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EVOLUTION

Chemical characterization of C_{31} sterols from sponges and Neoproterozoic fossil sterane counterparts

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Putative metazoan body fossils from the Precambrian are curiously lacking morphological characteristics that link them unambiguously to extant animal phyla, including sponges. Chemical fossils such as the rare C_{30} hydrocarbons 24-*iso*-propylcholestane (24-*i*pc) and 26-methylstigmastane (26-mes), however, have been proposed as evidence for the Neoproterozoic emergence of the Demospongiae (Porifera) due to their prevalence in rocks of this age and the occurrence of their sterol precursors in contemporary demosponges. However, there are alternative hypotheses which posit that diagenetic alteration products of algal sterols, or those from Rhizaria or other protists, account for the enigmatic steroid distributions observed in these ancient sedimentary rocks. Here, we report additional support for the Neoproterozoic rise of demosponges through the chemical characterization of two previously unrecognized C_{31} hydrocarbons—24-*n*-butylcholestane (24-*n*bc) and 24-*sec*-butylcholestane (24-*sec*bc). Precursor C_{31} sterols from contemporary demosponges, as well as a suite of synthesized C_{31} sterol standards were reduced to their sterane counterparts. Gas chromatography-tandem mass spectrometry analysis and collisionally activated dissociation mass spectra confirmed the presence of 24-*n*bc and 24-*sec*bc in well-preserved early Ediacaran rocks, and the coelution of these compounds with synthetic standards enhances the robustness of these findings. Co-occurrence of abundant 24-*i*pc and 24-*sec*bc was found for numerous Neoproterozoic-Cambrian rock/oil samples, closely mimicking the abundance patterns and high structural selectivity of major C_{30} and C_{31} sterols detected in numerous species of modern demosponges. These findings support the hypothesized first emergence of sponges during the Neoproterozoic Era.

demosponges | biomarkers | C_{31} steranes | eukaryote radiation | Neoproterozoic Era

Demosponges, an early diverging clade of animals, include extant counterparts that biosynthesize an array of unconventional sterols as abundant constituents of their membrane lipids (1–5). The emergence of these and other early eukaryotes had a significant impact on marine ecosystems, influencing the efficiency of the biological carbon pump and the ventilation of shelf environments in the Neoproterozoic Era (6). Dramatic changes in the assemblages of fossil steroids preserved in sedimentary rocks is another manifestation of the eukaryote across this time period radiation (7–9). Still, the timeframe over which animals, and especially sponges, first emerged is subject to debate.

The geological record of the chemical fossil 24-*iso*-propylcholestane (24-*i*pc), which has been hypothesized to indicate the emergence of demosponges, suggests their initial appearance sometime during the Cryogenian Period (~660 to 635 Mya) (10, 11). Phylogenomics and molecular clock studies also support an Ediacaran, Cryogenian, or Tonian (~1,000 to 541 Ma) origin of Porifera (12–17), while demosponge spicule fossils have been found near the Precambrian–Cambrian boundary thereby predating the Cambrian radiation (<~541 Ma) (18–20). Moreover, recent work (21) has shed light on the evolution of nonskeletonized sponges, revealing effective silica processing strategies that eventually led to biosilicification and the emergence of siliceous skeletons. The divergence of stem demosponges and hexactinellids prior to the advent of silica production highlights the independent evolutionary trajectories of silicifying protein mechanisms across distinct geological periods. This distinction provides a better alignment with the fossil record and addresses discrepancies with older molecular clock estimates. Adding to this understanding, the recent discovery of the late-Ediacaran fossil *Helicolocellus cantori* in South China, identified as a putative crown-group sponge with an organic latticework skeleton, marks a significant advance (22) that solidifies evidence for the existence of sponges in the Neoproterozoic Era and marking an early evolutionary stage of sponges that lacked biomineralized spicules. These data challenge the traditional reliance on

Significance

This study advances our understanding of the evolutionary timeline for early metazoans, particularly sponges, through the chemical identification of rare C_{31} steranes from Neoproterozoic rocks. We provide compelling evidence linking these ancient steranes to demosponges, with an onset significantly predating the Cambrian explosion. By comparing these fossil steranes with both reduced modern demosponge sterols and a range of synthetic standards, we enhance the robustness of our sterane biomarker analysis. Our findings not only reinforce the existing C_{30} sterane record for early animal life but also challenge previous interpretations of Neoproterozoic C_{30} sterane distributions where they are ascribed to diagenetic artifacts. This work underscores the potential of C_{30} and C_{31} steranes for confidently tracing the emergence of animals in Earth's history.

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The authors declare no competing interest.

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biomineralized spicules for identifying ancient sponge fossils and support biogeochemical evidence for the early emergence of sponges and a more complete and nuanced view of sponge evolution through geological time.

Chemical fossils that occur in well-preserved sedimentary rocks also serve as a tool to study organismic evolution in ways that are completely independent of physical fossils or genomic inferences. The rare and distinctive series of C_{30} steranes, namely 24-*n*-propylcholestane (24-*n*pc), 24-*iso*-propylcholestane (24-*i*pc), and 26-methylstigmastane (26-mes), found in Neoproterozoic sedimentary rocks and oils have drawn significant attention (10, 11, 23). Their carbon skeletons are the same as three of the most commonly occurring C_{30} sterols found in extant demosponges but which are rare or absent in other taxa (24). In the case of 24-*n*pc, multiple biogenic sources are possible because its sterol counterpart is found in foraminifera (25) and some pelagophyte algae, as well as in demosponges (10). Thus, 24-*n*pc has not been employed as an ancient animal marker. However, the only metazoans believed to have had the genetic ability to biosynthesize the sterol precursors of 24-*i*pc and 26-mes during the Neoproterozoic Era are demosponges (5, 16), as supported by sterol methyltransferase (SMT) gene sequence analysis combined with molecular clocks (26). Whether the SMTs that are effective for these specific bioalkylations and side-chain elongation reside in the sponge, their bacterial symbionts, or both, has yet to be established (27, 28).

Cryogenian–Cambrian hydrocarbon biomarker records from Oman and elsewhere (e.g., ref. 10) highlight an abundant and highly selective C_{30} sterane pattern in some but not all localities (29), often with strong preference for 24-*i*pc. Thus, the relative abundance of 24-*i*pc, when 24-*i*pc/24-*n*pc ratios exceed 0.5 (10), has been used as presumptive molecular fossil evidence to track the emergence of the earliest animal life. These uncommon C_{30} steroids also occur as covalently bound constituents fixed within the immobile kerogen phase of the same rocks, which confirms the stratigraphic placement and is an important indicator that these are not younger contaminant compounds that migrated into the sediments (10, 11).

However, the use of these compounds as a biomarker signature for early sponges has been questioned on two grounds. First, it has been suggested that the unicellular Rhizaria (Foraminifera, Cercozoa, Gromidae, Plasmodiophorida, and Radiolaria) could be a biological source of the C_{30} sterols precursors to the Neoproterozoic C_{30} steranes found in the geologic record (30). Rhizaria, however, only produce trace amounts of 24-*i*pc and/or 26-mes steroids, down to 0.01% of total steroids (30); so these cannot readily explain the high abundance of 24-*i*pc or 26-mes steranes found in the Neoproterozoic geologic record (10, 11). Second, it has been suggested that the Neoproterozoic C_{30} steranes are generated from a diagenetic methylation of Neoproterozoic C_{29} sterols (31, 32) derived from ancient algae, yet detectable C_{30} steranes do not always accompany C_{29} sterane signals in Ediacaran rocks (29) and the $\%C_{30}$ sterane contents are highly variable from sample to sample (10, 11). In light of these two critiques, our study sought to further investigate the unconventional steranes preserved in Neoproterozoic rocks. This led to the identification of two previously unrecognized ancient C_{31} steranes, whose structures match the sterane core in C_{31} sterol precursors found in various modern demosponges but are rare in other sources. To strengthen the chemical structural identification and verify the analytical approach, a suite of synthetic sterols, potential precursors of C_{31} steranes, was synthesized and employed as reference compounds. These findings enhance our understanding of unconventional steroid biosynthesis and the evolution of early animal life.

Results

C_{31} Steranes in Neoproterozoic Samples. Hydrocarbon fractions prepared from Neoproterozoic sedimentary rock bitumens and oils derived from the South Oman Salt Basin, eastern Siberia, and the Bikaner-Nagaur Basin, western India (*SI Appendix*, Table S1), were investigated for their sterane distributions using a customized GC-QQQ-MS methodology. The studied samples display an abundance of C_{30} steranes, comprising up to 15.5% of the total ($C_{27\text{-}30}$) sterane complement (*SI Appendix*, Table S1). The most prevalent form is 24-*i*pc where the ratio between the biologically inherited 5α(H),14α(H),17α(H)-20R isomers of 24-*i*pc and 24-*n*pc (24-*i*pc/24-*n*pc) reaches up to 21.2 in the sample OMR 027 (*SI Appendix*, Table S1). The mean value for (24-*i*pc/24-*n*pc) for a larger suite of Ediacaran rocks from Oman is 1.5 (10, 11, 33), and all are above 0.5. OMR 027 is from the Masirah Bay Formation, Oman (10), and is the least thermally altered Oman rock sample analyzed as reflected by its low ααα 20(S/[S + R]) C_{29} sterane ratio (0.30, *SI Appendix*, Table S1), consistent with residing at a very early stage of the oil window.

