Bacterial sterol methylation confounds eukaryotic biomarker interpretations

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Sterol lipids are required by most eukaryotes and are readily preserved as sterane molecular fossils. These geologic steranes are broadly interpreted as biomarkers for ancient eukaryotes[1,2] although diverse bacteria also produce sterols[3]. Steranes with side-chain methylations can act as more specific biomarkers[4] if their sterol precursors are limited to particular extant eukaryotes and are absent in bacteria. An abundance of one such sterane, 24-isopropylcholestane, in late Neoproterozoic rocks has been attributed to marine demosponges and potentially represents the earliest evidence for animals on Earth[5]. However, debates over this interpretation[6–14] continue given the potential for alternative sources of 24-isopropylcholestane and the lack of experimental evidence demonstrating the function of enzymes that methylate sterols to give the 24-isopropyl side-chain. Here we show that sterol methyltransferases from both sponges and bacteria are functional and identify three bacterial methyltransferases each capable of sequential methylations resulting in the 24-isopropyl sterol side-chain. We identified two of these propylating enzymes in a demosponge metagenome suggesting bacterial symbionts contribute to 24-isopropyl sterol biosynthesis in demosponges. Our results demonstrate yet-uncultured bacteria have the genomic capacity to synthesize side-chain alkylated sterols and should therefore be considered when interpreting side-chain alkylated sterane biomarkers in the rock record.

26 **Main text**

27 According to the sponge biomarker hypothesis, two side-chain alkylated sterane
28 biomarkers, 24-isopropylcholestane (24-ipc) and 26-methylstigmastane (26-mes), indicate
29 marine sponges of the Demospongiae class[5,15] (Fig. 1**a**). The co-occurrence of 24-ipc and
30 26-mes in the late Neoproterozoic (660-540 Ma)[5,15], preceding the earliest sponge
31 macrofossils by ~100 million years[7], may represent the first evidence of animals in the
32 geologic record. However, debate over this interpretation continues for several reasons[6–14]
33 including the potential for alternative sources. Demosponges are the only extant organisms
34 known to contain sterols with the 24-ipc and 26-mes side-chain structures as their major
35 sterols[15–21], but pelagophytes, which produce 24-*n*-propylcholesterol as their major sterol,
36 also contain 24-isopropyl sterols[22–25]. Trace amounts of 24-ipc and 26-mes were also
37 reported in hydrogenated lipid extracts from Rhizaria[10], although attempts to replicate these
38 results were not successful[11]. In laboratory pyrolysis experiments under conditions that
39 may mimic diagenesis, 24-ipc and 26-mes form from certain C_{29} sterols suggesting
40 Neoproterozoic 24-ipc and 26-mes could have formed abiotically from algal sterols[13,14].
41 Further, microbial symbionts, including bacteria from diverse phyla, can constitute up to
42 40% of sponge biomass[26]. Bacterial symbionts may therefore contribute to 24-isopropyl
43 and 26-methyl sterol biosynthesis in sponges, although bacterial enzymes that methylate
44 the sterol side-chain have not been previously identified.

45 One approach to constrain uncertainties in the sponge biomarker hypothesis is to
46 identify and characterize the enzymes responsible for these distinct alkylations at C-24 and
47 C-26. Sterol 24-C-methyltransferases (SMTs) alkylate the C-24 position at an isolated
48 double bond in an *S*-adenosylmethionine (SAM) dependent mechanism[27] and have been

49 characterized extensively in fungi and plants. Bioinformatic analyses addressing this
50 reaction in sponges prompted the hypotheses that at least two SMT copies in a genome are
51 required to produce 24-propyl sterols, and that sponge SMTs can perform multiple
52 methylations at C-24[28]. However, no experimental evidence verifying this activity by any
53 sponge-derived SMT currently exists, and SMTs that produce 24-propyl sterols have not
54 been identified in any extant organism.

55 **Sponge SMTs are functional.** Our first goal was to express putative sponge SMTs
56 and verify methylation activity in vitro. We chose eight SMT homologs identified in
57 genomes and transcriptomes from eight sponges of the Demospongiae,
58 Homoscleromorpha, and Calcarea classes, each of which have been shown to contain 24-
59 ethyl sterols at the species[29–32] or genus[32–34] level. We tested each SMT with two potential
60 substrates, desmosterol or 24-methylenecholesterol, and analyzed sterol products using gas
61 chromatography-mass spectrometry (GC-MS). All eight of the sponge SMTs methylated
62 desmosterol to produce 24-methyl sterols including 24-methylenecholesterol and
63 (epi)codisterol (Table 1). Six SMTs also methylated 24-methylenecholesterol to 24-ethyl
64 sterols including fucosterol, isofucosterol, and (epi)clerosterol (Fig. 1**d**). None of the
65 sponge SMTs produced 24-propyl sterols.

66 **Bacterial SMTs produce 24-isopropyl sterols**. Our analyses of sponge symbiont
67 metagenomes revealed several SMT homologs, many of which occurred in gene clusters
68 with homologs of other known sterol biosynthesis genes including the bacterial C-4
69 demethylases *sdmA* and *sdmB*[35] (Fig. 2). We therefore hypothesized bacterial symbionts
70 may contribute to side-chain alkylation in sponges. We chose 10 putative bacterial SMTs
71 identified in metagenomes from four demosponge species for analysis. Eight symbiont

72 SMTs methylated desmosterol to 24-methyl sterols including (epi)codisterol, 24-
73 methylenecholesterol, and 24-methyldesmosterol (Table 1, Fig. 1**c**). Three of these SMTs
74 also methylated 24-methylenecholesterol to 24-ethyl sterols including fucosterol,
75 isofucosterol, and (epi)clerosterol (Fig. 1**d**). Two SMTs identified in a metagenome from
76 *Aplysina aerophoba,* a demosponge known to contain trace 24-isopropyl sterols[21], also
77 methylated 24-methylenecholesterol to two distinct C_{30} sterols. The first symbiont SMT
78 produced 24*S*-24-isopropylcholest-5,25-dienol while the second produced 24-
79 isopropylcholest-5,24-dienol. Both propylating SMTs were capable of all three
80 methylation steps required to synthesize the 24-ipc side-chain via a single protein. One
81 propylating SMT was identified in a sterol biosynthesis gene cluster suggesting a yet-
82 unknown bacterial sponge symbiont has the genomic capacity to synthesize 24-isopropyl
83 sterols de novo (Fig. 2).

84 Identification of bacterial symbiont SMTs capable of side-chain alkylation led us
85 to question if bacteria from environments outside a sponge host could also produce side-
86 chain alkylated sterols. Through additional searches, we identified SMT homologs in a
87 variety of environmental metagenomes and metagenome-assembled genomes (MAGs).
88 We tested 14 of these SMT homologs from marine, freshwater, thermal spring, and coral
89 reef environments. Twelve environmental SMTs alkylated the sterol side-chain to produce
90 24-methyl and/or 24-ethyl sterols (Table 1). One SMT, identified in a drinking water filter
91 MAG assigned to the phylum Chlamydiae, produced both 24*S*-24-isopropylcholest-5,25-
92 dienol and 24-isopropylcholest-5,24-dienol (Fig. 1**a**).

93 The identification of functional metagenomic SMTs likely derived from bacteria,
94 including three that produce 24-isopropyl sterols, is significant given that no bacterial

95 proteins had previously been shown to alkylate the sterol side-chain. To confirm that these
96 SMTs are truly of bacterial origin, we performed phylogenetic analyses of the 24
97 metagenomic SMTs we tested. In our maximum-likelihood tree of SMT proteins (Fig. 3**a**),
98 the majority of the metagenomic SMTs are distinct and more closely related to each other
99 than to the SMTs of eukaryotes known to produce side-chain alkylated sterols. Further, 23
100 of the 24 putative bacterial SMTs, including those from sponge metagenomes, form two
101 clades separate from the sponge SMT clade. It is therefore unlikely that these metagenomic
102 SMTs were acquired from sponges via horizontal gene transfer. However, the observed
103 bootstrap values were not robust enough to draw any additional evolutionary conclusions,
104 nor to eliminate any remaining uncertainty of the bacterial origin of these SMTs. We
105 therefore used the recently developed classifier Whokaryote, which predicts whether a
106 metagenomic contig is likely eukaryotic or prokaryotic based on intergenic distance, gene
107 density, gene length, and k-mer frequencies. Whokaryote predicts all 19 of the contigs
108 containing the metagenomic SMTs tested here to be of prokaryotic origin (Extended Data
109 Table 2).

