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Steven J. Rowland *Faculty of Science and Engineering*

Paul A. Sutton *School of Geography, Earth and Environmental Sciences*

George A. Wolff *University of Liverpool*

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Biosynthesis of ambrein in ambergris: evidence from isotopic data and identification of possible intermediates

Steven J. Rowland[1]*, Paul A. Sutton[1] and George A. Wolff[2].

1. *Biogeochemistry Research Centre, School of Geography, Earth and Environmental Sciences, University of Plymouth, Drake Circus, Plymouth, PL4 8AA, UK.*
2. *Department of Earth, Ocean and Ecological Sciences, School of Environmental Sciences, University of Liverpool, Jane Herdman Building, Liverpool, L69 3GP, UK.*

***Corresponding Author:**

Phone: +44 (0)1752 584557

Fax: +44 (0)1752 584710

E-mail: srowland@plymouth.ac.uk

Abstract

Ambrein is found in ambergris, a coprolith occurring in the rectum of the sperm whale. *In vitro*, ambrein is produced by enzymatic cyclisation of squalene, via a monocyclic intermediate. However, little is known of the *in vivo* process.

In order to find evidence for the reaction *in vivo*, a comparison was made of the $\delta^{13}C$ relative isotopic ratios of ambrein in ambergris with those of co-occurring sterols. A statistically significant difference was noted. This suggests that ambrein originates *via* a different biosynthetic mechanism from that of the sterols. Examination of the minor constituents of a hydrogenolysed extract of ambergris revealed compounds with a bicyclic polypodane nucleus, rather than those with monocyclic structures.

It is hypothesised that *in vivo* biosynthesis of ambrein proceeds, at least in some cases, via bacterial production of bicyclic polypodenols. The latter are known products of non-concerted squalene (or squalene oxide) cyclisations in other organisms.

Keywords: Ambergris, polypodane, ambrein, Sperm whale, ambergris. *Physeter macrocephalus.*

Experimental

Materials

Ambergris samples from a sperm whale in the Antarctic in 1947 (Table S1), were provided by the Natural History Museum, London, as described by Rowland and Sutton (2017). Further samples from sperm whales in the Antarctic taken 1955-1978 (Table S1), were those described by Rowland et al. (2018b). Jetsam samples taken from beaches in 2017 from Somalia, New Zealand and Ireland (Table S1), were sourced as described by Rowland et al. (2018). A sample from Indonesia taken in 1939 (Table S1) and of unknown (jetsam or sperm whale) origin was also as described by Rowland et al. (2018).

For studies of the possible intermediates in ambrein biosynthesis, a piece of ambergris found on the beach on Chiloé Island, Chile in 2017, was used (Rowland et al. 2018). For hydrogenolysis studies, a piece of jetsam ambergris was provided from an unknown beach location in New Zealand, as described by Sutton and Rowland (2016).

Methods

A subsample of the Chilean sample (ca 0.7g) was taken herein with a spatula, dichloromethane added and the dispersion subjected to vortex mixing and ultrasonication. The resulting dispersion was filtered through a small plug of de-fatted cotton wool. The filtered extract was evaporated to dryness and subjected to column chromatography, as described by Rowland et al. (2018b).

Selected column chromatographic fractions and total dichloromethane extracts were treated with a N,O-bis(trimethylsilyl)acetamide+ trimethylchlorosilane + N-trimethylsilylimidazole (BSA+TMCS+TMSI mixture; 3:2:3; 'Sylon BTZ') purchased from Sigma–Aldrich (Poole, UK). Aliquots (nominal 20 μg) were first dried under a gentle stream of nitrogen (40°C) and then heated with the 'Sylon BTZ' (~50 μL; 70°C; 1 h) and diluted to 1 mL with dichloromethane (DCM), prior to analysis by GC-MS and CSIA.

GC-MS was carried out using an Agilent GC-MSD (Agilent Technologies, Wilmington, DE, USA). This comprised a 7890A gas chromatograph fitted with a 7683B Series autosampler and a 5975A quadrupole mass selective detector operated at 70eV ionisation voltage. The MS was autotuned using PFTBA (perfluorotributylamine) and the mass range was from *m/z* 50-550. The column was a HP-5MS fused silica capillary column (30 m x 0.25 mm i.d x 0.25 μm film thickness). The carrier gas was helium at a constant flow of 1.0 mL min^{-1}. A 1.0 μL sample was injected into a 250 °C splitless injector. There was no initial hold period and the injection was carried out in splitless mode. The oven temperature was programmed from 40 to 300 °C at 10 °C min^{-1} and held for 10 minutes and the transfer line and source temperatures maintained at 280°C and 230°C, respectively.