Those samples with an abundance of 24-*i*pc also contain two distinct and resolvable series of C_{31} sterane compounds identifiable on the basis of the 428 → 217 Da ion transition chromatograms using GC-QQQ-MS and the collisionally activated dissociation (CAD) mass spectrum for peak B (Fig. 1). Chromatographic peaks A and B correspond to the 5α(H),14α(H),17α(H)-20R isomers of 24-*n*-butylcholestane (24-*n*bc) and 24-*sec*-butylcholestane (24-*sec*bc) respectively. These structures were established by their cochromatography with authentic sterane standards on multiple capillary GC columns with differing stationary phase polarities (Fig. 2).

The C_{31} chromatographic peaks for 24-*sec*bc, in particular, are well defined in Neoproterozoic and Cambrian rocks and oils from Oman and Siberia and comprise 0.14 to 1.18% abundance relative to the dominant 5α(H),14α(H),17α(H)-20R isomer of (C_{29}) stigmastane (*SI Appendix*, Table S1). However, C_{31} steranes are more difficult to detect in Ordovician and younger rocks due to their extremely low absolute abundances and compared to the enhanced signals of the C_{26}-C_{30} sterane background (*SI Appendix*, Table S1). In the samples reported here, the distributions of the four primary C_{31} sterane stereoisomers (namely; αααS, αββ (S + R) and αααR) exhibit a systematic variation with the thermal maturity of the samples (*SI Appendix*, Figs. S1 and S2). As expected, the αααR isomer is the most abundant in the less thermally mature samples (low ααα 20(S/[S + R]) C_{29} sterane ratio of 0.3 for OMR 027) with higher values (ααα 20(S/[S + R]) ratios at equilibrium values close to 0.5) in the more mature samples (*SI Appendix*, Figs. S1 and S2 and Table S1).

Structural Characterization of the C_{31} Steranes. To confirm the proposed structures of the fossil C_{31} steranes, identified by the 428 → 217 Da ion chromatograms and the 428 Da CAD mass spectra, eight constitutional isomers for C_{31} sterols (1–8; see line structures for the sterols in *SI Appendix*, Fig. S3) were synthesized (see *Experimental Section* for the synthetic procedure) and evaluated. The synthetic sterols' chemical structures were confirmed using NMR (*SI Appendix*, Fig. S4) and then they were reduced to hydrocarbons using H_2 and Adams' catalyst at ambient temperature in hexane. Of these sterane products (I-VIII; see line structures for the steranes in Fig. 3) only two, corresponding to compounds VII and VIII (Fig. 3), showed identical GC retention times to fossil C_{31} steranes, for peaks A and B, present in chromatograms for the oils and sediment extracts. The model steranes (I–VIII; structures in Fig. 3) derived from the synthetic sterols were separately

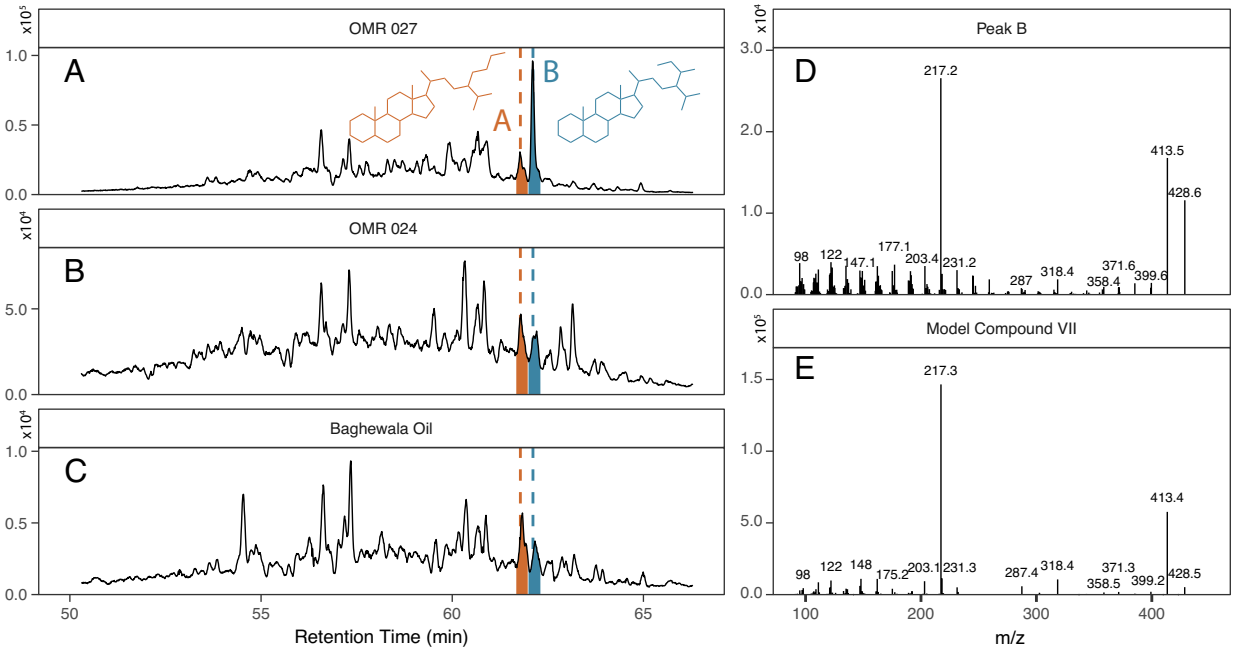

Fig. 1. Precursor-product (CAD) reaction chromatograms for the 428 → 217 Da ion transition in GC-QQQ-MS, featuring C_{31} sterane peak A (ααα-20R isomer of 24-*n*-butylcholestane (24-*n*bc), depicted in orange) and B (ααα-20R isomer of 24-*sec*-butylcholestane (24-*sec*bc), depicted in blue), for the free hydrocarbon fraction extracted from (*A*) OMR 027, (*B*) OMR 024 and (*C*) Baghewala oil, performed on a DB-1MS UI column. The corresponding CAD mass spectra of peak B ancient C_{31} sterane from (*D*) OMR 027, and (*E*) a sterane reference compound (24-*sec*-butylcholestane (24-*sec*bc); VII in Fig. 3), highlighting key characteristic molecular (428 Da) and 413 (M^{+*}-15) ions and diagnostic ions of 217 Da, and 318 Da that correspond to the complementary A + B + C ring and the C + D ring with side-chain fragments of the C_{31} sterane structure. Reproducibility of the CAD spectra is impacted by instrumental conditions, concentration differences, matrix effects, etc. (34, 35). CAD spectra performed on a DB-1MS UI column.

coinjected, with a hydrocarbon fraction from one of the geologic samples with prevalent C_{31} steranes as shown by well-defined peaks in GC-QQQ-MS (OMR 027) and with no evidence of peak distortion. The synthetic 5α(H),14α(H),17α(H)-20R 24-*n*bc (VIII; structure in Fig. 3) and 5α(H),14α(H),17α(H)-20R 24-*sec*bc (VII; structure in Fig. 3) coelute with peaks A and B, respectively, in the free hydrocarbon fraction of sample OMR 027 (Fig. 2). The elution order and the coelution experiments were verified using two additional GC capillary columns with different polarities (Fig. 2). This is an accepted practice in showing compound identity for trace components in complex mixtures where other analytical tools such as NMR are precluded (23, 34, 36, 37).

C_{31} Sterols in a Pelagophyte Alga. A culture of the "brown tide" pelagophyte alga *Aureococcus anophagefferens* (CCMP 1784) was also examined for the presence of C_{31} sterols, given that pelagophytes are established algal producers of abundant C_{30} sterols. Cells were solvent-extracted, the sterols concentrated using liquid column chromatography and analyzed by GC–MS. The sterol distribution observed in this analysis aligns with previously published findings (38) where sterol extracts primarily consist of 24-propylidenecholesterol (C_{30} precursor of 24-*n*pc), comprising approximately 67.5% of the total sterol content, followed by 24-methylcholesterol (a C_{28} sterol) at 28.7%. 24-ethylidenecholesterol and 24-ethylcholesterol (both C_{29} sterols) make up 3.8% collectively. Furthermore, our analysis identified traces of C_{31} sterols in the sterol extract of these pelagophytes, constituting 0.33% relative to the total sterol content (C_{28}-C_{30} sterols) (*SI Appendix*, Fig. S5 *A* and *B*).

