PALEOECOLOGY

# Cryogenian evolution of stigmasteroid biosynthesis

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**Sedimentary hydrocarbon remnants of eukaryotic $C_{26}$–$C_{30}$ sterols can be used to reconstruct early algal evolution. Enhanced $C_{29}$ sterol abundances provide algal cell membranes a density advantage in large temperature fluctuations. Here, we combined a literature review with new analyses to generate a comprehensive inventory of unambiguously syngenetic steranes in Neoproterozoic rocks. Our results show that the capacity for $C_{29}$ 24-ethyl-sterol biosynthesis emerged in the Cryogenian, that is, between 720 and 635 million years ago during the Neoproterozoic Snowball Earth glaciations, which were an evolutionary stimulant, not a bottleneck. This biochemical innovation heralded the rise of green algae to global dominance of marine ecosystems and highlights the environmental drivers for the evolution of sterol biosynthesis. The Cryogenian emergence of $C_{29}$ sterol biosynthesis places a benchmark for verifying older sterane signatures and sets a new framework for our understanding of early algal evolution.**

## INTRODUCTION

All modern eukaryotes biosynthesize sterols or acquire them through dietary uptake. Incorporated into the cell membrane, they are essential for homeostasis and cell signaling within this domain (*1*), and their concentration in lipid rafts plays a significant role for budding and endocytosis through affecting membrane curvature (*2*). Given the rarity of extended sterol biosynthesis in bacteria (*3*), steranes preserved in sediments and ancient rocks have been frequently used as fossil biomarkers diagnostic of ancient eukaryotes. The large diversity of sterols in living cells is principally defined by double bonds and functional moieties that show limited survival after cell death (*4*). Diagenetic processes during sedimentary burial largely reduce the structural diversity of residual steroids to variations in their (C-24) side-chain alkylation, leaving mostly three major saturated sterane hydrocarbons containing 27, 28, or 29 carbon atoms: cholestane, ergostane, and stigmastane. Hence, it is predominantly this tripartite diversity that allows for paleobiogeochemical reconstructions of past eukaryotic diversity.

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### The geological record of steranes

Systematic changes in the relative abundance of these three steranes have been observed over the course of the past ~550 million years of Earth history. A continuously increasing relative abundance of ergostanes (*5*), for example, has been attributed to the global rise and radiation of chlorophyll c–containing phytoplankton (*6*). Significantly less is known about the initial rise and early evolution of the eukaryote lineage. Molecular clocks place the last eukaryotic common ancestor at ~1.8 billion years ago (*7*), whereas the oldest unambiguously eukaryotic acritarch microfossils date to ca. 1.6 billion years ago (*8*). However, this consensus date had been distorted for more than one decade by false biomarker positives: Sedimentary steranes in Proterozoic rocks [for example, (*9*)] and up to 2.7 billion years in age (*10*) steered discussions of a much earlier eukaryotic dawning. Only the advent of enhanced contamination awareness (*11*, *12*) and an unprecedented clean drilling operation (*13*) persuasively unmasked Archean steranes as modern contaminants. As a positive corollary, the burden of proof for the detection of indigenous and syngenetic sterane biomarkers throughout the Precambrian was significantly raised and led us to systematically (re-)analyze both previously studied and new Proterozoic sedimentary sequences with an unexpected outcome. We here report on the heterogeneous diversity and palaeogeographic distribution of unambiguously indigenous (Supplementary Materials) steranes throughout the Neoproterozoic, a finding that changes our understanding of early eukaryotic steroid biosynthesis and evolution.

The composite molecular inventory of Phanerozoic rocks, petroleum, or modern environmental samples virtually always contains the $C_{27–29}$ steroid troika in varying relative abundances (*1*, *4*, *5*). In broad terms, cholesterol biosynthesis is dominant in metazoa and rhodophyceae, whereas elevated abundances of $C_{29}$ phytosterols are biosynthesized by green algae and higher plants. However, it is noteworthy that even pure cultures of eukaryotic algae only rarely display an exclusive steroidal end-member dominance (*4*, *5*, *14*). It is only the early rock record that deviates significantly from this balanced pattern: A $C_{27}$-only steroidal distribution in the Tonian Chuar Group (*15*) was recently reconfirmed and supplemented by two other similarly aged deposits, which appear devoid of any conventional $C_{28}$ or $C_{29}$ steranes (*16*). On the other