The stable isotope ratios of carbon ($\delta^{13}C$) of ambrein, coprostanol and epicoprostanol were determined in at least triplicate (Table S1) on their trimethylsilyl (TMS) ethers using a Delta V Advantage isotope ratio mass spectrometer (Thermo Fisher Scientific (TFS), Bremen, Germany) linked to a Trace 1310 gas chromatograph fitted with an AS 1310 Autosampler (TFS) with a ConFlo IV interface (Cu/Ni combustion reactor held at 1030 °C; Thermo Fisher). All samples were diluted with DCM and subsequently 1 μL of each sample was injected into a DB5-MS UI fused-silica column (30 m × 0.25 mm id × 0.25 μm film thickness; Agilent J&W). The temperature was set for 1 minute at 40 °C, and raised by 10 °C min^{-1} to 300 °C, where it was held for 20 minutes. The carrier gas was ultra-high-purity grade helium at a flow rate of 1.2 mL min^{-1}. The eluted products were combusted to CO_2 and ionised in the source of the

mass spectrometer by electron ionisation. The ion intensities of *m*/*z* 44, 45, and 46 were monitored in order to compute automatically the $^{13}C/^{12}C$ ratio of each peak in the extracts. Computations were performed with Isodat Gas Isotope Ratio MS Software (version 3.0; TFS) and were based on comparisons with a mixture of fatty acid methyl esters (FAMEs) of known $\delta^{13}C$ values (Schimmelmann Indiana FAMEs; Table S3). The results from the analysis (Table S1) are reported in ‰ relative to an international standard (V-PDB). Replicate measurements of the standard were used to determine the instrument precision (0.3 ‰) and accuracy (0.5 ‰). The values were also corrected subsequent to analysis to account for the addition of carbon through derivatisation of the alcohol group. The corrections were based on comparisons with repeat injections (x3) of an underivatised alcohol (1-octadocosanol) and its trimethylsilyl derivative (Table S2).

Table S1: Stable isotope ratios of carbon ($\delta^{13}C$, ‰) of sterols (epicoprostanol or coprostanol) and ambrein in extracts of ambergris from sperm whales and in jetsam. The mean effect on $\delta^{13}C$ caused by derivatisation with the same batch of Sylon BTZ reagent (-0.75‰; *cf* Table S2) was subtracted from each value measured on the TMS ethers. $\Delta\delta^{13}C$ values indicate the differences between $\delta^{13}C$ values for ambrein and sterols (Figure S1b).

Sample	Epicoprostanol/coprostanol	Ambrein	$\Delta\delta^{13}C$
Whale ambergris			
1947 Antarctic Whale (outer)	-28.79	-22.45	6.34
1947 Antarctic Whale (outer)	-28.69	-23.07	5.62
1947 Antarctic Whale (outer)	-28.34	-22.30	6.04
1947 Antarctic Whale (outer)	-28.64	-23.02	5.62
1947 Antarctic Whale (outer)	-28.34	-22.33	6.01
1947 Antarctic Whale (outer)	-28.14	-22.91	5.23
Mean	**-28.49**	**-22.68**	**5.81**
Standard Deviation	*0.25*	*0.35*	*0.40*
1947 Antarctic Whale (inner)	-28.86	-21.45	7.41
1947 Antarctic Whale (inner)	-28.28	-21.61	6.67
1947 Antarctic Whale (inner)	-28.11	-21.43	6.68
Mean	**-28.42**	**-21.50**	**6.92**
Standard Deviation	*0.39*	*0.1*	*0.42*
1955-78 Antarctic Whale	-26.27	-20.80	5.47
1955-78 Antarctic Whale	-25.82	-20.97	4.85
1955-78 Antarctic Whale	-26.31	-21.05	5.26
Mean	**-26.13**	**-20.94**	**5.19**
Standard Deviation	*0.27*	*0.13*	*0.36*
1955-78 Antarctic Whale	-28.10	-22.88	5.22
1955-78 Antarctic Whale	-28.12	-22.62	5.5
1955-78 Antarctic Whale	-28.03	-22.62	5.41
Mean	**-28.08**	**-22.71**	**5.38**
Standard Deviation	*0.05*	*0.15*	*0.14*