110 The genomic context of the SMTs we tested provides additional evidence of their
111 bacterial origin. Of the 24 metagenomic SMTs, nine were in gene clusters or MAGs with
112 a homolog of oxidosqualene cyclase (OSC), a key enzyme in sterol biosynthesis
113 responsible for cyclizing oxidosqualene to lanosterol[3]. OSCs cluster into robust
114 phylogenetic clades with high bootstrap support[36]. Thus, phylogenetic analyses of OSCs
115 associated with the SMTs we tested could confirm their bacterial origin. In our maximum-
116 likelihood tree of OSC proteins (Fig. 3**b**), we see that seven of the OSCs associated with
117 the SMTs analysed here, including four from demosponge metagenomes, fall into the

118 Group 1 clade which contains only bacterial OSCs. The remaining 2 OSCs, from soil and
119 hot spring sediment metagenomes, fall into bacterial Group 2. The position of these OSCs
120 provides additional evidence that the metagenomic SMTs we tested originate from a
121 bacterial source.

122 **Side-chain propylation mechanism.** It was surprising to observe that one SMT
123 was capable of propylating the sterol side-chain on its own as it has been hypothesized that
124 at least two SMTs would be required for this reaction[28]. To better understand this
125 unexpected biosynthetic pathway, we performed additional in vitro reactions with two of
126 the bacterial propylating SMTs using ^{13}C-labeled sterol substrates and analyzed the
127 resulting products by nuclear magnetic resonance (NMR; Extended Data Table 3). In
128 experiments with [28-^{13}C] 24-methylenecholesterol as the substrate of the *A. aerophoba*
129 symbiont SMT, the major C_{29} products were confirmed as isofucosterol and fucosterol
130 present in a 7:1 ratio. Trace epiclerosterol, but not clerosterol, was also detected. The
131 product of triple side-chain methylation was confirmed as *24S*-24-isopropylcholesta-5,25-
132 dienol. The label was found exclusively at C-25, and the *24R* isomer was absent (Extended
133 Data Fig. 2**a**). Fucosterol was not a substrate for the *A. aerophoba* symbiont SMT, but [29-
134 ^{13}C] isofucosterol yielded *24S*-24-isopropylcholesta-5,25-dienol with the label distributed
135 in a 4:1 label ratio between C-27 and C-26 (Extended Data Fig. 2**b**). This outcome shows
136 interconversion of the C-28 and C-25 cations by hydride-shifts is not involved in the
137 biosynthesis of this compound, and deprotonation of the initially formed cation is
138 unspecific.

139 The same set of reactions with the propylating Chlamydiae MAG SMT gave similar
140 results. Fucosterol was not a substrate and epiclerosterol, but not clerosterol, was detected.

141 However, minor differences were measured between these two bacterial SMTs. In
142 experiments with [29-^{13}C] isofucosterol and the Chlamydiae MAG SMT, the ratio of
143 isofucosterol and fucosterol was 30:1, and the ratio of label at C-27/C-26 of *24S*-24-
144 isopropylcholesta-5,25-dienol was 5:1. Additionally, the C_{30} Δ^{23} and Δ^{24} isomers, 24-
145 isopropylcholesta-5,23-dienol and 24-isopropylcholesta-5,24-dienol, were also detected.
146 The label was detected in the Z-vinylic methyl of 24-isopropylcholesta-5,24-dienol and in
147 one of the two methyls of the Z-isopropyl group of 24-isopropylcholesta-5,23-dienol.
148 These results provide evidence for one hydride-shift to generate the C-24 cation. The ratio
149 of *24S*-24-isopropylcholesta-5,25-dienol and its Δ^{24} and Δ^{23} isomers was estimated to be
150 20:4:1, respectively. Though minor mechanistic differences between these two propylating
151 SMTs exist, the predominant pathway to 24-isopropyl sterols observed here proceeds from
152 desmosterol to 24-methylenecholesterol to isofucosterol to 24*S*-24-isopropylcholest-5,25-
153 dienol (Fig. 1**b**).

154 **Discussion**. The results presented here experimentally verify that sponges do
155 indeed encode functional SMTs capable of sequentially methylating desmosterol to 24-
156 methylenecholesterol to 24-ethyl sterols, similarly to SMTs recently identified in annelid
157 worms[37]. However, as in Annelida[38], this activity was inconsistent among Porifera as the
158 SMTs from *Oscarella carmela* (Homoscleromorpha) and *Sycon ciliatum* (Calcarea) did
159 not sequentially methylate sterols, contrary to previous hypotheses[28]. Sponge-derived
160 SMTs that produce 24-ethyl sterols may therefore be restricted to certain species, although
161 we recognize these SMTs may require a different substrate than we provided in our assays.
162 Substrate specificity could also explain why no sponge SMT produced 24-isopropyl sterols
163 in our experiments, although this result was unsurprising given that we did not test SMTs

164 from sponges known to contain propylated sterols due to the lack of available genomic
165 data. However, we did show that bacterial symbionts have the genomic capacity to
166 synthesize these propylated lipids. Previous studies have provided evidence of de novo
167 sterol biosynthesis in demosponges[39–41], but the experiments were performed prior to the
168 development of 16S community sequencing using cell-free sponge extracts or whole
169 sponge tissue from which the presence of bacterial symbiont-derived enzymes cannot be
170 excluded. Additionally, transcripts for several key sterol biosynthesis genes in the
171 demosponge *Vaceletia* sp. were derived from bacteria[42]. Both sponge and bacterial
172 symbiont proteins may therefore be required to produce 24-isopropyl sterols in vivo.
173 However, the metagenomic gene clusters containing functional SMTs identified here,
174 along with steroid gene clusters containing SMT homologs previously identified in
175 bacterial MAGs associated with demosponges of the *Ircinia* genus[43], suggest bacterial
176 symbionts may produce 24-isopropyl sterols de novo without demosponge input. Our work
177 highlights the need for robust sequencing of both the sponge host and its microbiome
178 coupled to sterol analysis in demosponge species that contain 24-isopropyls as their major
179 sterols. This may reveal sponge-derived SMTs sufficient for the biosynthesis of these lipids
180 and confirm sponges and/or their bacterial symbionts as potential sources of 24-ipc
181 biomarkers.

182 The identification of three bacterial SMTs each capable of producing the 24-
183 isopropyl side-chain via a single protein is also significant. Molecular clock analyses
184 suggest demosponges were the likely source of Cryogenian 24-ipc biomarkers given that
185 demosponges acquired a second SMT copy prior to their deposition, while pelagophytes
186 did not acquire a third SMT copy until the Phanerozoic[28]. Additionally, researchers have

187 argued against Rhizaria as a source of 24-isopropyl sterols given that *Bigelowiella natans*
188 encodes only two SMT copies and is thus unlikely to synthesize 24-propyl sterols[11]. These
189 interpretations rely on the hypothesis that multiple SMT copies are required to produce 24-
190 isopropyl sterols. However, our results demonstrating one SMT is sufficient for side-chain
191 propylation make SMT copy number an unreliable predictor of an organism's ability to
192 produce 24-propyl sterols. Now that propylating SMTs have been identified, site-directed
193 mutagenesis as performed with yeast[44,45] and plant[46] SMTs may reveal specific amino acid
194 changes that allow an SMT protein to perform a third methylation. If such key residues are
195 identified, they could act as a more reliable predictor of 24-isopropyl sterol biosynthesis
196 directly from sequencing data than SMT copy number alone.