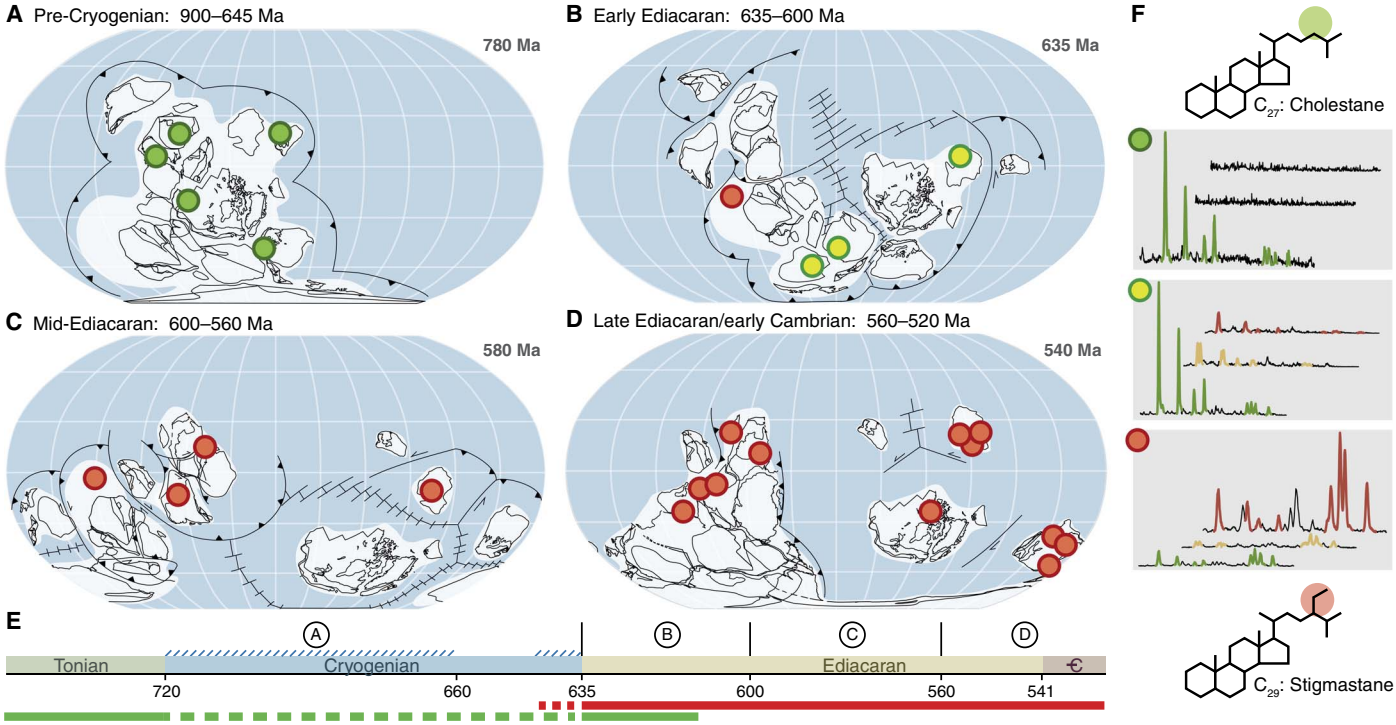


**Fig. 1. Evolutionary radiation of stigmasteroid (*24*) biosynthesis.** (**A**) Tonian rocks are nearly exclusively characterized by $C_{27}$ steranes [cholestane; see (F): Green symbols denote $C_{27}$ dominance and complete absence of $C_{29}$ steranes, with only sporadic traces of $C_{28}$ 4-desmethylsteranes]. (**B**) The oldest noncontaminated $C_{29}$ 24-ethylsteranes [stigmastane; see (F): yellow symbols stand for $C_{27} > C_{29}$, whereas red symbols indicate $C_{27} < C_{29}$ and often $C_{27} \ll C_{29}$] only appear during the latest Cryogenian (Marinoan deglaciation) and earliest Ediacaran in Oman, whereas contemporaneous rocks from three other localities are still strongly or exclusively dominated by $C_{27}$ steranes, indicating a 720- to 635-Ma Snowball Earth glacial origin of the stigmasteroid biosynthetic pathway—in agreement with the physiological membrane density advantage yielded by cellular phytosterols (*19*, *20*) and molecular clocks (*30*). (**C** and **D**) Global dominance of $C_{29}$ steroid biosynthesis by 600 Ma (table S1) highlights the evolutionary advantage of the likely chlorophyte host organism and a rapid rise to ecological dominance. (**E**) Schematic representation of Neoproterozoic time showing steroidal dominance (green, $C_{27}$ versus red, $C_{29}$) and the time brackets from (A) to (D). (**F**) Relevant molecular structures and schematic tandem-MS chromatograms of (bottom to top) $C_{27}$ [green, mass/charge ratio (*m/z*) 372 to 217], $C_{28}$ (yellow, *m/z* 386 to 217), and $C_{29}$ (red, *m/z* 400 to 217) steranes, emphasizing the color code used in (A) to (D). Ages on the top right of the maps comply with the palaeogeographic snapshots after (*31*).

