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

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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.
1. Introduction

Ambrein, until recently known only in ambergris, (von der Lühe et al. 2019), which is a natural product which occurs as large coproliths in about 1% of the population of the sperm whale, Physeter macrocephalus. It is a tricyclic diunsaturated triterpenoid alcohol (I; Figure 1; Lederer et al. 1946; reviewed by Clarke 2006). The structure of I has been cited as evidence of a biosynthetic origin from squalene (II; Figure 1), possibly mediated by bacteria in the rectum of the whale (Mori and Tamura 1990; reviewed by Clarke 2006). Since I does not have the polycondensed structures of regular triterpenes, it must be biosynthesised in an unusual, less concerted manner, via monocyclic, or possibly bicyclic intermediates (Domingo et al. 2009), or both.

In vitro, ambrein can certainly be produced from squalene by monocyclisation to deoxyachilleol A (DOA; III; Figure 1), then by further enzymatically-mediated cyclisation (Figure 1; Ueda et al. 2013). The only thing known of the biosynthesis of ambrein in vivo, arises from bioengineering of the same tetraprenyl-β-curcumene cyclase (TC) enzyme from Bacillus megaterium (BmeTC) used in the in vitro study, but instead modified and expressed in the yeast, Pichia pastoris (Moser et al. 2018). In that study, in addition to ambrein, both DOA and the bicyclic, 8α-hydroxypolypoda-13,17,21-triene (IV; Figure 1) were produced. The unique catalytic mechanism of TC apparently triggers different cyclization modes, depending on the substrate (Tenkovskaia et al. 2017).

In the present study, we took a two-fold, more indirect approach to the study of in vivo ambrein biosynthesis in ambergris in the sperm whale. First, we determined the relative isotopic carbon ratios ($\delta^{13}C$) of ambrein and of the co-occurring epicoprostanol (V; 5β-cholestan-3α-ol; Figure 1) or coprostanol (VI; 5β-cholestan-3β-ol; Figure 1) in natural ambergris samples, including in jetsam and in those taken directly from whales. Our hypothesis was that, if the ambrein and sterols were biosynthesised via different routes, they would have significantly different isotopic values.

Secondly, we examined extracts and a hydrogenolysed extract of jetsam ambergris samples, for possible intermediates in ambrein biosynthesis: bicyclic compounds with the polypodane skeleton (cf IV; Figure 1) were identified, rather than monocyclics (e.g. DOA, III; Figure 1).

2. Results and discussion
The bulk δ¹³C values (~16 to -25 ‰) of a small number of whole, unfractionated jetsam ambergris samples were determined previously (Rowland et al. 2018). However, the corresponding values for individual components of ambergris extracts have not been reported to our knowledge.

Trimethylsilylated fractions of the dichloromethane extracts of ambergris obtained directly from sperm whales (Rowland and Sutton, 2017; Rowland et al., 2018), or from jetsam samples (Rowland and Sutton, 2017; Rowland et al., 2018, 2018a), were examined herein by gas chromatography-isotope ratio monitoring-mass spectrometry (also known as compound specific isotope analysis, CSIA; Table S1). The small effect (mean -0.75 ‰, n=3), of the addition of the TMS derivatisation group on the isotope ratios thus determined, was measured by obtaining the isotopic values for n-octadecanol and n-octadecanol TMS ether, using the batch of Sylon silylation reagent used to derivatise the ambergris extracts (Table S2). This value was then subtracted from those measured for derivatised compounds (e.g. sterols and ambrein; Table S1). The CSIA instrument was also calibrated periodically by examination of a suite of fatty acid methyl esters of known isotopic values (Table S3).