These lipid extracts were also reduced in hexane, at ambient temperature and pressure, using Adams' catalyst and analyzed for their sterane distributions using GC-QQQ-MS (*SI Appendix*, Fig. S5*C*). The distribution of steranes in the reduced sterol extract correlates with the distribution of their sterol precursors (*SI Appendix*, Fig. S5 *A* and *C* and Table S2), with excellent retention of structural and stereochemical integrity of the hydrocarbon core. All C_{28}, C_{29}, and C_{30} pelagophyte steranes were detected as both ααα-20R and βαα-20R isomers, however the relative abundances reported below use the dominant ααα-20R isomer only for each carbon number. C_{30} steranes were the predominant product in the reduced fraction, constituting 57.2% of the total steranes (C_{28}-C_{30} sterane), followed by C_{28} steranes comprising 39.4%, and then C_{29} steranes comprising 3.4%. Additionally, two resolvable constitutional isomers of C_{31} steranes were identified in the reduced sterol fraction, together comprising ca. 0.6% relative to the C_{30} sterane (*SI Appendix*, Table S2). Based on the C_{30} contribution to the total steranes (*SI Appendix*, Table S2), this corresponds to ca. 0.3% relative to the total sterane content (*SI Appendix*, Fig. S5*C* and Table S2).

The primary pelagophyte C_{31} sterane produced by hydrogenation and hydrogenolysis corresponds to the 5α(H),14α(H),17α(H)-20R isomer of peak A (24-*n*bc) of the C_{31} sterane found in the geologic samples (*SI Appendix*, Fig. S6), which is the C_{31} sterane analog of 24-*n*pc. This identification was established by comparing the 428 Da CAD spectrum of the main C_{31} sterane in the reduced fraction with that of sterane model compound VIII (24-*n*bc), as shown in *SI Appendix*, Fig. S6 *C* and *D*. In addition, the C_{31} sterane that corresponds to the 5α(H),14α(H),17α(H)-20R isomer of peak B (24-*sec*bc) found in the geologic samples was also found in a trace amount (0.1% relative to total C_{30} steranes, dominated by to 24-*n*pc (*SI Appendix*, Table S2) in the reduced sterol fraction of *A. anophagefferens* (CCMP 1784) (*SI Appendix*, Fig. S6 and Table S2). The presence of 24-*n*bc and minor traces of 24-*sec*bc in the reduced sterol fraction of *A. anophagefferens* (CCMP 1784) was further substantiated through coinjection experiments with the

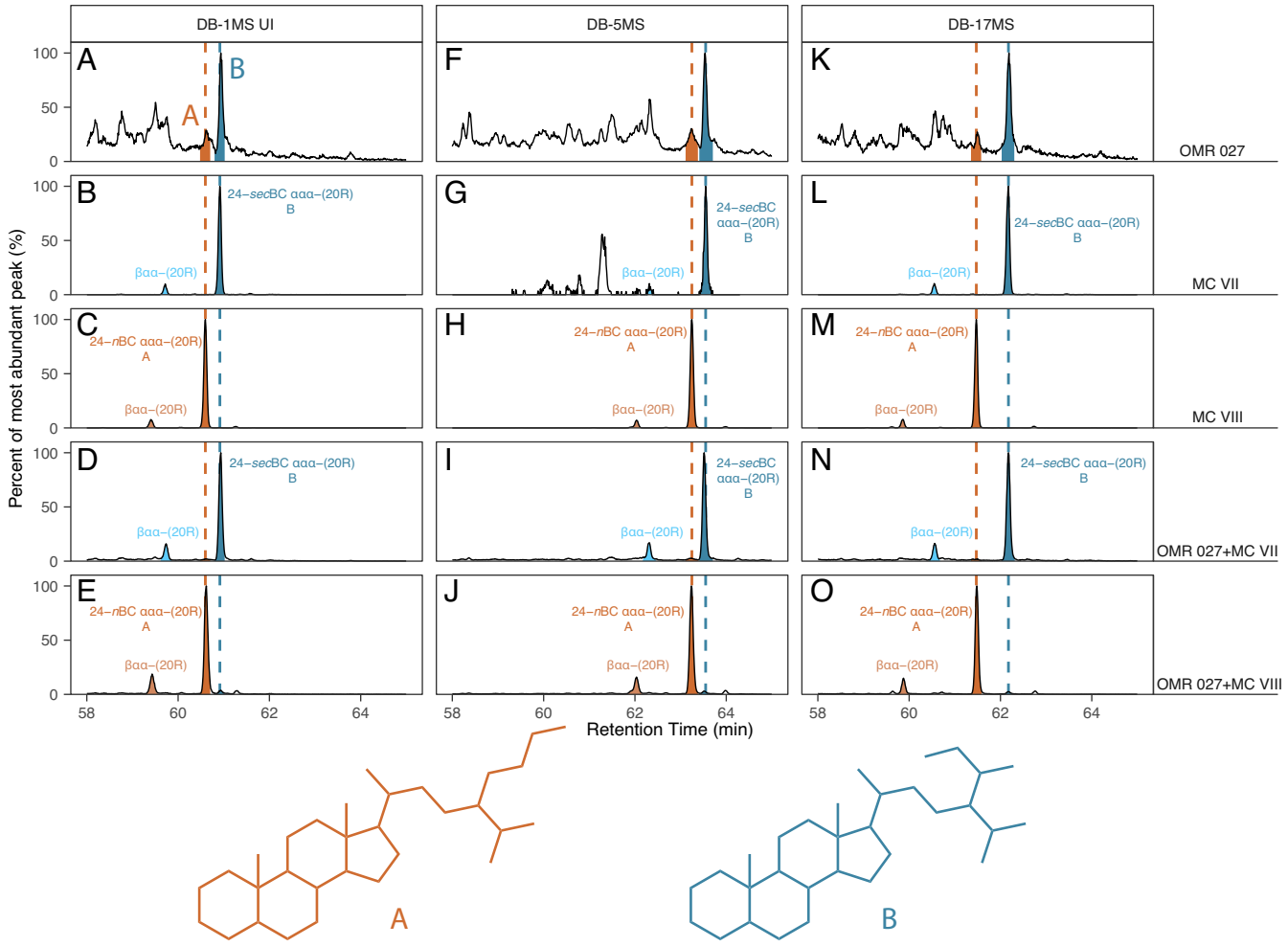

Fig. 2. Precursor-product (CAD) reaction chromatograms for the 428 → 217 Da ion transition in GC-QQQ-MS, featuring C_{31} sterane peak A (24-*n*-butylcholestane (24-*n*bc), depicted in orange) and B (24-*sec*-butylcholestane (24-*sec*bc) depicted in blue), from coinjection experiments on capillary columns of three different stationary phase polarities. The experiments involve coinjections of OMR 027 hydrocarbon fraction with sterane model compounds VII and VIII. Panels (*A*–*E*) represent coinjection results from a DB-1MS UI column, panels (*F*–*J*) from a DB-5MS column and panels (*K*–*O*) from a DB-17MS column, each illustrating the specific elution position and the outcomes of coelution experiments.

synthetic 5α(H),14α(H),17α(H)-20R 24-*n*bc (VIII; Fig. 3) and 5α(H),14α(H),17α(H)-20R 24-*sec*bc (VII; Fig. 3) on three GC capillary columns with different polarities (see elution order and coelution experiments on DB-5MS column, *SI Appendix*, Fig. S7).

C_{31} Sterols in Demosponges. A demosponge and a hexactinellid species (*SI Appendix*, Tables S2 and S3) that produce sterol precursors of conventional (C_{27}-C_{29}) steranes as well as a suite of demosponge species that produce sterol precursors for unconventional (C_{30}-C_{31}) steranes (11, 39–41), including 24-*i*pc and 26-mes, were

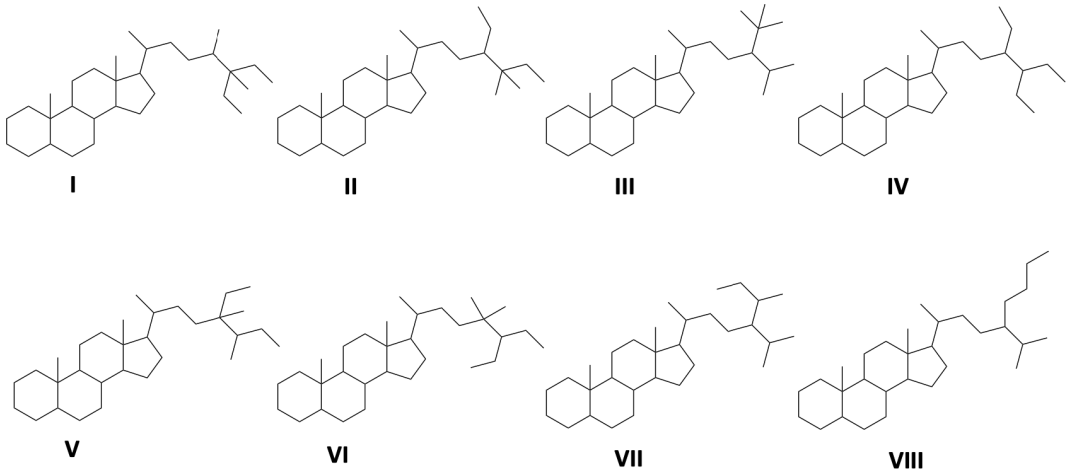

Fig. 3. The structures of eight C_{31} steranes derived from the reduction hydrogenation and hydrogenolysis of the synthesized constitutional isomers of C_{31} sterols. VII is 24-*sec*bc and VIII is 24-*n*bc, the two ancient C_{31} sterane compounds that we identified.

