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Sources of C₃₀ steroid biomarkers in Neoproterozoic–Cambrian rocks and oils

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Nettersheim et al.¹ propose that unicellular Rhizaria are the likely biological source of the C₃₀ steroidal hydrocarbons found abundantly in Neoproterozoic–Cambrian sedimentary rocks. Their hypothesis challenges earlier research arguing that 24-isopropylcholestane (24-ipc) and 26-methylstigmastane (26-mes) are produced by demosponges and, therefore, early animal biomarkers^{2–4}. Fundamental problems beset the new steroid biomarker data and its interpretation¹.

The primary problem is one of mass balance. C₃₀ steranes typically account for ~3%, on average, of the total C₂₇–C₃₀ steranes in many Neoproterozoic rocks and oils^{2,4} with 24-ipc, alone, accounting for >1% of the total sterane signal. Since all sterols typically follow similar preservation pathways, the carbon number distributions of C₂₇–C₃₀ steranes in the geological record should closely match proportions of sterol precursors of the source biota. Accordingly, plausible biological sources of ancient 24-ipc and 26-mes sterols must have

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Author contributions

J.A.Z. and G.D.L. performed the MRM GC–MS analyses at UC Riverside and wrote the manuscript in collaboration with R.E.S. J.A.Z., G.D.L. and R.E.S. interpreted the lipid biomarker data. P.C., E.A.S., M.R., E.G. and J.P.G. provided comments and edits on drafts of the manuscripts.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper and/or the cited references.

Competing interests

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been capable of biosynthesizing these compounds among their major sterols. The C₃₀ sterane percentage estimates listed in Supplementary Table 2 in Nettersheim et al.¹ are mostly <0.01% of total. In contrast, some contemporary demosponge taxa produce 24-ipc⁵ and 26-mes⁶ sterols among their major lipids, occasionally representing 10–99 wt% of total sterols. Some foraminifera, such as *Amphistegina lobifera* in Fig. 1 and as shown in the study of *Allogromia laticollaris* by Grabenstatter et al.⁷, are a possible Neoproterozoic source of 24-*n*-propylcholestane (24-npc), but this cannot explain the accompanying 24-ipc and 26-mes signals given the compound ratios (for example, 24-ipc typically 24-npc and above a threshold minimum ratio of 0.5) in samples from Oman, East Siberia and India^{2,4}. Of all the Rhizaria discussed by Nettersheim et al.¹, only the *Shepherdella*¹ and *Allogromia*⁷ forams produce C₃₀ sterols at >1% of total C₂₇–C₃₀ steroids and neither yield 24-ipc or 26-mes, the compounds hypothesized to be sponge biomarkers.

Most of the Rhizaria samples contain only traces of C₃₀ steroids¹, often down to <0.01% of total, at abundances not commonly reported. Even if such low levels could be quantified reliably, it is impossible to exclude steroids of dietary or other exogenous origins rather than these being from de novo biosynthesis. Evidence for this is the presence of 24-norcholestane, a biomarker associated with diatoms⁸ that is sometimes more abundant than the C₃₀ steroids in their data. Despite the reported low abundances of C₃₀ steroids, no controls to assess background or dietary steroid signals were conducted for the culture media, or the marine waters from which these rhizarians were grown or collected. The reliance on partial chromatograms, as in Fig. 2 in Nettersheim et al.¹, and without also displaying the complete sterane patterns or acknowledging multiple unassigned peaks, further disguises the mass balance problem.

A second problem concerns the indirectness of the approach. No analyses of protist sterols were performed to verify the sterane product distributions generated from catalytic hydrogenation. The 26-mes identifications¹ are unsupported by way of mass spectra or direct ties to specific sterol precursors.

Nettersheim et al. also propose that some Cercozoa can synthesize sterol precursors for all three of the major C₃₀ steranes (24-npc, 24-ipc, and 26-mes) found in Neoproterozoic rocks and oils. However, a previous investigation of sterol distributions from Cercozoa⁹ found only conventional sterols up to C₂₉, similar to those in green algae and plants. The presence of just two *sterol 24-C-methyltransferase* (SMT) biosynthesis gene homologues⁹ suggests that C₃₀ sterols are dietary, exogenous, or unintended trace biosynthetic products of these Rhizaria¹⁰. Further, we could not reproduce the noisy C₃₀ sterane profiles reported by Nettersheim et al.¹ for Cercozoa for a *Chlorarachnion reptans* hydrogenation product supplied to us (Fig. 1) and independently analysed in two different laboratories. All of this casts doubt on whether or not the C₃₀ steranes reported for the Cercozoan cultures are genuine signals or artefacts.

The vanishingly low C₃₀ steroid contents reported for the majority of Rhizaria highlights an intractable sterane mass balance problem that cannot be reconciled with the published ancient record. Failing to account for dietary steroids, a lack of comprehensive blank controls, and reproducibility issues for C₃₀ sterane data for Cercozoa cast doubt on the

hypothesis of Nettersheim et al.¹. In the absence of convincing evidence that Rhizaria can produce abundant 24-ipc and/or 26-mes, demosponges are currently the only known biological sources of 24-ipc and 26-mes sterol precursors that account for the patterns of C₃₀ steranes typically observed in the Neoproterozoic–Cambrian rock record.

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Fig. 1 |. Comparison of C30 sterane distributions obtained from hydrogenation of a demosponge (*R. distincta*) versus protists (*C. reptans* and *A. lobifera*).

Multiple reaction monitoring (MRM) gas chromatography–mass spectrometry (GC–MS) ion chromatograms showing sterane distributions (M⁺ → 217 Da ion transitions) for hydrogenation products from the demosponge *Rhabdastrella distincta*⁴ and two rhizarian protists (*A. lobifera* and *C. reptans*) supplied to us by Nettersheim et al.¹. While our MRM analyses confirmed the sterane distributions for *A. lobifera*, we could not reproduce the C₃₀ sterane signal or compound distributions reported for *C. reptans*¹. **a**, For C₃₀ steranes, repeat MRM analyses of *R. distincta*, even at low sample concentrations using high dilution factors, consistently yielded a well-defined 26-mes signal. In contrast, *C. reptans* principally generated noise even at optimum solution concentrations. A similar absence of robust C₃₀ sterane signal for *C. reptans* was also observed using GC–triple quadrupole (QQQ)–MS analysis performed at Massachusetts Institute of Technology. **b**, The total C₂₇–C₃₀ sterane distribution for *C. reptans* is dominated by C₂₈ and C₂₉ compounds, similar to green algae, as found previously for chlorarachniophyte protists⁹. **c**, Any trace C₃₀ sterane signal hidden within the noise may only be present at levels <0.002% of total steranes, which is at least

two orders of magnitude lower than the percentage abundances routinely reported in steroid assays. Compound assignments for any putative C₃₀ steranes at these trace levels is problematic and not commonly practiced. A dilution factor of 1 represents the optimum concentration for maximizing C₂₇–C₂₉ sterane signal.