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Synthesis of 26-methyl cholestane and identification of cryostanes in mid-Neoproterozoic sediments

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Abstract

The biomarker distributions of mid-Neoproterozoic sediments (800 and 717 million years) from various locations are characterized by the occurrence, besides cholestane isomers, of a novel series of C₂₈ steranes whereas classical C-24 alkylated steranes are absent. These unusual C₂₈ steranes, named cryostanes, seem to be restricted to pre-Snowball Earth sediments. We report here the conclusive identification of cryostanes as 26-methyl cholestanes based on comparison of chromatographic behavior and mass spectrum of the last eluting cryostane isomer with those of a 26-methyl 5 α (H),14 α (H),17 α (H),20*R*-cholestane standard obtained by synthesis. Sterols methylated at C-26 seem to be restricted to some demosponges, and based on molecular clock estimates, demosponges may have emerged in the time interval 800 to 700 Ma. A sponge origin of cryostanes is thus conceivable, making cryostanes the oldest molecular markers for animals. However, other biological sources need to be explored, including the large variety of eukaryotic protists.

28

29 Key words : mid-Neoproterozoic, Snowball Earth, 26-Methyl cholestane, Synthesis,
30 Cryostanes, Sponges

31 1. Introduction

32 The biomarker distributions of mid-Neoproterozoic sediments (800 and 650 million
33 years, Ma) from locations across supercontinent Rodinia are characterized by a
34 unique sterane pseudohomologue distribution with a cholestane predominance
35 approaching 100% and, in some sediment extracts, traces of ergostane and of a
36 novel series of C₂₈ steranes. By contrast, isomers of stigmastane, 24-*n*-propyl- and
37 24-isopropyl cholestanes as well as dinosteranes were not detected in the 800 to
38 650 Ma old sediments (Brocks et al. 2016; 2017). The unique sterane distribution
39 attests to an ecological state of mid-Neoproterozoic eukaryotes that was still
40 distinct from Ediacaran (635 to 541 Ma) and younger ecosystems (Brocks et al.,
41 2016). The name “cryostanes” was ascribed to the unusual new C₂₈ steranes which
42 are currently restricted to pre-Snowball Earth sediments (>717 Ma). Based on
43 considerations related to their behavior upon gas chromatographic analysis,
44 cryostanes have been postulated to correspond to sterane isomers having a C-26
45 methylated side-chain (Brocks et al., 2016). We report here the conclusive
46 identification of cryostanes based on comparison of chromatographic behavior and
47 mass spectrum of the last eluting cryostane isomer with those of a 26-methyl
48 5 α (H),14 α (H),17 α (H),20*R*-cholestane (noted 26-methyl $\alpha\alpha\alpha R$ -cholestane) 1
49 standard prepared by synthesis.

50 2. Experimental

51 2.1. Synthesis of 26-methyl $\alpha\alpha\alpha R$ -cholestane 1

52 2.1.1. Cholestan-26-al 3

An epimeric mixture of 25(*R*) + 25(*S*) cholestan-26-ol **2** (8.1 mg) was reacted with pyridinium dichromate (PDC; 62 mg) in dry CH₂Cl₂ (15 ml) at room temperature under vigorous stirring. After 90 min, additional PDC (32 mg) was introduced and the reaction stirred for further 2 h. The mixture was filtered on a small silica gel column to remove the reagent, the solvent was evaporated under reduced pressure, and chromatographed on a silica gel column using CH₂Cl₂ as eluent, yielding cholestan-26-al **3** (7.0 mg; 88%) as a 1/1 C-25 (*R*+*S*) epimeric mixture not separated by GC, but distinguishable by NMR. GC-MS (EI, 70 eV); *m/z* (relative intensity) 386 (*M*⁺, 18%), 371 (26), 358 (3), 276 (6), 217 (100), 203 (19), 149 (53), 95 (38). ¹H-NMR (500 MHz; CDCl₃): 0.620 (3H, s, H-18), 0.751 (3H, s, H-19), 0.874 (3H, d, *J* = 6.5 Hz, H-21), 1.065 (3H, d, *J* = 6.5 Hz, H-27)^a, 1.067 (3H, d, *J* = 6.5 Hz, H-27)^a, 1.77 (1H, m), 1.92 (1H, td, *J* = 3.5 Hz, *J* = 12.0 Hz), 2.31 (1H, m, H-25), 9.589 (1H, d, *J* = 2.0 Hz, H-26)^b, 9.592 (1H, d, *J* = 2.0 Hz, H-26)^b. ¹³C-NMR (125 MHz; CDCl₃): 12.3, 12.4, 13.6*, 18.8*, 21.0, 22.4, 23.7*, 24.4, 27.1, 28.5, 29.3, 29.3, 31.2*, 32.4, 35.6, 35.9*, 36.2*, 36.5, 38.9, 40.3, 42.8, 46.6*, 47.3, 55.0, 56.4, 56.8, 205.7.*

^a, ^b chemical shifts with the same superscript label correspond to the signal of the same proton from the two epimers at C-25.