197 In addition to its use as a demosponge biomarker, 24-ipc has also been used to
198 indicate marine input into terrestrial systems[47] given that 24-ipc precursor sterols are only
199 found in marine demosponges and algae. However, the production of 24-isopropyl sterols
200 by an SMT identified in a freshwater Chlamydiae MAG suggests these sterols may occur
201 in freshwater environments. Chlamydiae have not been shown to produce sterols, and
202 homologs of other sterol biosynthesis genes were absent in the Chlamydiae MAG. It is
203 therefore unlikely that this bacterium synthesizes 24-isopropyl sterols de novo. However,
204 some endosymbiotic Chlamydiae recruit sterols from their eukaryotic host into their
205 membranes[48]. Certain freshwater Chlamydiae may therefore methylate host-acquired
206 sterols to give the 24-isopropyl side-chain, which could explain the presence of 22-
207 dehydro-24-isopropylcholestanol throughout a freshwater sediment core from Motte Lake,
208 France[49]. We would therefore exercise caution when interpreting the presence of 24-ipc as
209 evidence for seawater incursion events.

210 Finally, although we identified SMTs that produce 24-isopropyl sterols, proteins
211 that methylate sterols to give other geologically important side-chain structures remain
212 undiscovered. Identification of enzymes that methylate sterols at the C-26 position to give
213 the 26-mes side-chain structure remains especially important when considering the
214 biomarker evidence for Precambrian sponges. SMTs from sponges and bacteria produced
215 (epi)codisterol suggesting both groups have the genomic capacity to produce substrates for
216 hypothetical 26-SMTs[39,50]. However, it remains to be seen if sponges and bacteria, sponge-
217 associated and otherwise, encode SMTs capable of methylation at C-26. Once identified,
218 in-depth kinetics studies with these enzymes and multiple substrates may reveal if SMTs
219 from sponges and/or bacteria are likely to produce 24-ipc and 26-mes sterol precursors in
220 ratios corresponding to the C_{30} sterane distributions observed in the Cryogenian. Verified
221 side-chain alkylated sterol biosynthesis in a bacterial isolate along with identification of
222 any unique isotopic signatures resulting from this reaction could also confirm bacteria as a
223 source these biomarkers. Until then, the co-occurrence of 24-ipc and 26-mes in high
224 relative abundance continues to provide compelling evidence for derived sponges in the
225 Cryogenian. However, sterol side-chain methylation by symbiotic and free-living bacteria
226 should now be considered when interpreting the presence of ergostane, stigmastane, and
227 24-isopropylcholestane in the geologic record.
228

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354 **Methods**

355 **Bioinformatics analysis.** SMT homologs were identified in the Joint Genome
356 Institute Integrated Microbial Genomes & Microbiomes (JGI IMG), GenBank, and
357 Compagen (http://www.compagen.org) databases using the *Saccharomyces cerevisiae*
358 S299C SMT amino acid sequence as the BLASTP search query (locus tag: YML008C,
359 maximum e-value: $1e^{-50}$, minimum percent identity: 30%). Additional demosponge SMT
360 sequences from *C. nucula*, *S. fasciculatas*, and *P. ficiformis* transcriptomes were provided
361 by Prof. David A. Gold[28]. Identifying sequence information is available in Extended Data
362 Table 1. Conserved sterol methyltransferase C-terminal and methyltransferase 11 domains
363 were identified using the HmmerWeb tool (version 2.41.1).

364 Oxidosqualene cyclase homologs were identified through BLASTP searches of the
365 metagenome scaffolds and MAGs containing the SMTs analyzed in this study and all
366 bacterial and eukaryotic genomes available in JGI IMG as of October 2019 using the

367 *Methylococcus capsulatus* Bath OSC protein sequence (locus tag: MCA2873, maximum
368 e-value: $1e^{-50}$, minimum percent identity: 20%, minimum sequence length: 350 aa).
369 Redundancy was decreased through manual removal of OSC homologs with 100%
370 similarity at the amino acid level. The N-termini of fused SMO/OSC homologs were
371 trimmed out of the dataset to begin the sequences at the squalene-hopene cyclase N-
372 terminal domain as identified using HmmerWeb.

373 All protein sequence datasets were aligned via MUSCLE. Conserved SMT domains
374 were aligned separately, then concatenated using Geneious. Maximum-likelihood trees
375 were generated using IQ-Tree on XSEDE with 1,000 ultra-fast bootstrap replicates using
376 the best amino acid substitution models, gamma shape parameters, and invariable sites
377 proportions under the Bayesian Information Criterion and Akaike Information Criterion
378 using ModelTest-NG on XSEDE (Extended Data Table 4). Resulting phylogenetic trees
379 were visualized in iTOL[51].

380 All contigs containing metagenomic SMTs were retrieved from JGI IMG or
381 GenBank and analyzed with Whokaryote[52] using the default inputs with the minimum
382 contig size decreased to 2,000 base pairs. Calculated features and predictions are shown in
383 Extended Data Table 2.

384 **Gene synthesis and molecular cloning.** SMT DNA sequences were codon-
385 optimized for expression in *E. coli* and artificially synthesized by GeneArt (ThermoFisher
386 Scientific) or through the Department of Energy Joint Genome Institute (DOE JGI) DNA
387 Synthesis Science Program. SMT sequences from DOE JGI were obtained in the IPTG-
388 inducible plasmid pSRKGm-*lac*UV5-rbs5[53] in *E. coli* TOP10. SMT sequences from
389 GeneArt were subcloned into pSRKGm-*lac*UV5-rbs5 by sequence and ligase independent

390 cloning (SLIC)[54] and transformed into electrocompetent *E. coli* DH10B (Invitrogen) using
391 a MicroPulser Electroporator (BioRad). Oligonucleotides were purchased from Integrated
392 DNA Technologies (Coralville, IA). PCR was performed according the to the
393 manufacturer's protocol using Phusion DNA Polymerase (New England Biolabs). DNA
394 fragments were purified using the GeneJET Gel Extraction Kit (ThermoFisher Scientific).
395 Plasmid DNA was isolated using the GeneJET Plasmid Miniprep Kit (ThermoFisher
396 Scientific). DNA was sequenced by ELIM Biopharm (Hayward, CA).

397 **Bacterial culture and heterologous expression.** *E. coli* expression strains were
398 cultured in 50 mL terrific broth (TB) supplemented with gentamycin (15 μg/mL) at 37 °C
399 while shaking at 225 rpm. Cultures were induced with 500 μM isopropyl *β*-D-1-
400 thiogalactopyranoside (IPTG) at an OD_{600} of ~0.6, then incubated an additional 4 hours at
401 30 °C while shaking at 225 rpm. Cells were harvested by centrifugation at 4,500 × *g* for 10
402 minutes at 4 °C. Cell pellets were stored at -80 °C until sonication.

403 **Sterol methyltransferase assay.** Cells pellets were resuspended in 5 mL buffer
404 containing the following: 50 mM Tris-HCl, 2 mM $MgCl_2$, 20% glycerol (v/v), and 0.1%
405 β-mercaptoethanol (v/v), pH 7.5. Cells were then lysed on ice via a Qsonica Sonicator
406 Q500 equipped with a 3.2 mm probe at 30% amplitude pulsing at 5 seconds on, 15 seconds
407 off for 8 minutes of total on time. Lysates were then partially clarified by centrifugation at
408 4,500 × *g* for 20 minutes at 4 °C. Total protein concentration in the resulting supernatant
409 was quantified using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific)
410 according to the manufacturer's Standard Microplate Protocol and a BioTek Synergy HT
411 Microplate Reader. SMT assays were then immediately prepared using enough supernatant
412 to give 4500 μg total protein. Other reaction components included 100 μM desmosterol

413 (Sigma-Aldrich), 24-methylenecholesterol (Avanti Polar Lipids, Inc.), [28-^{13}C] 24-
414 methylenecholesterol (Avanti Polar Lipids, Inc.), or synthesized [29-^{13}C] fucosterol or
415 isofucosterol, 100 μM *S*-(5′-sdenosyl)-*L*-methionine chloride dihydrochloride (Sigma-
416 Aldrich), and 0.1% Tween-80 (v/v) to a total reaction volume of 400 μL. Reactions were
417 held at 30 °C for 20 hours and then stored at -20 °C until lipid extraction.

