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Note

A novel method to measure the ^{13}C composition of intact bacteriohopanepolyols

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ABSTRACT

We present a novel method to measure the $^{13}C/^{12}C$ isotope ratio (reported as $\delta^{13}C$) of individual, intact bacteriohopanepolyols (BHPs) using semi-preparative ultrahigh pressure liquid chromatography (UPLC) followed by high temperature gas chromatography–isotope ratio mass spectrometry (HT-GC–IRMS). The method is reproducible, as indicated by the precision of $\delta^{13}C$ values for bacteriohopanetetrol (BHT) extracted from *R. palustris* biomass and analyzed across an order-of-magnitude range of IRMS peak areas ($\delta^{13}C = -33.4 \pm 0.6‰$ VPDB, $n = 94$, $\pm 1\sigma$). To show that this method successfully separates individual BHPs from complex environmental matrices, we report $\delta^{13}C$ values for BHT and the BHT stereoisomer (BHT-II) from a ca. 2.9 Ma, organic-rich Mediterranean Sea sediment sample. These analyses suggest that intact BHP $\delta^{13}C$ measurements can greatly improve the interpretation of environmental signals by alleviating the need for side-chain cleavage which reduces BHP source-specificity.

1. Introduction

Bacteriohopanepolyols (BHPs) are structurally diverse, pentacyclic triterpenoid lipids produced by a wide range of bacteria, including cyanobacteria (Rohmer et al., 1984). Whereas some BHPs such as bacteriohopanetetrol (BHT) are ubiquitous, others are associated with specific environments or specific microbial processes. For example, BHT methylated at the C-2 position (2Me-BHT) historically has been treated as a cyanobacterial biomarker (Summons et al., 1999), although this is now challenged by the presence of 2Me-BHT in the anoxygenic, phototrophic α-proteobacterium *Rhodopseudomonas palustris* (Rashby et al., 2007). Similarly, a BHT stereoisomer (BHT-II) associated with marine oxygen minimum zones (Sáenz et al., 2011) is proposed to be a biomarker for anaerobic ammonia oxidizing (anammox) bacteria (Rush et al., 2014).

While the relative abundances of specific BHPs in environmental samples are informative, the interpretation of BHP sources would be enhanced by concomitant measurement of stable carbon isotope ratios ($^{13}C/^{12}C$, reported as $\delta^{13}C$). To date, isotope compositions of intact BHPs remain elusive due to their long, polar side chains, which render these compounds unamenable to traditional gas chromatographic (GC) analysis. Rather, BHP side chains are often cleaved, resulting in hopanols whose ^{13}C composition can be readily analyzed by GC isotope ratio mass spectrometry (GC–IRMS; Summons et al., 1994). However, cleavage removes side-chain structural diversity, thus combining multiple BHPs into a single hopanol and yielding ^{13}C compositions that reflect the weighted average of precursor BHPs. Because precursor BHPs can be sourced from a range of organisms with unique ^{13}C signatures (e.g., Sáenz et al., 2011; Rush et al., 2014), $\delta^{13}C$ values of cleaved hopanols cannot be uniquely interpreted, hindering the utility of BHPs to be used as source-specific biomarkers.

To alleviate this issue, here we describe a novel method to measure the ^{13}C composition of individual, intact BHPs by coupling semi-preparative, ultrahigh pressure liquid chromatography (UPLC) with high temperature (HT-GC–IRMS (e.g., Sessions et al., 2013). We analyzed the ^{13}C compositions of BHT and 2Me-BHT extracted from *R. palustris* biomass, demonstrating robustness over a range of HT-GC–IRMS injection concentrations. Finally, to validate the utility of this method for environmental samples, we present BHT and BHT-II $\delta^{13}C$ values from a ca. 2.9 Ma Mediterranean Sea sediment.

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2. Experimental

2.1. Sample collection and processing

2.1.1. Sample collection

To generate sufficient amounts of BHPs to be used as laboratory standards, 1 L batch cultures of *R. palustris* strain CGA009 were grown photo-heterotrophically on YPMS medium amended with acetate (50 mM MOPS, 20 mM succinate, 40 mM acetate, 0.3% yeast extract, 0.3% peptone, buffered to pH 7.0 using NaOH) and were continuously shaken (120 rpm) at 30 °C in ambient light (Wu et al., 2015). Cultures were propagated by inoculating 1 L of fresh medium with 50 mL of inoculum in mid-log phase. All batches were harvested by centrifugation at the onset of stationary phase, when OD600 reached ~1.4 (Wu et al., 2015), and stored frozen at −20 °C. Additionally, a single sediment sample (ca. 1 g) was analyzed from ODP core 964 E6H4 (5–6 cm), deposited during sapropel event i-282 and recovered from the Eastern Mediterranean Sea during ODP leg 160, freeze-dried and homogenized by mortar and pestle.

2.1.2. Extraction and separation

R. palustris biomass was extracted at 100 °C for 20 min in 20 mL of dichloromethane/methanol (9:1, v/v) using a Microwave Accelerated Reaction System (MARS, CEM Corporation). Mediterranean sediment was similarly extracted using a MARS at 100 °C, but with a three-step protocol to maximize lipid yields: (i) dichloromethane/methanol (1:1, v/v), (ii) dichloromethane/methanol (9:1, v/v), (iii) 100% dichloromethane (20 min each). After extraction, each sample was rinsed 3× with dichloromethane. Rinses and extracts were combined into a total lipid extract (TLE) and, similar to Pearson et al. (2016), were separated into fractions over 10 mL columns of pre-combusted SiO_2 (130–270 mesh): (i) F1: 12 mL of 100% *n*-hexane; (ii) F2–5: 12 mL of *n*-hexane/ethyl acetate (9:1, v/v), (iii) F6–8: 12 mL of *n*-hexane/ethyl acetate (4:1, v/v); (iv) F8.5: 15 mL of *n*-hexane/ethyl acetate (1:1, v/v); (v) F9–10: 7.5 mL of 100% ethyl acetate followed by 7.5 mL of 100% methanol. BHPs were contained in F9–10.