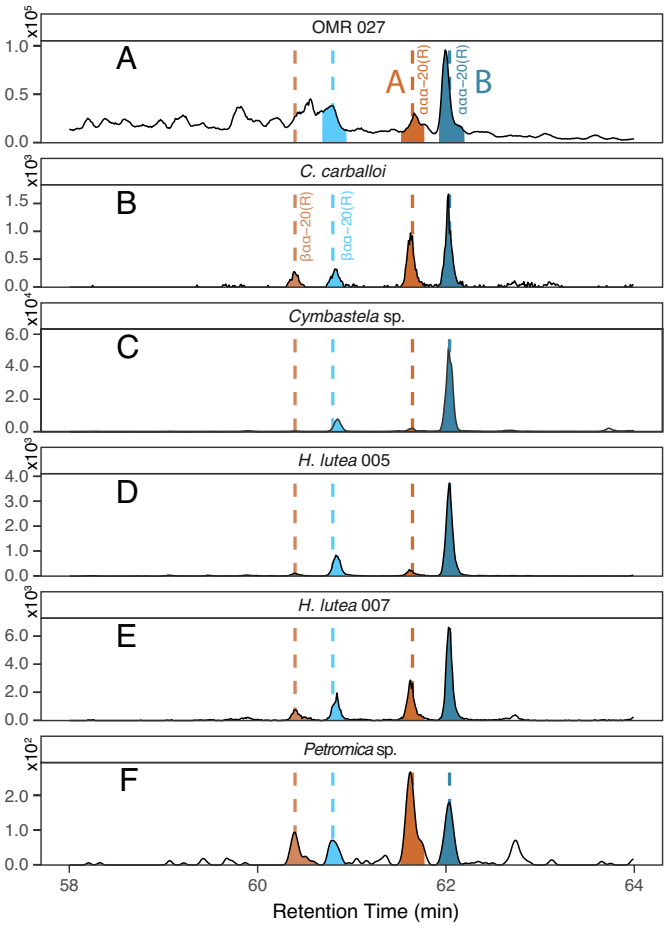

Fig. 4. Precursor-product (CAD) reaction chromatograms for the 428 → 217 Da ion transition from GC-QQQ-MS, featuring C_{31} sterane peak A (βαα-20R and ααα-20R peaks of 24-*n*-butylcholestane (24-*n*bc), depicted in orange) and B (βαα-20R and ααα-20R of 24-*sec*-butylcholestane (24-*sec*bc), depicted in blue), (*A*) for the free hydrocarbon fraction extracted from OMR 027, and for the reduced sterol fraction extracted from (*B*) *Ciocalypta carballoi* UPSZMC 193109 (*C. carballoi*.), (*C*) *Cymbastela* sp. UPSZMC 191072 (*Cymbastela* sp.), (*D*) *Halichondria lutea* 17-V-88-4-005 (*H. lutea* 005), (*E*) *Halichondria lutea* 17-V-88-4-007 (*H. lutea* 007) and f) *Petromica* sp. 4-VI-88-1-003 performed on a DB-1MS UI column.

solvent-extracted. The extracted sterol fractions were purified using silica gel column chromatography and analyzed to search for possible C_{31} sterols using the same approaches as described above. A suite of mono-, di-, and tri-unsaturated C_{31} sterols was detected in sterol fractions, with the di- and tri-unsaturated forms being the most prevalent in a *Cymbastela* species (*SI Appendix*, Fig. S8). As before, these sterol fractions were reduced at ambient temperature and pressure using Adams' catalyst or hydropyrolysis (see *SI Appendix*, Table S2 for reduction method), and the resulting steranes were analyzed by GC-QQQ-MS. On the basis of the 428 → 217 Da precursor-product reaction chromatograms, a series of C_{31} steranes were confirmed as being present in multiple demosponge species. Close examination of these chromatograms showed that the two most common C_{31} sterane peaks for modern sponges correspond precisely to peaks A and B present in the geologic samples (Fig. 4) and, in turn, to the synthetic 5α(H),14α(H),17α(H)-20R 24-*n*bc (VIII; Fig. 3) and 5α(H),14α(H),17α(H)-20R 24-*sec*bc (VII; Fig. 3), present in the reduced sterol fractions.

The abundances of the derived C_{31} steranes (24-*n*bc and 24-*sec*bc) in the reduced sterol fraction differed among various demosponge species, with levels reaching up to 2.4% for 24-*sec*bc, and 14.1% for 24-*n*bc relative to the co-occurring C_{30} steranes (*SI Appendix*, Table S2). The *Geodia hentscheli* specimen yielded conventional (C_{27}-C_{31}) steranes, with 24-*n*bc as the dominant C_{31} sterane product, accompanying low amounts of C_{30} steranes composed of exclusively 24-*n*pc. Notably, the six demosponge species that produce abundant 24-*i*pc sterols (*C. carballoi, Cymbastela* sp., *Halichondria lutea, Petromica* sp., *Topsentia* sp., *T. ophiraphidites*), all yielded detectable amounts of both 24-*sec*bc and 24-*n*bc as C_{31} steranes from reduction. The 24-*sec*bc is usually the most abundant C_{31} sterane generated from this group of demosponges (Fig. 4 and *SI Appendix*, Table S2).

In addition, 24-ethyl-26-methyl-27-methylcholestane (28-methylxestosterane; ~0.3% relative to the co-occurring C_{30} steranes) was identified in the reduced fraction of sterols extracted from *Petrosia (Strongylophora) corticata*, which produces a 26-mes sterol precursor (strongylosterol, C_{30}) as its main sterol (*SI Appendix*, Fig. S9). The presence of 24-ethyl-26-methyl-27-methylcholestane (28-methylxestosterane) in the reduced sterol fraction of *P. (S.) corticata*, along with two additional trace C_{31} sterane compounds (25-methylxestosterane (I; structure in Fig. 3) and xestospongesterane (VI; structure in Fig. 3); see *Discussion*), was further substantiated through coinjection experiments with the synthetic 5α(H),14α(H),17α(H)-20R 24-ethyl-26-methyl-27-methylcholestane (IV; structure in Fig. 3) on three columns with different polarities (see elution order and coelution experiments on DB-17MS column; *SI Appendix*, Fig. S10)

Discussion

In this study, we report the structures of two previously unrecognized fossil C_{31} steranes (VII and VIII structure in Fig. 3, equivalent to peaks B and A, respectively, in Fig. 1) in ancient sediment samples. These steranes are particularly prominent in the Ediacaran and Cambrian period in sedimentary rocks and in some derived oils from the South Oman Salt Basin, Eastern Siberian Platform, and the Bikaner-Nagaur Basin, western India (Fig. 1 and *SI Appendix*, Table S1 and Fig. S1). Owing to the very low concentrations of these compounds, and their presence in the complex matrices of sedimentary hydrocarbon mixtures, the identifications were only possible using targeted methods based on GC-QQQ-MS and chromatographic comparisons with authentic standards prepared by chemical synthesis. On the basis of 428 → 217 precursor-product ion chromatograms and CAD mass spectra (Fig. 1 and *SI Appendix*, Fig. S6), peak A in Fig. 1 was identified as a 5α(H),14α(H),17α(H)-20R C_{31} sterane with *n*-butyl substituent at C-24 (24-*n*bc; VIII). Peak B in Fig. 1 was identified as a 5α(H),14α(H),17α(H)-20R C_{31} sterane with a *sec*-butyl group substitute at C-24 (24-*sec*bc; VII).

These previously unrecognized C_{31} steranes exhibit a highly selective pattern with respect to their compound distributions given the possibility of many feasible stable structural forms in the geologic record; they are present as specific constitutional isomers and only occur abundantly across a limited stratigraphic range (Fig. 1 and *SI Appendix*, Table S1 and Fig. S1). These observations indicate a biogenic origin for these fossil steranes, as diagenetic and catagenetic processes are expected to yield a complex assemblage of numerous structural isomers, arising through non-selective side-chain modifications of C_{27}-C_{30} steranes, rather than the observed selective set of distinctive and well-defined chromatographic peaks for only two dominant C_{31} sterane compounds out of many more possible C_{31} structural isomers. Furthermore, the distributions of the four primary C_{31} sterane isomers (namely, αααS, αββ(S + R) and αααR) exhibit a systematic variation with the thermal maturity of the samples (*SI Appendix*, Figs. S1 and S2). As expected, the αααR isomer is the most abundant in the

less thermally mature samples, giving a low ααα 20(S/[S + R]) C_{29} sterane ratio but with values close to 0.50 in more mature samples (42) (*SI Appendix*, Figs. S1 and S2 and Table S1). This strengthens the case for the ancient C_{31} steranes having a biogenic origin and experiencing the same burial history as the other (C_{27}-C_{30}) sterane constituents, rather than being derived diagenetically from side-chain modification of algal steranes in marine sediments.