Jetsam ambergris			
Somalia	-30.85	-22.67	8.18
Somalia	-30.84	-22.73	8.11
Somalia	-30.95	-22.75	8.20
Mean	**-30.88**	**-22.72**	**8.16**
Standard Deviation	*0.06*	*0.04*	*0.05*
New Zealand	-31.12	-25.20	5.92
New Zealand	-30.64	-25.15	5.49
New Zealand	-31.38	-25.24	6.14
Mean	**-31.05**	**-25.20**	**5.85**
Standard Deviation	*0.38*	*0.05*	*0.33*
Co. Clare, Ireland	-28.82	-23.08	5.74
Co. Clare, Ireland	-28.80	-22.52	6.28
Co. Clare, Ireland	-28.50	-22.56	5.94
Mean	**-28.71**	**-22.72**	**5.99**
Standard Deviation	*0.18*	*0.31*	*0.27*
1939 Indonesia	-25.09	-20.16	4.93
1939 Indonesia	-25.15	-20.23	4.92
1939 Indonesia	-25.12	-20.11	5.01
Mean	**-25.12**	**-20.17**	**4.95**
Standard Deviation	*0.03*	*0.06*	*0.05*

Table S1

Table S2: Stable isotope ratios of carbon ($\delta^{13}C$, ‰) of 1-octadocosanol and 1-octadocosanol trimethyl silyl (TMS) ether (n=5) and mean difference caused by derivatisation with same batch of Sylon BTZ reagent used for derivatisation of ambergris extracts (*cf* Table S1).

	1-Octadocosanol	**1-Octadocosanol TMS ether**
	6.95	7.79
	7.23	7.80
	7.11	7.94
Mean	**7.10**	**7.84**
Standard Deviation	*0.14*	*0.09*
Mean difference	**-0.75**	

Table S2

Table S3: Indiana Schimmellman fatty acid methyl ester (FAME) international standards and their specified and measured isotopic values.

Fatty Acid Methyl Ester	**$\delta^{13}C$ mean value in ‰ vs V-PDB, (± 1σ) (range) (# of measurements)**
Tetradecanoic acid methyl ester (C14:0) #n14M, methyl myristate #n14M, $C_{15}H_{30}O_2$,	-29.98 ± 0.02‰, from -29.96‰ to -29.99‰ n = 3
Hexadecanoic acid methyl ester (C16:0) #n16M, methyl palmitate #n16M, $C_{17}H_{34}O_2$,	-29.90 ± 0.03‰, from -29.87‰ to -29.94‰ n = 3
Octadecanoic acid methyl ester (C18:0) #n18M, methyl stearate #n18M, $C_{19}H_{38}O_2$,	-23.24 ± 0.01‰, from -23.23‰ to -23.35‰ n = 4

Table S3

Figure legends:

Figure S1a: Bar chart illustrating the mean values of $\delta^{13}C$ of sterols and ambrein in all ambergris samples (Table S1).

Figure S1b: Bar chart illustrating the differences in mean values of $\delta^{13}C$ of sterols compared to those of ambrein in all ambergris samples (Table S1).

Figure S2: Bar charts illustrating the differences in mean values of $\delta^{13}C$ of sterols compared to those of ambrein in sperm whale ambergris and jetsam ambergris samples; inner and outer portions of sperm whale ambergris from 1947 whale; sperm whale ambergris from 1947 and 1955-78; jetsam ambergris from different locations (*n.b*. it is not known if the 1939 sample from Indonesia was from jetsam or taken directly from a whale).