The mean δ¹³C value of the two sterols in the ambergris extracts was -28.37 ± 1.85 ‰ (n=27), whereas that of the ambrein in the same extracts was significantly different (P<0.001), with a mean of -22.34 ± 1.38‰ (n=27). These data are illustrated in Figure S1a. The mean difference between the δ¹³C values of the two sterols and ambrein (Δδ¹³C; Figure S1b) was 6.01 ± 0.98 ‰ (n=27). In some extracts the major sterol was epicoprostanol, in others it was coprostanol: there was only a little difference (Figure S1b) in the Δδ¹³C values, whichever sterol was measured (6.29 ± 1.05 ‰ and 5.44 ± 0.48 ‰).

These data suggest that ambrein was made by a different biosynthetic route to that of the sterols, which are known bacterial metabolites of cholesterol and thus probably isotopically similar to cholesterol.

The isotopic difference (Δδ¹³C value) between the two different compound classes was maintained at approximately 6 ‰, whether sperm whale or jetsam ambergris was measured (Figure S2a).

There were however, small differences in the Δδ¹³C values, even within a single ambergris boulder. For example, for samples taken from the outer or inner part of a large coprolith of a sperm whale in 1947 (Figure S2b; Clarke 2006) the mean Δδ values were 5.8 and 6.9 ‰. Similarly, there was a small difference in the mean Δδ¹³C values obtained for ambergris from
the Antarctic whale in 1947 (6.2 ‰; Figure 2c) compared to those from whales taken in 1955-1978 (5.3 ‰; Figure S2c).

Somewhat larger variability in Δδ 13C values was observed (Figure S2d) in some individual jetsam ambergris samples from different locations (Figure S2d). Notably those of a coprolith from Somalia were higher, and those from Indonesia lower, than those from Ireland or New Zealand. However, the differences between the isotope ratios of the sterol and ambrein (i.e. Δδ 13C values) were always at least 5 ‰ and varied only from approximately 5 to approximately 8 ‰ (each n=3; Figure S2d). Thus, these data all show that the isotopic values for carbon in ambrein were significantly different (heavier) from those of the sterols, in all cases (Figures S1 and S2).

Given that these data support the hypothesis that ambrein was made by a different biosynthetic route to that of the sterols, the question then arises; what is that route?

Examination by gas chromatography-mass spectrometry (GC-MS), of fractions obtained by column chromatography of an extract of a jetsam ambergris sample found in Chile in 2017 (Rowland et al. 2018) revealed a number of previously unreported components, in addition to several well-known compounds.

A fraction eluting with hexane (3 mg, 0.4%), and therefore expected to contain hydrocarbons, was dominated by three C30:6 ambratrienes (96%), identified by comparison of the mass spectra with those reported previously (Governo et al. 1977). These probably arose from dehydration of ambrein on the silica gel chromatography column (Governo et al. 1977). In addition, a minor component, also with a mass spectrum consistent with a C30:6 alkene, was present. The mass spectrum (M+ 410, 191, 149, 81, (100%)), was similar to that of γ-polypodatetraene II (VII; Figure S4; Hoshino and Sato, 1999). It is possible that VII also resulted from dehydration of a corresponding triunsaturated alcohol (e.g. IV; Figure 1).

A search of the GC-MS data for this fraction was also made for the monocyclic hydrocarbon DOA (III: Figure 1), reported as the in vitro product of incubation of squalene with a mutant SHC (Ueda et al. 2013) and in vivo product of an engineered yeast (Moser et al. 2018). The mass spectrum of III has been reported by Moser et al. (2018), but is rather nondescript and is similar to that of squalene. The GC-MS retention position, relative to squalene, has been reported by Moser et al. (2018) and by Ueda et al. (2013). Significant ions in the mass spectrum of III are reported to occur at m/z 410 (M+) and m/z 177/149/123 (Moser et al. 2018). A mass chromatogram of the sum of these ions at the relevant retention times, revealed no detectable DOA in this fraction of ambergris extract.
A further, more polar column chromatography fraction, eluting with diethyl ether (29 mg, 4%) contained ambreinolide (VIII: Figure S4) and epicoprostanol (V; Figure 1), both identified by comparison of the mass spectra with those of a NIST library (NIST 2011). In addition, two minor components were detected, which were characterised by GC-MS (M+ 498, 483 (M+-Me), 143, (100%)) and assigned as TMS ethers of an unknown C30:6 alcohol. These were tentatively assigned on the basis of the mass spectrum, as the TMS ethers of a polypodatetraenol, similar to those reported previously in squalene hopene cyclase (SHC) mutants from Alicyclobacillus acidocaldarius and in species of the Polypodaceae, Burseraceae and Hypericaaceae plant families (Nguyen and Harrison 1999; Bennett et al. 1993; reviewed by Domingo et al. 2009).

