-elution of the 5\(\alpha\)t,14\(\alpha\),17\(\alpha\)(H)-20R stereoisomer with the same isomer produced from *R. globostellata* and other extant demosponges (Fig. 1). This co-elution was further confirmed using two different gas chromatography–mass spectrometry (GC-MS) techniques in two different laboratories using different gas chromatography column stationary phases (see Methods).

To better constrain the taxonomic distribution of 26-mes, we supplemented literature reports with targeted analyses of extant
sponges using HyPy to directly convert sterols into steranes (Supplementary Table 5). Species of Rhabdastrella and Geodia both produced appreciable amounts of 26-mes after the reductive conversion of sterols to steranes via HyPy treatment (1–9% of total C27–30 steranes, Supplementary Table 5). Apart from Geodia hentscheli, which only makes conventional sterols for which side chain alkylation is restricted to the C-24 position, 26-mes was the predominant C26 sterane product in our Rhabdastrella and Geodia specimens. Molecular phylogenetic results indicate that these species are closely related within Geodiidae (order Tetractinellida). Additionally, we also detected trace amounts of 26-mes steranes along with 24-ipc and 24-ncp in four species of Aplysina and Verongula (order Verongiida) and one species of Cymbaxinella (order Agelasida) (Supplementary Table 5). No 26-mes precursors were detected in a Jasps species, where stelliferasterol and isostelliferasterol were supposedly originally discovered. This is consistent with the belief that the original Great Barrier Reef ‘Jasps stellifer’ specimens were misidentified and were in fact R. globostellata2. Our HyPy results for three specimens of R. globostellata confirmed that 26-mes sterol precursors were present, as well as in two other Rhabdastrella species. Other than the Geodiidae, another known major source of 26-mes steroids is Petrosia (Strongylophora) cf. durissima3 (order Haplosclerida), which can synthesize strongylosterol (Fig. 2) as its dominant single sterol. As not all demosomes make 26-mes, Geodiidae and P. (S.) cf. durissima may have retained the ancestral capacity to make terminally methylated C30 steroids as major membrane lipids, which has been lost in other demosome groups.

Our new findings of 26-mes production in Geodia, Rhabdastrella, Aplysina (aspiculate), Verongula (aspiculate) and Cymbaxinella species suggest that a wider range of demosome groups might possibly make 26-mes, as well as other terminally methylated steroids, but have not yet been identified. These demosome species and others can make various unusual C24 and C30 sterols with terminal methylation in the side chain (Supplementary Information). For example, Thymosiopsis conglomerans (order Chondrillida) makes a distinctive C30 sterol24, yielding a different sterane skeleton that has not been detected in the ancient record (Fig. 1). The finding of this extra terminal carbon atom in a variety of sterols from diverse extant demosponges suggests that the capability for 26-methylated sterol side chains probably has a deep origin within the clade. In the case of both 24-ipc and 26-mes, the ability to make these sterols is phylogenetically widespread within demosomes. Notably, the known extant demosponge species that contain 26-mes as the dominant hydrocarbon core of their C30 steranes are different from those that make 24-ipc as major steroids. Specifically, the demosponge family Halichondriidae (Gioia/calypta (previously Collocalypta), Halichondria and Epipolaxis) and the genus Topsentia make 24-ipc among their most abundant sterols22 while 24-ipc constitutes >99% of sterols in Cymbastela coralliophila (Pseudoa/xynissia species in the original publication) from the family Axinellidae.

From a comprehensive database of steroid assays performed on extant organisms from decades of lipid research, alongside our targeted assays here, 26-mes precursor sterols are found only in certain demosponge groups—but to our knowledge—have never been reported from any other group of eukaryotes (Supplementary Information). This evidence of absence applies to diverse groups of algae35,36, hexactinellid sponges (ref. 2 and this study) calcisponges (refs 2,29 and this study) homocelomorphs (this study) and unicellular animal outgroups37. Indeed, only steroids possessing conventional side chains (with methyl, ethyl or propyl groups or a hydrogen substituent at C-24) have been reported for other sponge classes, heterotrophic protists and these unicellular animal outgroups38,39,40, but not steroids with unconventional side chains of any variety. Thus, the finding of 26-mes, together with the 24-ipc steranes in Neoproterozoic rocks and oils, is most parsimoniously explained by an origin from demosomes living in marine settings.