418 **Lipid extraction.** Lipids were extracted using a modified Bligh-Dyer method[55,56]
419 with the completed in vitro reactions as the water phase. Reactions were sonicated in 10:5:4
420 (vol:vol:vol) methanol:dichloromethane:water for 1 hour. The organic phase was then
421 separated with twice the volume of 1:1 (vol:vol) dichloromethane:water followed by
422 storage at -20 °C for >1 hr. Following centrifugation at 2,800 x *g* for 10 min at 4 °C, the
423 organic phase was transferred and evaporated under N_2 to give total lipid extracts (TLEs).
424 TLEs were derivatized to trimethylsilyl ethers in 1:1 (vol:vol)
425 pyridine:Bis(trimethylsilyl)trifluoroacetamide for 1 hr at 70 °C prior to GC-MS analysis.

426 **GC-MS analysis.** Lipids were separated with an Agilent 7890B Series GC
427 equipped with two Agilent DB-17HT columns (30 m x 0.25 mm i.d. x 0.15 μm film
428 thickness) in tandem with helium as the carrier gas at a constant flow of 1.1 ml/min and
429 programmed as follows: 100°C for 2 min, then 12°C/min to 250°C and held for 10 min,
430 then 10°C/min to 330°C and held for 17.5 min. 2 uL of each sample was injected in splitless
431 mode at 250°C. The GC was coupled to an Agilent 5977A Series MSD with the ion source
432 at 230°C and operated at 70 eV in EI mode scanning from 50 to 850 Da in 0.5 s. Sterols
433 were identified based on retention time and comparison to previously published spectra
434 and laboratory standards.

435 **NMR analysis.** In addition to GC-MS analysis, structures of sterol products formed
436 by the metagenomic sterol methyltransferases referred to as "Aplysina aerophoba
437 JGIcombinedJ30088_10000835 1 of 2" and "Chlamydiae sp., drinking water 1 of 2" were
438 confirmed by NMR. ^{13}C-labeling experiments were used to assist the identification of the
439 biosynthetic products by 2D-NMR (heteronuclear single quantum coherence (HSQC) and
440 heteronuclear multiple bond correlation (HMBC)) by increasing the amount of ^{13}C at a
441 specific position by two orders of magnitude compared to natural abundance. The two
442 terminal isopropyl groups of 24-isopropyl sterols come from biosynthetically disparate
443 origins, the isoprenoid pathway and SAM methylation. Because of the possibilities of
444 hydride-shifts and different positions of deprotonation, each of the four terminal methyl
445 groups can have multiple different origins within the product[20]. In addition to enhanced
446 sensitivity, ^{13}C-labeling provided mechanistic details of a pathway which, because of the
447 intrinsic symmetry of 24-isopropyl sterols, would be otherwise hidden.

448 Total lipid extracts from pooled in vitro reactions using [28-^{13}C] 24-
449 methylenecholesterol or [29-^{13}C] isofucosterol as the substrate for both SMTs were
450 fractionated using small silica pipet columns (1 mL) eluting with 5 mL each of
451 hexanes:ethyl acetate 9:1, 2:1, and 1:1 (vol:vol). Steroidal constituents were found in the
452 2:1 solvent fraction and concentrated with a stream of N_2 prior to further purification by
453 preparative thin-layer chromatography (TLC; 4:1 hexanes:ethyl acetate). Sterols were
454 separated by high-performance liquid chromatography (HPLC) and the fractions were
455 analyzed by 800 MHz NMR[35]. Sterols were identified by comparison of the HSQC-DEPT
456 and HMBC spectra with reference compounds.

457 In experiments with [28-^{13}C] 24-methylenecholesterol, the presence of
458 isofucosterol was confirmed by a ^{1}H-^{13}C cross peak corresponding to C-28 at 5.108 and
459 116.48 ppm. The presence of fucosterol was shown by an HSQC cross peak at 5.183 and
460 115.58 ppm. Ratios of isofucosterol to fucosterol were determined by comparison of the
461 integral ratios of the cross peaks[35] for these two isomers. The presence of epiclerosterol
462 was confirmed by cross peaks for C-28 at 1.290/1.380 and 26.01 ppm. Structures of the
463 C_{29} sterol products were confirmed by long-range HMBC correlations from the 29-methyl
464 group. Isofucosterol showed an HMBC cross peak for H-29 and C-28 at 1.59 and 115.58
465 ppm; fucosterol and epiclerosterol showed HMBC cross peaks at 1.57 and 116.48 ppm and
466 0.799 and 26.01 ppm, respectively. The presence of *24S*-24-isopropylcholesta-5,25-dienol
467 labeled at the C-25 position was confirmed by HMBC from the 27-methyl group (cross
468 peak between 1.570 and 147.38 ppm). The presence of 24-isopropylcholesta-5,24-dienol
469 was confirmed by HMBC showing two adjacent cross peaks correlating the C-26 and C-
470 27 methyl protons (1.633/1.651 ppm) with C-25 (123.1 ppm).
471 In experiments with [29-^{13}C] isofucosterol, the label distribution between the C-27
472 and C-26 positions of *24S*-24-isopropylcholesta-5,25-dienol was shown by integration of
473 the HSQC signals for C-27 (1.570 and 18.95 ppm) and C-30 (4.602/4.740 and 111.85). The
474 label on the Z-vinylic methyl of 24-isopropylcholesta-5,24-dienol was detected by HSQC
475 (1.651 and 19.73 ppm). 24-Isopropylcholesta-5,23-dienol was detected by label at 20.96
476 ppm exclusively, which is one of the two methyls of the Z-isopropyl group. The ratio of

477 *24S*-24-isopropylcholesta-5,25-dienol, 24-isopropylcholesta-5,24-dienol, and 24-
478 isopropylcholesta-5,23-dienol was estimated by integration of the HSQC.

479 **Preparation of [29-^{13}C] fucosterol and isofucosterol.** Specifically labeled C_{29}
480 sterol precursors were prepared by modification of the procedure reported in[57]. The
481 precursor 24-formylcholesterol i-methyl ether was obtained either through hydroboration
482 of i-Me 24-methylenecholesterol followed by Dess-Martin periodinane oxidation or
483 ozonolysis of i-Me 24-vinylcholesterol. The latter reaction was carried out in 90:9:1
484 (vol:vol:vol) dichloromethane/methanol/pyridine and yielded the desired 28-
485 formylcholesterol *i*-methyl ether and its methyl hemiacetal in a 1:5 ratio. The hemiacetal
486 was isolated as a mixture of four isomers. ^{1}H-NMR (600MHz): 8.06-8.03 (1H, 28-OH),
487 4.68-4.63 (1H, 28-H), 3.56-3.52 (3H, 28-OMe) 3.332 (s, 3H, 6-OMe), 2.767 (s, 1H, 6-H),
488 1.108 (s, 3H, 19-H), 0.710 (s, 3H, 18-H). This mixture was reacted with ^{13}C-
489 methylmagnesium iodide to give four isomers of [29-^{13}C] i-Me 28-hydroxysitosterol:
490 24R,28R (H-28: 3.938 ppm; C-29: 21.97 ppm), 24S,28S (H-28: 3.938 ppm; C-29: 22.20
491 ppm), 24R,28S (H-28: 3.820 ppm; C-29: 21.05 ppm), and 24S,28R (H-28: 3.803 ppm; C-
492 29: 21.01 ppm). Preparative TLC (4:1 hexanes:ethyl acetate) gave two bands
493 corresponding to a mixture of the RR and SS isomers (higher Rf) and a mixture of the RS
494 and SR isomers (lower Rf) in ca. 2:1 ratio. These two mixtures were separately dehydrated
495 with phosphorus oxychloride to obtain after deprotection [29 ^{13}C] fucosterol (C-29: 13.16
496 ppm) and [29 ^{13}C] isofucosterol (C-29: 12.76 ppm), respectively.