Our analyses confirm that the samples where these ancient C_{31} steranes are most abundant (*SI Appendix*, Table S1) possess an additional distinctive quality in that they also show appreciable moderate-high abundances of C_{30} steranes, particularly for those with a preference for 24-*i*pc. Neoproterozoic Oman (10, 33) and eastern Siberia (43) samples typically yield appreciable C_{30} sterane content accounting for 1-4% of total steranes but occasionally reaching 5% or higher, consistent with our data. Given this, our investigation turned toward identifying the sterol precursors in organisms known to produce sterols with extended side-chains. For instance, pelagophyte algae are recognized for producing 24-*n*-propylidenecholesterol and 24-*n*-propylcholesterol (38, 44–46) as major sterols but only traces of 24-*iso*propylcholestadienol (47, 48) which can be the geologic precursor of the fossil counterpart of the 24-*n*pc and 24-*i*pc, respectively, identified in the geologic record. Our analysis further identified traces of C_{31} sterols in the extract of a "brown tide" pelagophyte alga *A. anophagefferens* (CCMP 1784) (*SI Appendix*, Fig. S5 *A* and *B*). Reduction of these sterols afforded the C_{30} sterane 24-*n*pc together with low but notable presence of a peak corresponding to the 5α(H), 14α(H),17α(H)-20R isomer of compound VIII (24-*n*bc; 0.46% relative to C_{30} steranes; *SI Appendix*, Fig. S5*C* and Table S2) which matches peak A in Ediacaran geologic samples (*SI Appendix*, Fig. S6). Traces of the 5α(H),14α(H),17α(H)-20R isomer of compound VII (24-*sec*bc) were also detected, amounting to 0.1% relative to total C_{30} steranes, predominantly 24-*n*pc (*SI Appendix*, Table S2), which would be undetectable in the geological samples due to insufficient abundance.

In contrast, 24-*sec*bc can range from 7.8 to 87.5%, relative to 24-*n*pc, in some rocks and oils from the Ediacaran to Early Cambrian (*SI Appendix*, Table S1). The exception is an Ediacaran rock sample from western Russia (UZ-1-21) that contained negligible C_{30} and C_{31} sterane content (<0.1%) despite containing abundant C_{29} steranes (29). The (24-*sec*bc/24-*n*pc) ratio highlights how much more abundant 24-*sec*bc is in these ancient samples compared to the trace amounts produced by the pelagophytes (0.1% of 24-*n*pc). Furthermore, pelagophytes and their marine algal ancestors did not acquire the capability to produce C_{30} sterols until significantly later in the Paleozoic Era (26), so these are not a likely biogenic source of Neoproterozoic-Cambrian C_{30} and C_{31} steranes. Taken together, both the extremely low levels of 24-*sec*bc in pelagophytes and the timing of their evolutionary emergence indicate that these algae cannot account for the high concentrations of 24-*sec*bc, co-occurring with high 24-*i*pc, observed here in the Ediacaran-Cambrian samples. While a marine algal source of 24-*n*pc and 24-*n*bc is highly plausible for Devonian and younger samples (*SI Appendix*, Table S1), such a biogenic source cannot readily account for these steranes in Ordovician or older samples (49).

Another plausible biogenic steroid precursor of ancient C_{31} steranes, particularly 24-*sec*bc, occurs in a group of contemporary demosponges that also produce the sterol precursors of 24-*i*pc found in the geologic record. Species within the *Topsentia, Ciocalypta, Cymbastela, Halichondria,* and *Petromica* genera of demosponges with 24-*i*pc as their major membrane sterols were solvent extracted, purified using liquid chromatography and analyzed for C_{31} sterols (*SI Appendix*, Tables S2 and S3). Our analyses showed that the species that contain 24-*i*pc as their major sterols also produce C_{31} sterols that share a carbon skeleton identical to that of the ancient C_{31} steranes (24-*n*bc and 24-*sec*bc, Peak A and B respectively in Fig. 4) found in the geologic record. The precursor sterols in these modern demosponges are typically mono- and di-unsaturated for the C_{30} sterols [24-isopropylcholesterol and 22-dehydro-24-isopropylcholesterol (24)], but exist in mono-, di-, and tri-unsaturated forms for the C_{31} sterols. The C_{31} tri-unsaturated sterol, readily detectable in *Cymbastela* sp. (*SI Appendix*, Tables S2 and S3) from full scan GC–MS (*SI Appendix*, Fig. S8), is most likely ophirasterol (40) based on mass spectral features reported previously. Ophirasterol was first identified in *Topsentia ophiraphidites* (40), which is known to produce 24-*i*pc sterols in high abundance (*SI Appendix*, Table S2).

The ability of demosponges to produce C_{31} sterol precursors varies among taxa. In some species, the abundance of C_{31} precursor sterols and their corresponding C_{31} steranes can reach significant levels. The levels of 24-*n*bc C_{31} sterane in the reduced sterol fraction reach up to 14.1% relative to C_{30} steranes and 20.5% relative to 24-*n*pc. The abundance of 24-secbc, a major C_{31} sterane produced by reduction of demosponge sterol fractions, reached 2.4% relative to C_{30} steranes and >1,000% relative to 24-*npc* (*SI Appendix*, Table S2). Indeed, all the demosponge species in our sample set that contained 24-*i*pc as major sterols yielded detectable 24-*sec*bc in the reduction products but, notably, this compound was not found in the other sponges tested (*Geodia hentscheli*, *Petrosia (S) corticata*, *Vazella pourtalesii*) which are devoid of appreciable 24-*i*pc. Notably, *Vazella pourtalesii* (a hexactinellid), that was shown to produce mainly C_{27}-C_{29} stanols, along with traces of 24-*n*pc stanol, contains no detectable C_{31} sterols/stanols or steranes in the reduced fraction (*SI Appendix*, Table S2). The hexactinellid sponges are known to obtain steroids from their diet and modify them into specific forms, particularly stanols, through a series of reductive steps (50). Thus, the absence of any C_{31} sterol or any C_{31} steranes in the reduced fractions implies that there is no significant background signal of C_{31} sterols or steranes that can be readily picked up from the host marine environment through feeding or produced from laboratory reduction artifacts. Previous research (40, 41) has documented that in certain demosponges that make abundant 24-*i*pc sterols, different C_{31} monohydroxy sterols, including compounds with *t*-butyl substituents and others with more geologically stable side-chains, may constitute a major portion (up to 64%) of the total sterol content. This substantial presence underscores that these C_{30} and C_{31} sterols are not accidental byproducts of metabolic pathways but are major biosynthetic products, serving crucial roles in these organisms. These observations (40, 41) align with our findings, which demonstrate the presence of high levels of C_{30} steroids including 24-*i*pc, and appreciable C_{31} steroids in some but not all demosponge species. Demosponges are capable of producing a diverse array of sterols for specific biological functions, further indicating that these lipids are significant and purposeful products of their metabolic pathways.

Furthermore, the detection of three additional C_{31} sterane structural isomers (including 25-methylxestosterane (I; structure in Fig. 3), xestospongesterane (VI; structure in Fig. 3), and 28-methylxestosterane (IV; structure in Fig. 3) (*SI Appendix*, Figs. S9 and S10), in the reduced sterol fraction from *Petrosia (S.) corticata*, extends the diversity of known demosponge-derived steranes. Among these additional C_{31} sterane compounds, 28-methylxestosterane was the most prominent, although still representing only 0.3% relative to co-occurring C_{30} steranes. A previous study on a closely related

species (51) has reported that sponges known to produce 26-mes sterols as their main sterol are able to produce traces of 24-ethyl-26-methyl-27-methylcholesterol (28-methylxestosterol), a likely precursor for 28-methylxestosterane, as well as 25-methylxestosterol and xestospongesterol, the likely precursors for 25-methylxestosterane (I; Fig. 3) and xestospongesterane (VI; Fig. 3) respectively. However, this particular C_{31} sterane isomer, 28-methylxestosterane, has not yet been found in ancient samples. This could be attributed to the trace constituents of their sterol precursor in the parent sponge (51), less than 1% of 26-mes, which itself is usually significantly rarer and less abundant than 24-*i*pc in the ancient rock record (11). Since 28-methylxestosterol is not a major sterol in any sponge or other parent organism, it is unlikely to be preserved or noticed in significant quantities in the rock record. An additional significant impediment to preservation and detection of any 25-methylxestosterane (I; structure in Fig. 3) and/or xestospongesterane (VI; structure in Fig. 3) over geologic timescales is the quaternary carbon in the side-chain of the sterol precursors, which is thermally labile and susceptible to bond cleavage during burial maturation of host sediments.