In terms of possible older occurrences of sponge biomarkers, robust evidence for steranes has been reported in some 800–700 Myr Neoproterozoic rocks29,30 from the Chuar Group (USA) and Visingsö Group (Sweden). These rocks contain an unusual C26 sterane, 26-methylcholestanone, informally designated as cryostane (Fig. 3), which has been proposed as a possible ancient sponge or unicellular metazoan marker. Cryostane is also characterized by the unusual terminal side chain methylation at C-26, making it a structural analogue of 26-mes and adding credence to the case for cryostane being a plausible ancient sponge biomarker. However, plausible precursor steroids for cryostane have not yet been found in any extant organisms, despite the discovery of a wide variety of other unconventional steroid structures in modern sponges. The Chuar and Visingsö Group rocks, like all pre-Sturtian-aged samples reported so far, are devoid of 24-ipc, 24-ncp and 26-mes steranes. Thus, cryostane cannot currently be applied as a robust animal biomarker until more is known about its biological origins and whether the biosynthetic capacity to make unconventional 26-methylated steroids (Fig. 4) is restricted to demosomes or otherwise. The origins of cryostane are intriguing and a bridging of the cryostane and 26-mes/24-ipc records may signify a continuity of sponge markers persisting through the two Neoproterozoic glaciation events (Fig. 3), but this requires further investigation.

Conclusions

The co-occurrence of 24-ipc and 26-mes steranes constitutes the earliest robust biomarker evidence for Neoproterozoic animals, first detected in the Cryogenian period before the deposition of the Marinoan cap carbonate (>635 Ma), but following the Sturtian glaciation (beginning at <717 Ma and terminating around 660 Ma)24. This suggests that demosomes first achieved ecological prominence in Neoproterozoic marine paleoenvironments, at least between 660 and 635 Ma, which is consistent with recent molecular clock predictions for their first appearance13,14,15. This view from molecular clocks and biomarkers remains to be reconciled.
with the fossil spicule record, which suggests a later (Cambrian) origin. Future sampling of modern taxa may reveal other sources of 26-mes steroids (see Supplementary Information), but multiple possibilities for taphonomic mega-bias of early sponge body fossils have been identified (see ref. 31 and the references therein), perhaps related to sparse biomineralization and/or silica dissolution and reprecipitation in low-oxygen marine conditions, despite overall higher Proterozoic oceanic silica levels. The records could also be reconciled if demosponge spicules evolved convergently in the Cambrian or if aspiculate demosponges were dominant producers of Neoproterozoic 26-mes and 24-ipc.

All available current data indicate that 26-mes steranes are made by diverse species of modern demosponges, and apparently not by any other sponge class (Hexactinellida, Homoscleromorpha or Calcarea) or other extant eukaryote, implying that Neoproterozoic total-group demosponges were the most probable source biota for these biomarkers. As demosponges are derived within Porifera, these data consequently predict the presence of sponges at this time irrespective of whether sponges or ctenophores are the sister group of all other animals. Thus, this new Neoproterozoic steroid biomarker evidence for demosponges provides a conservative minimum time estimate for the origin of animal multicellularity and the sponge body plan involving feeding with a water canal system.