497 **Data availability**

498 The data that support the findings of this study are available within this paper and its
499 supplementary information files. Additional (raw) data are available from the
500 corresponding author upon reasonable request.

501 **Methods references**

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526 **Author contributions.** M.O.B. wrote the manuscript with input from all co-authors.
527 M.O.B. performed the molecular biology experiments, lipid extractions, and GC-MS
528 analyses. M.O.B. and P.V.W. performed the bioinformatic analyses. B.O.O. and J.-L.G.
529 synthesized the ^{13}C labeled sterols and performed the NMR analyses. M.O.B analyzed and
530 interpreted the bioinformatic and GC-MS data. B.O.O. and J.-L.G. analyzed and
531 interpreted the NMR data. P.V.W. and M.O.B. conceived the project with contribution
532 from J.-L.G.

533 **Competing interest declaration.** The authors declare no conflict of interest.

534 **Corresponding author**: welander@stanford.edu

535 **Table 1. Sterols identified as products of sponge and bacterial sterol**

536 **methyltransferases.**

	C_{28}			C_{29}				C_{30}	
	(epi)codisterol (II)	24-methylenecholesterol (III)	24-methylidesmosterol (V)	(epi)clerosterol (IV)*	fucosterol (VI)	isofucosterol (VII)	24S-24-isopropylcholest-5,25-dienol (VIII)	24-isopropylcholest-5,24-dienol (IX)
DEMOSPONGIAE								
Amphimedon queenslandica		+			+			
Chondrilla nucula	+	+		+				
Sarcotragus fasciculatus	+	+			+			
Haliclona amboinensis	+	+		+	+	+		
Haliclona tubifera	+	+		+				
HOMOSCLEROMORPHA								
Oscarella pearsei	+	+		+	+	+		
Oscarella carmela	+	+						
CALCAREA								
Sycon ciliatum	+	+						
DEMOSPONGE METAGENOME								
Aplysina aerophoba JGIcombinedJ30088_10000835 1 of 2	+	+		+	+	+	+	
Aplysina aerophoba JGIcombinedJ30088_10000835 2 of 2								
Aplysina aerophoba Ga0209021_1000146	+	+	+		+	+		+
Petrosia ficiformis LXNJ01014139		+						
Petrosia ficiformis LXNJ01003202		+						
Petrosia ficiformis LXNJ01000389	+	+	+		+			
Petrosia ficiformis LXNJ01004423								
Sarcotragus foetidus		+						
DEMOSPONGE METAGENOME-ASSEMBLED GENOME								
Theonella swinhoei associated Chromatiales sp. 1 of 2		+						
Theonella swinhoei associated Chromatiales sp. 2 of 2								
OTHER METAGENOME								
Marine sediment, Gulf of Thailand		+						
Marine sediment, Helgoland, North Sea		+						
Western Arctic Ocean	+	+		+	+	+		
Freshwater, Lake Fryxell, Antarctica	+	+			+	+		
Lake sediment, Walker Lake, Nevada		+						
Hot spring, Beatty, Nevada								
Hot spring sediment, Dewer Creek, BC		+						
OTHER METAGENOME-ASSEMBLED GENOME								
Chlamydiae sp., drinking water 1 of 2**	+	+	+	+	+	+	+	+
Chlamydiae sp., drinking water 2 of 2	+		+					
Nitrospira sp., coral reef 1 of 2	+	+		+				
Nitrospira sp., coral reef 2 of 2								
Sandaracinus sp., marine 1 of 2	+	+						
Sandaracinus sp., marine 2 of 2	+	+		+	+	+		
Spirochaetes sp., soil		+						

537

538 *NMR confirmed epiclerosterol and not clerosterol as products of the "*Aplysina aerophoba*

539 JGIcombinedJ30088_10000835 1 of 2" and "Chlamydiae sp., drinking water 1 of 2" sterol

540 methyltransferases.

541 **24-isopropylcholesta-5,23-dienol was also detected as a product of the "Chlamydiae sp.,

542 drinking water 1 of 2" sterol methyltransferase by NMR, but was not detected by GC-MS.