To summarize, the prevalence of the C_{31} steranes, 24-*sec*bc in particular, in Neoproterozoic samples that also contain the putative sponge biomarkers 24-*i*pc and 26-mes, strongly implicates demosponges as the likely source of this suite of rare C_{30} and C_{31} steranes (Fig. 5). More specifically, co-occurrence of abundant 24-*i*pc with readily detectable 24-*sec*bc in the same ancient rocks and oils (*SI Appendix*, Table S1) is a compelling molecular fingerprint for ancient demosponges. Such a close match with the main C_{30} and C_{31} sterol distributions found in modern demosponges that make 24-*i*pc among their major sterols (*SI Appendix*, Tables S2 and S3) is more than coincidence. This C_{30} and C_{31} ancient sterane pattern is found in some of the oldest rock samples analyzed from south Oman, such as those from the Masirah Bay (early Ediacaran) Formation. This includes the best thermally preserved ancient rock sample from Oman (OMR 027, Masirah Bay Fm.), which yielded the highest selectivity for both 24-*i*pc and 24-*sec*bc steranes for any ancient rock or oil, mimicking the sterol pattern for numerous modern demosponge species. The 24-*i*pc and 24-*sec*bc steranes can be considered as a pair given their biosynthetic relationship and, therefore, useful as tandem lipid biomarkers for tracking ancient sponge inputs. The prevalence of these hydrocarbons in ancient samples from the Ediacaran Period is evident in comparison to younger rocks (*SI Appendix*, Table S1). A very low abundance of C_{30} steranes in rocks from the late Cambrian and through the Ordovician (49), is consistent with a radiation of suspension feeding eumetazoans which replaced sponges as major primary consumers in Paleozoic marine shelf habitats.

Currently, the published Cryogenian biomarker record is very sparse in terms of sample numbers (7, 10), and in the availability of well-dated stratigraphic intervals. Due to these constraints, including possible unconformities in key Cryogenian-early Ediacaran units (52), we cannot confidently evaluate yet whether C_{30} and C_{31} steranes first emerge in Cryogenian rock successions (Fig. 5 and *SI Appendix*, Table S1).

The abundance ratios of 24-*i*pc/24-*n*pc for Ediacaran rocks and oils from Oman, Siberia, and India all exceed 0.5 (and reach as high as 21.2). Similarly, the (24-*sec*bc/C_{29} sterane) sterane ratios consistently surpass the 0.1% threshold for reliable identification by up to an order of magnitude (with elevated values reaching up to 58% found for some closely related modern demosponge species; *SI Appendix*, Table S2). In contrast, a small suite of Ordovician and younger rocks (*SI Appendix*, Table S2) generally yielded negligible amounts of 24-*sec*bc, with values below the 0.1% cut off for the (24-*sec*bc/C_{29} sterane) ratio. These sterane distributions collectively support a biogenic origin from demosponges and/or their microbial symbionts (27). Molecular clock predictions (13–17, 55, 56) of Neoproterozoic porifera as a component of the fauna of this time is strongly supported from this combined C_{30} and C_{31} sterane biomarker record (Fig. 5). The two series of C_{30} sponge steranes are demonstrably absent in older Neoproterozoic sedimentary rocks (8) that predate the Sturtian glaciation event. This supports the hypothesis (57) that protracted Neoproterozoic Snowball Earth cooling presaged global scale ecological and environmental upheaval that provided opportunities for the later rise of complex multicellular life in the ocean.

Experimental Section

Geologic Samples. Rocks and oils from the Huqf Supergroup in the South Oman Salt Basin, oils from eastern Siberia, and one oil (Baghewala oil) from the Marwar Supergroup in India were analyzed for their free hydrocarbons. Details about the geologic setting of these samples can be found in previously published work (33, 43, 58). Additionally, a suite of six thermally immature Phanerozoic rocks, from Ordovician to Jurassic-age, were also analyzed for comparison. Their biomarker ratios are reported in *SI Appendix*, Table S1 alongside those for Ediacaran and Early Cambrian rocks and oils.

Rock Bitumen Extraction, Oil and Bitumen Fractionation. Rock powders were extracted with a mixture of CH_2Cl_2 and CH_3OH (9:1, v/v, 7 mL solvent/gr rock powder) extracted using a microwave accelerated solvent extractor (MARS6 Express, CEM Corporation, USA) at 120° C for 30 min. The extracted bitumen was then dried with anhydrous magnesium sulfate (activated at 120° C overnight), filtered, and then concentrated under nitrogen flow. The total lipid extracts/oils were fractionated by silica gel adsorption chromatography, eluting successively with hexane/CH_2Cl_2 (9:1, v/v) and CH_2Cl_2/CH_3OH (4:1, v/v) to yield nonpolars (saturated and aromatic hydrocarbons combined), and polar fractions, respectively. The hydrocarbon (nonpolar) fractions were then analyzed by GC-QQQ-MS at MIT to determine their sterane distributions (see below).

The bitumens extracted by microwave accelerated solvent extraction for six thermally immature Phanerozoic rock samples, ranging from the Ordovician to Jurassic in age, as well as for two Neoproterozoic rock samples (GM-1 and UZ-1-21; see *SI Appendix*, Table S1), were separated at UCR using a different column chromatographic method. In this case, the whole extracts were fractionated by silica gel column chromatography using a solvent elution scheme to yield three distinct fractions; saturated hydrocarbons, aromatic hydrocarbons, and polar fractions, according to established analytical procedures described previously (8, 11). The saturated hydrocarbon fractions were then analyzed by GC-QQQ-MS at MIT to determine their sterane distributions, as described below.

Extraction and Analysis of Sterols in Modern Sponge Cells and a Pelagophyte. The brown tide pelagophyte alga *Aureococcus anophagefferens* (CCMP 1784) and modern sponge sterol fractions were extracted from the collection summarized in *SI Appendix*, Table S3, following previously established methods (11). Additional methodological details are provided in the *SI Appendix*.