Methods

Catalytic HyPy of sponge biomass. Continuous-flow HyPy experiments were performed on 30–150 mg of catalyst-loaded sponge biomass at the University of California, Riverside (UCR) as described previously2,19. Freeze-dried sponge biomass was initially impregnated with an aqueous methanol solution of ammonium dicyandiamidomolybdate ((NH₄)₂MoO₂S₂) to give a nominal loading of 3–10 wt% catalyst. Ammonium dicyandiamidomolybdate reductively decomposes in situ under HyPy conditions above 250 °C to form a catalytically active ammonium ammonium dioxide molybdate (NH₄)₂MoO₂S₂ phase. The catalyst-loaded samples were heated in a stainless-steel (316 grade) reactor tube from ambient temperature to 250 °C at 100 °C min⁻¹, then to 460 °C at 8 °C min⁻¹. A hydrogen sweep gas flow rate of 6 dm³ min⁻¹, measured at ambient temperature and pressure, through the reactor bed ensured that the residence times of volatiles generated were of the order of only a few seconds. Products were collected on a silica gel trap cooled with dry ice and recovered for subsequent fractionation using silica gel adsorption chromatography.

HyPy products (hydropyrolysates) of sponge biomass were separated by silica gel adsorption chromatography into aliphatic (alkane + alkene), aromatic and polar (or nitrogen, sulfur or oxygen) compounds by elution with n-hexane, n-hexane:dichloromethane (DCM) (1:1 v/v) and DCM:methanol (3:1 v/v), respectively. For hydropyrolysates, solvent-extracted activated copper turnings were added to concentrated solutions of aliphatic hydrocarbon fractions to remove all traces of elemental sulfur, which is formed from disproportionation of the catalyst during HyPy. Aliphatic fractions were further purified to a saturated hydrocarbon fraction by the removal of any unsaturated products (alkenes) via silver nitrate-impregnated silica gel adsorption chromatography and elution with n-hexane.

Lipid biomarker analysis of ancient rocks/oils. Detailed methods for the extraction and analysis of sedimentary rocks and oils at UCR were described previously2,24 and data are shown in Supplementary Table 3. Rock pieces were first trimmed with a water-cooled rock saw to remove outer weathered surfaces (of at least a few mm thickness) and expose a solid inner portion. They were then sonicated in a sequence of ultrapure water, methanol, DCM and hexane before a final rinse with DCM before powdering and bitumen extraction. Rock fragments were powdered in a zirconia ceramic puck mill in a SPEX 8515 shatterbox,
and cleaned between samples by powdering two batches of fired sand (850 °C overnight) and rinsing with the above series of solvents. Typically, 5 g of crushed rock was extracted in a CEM Microwave Accelerated Reaction System at 100 °C in a 45:55 methanol:methylene chloride mixture for 15 min. 26-mes sterane in our ancient oils was detected using 404 Da and 24-ipc in 414 Da ion chromatograms from C30 and C31 hopanes was used in the MRM-GC-MS instrument at UCR. GC-QQQ-MS was performed at GeoMark Research (Houston, Texas) on an Agilent 7000A Triple Quad interfaced with an Agilent 7890A gas chromatograph equipped with a J&W Scientific capillary column (DB-5MS, 0.25 mm i.d., 0.25 μm film thickness, 10 m length, 0.25 mm i.d., 0.25 μm film thickness, 10 m length, 60 m × 0.25 mm i.d., 0.25 μm film) using hexulim as the carrier gas. Typically, 1 μl of a hexulim fraction dissolved in hexulim was injected onto the gas chromatography column in splitless injection mode. The gas chromatography temperature programme consisted of a constant hold at 60 °C for 2 min, heating to 150 °C at 10 °C min⁻¹ followed by heating to 320 °C at 3 °C min⁻¹, and a final hold for 22 min. Analyses were performed via splitless injection in electron impact mode, with an ionization energy of 70 eV and an accelerating voltage of 8 kV. MRM transitions for C30–C32 hopanes, C19–C26 methylhopanes, C17–C19 steranes, C20–C22 steranes, and C23–C26 steranes were monitored. Polycyclic hydrocarbons were detected using 24-ipc in 325 °C at 2 °C min⁻¹) and a hold for 20 min. Data was analysed using ChemStation for the three 26-mes precursors (stelliferasterol (B13), isostelliferasterol (B14) and strongysterol (B15); see also Fig. 2) in certain Rhubadastella and Geodia sponge species were identified from published mass spectral features and relative retention times. Stelliferasterol was the dominant C20 sterol in R. globostellata (PC922; Supplementary Figs. 1–2), while strongysterol, stelliferasterol and isostelliferasterol were found in Geodia parva (Gp11).