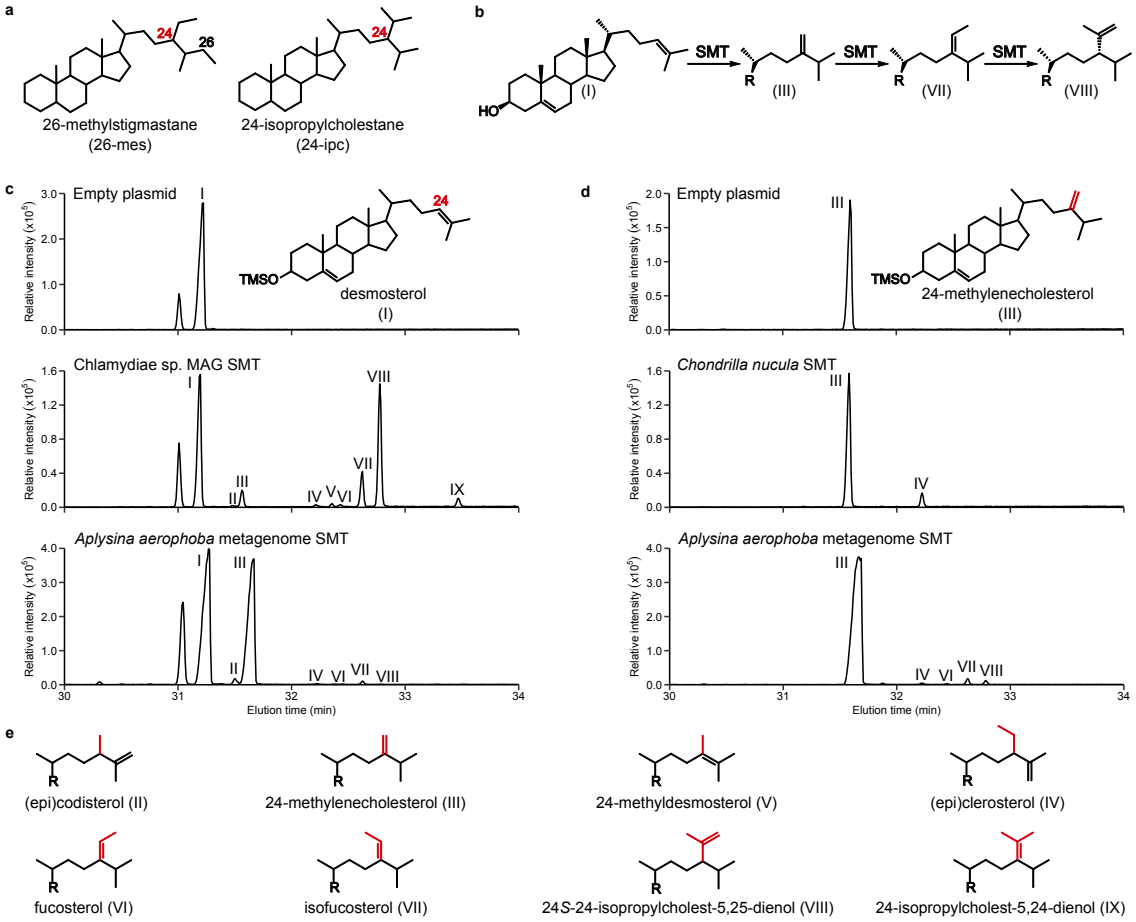


Figure 1. Sterol methyltransferases from sponges and bacteria perform multiple side-chain methylations. a, Structures of the sponge biomarkers 26-methylstigmastane (26-mes) and 24-isopropylcholestane (24-ipc). **b**, Predominant pathway to 24-isopropyl sterols by bacteria sterol methyltransferases (SMTs). **c**, Representative extracted ion chromatograms (*m/z* 456, 470, 484, 498) of total lipid extracts from in vitro reactions performed with *E. coli* lysates containing an empty plasmid (**upper**), a sterol methyltransferase (SMT) identified in a freshwater metagenome-assembled genome assigned to the phylum Chlamydiae (**middle**), and an SMT identified in a metagenome from the microbiome of the demosponge *Aplysina aerophoba* (**lower**) with desmosterol as the substrate. **d**, Representative extracted ion chromatograms (*m/z* 456, 470, 484, 498) of total lipid extracts from in vitro reactions performed with *E. coli* lysates containing an empty plasmid (**upper**), an SMT identified in a transcriptome of the demosponge *Chondrilla nucula* (**middle**), and the same SMT identified in the *A. aerophoba* metagenome as in **a** (**lower**) with 24-methylenecholesterol as the substrate. **e**, Side-chain structures of sterols identified in **a** and **b**. All lipids were derivatized to trimethylsilyls prior to GC-MS analysis. Mass spectra of identified sterols are shown in Extended Data Fig. 1.

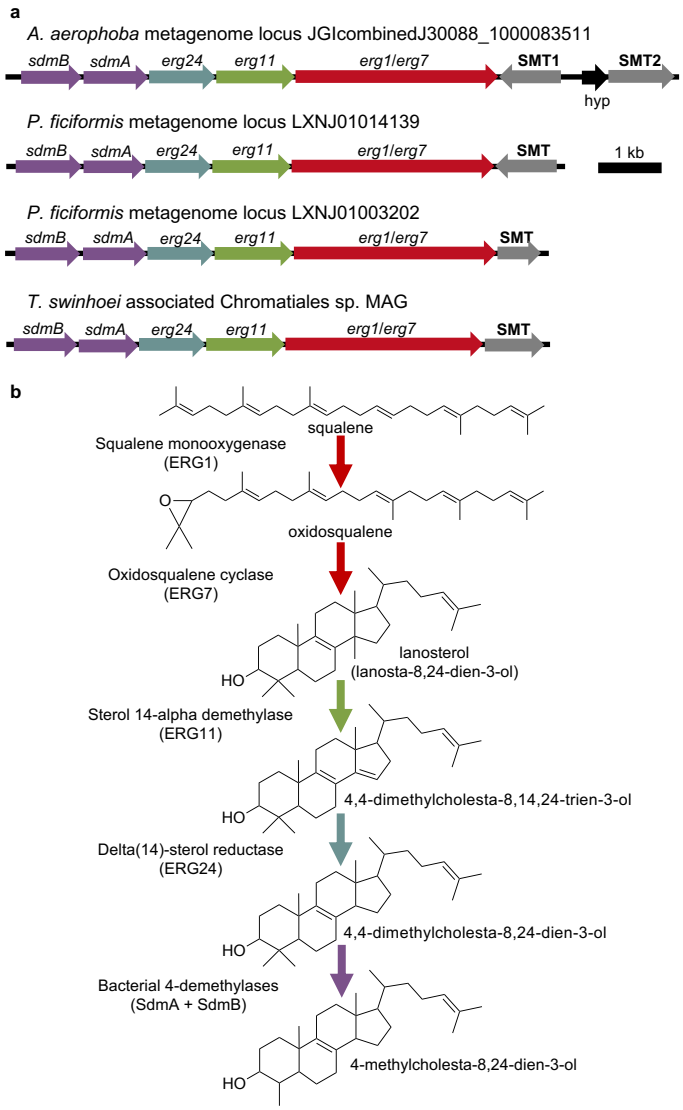


Figure 2. Sterol gene clusters in demosponge metagenomes suggest de novo biosynthesis by bacterial symbionts. a, Putative sterol biosynthesis gene clusters identified in metagenomes from the microbiomes of the demosponge species *Aplysina aerophoba, Petrosia ficiformis*, and *Theonella swinhoei*, each containing a functional sterol methyltransferase. **b***,* Schematic of the bacterial sterol biosynthesis pathway from squalene to 4-methylcholesta-8,24,-dien-3-ol. Arrow colors at each step correspond to the homologs identifiied in **a**. Detailed information on the source of each metagenomic locus is available in Extended Data Table 1.

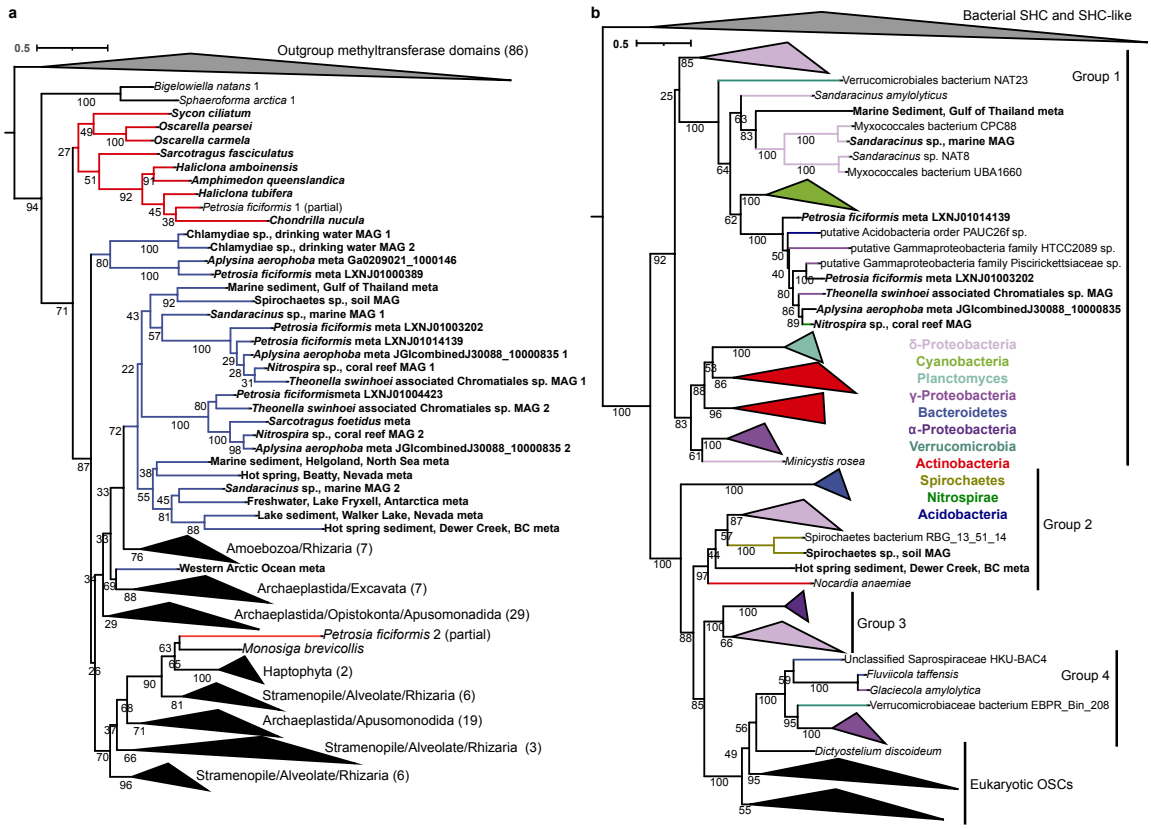
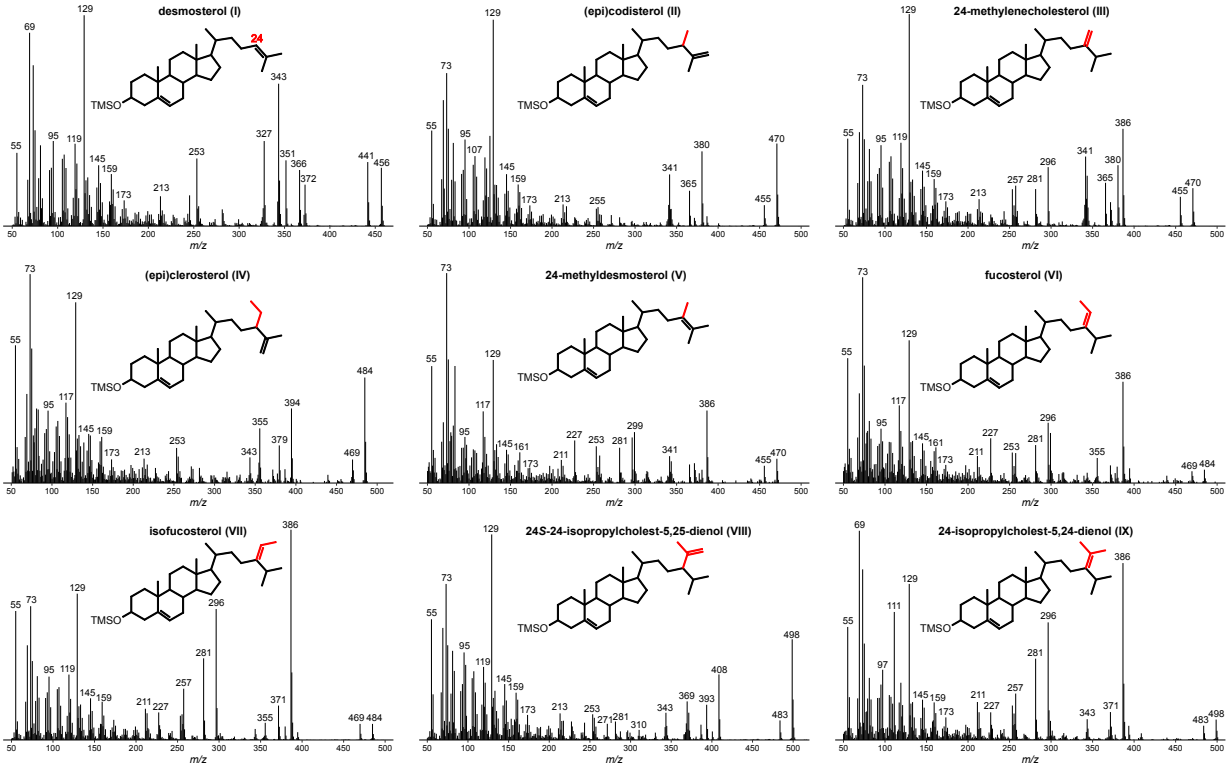