Figure S3: (Inset) Partial GC-MS total ion current chromatograms of a. hydrogenolysed ambergris extract from New Zealand jetsam ambergris (*cf* Sutton and Rowland 2016); b. synthetic polypodane mixture (*cf* Robson and Rowland, 1994). Mass spectra are shown for A. component with retention time of 24.4 minutes in hydrogenolysed ambergris extract from New Zealand jetsam ambergris and B. a synthetic polypodane, also with a retention time of 24.4 minutes in a synthetic polypodane mixture (*cf* Robson and Rowland 1994). Components with retention times of 24.5 and 24.8 minutes are also polypodane isomers (the latter was assigned as the 8α (H) isomer; Robson and Rowland 1994). The synthetic component with retention time of 24.3 minutes is due to unhydrogenated alkene (Robson and Rowland 1994). The data indicate that hydrogenolysis of ambergris produces, not only the major tricyclic ambrane reported previously (Sutton and Robson 2016), but also minor bicyclic polypodanes.

Figure S4: Structures of other compounds discussed in the text.

Figure S1a

$\delta\ ^{13}C$

‰

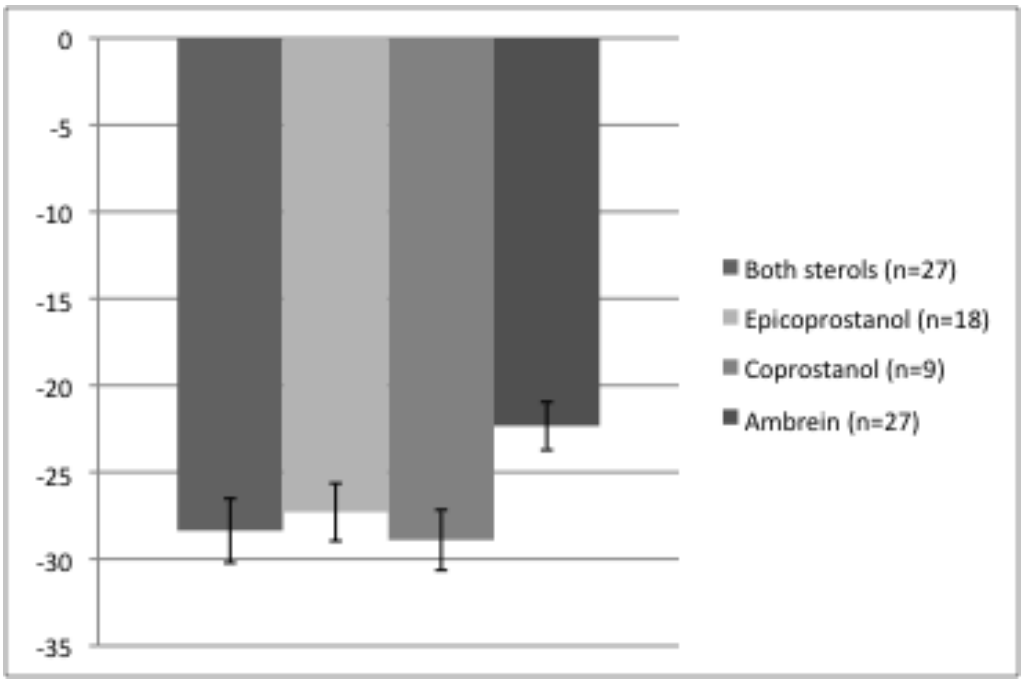


Figure S1b

$\Delta\delta^{13}C$

‰

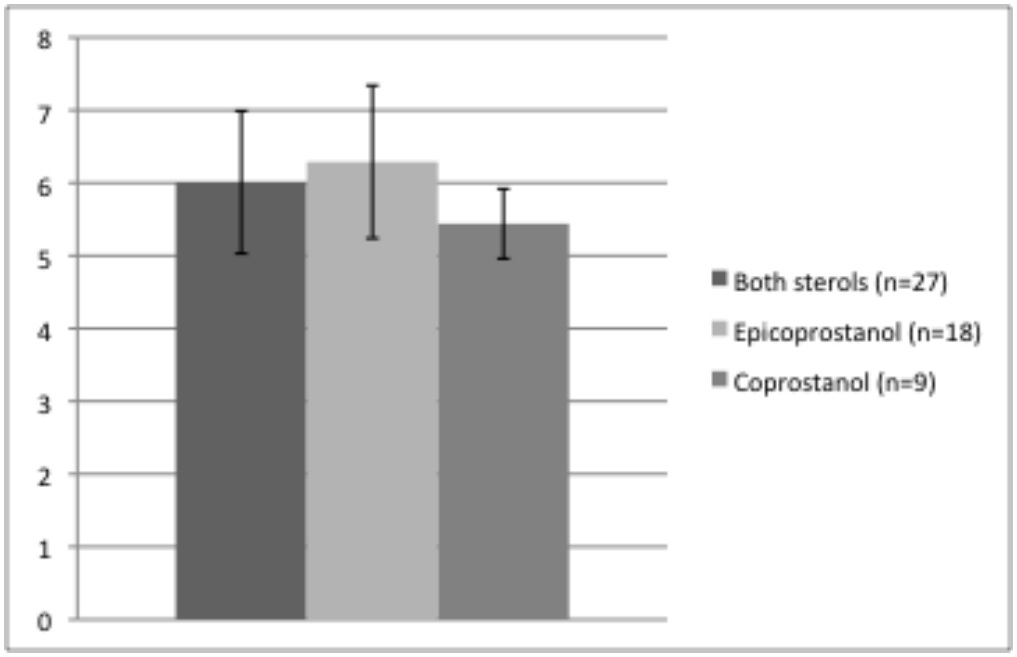
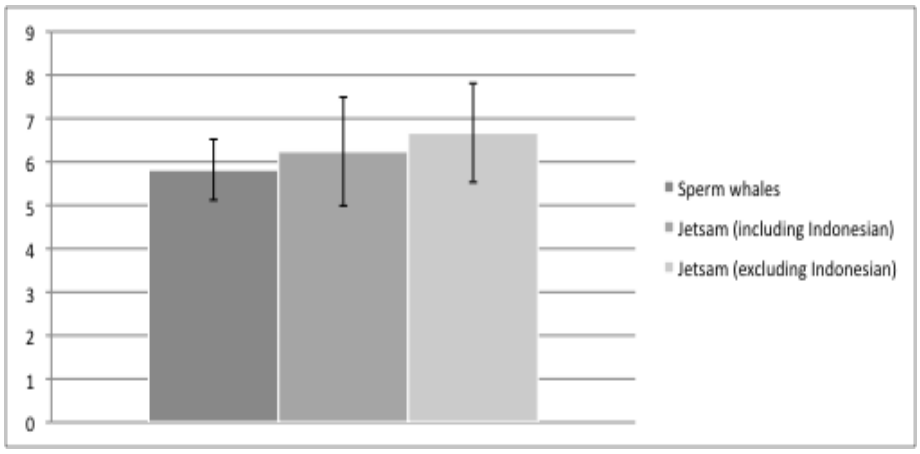
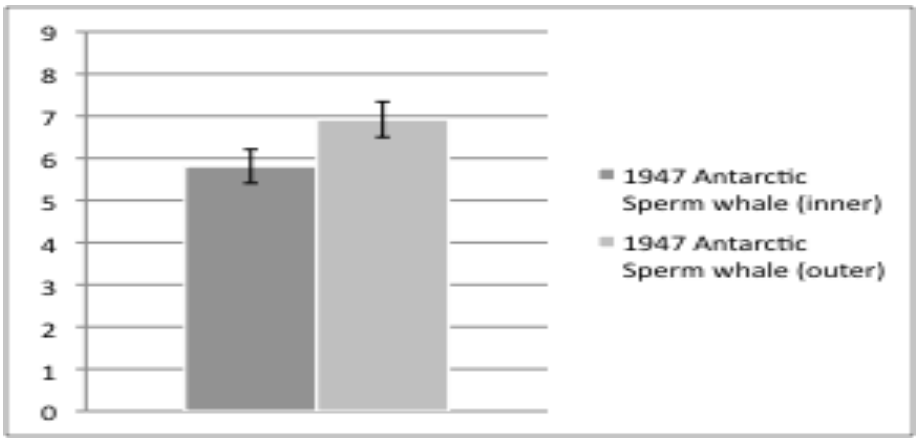