* splitted signals in the ¹³C-NMR spectrum reflecting the presence of two epimers at C-25.

2.1.2. 26-Methylene-cholestane **4** (epimeric mixture at C-25)

An aliquot (500 µl) of a solution of triphenylphosphine (PPh₃; 295 mg), isopropanol (75 µl) and tris(triphenylphosphine)rhodium(I) chloride (Wilkinson's catalyst; 31 mg) in tetrahydrofuran (THF; 10 ml) and trimethylsilyldiazomethane (TMSCHN₂; 2 N in diethylether, 80 µl) were added, successively, on a mixture containing cholestan-26-al **3** (2.4 mg) and 16-oxo-methylhexadecanoate (6.5 mg). After 45 min, the color of the reaction mixture turned from yellow to red with the formation of

N₂ bubbles. After a further 60 min, the excess of TMSCHN₂ was eliminated using a few drops of trifluoroacetic acid in CH₂Cl₂ (10%, v/v). The solvent was removed under reduced pressure and 26-methylene-cholestane **4** (1.5 mg, 62%) was recovered from the crude mixture by silica gel column chromatography using cyclohexane as eluent. GC-MS (EI, 70 eV); *m/z* (relative intensity) 384 (M⁺, 26%), 369 (29), 274 (7), 257 (26), 217 (100), 203 (22), 149 (57), 95 (53). ¹H-NMR (500 MHz; CDCl₃): 0.619 (3H, s, H-18), 0.752 (3H, s, H-19), 0.868 (3H, d, *J* = 6.5 Hz, H-21)^a, 0.870 (3H, d, *J* = 6.5 Hz, H-21)^a, 0.952 (3H, d, *J* = 7.0 Hz, H-27)^b, 0.953 (3H, d, *J* = 7.0 Hz, H-27)^b, 1.77 (1H, m), 1.93 (1H, td, *J* = 3.5 Hz, *J* = 12.0 Hz), 2.08 (1H, m, H-25), 4.88 (1H, d, *J* = 10.5 Hz, H-28a), 4.92 (1H, d, *J* = 16.0 Hz, H-28b), 5.660 (1H, ddd, *J* = 7.0 Hz *J* = 10.5 Hz, *J* = 16.0 Hz, H-26)^c, 5.665 (1H, ddd, *J* = 7.0 Hz *J* = 10.5 Hz, *J* = 16.0 Hz, H-26)^c. ¹³C-NMR (125 MHz; CDCl₃): 12.3, 12.5, 18.9, 20.4*, 21.0, 22.4, 23.9, 24.4, 27.1, 28.5, 29.3, 29.3, 32.4, 35.8, 36.0*, 36.2, 36.5, 37.4, 38.0*, 38.9, 40.3, 42.8, 47.3, 55.0, 56.5, 56.9, 112.4*, 145.4*.

^a, ^b, ^c chemical shifts with the same superscript label correspond to the signal of the same proton from the two epimers at C-25.

* splitted signals in the ¹³C-NMR spectrum reflecting the presence of two epimers at C-25.

2.1.3. 26-Methyl $\alpha\alpha\alpha$ R-cholestane **1** (epimeric mixture at C-25)

PtO₂ (ca. 10 mg) was added to an aliquot of 26-methylene-cholestane (0.8 mg) dissolved in EtOAc (3 ml). After stirring under H₂ atmosphere for 90 min, the mixture was put under N₂ atmosphere, and the catalyst was filtered off and washed with CH₂Cl₂. 26-Me $\alpha\alpha\alpha$ R-cholestane **1** was obtained in quantitative yield. GC-MS (EI, 70 eV); *m/z* (relative intensity) 386 (M⁺, 17%), 371 (32), 276 (11), 217 (100), 203 (20), 149 (42), 95 (17). ¹H-NMR (500 MHz; CDCl₃): 0.623 (3H, s, H-18), 0.753 (3H, s, H-19), 0.816 (3H, d, *J* = 6.5 Hz, H-27)^a, 0.820 (3H, d, *J* = 6.5 Hz, H-