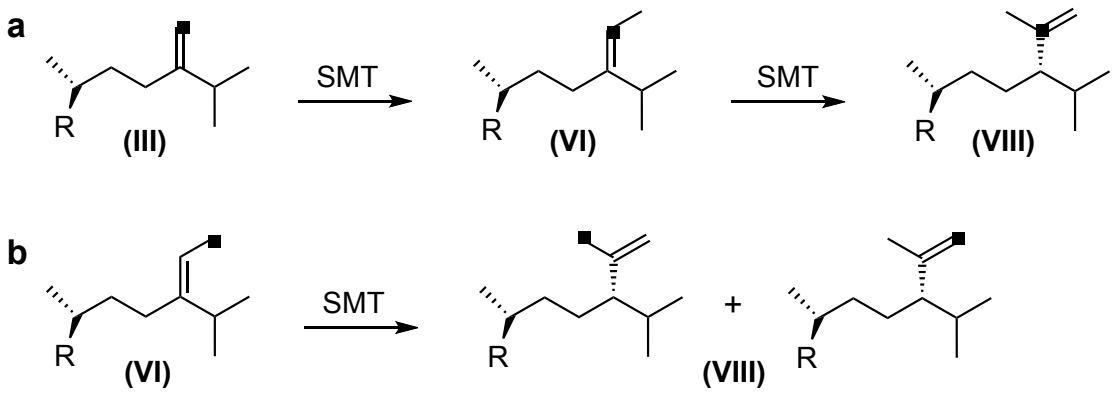
Figure 3. Metagenomic SMTs originate from bacteria. a, Maximum likelihood tree generated from a concatenated alignment of the conserved methyltransferase and C-terminal domains of SMT proteins. Metagenomic SMTs are in blue, sponge SMTs are in red, and other eukaryotic SMTs are in black. The numbers in parentheses indicate the number of proteins in collapsed clades. Bolded labels indicate SMTs tested in this study. **b**, Maximum likelihood tree of oxidosqualene cyclases (OSCs). OSC groups are assigned as in[36]. Bolded labels indicate OSCs in metagenomic gene clusters or metagenome-assembled genomes with SMTs tested in this study.



575 **Extended Data Figure 1**. **Mass spectra of sterols identified in the main text.** Total lipid

576 extracts were derivatized to trimethylsilyl ethers. Detailed GC-MS methods can be found

577 in the Methods section of the main text.



578

579 **Extended Data Figure 2. ^{13}C-labeling experiments with propylating SMTs from an**

580 ***Aplysina aerophoba* symbiont and a Chlamydiae sp.** Positions of the ^{13}C labels are

581 shown for the predominant propylation pathway proceeding from 24-methylenecholesterol

582 (III) to isofucosterol (VI) to 24S-24-isopropylcholest-5,25,dienol (VIII). **a**, Experiments

583 with [28-^{13}C] 24-methylenecholsterol as the substrate. **b**, Experiments with [29-^{13}C]

584 isofucosterol as the substrate.

585

586 **Extended Data Table 1.** Sources of sterol methyltransferases analyzed in this study.

SMT	Source	Genome ID/ BioProject	Locus Tag/Locus
DEMOSPONGIAE			
Amphimedon queenslandica	JGI IMG	2507525020	anonymous.512
Chondrilla nucula	(28)		
Sarcotragus fasciculatus	(28)		
Haliclona amboinensis	Compagen		
Haliclona tubifera	Compagen		
HOMOSCLEROMORPHA			
Oscarella pearsei	Compagen		
Oscarella carmela	Compagen		
CALCAREA			
Sycon ciliatum	Compagen		
DEMOSPONGE METAGENOME			
Aplysina aerophoba JGIcombinedJ30088_10000835 1 of 2	JGI IMG	3300002448	JGIcombinedJ30088_1000083511
Aplysina aerophoba JGIcombinedJ30088_10000835 2 of 2	JGI IMG	3300002448	JGIcombinedJ30088_100008359
Aplysina aerophoba Ga0209021_1000146	JGI IMG	3300027386	Ga0209021_100014628
Petrosia ficiformis LXNJ01014139	GenBank	PRJNA318959	LXNJ01014139.1
Petrosia ficiformis LXNJ01003202	GenBank	PRJNA318959	LXNJ01003202.1
Petrosia ficiformis LXNJ01000389	GenBank	PRJNA318959	LXNJ01000389.1
Petrosia ficiformis LXNJ01004423	GenBank	PRJNA318959	LXNJ01004423.1
Sarcotragus foetidus	GenBank	PRJNA318959	LXNI01004480.1
DEMOSPONGE METAGENOME-ASSEMBLED GENOME			
Theonella swinhoei associated Chromatiales sp. 1 of 2	JGI IMG	2690315631	Ga0136241_103361
Theonella swinhoei associated Chromatiales sp. 2 of 2	JGI IMG	2690315631	Ga0136241_10845
OTHER METAGENOME			
Marine sediment, Gulf of Thailand	JGI IMG	3300010430	Ga0118733_1000126028
Marine sediment, Helgoland, North Sea	JGI IMG	3300004097	Ga0055584_1001717352
Western Arctic Ocean	JGI IMG	3300010883	Ga0133547_104202862
Freshwater, Lake Fryxell, Antarctica	JGI IMG	3300009032	Ga0105048_100346915
Lake sediment, Walker Lake, Nevada	JGI IMG	3300009504	Ga0114946_100084975
Hot spring, Beatty, Nevada	JGI IMG	3300009444	Ga0114945_100210811
Hot spring sediment, Dewer Creek, BC	JGI IMG	3300007072	Ga0073932_10040971
OTHER METAGENOME-ASSEMBLED GENOME			
Chlamydiae sp., drinking water 1 of 2	JGI IMG	2619618861	Ga0074140_11584
Chlamydiae sp., drinking water 2 of 2	JGI IMG	2619618861	Ga0074140_11583
Nitrospira sp., coral reef 1 of 2	JGI IMG	2681812944	Ga0131816_12726
Nitrospira sp., coral reef 2 of 2	JGI IMG	2681812944	Ga0131816_12722
Sandaracinus sp., marine 1 of 2	JGI IMG	2775506781	Ga0248530_10706
Sandaracinus sp., marine 2 of 2	JGI IMG	2775506781	Ga0248530_10707
Spirochaetes sp., soil	JGI IMG	2708742746	Ga0154758_10818