Figure S2

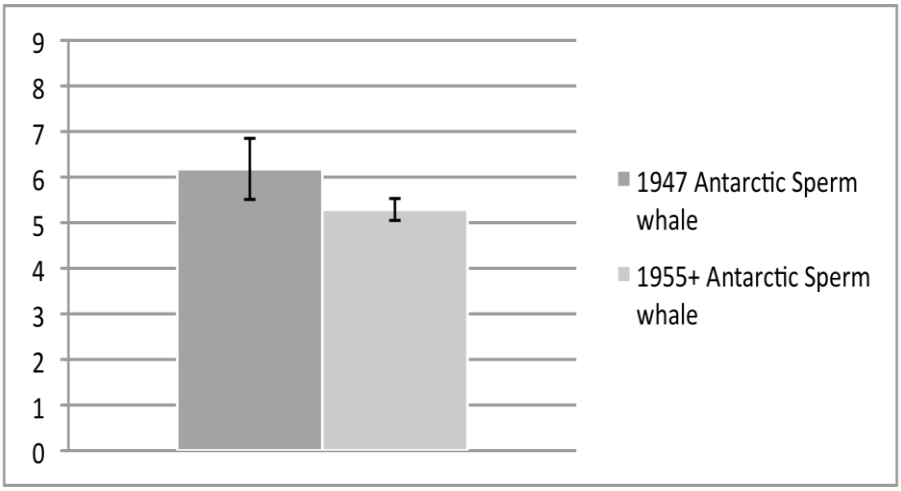
a



b



c



d

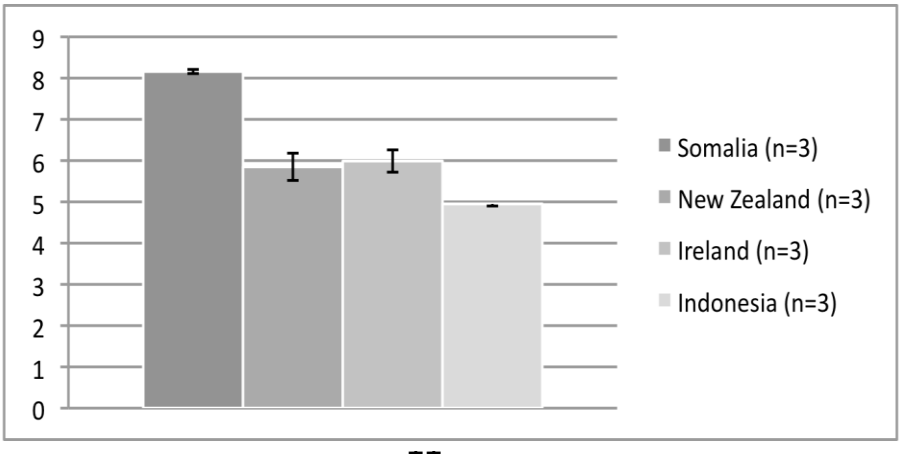


Figure S3

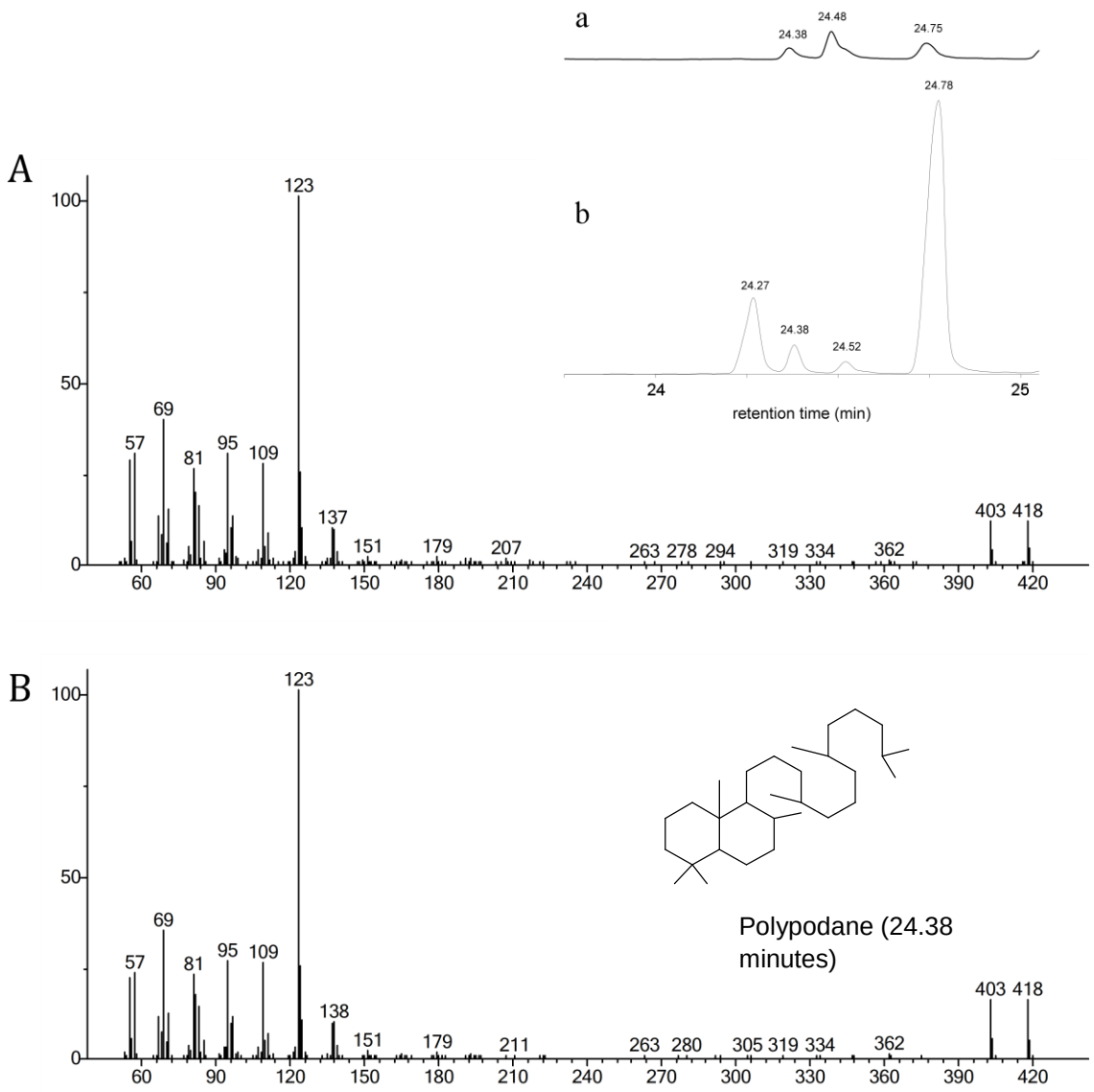
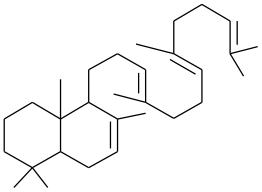
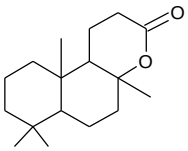

a
24.38
24.48
24.75
b
24.27
24.38
24.52
24.78
24
25
retention time (min)
A
100
50
0
123
69
57
81
95
109
137
151
179
207
263 278 294
319 334
362
403 418
60 90 120 150 180 210 240 270 300 330 360 390 420
B
100
50
0
123
69
57
81
95
109
138
151
179
211
263 280
305 319 334
362
403 418
60 90 120 150 180 210 240 270 300 330 360 390 420
Polypodane (24.38 minutes)

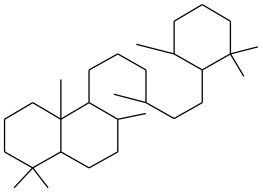
Figure S4



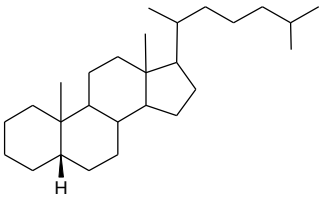
VII: g-polypodatetraene



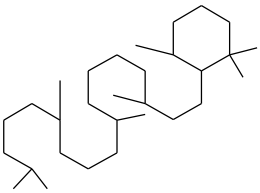
VIII: ambreinolide



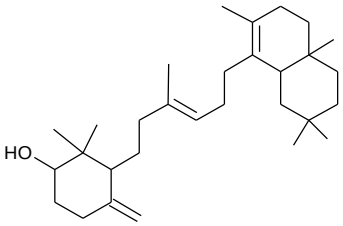
IX: ambrane



X: coprostane



XI: deoxyachillane



XII: achilleol B