27)^a, 0.846 (3H, t, $J = 7.0$ Hz, H-28), 0.878 (3H, d, $J = 6.5$ Hz, H-21), 1.78 (1H, m),
1.93 (1H, td, $J = 3.5$ Hz, $J = 12.0$ Hz). ¹³C-NMR (125 MHz; CDCl₃): 11.6*, 12.3,
12.5, 14.3, 18.9*, 19.5*, 21.0, 22.4, 23.8*, 24.4, 27.1, 28.5, 29.3*, 29.6, 29.9, 32.4,
34.7*, 35.8, 36.1, 36.5*, 37.4*, 38.9, 40.4, 42.8*, 47.3, 55.0, 56.5*, 56.9.

^a chemical shifts with the same superscript label correspond to the signal of the
same proton from the two epimers at C-25.

* splitted signals in the ¹³C-NMR spectrum reflecting the presence of two epimers
at C-25.

2.2. Coinjection of 26-methyl $\alpha\alpha\alpha$ R-cholestane

A coinjection experiment of 26-methyl $\alpha\alpha\alpha$ R-cholestane **1** and a bitumen
containing cryostane was performed on two gas chromatographic columns. The
bitumen was extracted from black shale (sample 10J093) of the 740 Ma old Chuar
Group collected in the Grand Canyon 1544 meters above the base of the group.
Details of the geology and sampling locality are given in Brocks et al. (2016). Any
surficial contaminants were removed from the rock sample using the microablation
protocol, and bitumen was extracted and fractionated according to procedures
outlined by Jarrett et al. (2013).

2.3. Analytical methods

2.3.1. GC-MS

GC-MS of synthetic products was carried out with a Thermo Scientific Trace Ultra
gas chromatograph coupled to a Thermo Scientific TSQ Quantum mass
spectrometer equipped with an autosampler Tri Plus and a programmed
temperature vaporizing (PTV) injector. The temperature of the source was set at
220 °C. The mass spectrometer was operating in the electron impact (EI) mode at
70 eV and scanning m/z 50 to 700. Gas chromatographic separations were
performed on a HP5-MS column (30 m x 0.25 mm; 0.1 μ m film thickness) using He

as carrier gas. Temperature program: 70 °C-200 °C (10 °C min⁻¹), 200 °C-300 °C (4 °C min⁻¹), isothermal at 300 °C for 40 min. For the co-injection experiments, GC-MS was carried out on an Agilent 6890 gas chromatograph coupled to a Micromass Autospec Premier double sector mass spectrometer (Waters, USA). Detailed MS operating conditions are given in Jarrett et al. (2013). For the co-injection experiments, a 60 m DB-5 MS (0.25 mm i.d., 0.25 µm film thickness, Agilent JW Scientific, USA) and a 60 m DB-1 MS (0.25 mm i.d., 0.25 µm film thickness, Agilent JW Scientific, USA) capillary columns were used with helium as carrier gas (constant flow of 1 ml/min). Samples were injected in splitless mode into a Gerstel PTV injector at 60°C (held for 0.1 min) and heated at 260°C/min to 300°C. The GC oven was programmed from 60°C (held for 4 min) to 315°C at 4°C/min, with total run time of 100 min. Steranes were monitored in MRM mode in the 386 → 217 transition. Samples were co-injected manually.

2.3.2. NMR

The NMR spectra were recorded on a Bruker Avance I 500 MHz spectrometer (500 MHz for ¹H; 125 MHz for ¹³C). The chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane with the solvent used as internal standard (CHCl₃: δ¹H 7.26 ppm; CDCl₃: δ¹³C 77.23 ppm) and the coupling constants are expressed in Hz.