587 **Extended Data Table 2.** Whokaryote predicts all metagenomic contigs containing sterol

588 methyltransferases analyzed in this study as prokaryotic.

SMT	contig	contig length	nr genes	ratio same orientation	ID general avg	ID general std	gene density	gene length	ID Q1	ID Q3	rbs ratio	tiara pred	predicted
Aplysina aerophoba JGIcombinedJ30088_10000835	JGIcombinedJ30088_10000835	32339	23	0.68	128.14	183.70	0.91	1285.09	3.75	181.00	0.96	1.0	prokaryote
Aplysina aerophoba Ga0209021_1000146	Ga0209021_1000146	97163	103	0.68	115.34	117.55	0.88	829.83	31.00	173.75	0.90	1.0	prokaryote
Petrosia ficiformis LXNJ01014139	LXNJ01014139.1	3313	4	0.00	100.67	62.53	0.91	752.00	56.50	124.50	0.25	1.0	prokaryote
Petrosia ficiformis LXNJ01003202	LXNJ01003202.1	8597	8	0.57	115.57	167.57	0.91	975.50	0.00	131.00	0.88	1.0	prokaryote
Petrosia ficiformis LXNJ01000389	LXNJ01000389.1	31336	22	0.67	79.90	78.14	0.94	1341.91	4.00	147.00	0.77	1.0	prokaryote
Petrosia ficiformis LXNJ01004423	LXNJ01004423.1	7072	6	0.60	102.20	58.00	0.93	1093.00	63.00	126.00	0.67	1.0	prokaryote
Sarcotragus foetidus	LXNI01004480.1	7225	5	0.50	105.25	156.88	0.94	1359.20	6.75	121.00	0.80	1.0	prokaryote
Theonella swinhoei associated Chromatiales sp. 1 of 2	2690316284	444506	427	0.74	74.66	122.32	0.93	968.13	1.00	104.25	0.68	1.0	prokaryote
Theonella swinhoei associated Chromatiales sp. 2 of 2	2690316289	117771	97	0.83	74.25	116.07	0.94	1142.96	0.00	97.50	0.65	1.0	prokaryote
Marine sediment, Gulf of Thailand	Ga0118733_100012602	21160	19	0.89	72.06	133.71	0.94	1042.53	0.00	65.50	0.84	1.0	prokaryote
Marine sediment, Helgoland, North Sea	Ga0055584_100171735	2192	3	0.50	80.50	75.50	0.93	676.00	42.75	118.25	0.00	1.0	prokaryote
Western Arctic Ocean	Ga0133547_10420286	2727	3	0.00	147.00	126.00	0.89	810.00	84.00	210.00	0.33	1.0	prokaryote
Freshwater, Lake Fryxell, Antarctica	Ga0105048_10034691	7428	9	0.63	86.00	80.31	0.88	722.33	13.50	104.75	0.44	1.0	prokaryote
Lake sediment, Walker Lake, Nevada	Ga0114946_10008497	6500	5	0.50	77.25	71.98	0.93	1212.80	22.50	103.75	0.40	1.0	prokaryote
Hot spring, Beatty, Nevada	Ga0114945_10021081	3480	3	0.00	63.00	7.00	0.96	1108.00	59.50	66.50	0.33	1.0	prokaryote
Hot spring sediment, Dewer Creek, BC	Ga0073932_1004097	18020	12	0.82	164.73	132.22	0.89	1342.75	67.00	269.00	0.92	1.0	prokaryote
Chlamydiae sp., drinking water 1 and 2	2619626156	1146800	1105	0.68	85.18	128.28	0.92	956.10	0.00	118.00	0.42	1.0	prokaryote
Nitrospira sp., coral reef 1 and 2	2681831735	70077	63	0.74	147.08	145.66	0.87	962.71	27.00	196.00	0.89	1.0	prokaryote
Sandaracinus sp., marine 1 and 2	2775515747	24518	17	0.88	112.25	106.29	0.89	1282.47	22.50	163.50	0.76	1.0	prokaryote
Spirochaetes sp., soil	2708772188	70818	55	0.54	151.69	167.86	0.89	1142.60	6.25	242.50	0.24	1.0	prokaryote

589 **Extended Data Table 3.** 800 MHz ^{1}H and 201 MHz ^{13}C NMR assignments of selected

590 sterol side-chain positions at 30 °C.

	C-24	C-25	C-26	C-27	C-28	C-29	C-30
Fucosterol	**145.6**	2.199 **34.7**			5.183 **115.58**	1.574 **13.16**	
Isofucosterol	**147.1**	2.829 **28.6**			5.108 **116.48**	1.591 **12.76**	
Clerosterol*	1.838 **49.54**	**147.61**	4.730, 4.642 **111.33**	1.570 **17.85**	1.305, 1.341 **26.53**	0.805 **12.04**	
Epiclerosterol	1.81 **49.75**	**147.98**	4.725, 4.636 **111.06**	1.576 **18.13**	1.290, 1.380 **26.01**	0.800 **11.96**	
24*R*-24-Isopropylcholest-5,25-dienol*	1.56 **55.03**	**147.29**	4.613, 4.738 **112.02**	1.567 **18.63**	1.49 **30.25**	0.911 **20.85**	0.803 **21.60**
24*S*-24-Isopropylcholest-5,25-dienol	1.52 **55.50**	**147.38**	4.602 4.740 **111.85**	1.570 **18.95**	1.51 **30.24**	0.913 **20.85**	0.807 **21.49**
24-Isopropylcholest-5,23-dienol			1.013 **24.77**	1.013 **24.79**		0.980 **20.96**	0.980 **21.18**
24-Isopropylcholest-5,24-dienol	**137.0**	**123.1**	1.633 **20.81**	1.651 **19.73**		0.938 **21.34**	0.938 **21.41**

591 ^{13}C data are in boldface. The assignments were determined by HSQC-DEPT, HMBC, and

592 COSY 2D-NMR experiments. ^{1}H and ^{13}C chemical shifts are given to three and two

593 decimal places, respectively, if directly observed, two and one, respectively, if determined

594 from 2D spectra. Diastereotopic signals are not assigned. *Clerosterol and 24*R*-24-

595 isopropylcholest-5,25-dienol were not detected as products of the *A. aerophoba* symbiont

596 and Chlamydiae SMTs but are shown for comparative purposes.

597 **Extended Data Table 4.** Best amino acid substitution models, gamma shape parameters,
598 and invariable sites proportions under the Bayesian Information Criterion and Akaike
599 Information Criterion using ModelTest-NG on XSEDE for generation of sterol
600 methyltransferase and oxidosqualene cyclase maximum-likelihood trees.

Protein	Substitution Model	Gamma Shape Parameter	Invariable Sites Proportion
sterol methyltransferase	LG+I+G	1.260	0.020
oxidosqualene cyclase	LG+I+G	1.212	0.022