end of the $C_{27–29}$ steroidal spectrum, it has long been known that certain Ediacaran to Early Cambrian oils in Oman, Siberia, and India are strongly dominated by $C_{29}$ steranes (*6*). However, because of the migrated nature of these petroleum fluids and the large temporal data gap spanning the Ediacaran, virtually nothing has been known on the timing and distribution of rising $C_{29}$ steroid abundances or what this peculiar signal could mean.

## RESULTS AND DISCUSSION

With additional analyses and a literature survey, we now convincingly show that the Late Ediacaran dominance of $C_{29}$ steranes does not represent rare or isolated depositional environments. This signature is not restricted merely to particular lithologies, latitudes, or facies zones (table S1), but instead seems representative of a highly uniform global steroid metabolism during the Late Ediacaran (Fig. 1), as confirmed by 67 samples from 14 localities (Supplementary Materials). Despite the rarity of thermally well-preserved and organic rich sedimentary strata of pre-Cryogenian age, we find that also in this time slice [ca. 900 to 720 million years ago (Ma)], the predominance of $C_{27}$ steranes is not dependent on environmental factors (table S1) but appears inherent to global steroid metabolism before the Cryogenian, whereas common $C_{29}$ steranes are systematically absent in all 35 samples from five localities spanning the globe (Fig. 1).

### Steroid biosynthesis and physiology

Across all clades—including some bacteria—steroid biosynthesis starts with the epoxidation of squalene (*17*), followed by enzymatic cyclization to one of two possible $C_{30}$ protosterols: lanosterol or cycloartenol (*1*, *4*). Although the evolutionary relationship between the lanosterol and cycloartenol cyclases has recently been found to be more complex than previously assumed (*3*), downstream modifications of lanosterol to both $C_{27}$ and $C_{28}$ sterols conserve the same reaction order of demethylations (C-4 and C-14) performed by the same enzymes after methylation at C-24 (*4*), but do not lead to 24-ethyl ($C_{29}$) steroids. Cycloartenol, on the contrary, can be a precursor to $C_{27}$–$C_{29}$ sterols (*18*) following a different reaction order, thus pointing to a separate evolutionary origin of this pathway and implying that cycloartenol biosynthesis likely preceded the emergence of $C_{29}$ steroids. This provides a plausible explanation for the sporadic co-occurrence of $C_{28}$ ergostane traces alongside the pre-Cryogenian $C_{27}$ signal (table S1), whereas $C_{29}$ steranes are systematically absent. A high compositional diversity of lipids is thought to ensure a stable and impermeable membrane even when cellular composition, osmolarity, or pH is changed because of physiological or pathological events (*2*, *4*). The presence of $C_{29}$ sterols in "raft-like" model membranes significantly lessens temperature dependence of membrane dynamics, as compared to systems with $C_{27}$ and $C_{28}$ sterols, suggesting that $C_{29}$ 24-ethylsterols are produced to extend the temperature range in which membrane-associated

biological processes can take place (*19*). Hence, the use of $C_{29}$ sterols yields an important advantage to large temperature fluctuations, and it has been hypothesized that this adaptive "membrane tuning" represents an evolutionary response to large temperature variations (*20*).

The Neoproterozoic Snowball Earth events—two severe glacial episodes within the time interval 717 to 632 Ma (*21*, *22*), whose globally distributed diamictite remnants reflect the magnitude and spatial extent of these glaciations (*23*)—represent the most pervasive climatic perturbation in all of Earth's history. However, the biological consequences, in particular whether Neoproterozoic life experienced an evolutionary bottleneck or a catalyst, are unclear. $C_{29}$ 24-ethylsteranes (*24*) are systematically absent from sediments deposited before the onset of the Snowball Earth events (Fig. 1A) but are present in rocks deposited during and directly after the Marinoan deglaciation (Fig. 1B), implying an origin of stigmasteroid (*24*) biosynthesis during the glaciation. Whereas subglacial water temperatures during the Cryogenian glaciations were relatively constant as a result of vigorous convective mixing (*25*), large diurnal temperature variations would have existed in low-latitude cryoconite pans (*26*). Recent climate models imply ice-free tropical continents (*27*), whose wind-blown dust would have created ablated glacial surfaces with meltwater above low-albedo accumulations of dust, biomass, and degraded organic matter (*28*). The modern equivalents of these cryoconite holes host diverse bacterial and eukaryotic communities and can be considered individual ecosystems (*29*). Hence, small and isolated eukaryotic populations were exposed to strong environmental forcing by frequent large temperature variations (*27*) in habitats with limited or periodic population mixing—providing an ideal scenario for beneficial mutation and localized evolutionary selection toward more successful $C_{29}$-sterol producing algae.