3. Results and discussion

3.1. Synthesis of 26-methyl αααR-cholestane 1

26-Methyl αααR-cholestane **1** was synthesized in 3 steps (Fig. 1a) starting from 26-hydroxy αααR-cholestane **2** (occurring as a *R+S* epimeric mixture at C-25), a compound previously prepared starting from stigmasterol following a 14-step synthesis (Paulus, 1993). The first step of the present synthesis consisted of oxidizing **2** into its related aldehyde **3** using PDC (Corey and Schmidt, 1979). For

the introduction of an additional carbon atom at C-26, the rhodium-catalyzed methylenation of aldehydes described by Lebel and Paquet (2004) was used, leading to the alkene **4** (Fig. 1a). This procedure involved PPh₃, Wilkinson's catalyst, and TMSCHN₂ as the carbon source. A main difficulty was the low amounts of substrate available (7 mg) for this step, which required a significant downscaling of the procedure described by Lebel and Paquet (2004). Since the reaction cannot be performed using reagents in large excess, the synthesis on 2-3 mg aliquots of aldehyde **3** was performed in the presence of 6-7 mg of another aldehyde acting as a load and which reacts in the same manner as **3**. 16-oxo-methylhexadecanoate obtained by PDC oxidation of commercial 16-hydroxy-methylhexadecanoate was chosen as the load since this compound leads upon methylenation to 16-methylene-methylhexadecanoate, a compound easily separated from **4** by silica gel chromatography. Using this procedure, we synthesized 26-methylene-cholestane **4** from aldehyde **3** in 62% yield. Finally, 26-methyl $\alpha\alpha\alpha R$ -cholestane **1** was obtained in almost quantitative yield by hydrogenation of **4** using PtO₂ as catalyst. In this synthesis, since the starting product was present as a C-25 *R* + *S* epimeric mixture, the synthetic intermediates as well as the final compound were obtained as C-25 epimers which co-elute in GC under the conditions used (see experimental), but can be partly distinguished upon NMR analysis (presence of ¹H and ¹³C splitted signals indicating a 25*R*/25*S* ratio of ca. 1).

3.2 Identification of the geochemical cryostanes as 26-methyl cholestane isomers

The identification of the geochemical cryostanes as 26-methyl cholestane isomers relies on the fact that the mass spectrum of synthetic 26-methyl $\alpha\alpha\alpha R$ -cholestane **1** (Fig. 1b) is identical to that of the last eluting geological cryostane isomer. In addition, both compounds coelute in GC on two different columns (Fig. 1c). Given

the similarity with “classical” geological sterane distributions, the three-early eluting geochemical isomers (Fig. 1c) most likely correspond to, respectively, $\alpha\alpha\alpha S$, $\alpha\beta\beta R$ and $\alpha\beta\beta S$ isomers of **1**. It is noteworthy that the first eluting cryostane isomer coelutes with a minor synthetic 20*S* isomer of **1** (< 3 % related to **1** determined by GC-MS). Its co-occurrence with **1** can be explained by the fact that the synthesis of cholestan-26-ol **2** (Paulus, 1993) involved a short chain steroid aldehyde intermediate **5** (Fig. 1a) likely to have isomerized to a small extent at the C-20 (enol) position.

Biological sterols with the cryostane side-chain are not known. Sterols methylated at C-26 and bearing additional alkylations at other positions (e.g. C-24) have however been identified in living organisms, and seem to be restricted to some demosponges (Giner, 1993). Most demosponges synthesize 26-methylsterols via methylation of dietary sterols, and this capability was presumably present in the last common ancestor of all Demospongiae. Based on molecular clock estimates, demosponges may have emerged in the time interval 800 to 700 Ma, so a sponge origin of cryostanes is conceivable and would represent the oldest molecular evidence for animals (Brocks et al., 2016). However, other sources need to be explored, including the large variety of eukaryotic protists that have a poorly investigated lipidome, and the possibility of targeted microbial alteration of sedimentary sterols.

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225

226 **Figure caption**

227 **Fig. 1.** (a) Synthesis of 26-methyl $\alpha\alpha\alpha R$ -cholestane **1** from 26-hydroxy $\alpha\alpha\alpha R$ -
 228 cholestane **2** (i) PDC, CH_2Cl_2 , (ii) 16-oxo-methylhexadecanoate, $i\text{PrOH}$, PPh_3 ,
 229 Wilkinson's catalyst, THF, (iii) TMSCHN_2 , THF, (iv) H_2 , PtO_2 , EtOAc . (b) Mass
 230 spectrum (EI, 70 eV) of 26-methyl $\alpha\alpha\alpha R$ -cholestane **1**. (c) MRM chromatograms of
 231 $386 \rightarrow 217$ precursor-product transitions showing a co-injection experiment on a
 232 DB-5MS chromatographic column for the identification of cryostane. **(I)** Chuar
 233 Group sample 10J093 (outcrop, 1544 meters above base of Chuar Group); **(II)** co-
 234 injection of the above samples.

Figure 1