Since the amounts of polypodane-related compounds were low in these fractions, studies by nuclear magnetic resonance (NMR) spectroscopy (cf Ueda et al. 2013; Moser et al. 2018; Rowland et al. 2018a) were precluded. In any event, the mass spectra of many C30 alkenes and polypodols are very similar to one another and are also rather non-specific for identification (Moser et al. 2018). Requests for samples of compounds for co-chromatography and authenticated in previous studies (Moser et al. 2018) were denied on commercial interest grounds.

Therefore, an aliquot of the total extract of a sample of jetsam ambergris from New Zealand (sample described by Rowland and Sutton 2017) was subjected to hydrogenation/hydrogenolysis, as reported previously (Sutton and Rowland 2016). In addition to the major tricyclic product ambrane (IX; Figure S4) reported previously, two alkanes with mass spectra indistinguishable from those of two synthesised isomeric polypodanes (Figure S3; cf Robson and Rowland 1994) were present. The unknowns also co-chromatographed with at least two of these synthetic polypodanes (Figure S3). A small amount of coprostone (5β(H) cholestane; X) was also present, identified by comparison of the mass spectrum with that of the a NIST library (NIST 2011). A search of the GC-MS data was also made for spectra consistent with that predicted for the unknown deoxyachillane A (XI), which would result from hydrogenation of DOA, if that were present in the extract: none was detected.

It is possible that the bicyclic functionalised polypodanes tentatively identified herein are end metabolites of isoprene biosynthesis, rather than intermediates in the biosynthesis of ambrein, or that they are intermediates in the biosynthesis of compounds other than ambrein. Certainly they are also known to produce polycyclic onceroids in vitro (Ueda et al. 2013). Such natural products have not been identified to date in ambergris extracts, although they may be in future studies.
The occurrence of compounds with the bicyclic polypodane skeleton in two samples of ambergris which differ in age by over 1000 years (Rowland et al. 2018), suggests that the compounds may be common in ambergris. Thus, a possible pathway \textit{in vivo} is as shown (Figure 1).

Whilst compounds somewhat seemingly structurally analogous to ambrein, such as achilleol B (XII: Figure S4), are known in higher plants, including yarrow and rice (Barrero et al. 1990; Ito et al. 2011), they likely have a different biosynthetic origin. For example, achilleol B has been suggested to be formed respectively from monocyclic achilleol A (Barrero et al. 1990), or by cyclisation of (3S)-2,3-epoxy-2,3-dihydrosqualene to form a pentacyclic olean-13-yl cation with subsequent cleavage of two of the rings to yield the tricyclic XII (Figure S4; Ito et al. 2011).

3. Conclusions

A comparison of the $\delta^{13}$C relative isotopic composition of ambrein originating from the sperm whale, with those of co-occurring sterols, showed a significant difference between them of about 6‰. 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 the monocyclics found in \textit{in vitro} experiments.

It is hypothesised that \textit{in vivo} biosynthesis of ambrein proceeds, at least in some cases, via bacterial production of bicyclic polypodanes. The latter are known products of non-concerted squalene (or squalene oxide) cyclisations in other organisms. Alternatively, the bicyclics may be minor natural products unrelated to ambrein. Much remains to be learned.

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\textbf{References}