### The ecological rise of green algae

The localized evolution of stigmasterol (*24*) biosynthesis is suggested by a postglacial gradient: Around 635 Ma, that is, during and directly after the Marinoan deglaciation, eukaryotes in the South Oman Salt Basin (SOSB) were already dominated by $C_{29}$ sterol–producing species, whereas the algal community in three other coeval locations still largely consisted of eukaryotes synthesizing $C_{27}$ sterols (Fig. 1B). A $C_{29}$ sterane dominance in carbonates and shales that are embedded within the Marinoan diamictite in the SOSB—thus possibly up to 645 Ma in age (Supplementary Materials)—firmly places the rise of this biosynthetic capacity within, and not directly after, the Cryogenian. Although $C_{29}$ sterol–synthesizing algae are dominant in only one locality between 635 and 600 Ma, they had reached global dominance at the latest by 600 to 560 Ma (Fig. 1C). This rapid global radiation testifies to the exceptional ecological success of stigmasteroid-producing algae and, in turn, suggests that this biosynthetic capacity must have emerged during the Cryogenian glaciations and not earlier. Green algae predominantly biosynthesize $C_{29}$ sterols (*1*, *4*–*6*, *14*), dominate modern cryoconites (*29*), and likely existed before the Cryogenian (*7*). With molecular clocks indicating a Late Cryogenian divergence of green algal SMT genes (*carbon-24/28-sterol methyltransferase* responsible for C-24 methylation of sterols) from those of other Archaeplastida (fig. S1) (*30*), our data suggest that stigmasteroid biosynthesis emerged in an ancestral green alga and subsequently led to the rise of this group to ecological dominance.

## CONCLUSIONS

Dating the evolving sterol biosynthetic pathway not only provides a unique and precise calibration point for molecular clock studies of biochemical processes but also places a new benchmark for reliably recognizing steroid contamination in pre-Cryogenian sediments. Considering biochemical innovation and the physiological function of membrane lipids within a well-dated geological framework brings us closer to understanding the environmental drivers of evolving organismic complexity and sets a new framework for our understanding of early eukaryotic evolution.

## MATERIALS AND METHODS

For this study, we summarized selected biomarker data from the literature, where the indigeneity of components was evident or highly likely (see the Supplementary Materials for approach and rationale), and performed laboratory analyses on sedimentary rock samples. We analyzed the molecular inventory of 158 Neoproterozoic rocks, of which 61 yielded trustworthy and reliable steroidal signatures that were deemed as unambiguously syngenetic (table S1, Supplementary Materials). Five studied formations did not yield a single sample with uncontaminated steroidal signatures (table S2). Furthermore, 68 selectively chosen Neoproterozoic sterane values from the literature were used (table S1).

### Rock sample preparation and workup

Samples were analyzed using standard organic geochemical techniques under measures of extreme precaution, similar to techniques that we previously reported (*13*, *32*). In brief, samples were first separated into interior and exterior portions, either by sawing using a diamond-rimmed blade–fitted stainless steel saw [Lortone; blade cleaned by ultrasound-assisted solvent extraction in dichloromethane (DCM) and by baking at 450°C for 8 hours] or by the microablation technique (*33*). Sample interiors and exteriors were separately crushed and ground to a fine powder using a shatterbox (Siebtechnik, Scheibenschwingmühle) fitted with a custom-made stainless steel puck and mill, which were cleaned by baking at 500°C for 8 hours. In between samples, the puck and mill were cleaned by grinding and discarding clean quartz sand five times and by solvent rinsing with DCM. The resulting sample powders were solvent-extracted (DCM) by ultrasound agitation in Teflon vessels or by using a CEM Mars 6 microwave extraction approach (up to 30 g of powder extracted with three times 30 ml of DCM under stirring at 120°C for 20 min each). The resulting total lipid extracts were desulfurized using activated (with $HCl_{aq}$), neutralized, and solvent-extracted copper pellets and fractionated on silica gel columns into saturated hydrocarbons (SAT), aromatic hydrocarbons, and polar compounds, as described in more detail elsewhere (*32*). SAT were spiked with internal standards ($C_{30}D_{62}$ triacontane and $d_4$-5α-cholestane) and analyzed using coupled gas chromatography (GC) and mass spectrometry (MS).