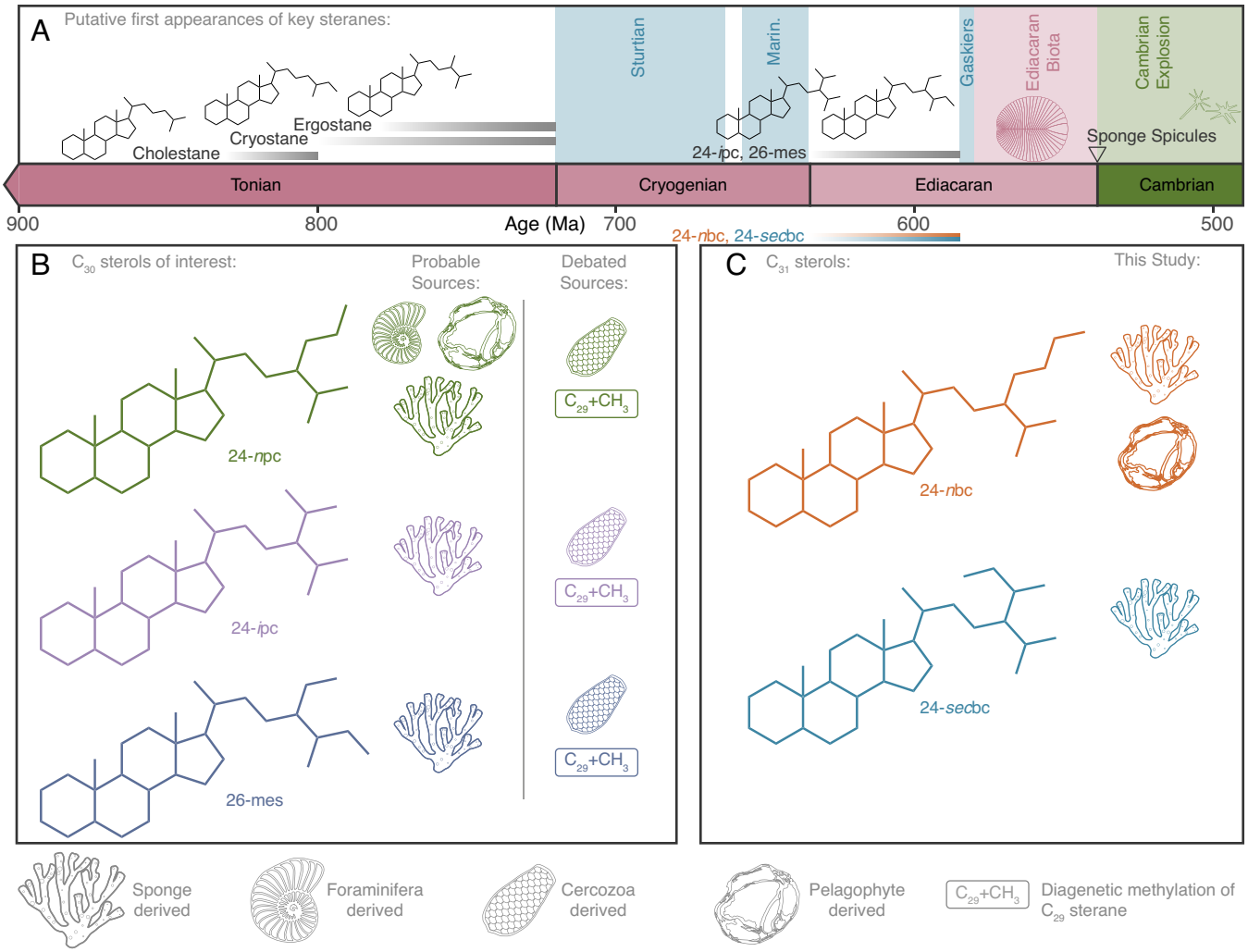

Fig. 5. Pictorial representation of the timeline for ancient steranes, highlighting key compounds and their possible biogenic sources. Panel (*A*) shows the time interval for the putative first appearance of metazoan chemical fossils (gray bar for C_{30} steranes and half-blue half-orange bar for the C_{31} steranes (this study) alongside body fossils in the geological record. The GM-1 core sample (*SI Appendix*, Table S1), was previously interpreted as interglacial Cryogenian-age [~660–635 Ma; (10)], but this requires verification due to uncertainties in age models for South Oman (e.g., ref. 52), some of which suggest a possible unconformity between the Abu Mahara and Nafun Groups may span tens of millions of years. More broadly, the Cryogenian biomarker record is currently sparse and requires further investigation using multiple rock samples from different locations, with depositional ages constrained by geochronology. Thus, to be conservative, our timeline for ancient C_{30} and C_{31} steranes begins in the early Ediacaran pending further work on Cryogenian rocks. This panel also shows the time intervals (gray bars) for the first appearance of cholestane, cryostane as reported in (53, 54), and ergostane as reported in (8). Fading on the bars reflects geochronological uncertainties. Panel (*B*) focuses on the Neoproterozoic origin of key steranes (24-*n*pc, 24-*i*pc, and 26-mes) highlighting their accepted and debated biological sources. Whereas 24-*n*pc can be derived from both foraminifera and sponges, 24-*i*pc and 26-mes are more selective chemical fossils for sponges. The identification of a previously unrecognized C_{31} sterane (24-*sec*bc) in the same Neoproterozoic samples and the occurrence of their C_{31} sterol counterparts in multiple modern demosponge species further support the sponge biomarker hypothesis. This presence, especially in samples containing abundant 24-*i*pc and 26-mes, underscores the likelihood that these hydrocarbons collectively originated from sponges. Panel (*C*) depicts the chemical structures of newly identified C_{31} compounds from this study, highlighting 24-*sec*bc as a demosponge-derived biomarker. While 24-*n*bc can originate from both pelagophytes and demosponges, its Neoproterozoic occurrence likely reflects only demosponge sources.

Reduction (hydrogenation and hydrogenolysis) of Sterols Extracted from Sponge Cells and a Pelagophyte over Adams' Catalyst. Sterol fractions extracted from selected modern sponge species: *Ciocalypta carballoi*, *Cymbastela* sp. (DR15-852), *Halichondria lutea* (17-V-88-4-007), *Topsentia ophiraphidites*, *Petrosia (S.) corticata*, and from the pelagophyte *Aureococcus anophagefferens* (CCMP 1784) were hydrogenated and hydrogenolyzed using Adams' catalyst (*SI Appendix*, Tables S2 and S3). The sterol fraction was dissolved in hexane and reduced by bubbling a gentle stream of H_2 for 2 h while stirring with ~0.2 mg of platinum (IV) oxide. The reduced samples, after catalyst removal, were separated into nonpolar and polar factions as described above. Reduced sponge sterols were also obtained using catalytic hydropyrolysis.

Catalytic Hydropyrolysis of Sponge Biomass. Continuous-flow hydropyrolysis (HyPy) experiments were performed on five freeze-dried sponge species: *Halichondria lutea* (17-V-88-4-005), *Petromica* sp. (4-VI-88-1-003), *Topsentia* sp. (GW3359), *Geodia hentscheli* (GhII), and *Vazella pourtalesii* (*SI Appendix*, Tables S2 and S3). Approximately 30 to 150 mg of catalyst-loaded sponge biomass as described previously (11). Additional methodological details are provided in the *SI Appendix*.

Sterane Analysis by Gas Chromatography Triple Quadrupole Mass Spectrometry (GC–QQQ–MS). For the identification of steranes a gas chromatograph coupled to a triple quadrupole mass spectrometer (GC-QQQ-MS; Agilent 7890B/7010, USA) was used. Samples (1μL) were injected into the device via a multimode

inlet using an autosampler. The inlet temperature was ramped from 60 °C to 340 °C at a rate of 70 °C/min. The injection was carried out in a splitless mode. The carrier gas was at a constant rate flow of 1.5 mL/min. The GC oven temperature was held at 60 °C for 2 min then programmed at 8 °C/min to 220 °C, then 2 °C/min to 320 °C min and held for 28 min. The transfer line temperature was held at 320 °C and the quadrupole analyzer and the MS source were operated at 150 °C and 270 °C, respectively with the source in electron ionization mode at 70 eV. The GC–MS system was equipped with either Agilent DB-5MS, DB-1MS UI, or DB-17MS capillary columns (60 m × 250 μm × 0.25 μm).

To accurately assess the C_{31} sterane signals, we monitored a suite of mass transitions for A-ring alkylated steranes (e.g., Da 428→245, and 428→259) using our QQQ method. This careful evaluation was necessary to distinguish genuine C_{31} sterane peaks from cross-talk signals predominantly contributed by other C_{31} hydrocarbons (e.g. ethylstigmastanes) in 428 → 217 Da ion transition chromatograms, a common issue in our Phanerozoic samples where the 24-*sec*bc/C_{29} sterane ratios were typically around or less than 0.1%. Relative retention times and peak areas were critically assessed to ensure accurate sterane analysis. Further, CAD mass spectra confirmed that the 217 product ions of the C_{31} steranes arose through unimolecular dissociation of a 428 precursor. These spectra were generated in the QQQ collision cell and reflect fragments formed directly from molecular ions, not from electron impact (34). This confirms that the 428 → 217 transition observed in MRM originates specifically from C_{31} steranes and not from coeluting compounds such as methylsteranes or triterpanes.

Sterol Analysis by Gas Chromatography Mass Spectrometry (GC–MS). For the identification of sterols after they were derivatized with 50 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) in 50 μL of pyridine at 70 °C for 30 min a gas chromatograph-mass spectrometer system (GC–MS; Agilent 7890A/5975, USA) was used. Samples (1μL) were injected into the device via a multimode inlet using an autosampler. The inlet temperature was 300 °C. The injection was carried out in a splitless mode. The carrier gas was at a constant rate flow of 1.5 mL/min. The GC oven temperature was held at 60 °C for 2 min then programmed at 8 °C/min to 220 °C, then 2 °C/min to 320 °C min and held for 28 min. The transfer line temperature was held at 300 °C and the quadrupole analyzer and the MS source were operated at 150 and 250 °C, respectively with the source in electron ionization mode at 70 eV. The GC-MS system was equipped with Agilent DB-5MS capillary columns (60 m × 250 μm × 0.25 μm).

NMR Spectra. Eight constitutional isomers for C_{31} sterols (1–8; see line structures in *SI Appendix*, Fig. S3) were synthesized, and their structures were confirmed using NMR spectra (*SI Appendix*, Fig. S4). NMR spectra were acquired using a Bruker Avance III 600 or 800 MHz spectrometer at 25 °C. Calibration was by the residual solvent signal ($CDCl_3$: 1H = 7.26 ppm, ^{13}C = 77.0 ppm). Preparative TLC was performed on glass-backed plates (10 cm in length) coated with a 0.25 mm layer of silica gel 60 F254. High-Performance Liquid Chromatography (HPLC) was carried out using a Waters 6000A pump, Waters 410 differential refractometer, and two Altex Ultrasphere ODS 5 μm 10 × 250 mm columns in series using a flow rate of 3 mL/min.

C_{31} Sterols Synthesis. Eight constitutional isomers for C_{31} sterols (1–8; see line structures in *SI Appendix*, Fig. S3) were synthesized according to the following procedure (more detailed reaction schemes are given in the *SI Appendix*, Scheme S1 *A*–*E*).

25,26,27-Trimethylergosta-5,24(28)-dien-3-ol (25-Methylxestosterol, 1 in SI Appendix, Fig. S3). This was isolated from a species of *Xestospongia* (59) and confirmed synthetically (59, 60).

E-25,26-Dimethylstigmasta-5,24(28)-dien-3-ol (2 in SI Appendix, Fig. S3). Using the sequence given for sterol ***3*** with 6β-methoxy-3α,5-cyclo-5α-25,26-dimethylcholestan-24-one (61) and vinylmagnesium bromide led to the title compound (*SI Appendix*, Scheme S1*A*). ^{1}H-NMR ($CDCl_3$, 600 MHz): 5.352 (1H, m), 5.214 (1H, q, J = 6.7 Hz), 3.525 (1H, tt, J = 11.1, 4.7 Hz), 2.561 (1H hex), 1.600 (3H, d, J = 6.8 Hz), 1.010 (3H, s), 1.001 (3H, d, J = 6.8), 0.961 (6H, s), 0.660 (3H, s), 0.674 (3H, t, J = 7.5).