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

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files.

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References


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Author contributions

J.A.Z. and G.D.L. planned the investigation and wrote the manuscript with input from J.A.Z. and G.D.L. planned the investigation and wrote the manuscript with input from the Marine Conservation Target fund of the Department of Fisheries and Oceans Canada. Other specimens were provided by E. Kenchington through funding from the Marine Conservation Target fund of the Department of Fisheries and Oceans Canada. Other specimens were provided by E. Kenchington as Canadian lead for the NEREEDA (NAFO Potential Vulnerable Marine Ecosystems-Impacts of Deep-sea Fisheries) project led by Spain and Canada. We thank K. Ubyhaysakera (Department of Chemistry, Uppsala University) for GC-MS analyses, and S. Rajendran and T. Aljaazar (Department of Medicinal Chemistry, Uppsala University) for help with the isolation of sponge sterols. We thank Petroleum Development Oman for Neoproterozoic–Cambrian rock samples from the South Oman Salt Basin for ancient biomarker analysis. We are grateful to D. Rocher for GC-QQQ-MS analysis and GeoMark Research (Houston, TX) for providing oil samples from Eastern Siberia.

Competing interests

The authors declare no competing interests.

Additional information

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Software and code

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| Data collection | No such software was used |
| Data analysis   | No such software was used |

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Ecological, evolutionary & environmental sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Study description | We analyzed and quantified the lipid biomarker content of a large suite of ancient sedimentary rocks and oils, specifically targeting ancient steroid biomarker compounds sourced by demosponges of Neoproterozoic-Cambrian age. |
| Research sample | We targeted ancient sedimentary rocks of low thermal maturity which allowed excellent preservation of lipid biomarker constituents |
| Sampling strategy | 64 ancient sedimentary rocks samples of Cryogenian-Cambrian age were analyzed to provide stratigraphic coverage of all formations from the Huqf Supergroup, South Oman Salt Basin. Ediacaran-Cambrian oils from Siberia and India, as well as some Phanerozoic source rocks, were also analyzed for comparison. |
| Data collection | Lipid biomarker analysis was performed at UC Riverside and MIT |
| Timing and spatial scale | Sedimentary rock and oils were obtained from research projects between 2005 and 2017, mostly supplied by oil companies from sub-surface drilling of wells. The rock and oils used are grouped by geological age and provenance. |
| Data exclusions | No exclusions, not applicable. |
| Reproducibility | Our reproducibility for molecular biomarker ratios and yields was assessed using oil hydrocarbon standards ran with each batch of analyses. |
| Randomization | The rocks and oil samples are grouped by geological age and provenance |
| Blinding | Not applicable |

Did the study involve field work? ☑ Yes ☑ No

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
| ☑ ☑ | Unique biological materials |
| ☑ ☑ | Antibodies |
| ☑ ☑ | Eukaryotic cell lines |
| ☑ ☑ | Palaeontology |
| ☑ ☑ | Animals and other organisms |
| ☑ ☑ | Human research participants |

Methods

| n/a | Involved in the study |
| ☑ ☑ | ChiP-seq |
| ☑ ☑ | Flow cytometry |
| ☑ ☑ | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

Modern sponge samples are available from the collections of two of the co-authors, Sperling and Cardenas.

Palaeontology

Specimen provenance

Sedimentary rock and oil samples were supplied by Petroleum Development Oman from wells drilled. PDO have approved publication of the data.
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