### GC and MS

Full-scan analyses were performed on a Trace GC Ultra (Thermo Scientific) coupled to an ALMSCO BenchTOF-dx mass spectrometer. The gas chromatograph was fitted with a VF-1 MS column [40 m; inner diameter (i.d.), 0.15 mm; film thickness, 0.15 μm] and operated with a constant flow (1.4 ml/min) of helium (99.999 % pure, Westfalen AG) as a carrier gas. Samples (between 1 out of 50 and 1 out of 500 μl) were injected in splitless mode using a programmed temperature vaporizer injector (ramped from 60° to 315°C at 14.5°/s). The GC oven was held at 60°C (2 min) before ramping at 4.5°/min to a final temperature of 325°C, which was held for 10 min. Ionization was

achieved at 70 eV (electron impact) and 250°C with a filament current of ca. 4 A. Data were measured from *m/z* 30 to 800 but only recorded from *m/z* 50 to 550 at ca. 1000 mass resolution using 2469 scans per scanset and a scanset period of 250 ms. Analytes were quantified by comparison to internal standards without correcting for individual response factors.

Target compound analysis for biomarkers (including steranes) was performed on a Thermo Quantum XLS Ultra triple-quadrupole mass spectrometer coupled to a Thermo Trace GC Ultra, fitted with a DB-XLB capillary column (60 m; i.d., 0.25 mm; film thickness, 0.25 µm) and a deactivated precolumn (10 m; i.d., 0.53 mm). A constant flow (1.3 ml/min) of helium (99.999 % pure, Westfalen AG) was used as a carrier gas. Volumes of typically 1 or 2 µl out of 50 to 200 µl were injected on column at 70°C. The oven was held isothermal at 70°C (5 min), then heated to 335°C at 4°/min, and held at the final temperature for 9 min. Ionization was achieved by electron impact at 70 eV and 250°C, with an emission current of 50 µA. Q1 and Q3 were each operated in 0.7-Da resolution with a cycle time of 0.5 s. Q2 was operated with Argon 5.0 collision gas at a pressure of 1.1 mtorr and varying collision voltages depending on the target analyte. Compounds were quantified on characteristic parent-to-daughter ion mass transitions (for $C_{27}$–$C_{29}$ steranes: *m/z* 372, 386, and 400 fragmenting to *m/z* 217) relative to $d_4$-5α-cholestane (*m/z* 376 fragmenting to *m/z* 221) without correcting for differential response factors.

### Hydropyrolysis of kerogens

For hydropyrolysis (University of Nottingham), aliquots of 60 to 125 mg of purified kerogen were mixed with ammonium dioxydithiomolybdate catalyst. First, a procedural blank was run and tested negative (GC-MS at the University of Nottingham) for steranes. Subsequently, each sample/catalyst mixture was interspersed with ~100 mg of pre-extracted and calcined acid-washed quartz sand to "bulk out" the sample and prevent reactor blockage. A thermal desorption step was run from ambient to 250°C at 300°/min and subsequently to 350°C at 8°/min and held for 2 min, during which released "free" hydrocarbons were trapped on silica. After changing the trap silica, a pyrolysis step was run from ambient to 350°C at 300°C/min, then to 520°C at 8°/min, and held for 2 min, during which covalently bound hydrocarbons were "cracked" and trapped on silica. Between samples, the system was cleaned thoroughly: Reactor tubes and fittings were cleaned by ultrasound-assisted extraction in DCM, followed by heating to 550°C (30 min) in the HyPy system.

### Identification of SMT genes

Genes coding for two SMT enzymes in the green alga *Ostreococcus lucimarinus* were identified from the literature (*30*) and via GenBank (www.ncbi.nlm.nih.gov/). A homologous sequence search in green algae was carried out in GenBank using BLASTp with a cutoff threshold of $<1 \times 10^{-5}$.

## SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/3/9/e1700887/DC1

Supplementary Text

fig. S1. Molecular clock analysis of SMT genes (*30*) reveals a divergence of green algae during the Late Cryogenian.

fig. S2. Studied sample locations on a modern continental configuration.

fig. S3. Studied sample locations in a palaeogeographic context after (*31*).

fig. S4. Estimated maximum time brackets of the studied samples.

fig. S5. Hydropyrolysis of an earliest Ediacaran cap carbonate sample from the Mirassol d'Oeste Fm verifies the $C_{27}$ sterane–dominated signature found in other samples from this unit and confirms their syngenicity to the host rock.

table S1. Steranes in Neoproterozoic rocks.

table S2. Unsuitable samples.

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