28,28-Dimethylstigmasta-5,24-dien-3-ol (3 in SI Appendix, Fig. S3). 6β-Methoxy-3α,5-cyclo-5α-25-methylcholestan-24-one (11.0 mg) (62) was treated with a 0.5 M THF solution of isopropenylmagnesium bromide (0.8 mL) at 40 °C for 4.5 h (*SI Appendix*, Scheme S1*B*). Saturated aqueous solutions of NH_4Cl and NaCl were added and the mixture extracted with hexane/EtOAc 4:1 (*SI Appendix*, Scheme S1*B*). The organic layers were filtered through silica gel, the solvent was evaporated, and the products were separated by preparative TLC (hexanes/EtOAc 9:1). A 2:1 ratio of starting material to products was obtained which did not improve with time or heating (this is thought to be due to competing enolization).

The products were acetylated with 1 mL of DCM/py/Ac_2O 4:2:1 containing 3% DMAP at 40 °C ON (*SI Appendix*, Scheme S1*B*). A mixture was obtained after evaporation of the solvents which contained the anticipated tertiary allylic acetates together with what is thought to be acetoacetates and the acetates of the enol forms. It was unnecessary to separate these side products because they gave the same product in the next step.

The allylic acetates were treated with 0.5 cm Li wire in 8 mL NH_3 (liq.) containing 2.5 mL Et_2O for 30 min (*SI Appendix*, Scheme S1*B*). After quenching with EtOH, and evaporation of the solvents, the residue was extracted with saturated aqueous NH_4Cl and NaCl and hexane/EtOAc 4:1. The organic layers were filtered through silica gel, the solvent was evaporated, and the products were purified by preparative TLC (hexanes/EtOAc 39:1).

Deprotection of the *i*-methylether was carried out as follows: Approximately 1 mg of protected sterol was treated with 1.0 mL 1% TFA/DCM for 10 min. After the addition of 10 μL pyridine, the solvent was evaporated with a stream of N_2. The resulting TFA ester was removed by treatment with 1 mL 10% TEA/MeOH at 40 °C for 30 min (*SI Appendix*, Scheme S1*B*). The solvent was evaporated with a stream of N_2 and the product was purified by preparative TLC (hexanes/EtOAc 4:1) and HPLC using MeOH as the solvent. ^{1}H-NMR ($CDCl_3$, 600 MHz): 5.351 (1H, dt, J = 5.2, 1.8 Hz), 3.524 (1H, tt, J = 11.1, 4.5 Hz), 1.797 (3H, s), 1.659 (3H, s), 1.163 (9H, s), 1.008 (3H, s), 0.974 (3H, d, J = 6.6), 0.685 (3H, s).

E-26,27-Dimethylstigmasta-5,24(28)-dien-3-ol (E-28-Methylxestosterol, 4 in SI Appendix, Fig. S3). This was isolated from *Strongylophora durissima* and confirmed synthetically (51).

24,26-Dimethylstigmastanol (5 in SI Appendix, Fig. S3). Using the sequence given for sterol ***3*** with 6β-methoxy-3α,5-cyclo-5α-26-methylcholestan-24-one (63) and vinylmagnesium bromide led to 6β-methoxy-3α,5-cyclo-5α-26-methylstigmast-24(28)-ene as a 1:1 mixture of C-25 stereoisomers and an *E*/*Z* ratio of 5:1 (*SI Appendix*, Scheme S1*C*).

After dichlorocyclopropanation of 5 mg with 2 mL $CHCl_3$, 1.5 mL 12 M NaOH and 15 mg BTEAC for 12 hr., 20% of the starting material remained as exclusively the *E*-isomer (*SI Appendix*, Scheme S1*C*). After separation by preparative TLC (hexanes/EtOAc 79:1 2x), the starting material was deprotected as above to give *E*-26-methylstigmasta-5,24(28)-dien-3-ol as a 1:1 mixture of C-25 stereoisomers (*SI Appendix*, Scheme S1*C*). ^{1}H-NMR

($CDCl_3$, 600 MHz): 5.353 (1H, m), 5.147 (1H, q, J = 6.6 Hz), 3.525 (1H, m), 1.580 (3H, d, J = 6.8 Hz), 1.011 (3H, s), 0.965 (3H, d, J = 6.5), 0.960 (1.5H, d, J = 6.9), 0.956 (1.5H, d, J = 6.9), 0.804 (1.5H, t, J = 7.4), 0.800 (1.5H, t, J = 7.4), 0.687 (3H, s). The *Z*-, 24*S*-isomer (isostelliferasterol) has been isolated from *Rhabdastrella globostellata* (63) [wrongly identified as *Jaspis stellifera* (11)].

Dechlorination of the dichlorocyclopropyl fraction with Li/NH_3 also led to 10% dehydrogenation to give a $\Delta^{20(21)}$ by-product that was separated by preparative TLC. Its structure was determined by HMBC correlations between the olefinic C-21 signals (4.854 and 4.751, 4.815 and 4.733 ppm) and C-18 (0.619 and 0.606 ppm). This was not previously detected in analogous reactions without the C-26 methyl group (64).

In order to avoid the conversion of the *i*-methylether to the sterane during hydrogenolytic ring opening of the side chain cyclopropyl ring, the mixture was first deprotected to give the cyclopropyl Δ^5 sterol as a mixture of 8 stereoisomers, 4 of which, coming from the *Z*-isomer, were minor. The major ^{1}H-NMR signals (800 MHz) were 5.351, 3.523, 1.006, and 0.670 ppm corresponding to C-3, C-6, C-19, and C-18, respectively. HMBC and COSY spectra showed the major cyclopropyl C-28 methyls to cluster at 1.06 to 1.02 ppm, C-21 at 0.92 to 0.90 ppm, and the terminal side chain methyls at 0.88 to 0.82 ppm. The major cyclopropyl signals were at 0.6 to 0.5 ppm (CH); the CH_2 signals were at 0.45 to 0.42, 0.32 to 0.27, and –0.16 to –0.22 ppm. Signals arising from the minor *Z*-isomer were detected at 1.003 (C-19), 0.658 (C-18), 0.453, and -0.276 ppm (cyclopropane CH_2).

Hydrogenation in MeOH/AcOH 4:1 for 5 d with PtO_2 led to complete reduction of the Δ^5-bond, cyclopropyl ring cleavage, and 25% deoxygenation (sterane) (*SI Appendix*, Scheme S1*C*). Cyclopropyl ring cleavage reduced the number of stereoisomers from 8 to 4; nevertheless, the terminal side chain methyl groups were not resolved and were assigned by HMBC. ^{1}H-NMR ($CDCl_3$, 600 MHz): 3.587 (1H, m), 0.902 (3H, d, J = 6.5), 0.89 to 0.86 (3H 26-Me), 0.800 (3H, s), 0.78 to 0.75 (3H C-27), 0.76 to 0.72 (3H C-28), 0.679 (3H, s), 0.644 (3H, s).

***Z*-24,26,27-Dimethylergosta-5,25-dien-3-ol (Xestospongesterol, 6 in SI Appendix, Fig. S3).** This was isolated from a species of *Xestospongia* and confirmed synthetically (65).

28-Ethylstigmasta-5,24-dien-3-ol (7 in SI Appendix, Fig. S3). Using the sequence given for sterol **3** with 6β-methoxy-3α,5-cyclo-5α-26-methylcholestan-24-one (63) led to the title compound as a 1:1 mixture of 2 stereoisomers (*SI Appendix*, Scheme S1*D*). ^{1}H-NMR ($CDCl_3$, 600 MHz): 5.350 (1H, m), 3.524 (1H, m), 2.561 (1H hex, J = 6.9 Hz), 1.650 (3H, s), 1.639 (3H, s), 1.008 (3H, s), 0.984 (3H, d, J = 6.6 Hz), 0.926 (1.5H, d, J = 6.6 Hz), 0.916 (1.5H, d, J = 6.6 Hz), 0.791 (1.5H, t, J = 7.3 Hz), 0.784 (1.5H, t, J = 7.3 Hz), 0.684 (3H, s).

24-Butylcholesterol (8 in SI Appendix, Fig. S3). Hydrogenation of 24-(3-butenyl)-6β-methoxy-3α,5-cyclo-5α-cyclocholestane (66) in EtOAc and 10% Pd/C using a balloon, followed by deprotection of the *i*-methyl ether as above, gave the product as a 1:1 mixture of stereoisomers (*SI Appendix*, Scheme S1*E*). ^{1}H-NMR ($CDCl_3$, 600 MHz): 5.352 (1H, m), 3.525 (1H, m), 1.008 (3H, s), 0.922 (1.5H, d, J = 6.6), 0.919 (1.5H, d, J = 6.6), 0.891 (3H, t, J = 7.1), 0.828 (3H, d, J = 6.9), 0.810 (1.5H, d, J = 6.8), 0.803 (1.5H, d, J = 6.8), 0.678 (3H, s).

These C_{31} sterols (1–8; see *SI Appendix*, Fig. S3) were reduced to their sterane counterparts (I–VIII; see Fig. 3) with H_2 and Adams' catalyst at ambient temperature and pressure as described earlier in the *Experimental Section*.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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