2.1.3. Derivatization and acetylation standard

Before derivatization, ca. 25 μg of a synthetic sterol standard with a known ^{13}C composition (determined by elemental analyzer IRMS: $\delta^{13}C = -28.57 \pm 0.03$‰ VPDB; $n = 10$) was added to F9–10 to monitor the acetylation ^{13}C effect in a sample-specific manner (Pearson, 1999). Cholestane-3β,5α,6β-triol ("CT standard"; Avanti Polar Lipids), was chosen for three reasons: (i) it has a similar polarity to BHPs; (ii) it does not co-elute with BHPs during semi-preparative UPLC collection; and (iii) it contains three hydroxyl functional groups and a C_{27} backbone, maximizing the acetate-derived carbon signal (i.e., 18% of total carbon atoms in the acetylated form). After CT standard addition, samples were evaporated to dryness, reconstituted in 200 μL of 1:1 pyridine/acetic anhydride, and derivatized for ≥12 h at 25 °C. After derivatization, samples were again evaporated to dryness, reconstituted in methanol/isopropanol (30:70, v/v), filtered through 0.45 μm syringe-tip PTFE filters, and stored at −20 °C.

2.2. Analytical methods

2.2.1. Semi-preparative ultrahigh pressure liquid chromatography (UPLC)

Individual compounds were isolated using an Agilent 1290 Infinity series UPLC coupled to an Agilent 1260 Infinity series fraction collector. Following Kusch et al. (2018), samples were separated by reverse phase using three Phenomenex Kinetex C_{18} columns in series (4.6 × 150 mm; 2.6 μm particle size) at 10 °C. Samples were injected in solvent A (20 μL injection) and separated isocratically using 60:40 A/B mixture for 75 min, where A is methanol/isopropanol (30:70, v/v) and B is methanol/water (90:10, v/v). Columns were then back-flushed for 20 min with 100% solvent C followed by 20 min with 60:40 A/B, where C is ethyl acetate/methanol (90:10, v/v). Finally, columns were re-equilibrated for 15 min using 60:40 A/B, leading to a total run time of 130 min. Flow rate was maintained at 350 μL min^{-1} throughout. Multiple injections were performed for each sample and fractions from repeat injections were combined to prepare sufficient compound masses for isotope analysis.

To constrain elution times for the CT standard and individual BHPs, timing runs were performed prior to each semi-preparative UPLC sequence. BHPs were identified using an Agilent 6410 triple quadrupole (QQQ) mass spectrometer equipped with an atmospheric-pressure chemical ionization (APCI) source run in positive ion mode using multiple reaction monitoring. APCI source parameters, QQQ settings, and compound identification methods are described in Kusch et al. (2018). Finally, BHP recovery and purity were assessed by analyzing ca. 5% of each preparatory fraction using flow injection analysis and APCI-QQQ mass spectrometric detection (MS2 scan mode, 350–850 Da range).

2.2.2. High temperature gas chromatography mass spectrometry (HT-GC–MS)

Prior to isotope analysis, compound purities were further validated and HT-GC elution times were determined using an Agilent 6890 gas chromatograph equipped with a programmable temperature vaporizing (PTV) inlet and coupled to an Agilent 5973 inert quadrupole mass spectrometer. Similar to Sessions et al. (2013), compounds were separated using a Zebron ZB-5HT high temperature column (30 m × 0.25 mm i.d.; 0.10 μm film thickness) with a He carrier gas flow rate of 1.3 mL min^{-1} and the following oven temperature program: hold at 50 °C for 1.5 min, ramp to 300 °C at 15 °C min^{-1}, ramp to 350 °C at 10 °C min^{-1}, hold for 11.83 min (35 min total). Samples were injected in ethyl acetate (1 μL) with the following PTV inlet program (splitless mode): hold at 55 °C for 0.05 min, ramp to 325 °C at 14 °C s^{-1}, hold for 1.5 min. The split valve was opened (15 mL min^{-1} split flow) and the inlet was cleaned at 360 °C for 1 min before returning to 55 °C for the duration of the run. Compounds were detected in scan mode (50–750 Da range; 70 eV) with a transfer line temperature of 320 °C and a source temperature of 225 °C.

2.2.3. High temperature gas chromatography isotope ratio mass spectrometry (HT-GC–IRMS)

Compound-specific $\delta^{13}C$ values were determined using a Thermo Scientific Trace GC Ultra coupled to a Thermo Scientific Delta V Advantage IRMS via a GC Isolink system. All inlet and GC parameters were identical to those described above for HT-GC–MS analysis (Section 2.2.2). The GC Isolink combustion reactor temperature was held at 1000 °C and $^{13}C/^{12}C$ ratios were calibrated against CO_2 gas with a known $\delta^{13}C$ value. All samples were injected at least in triplicate (Table 1). To monitor instrument performance, 10–20 ng of an internal standard (*n*-C_{38} alkane) with known isotope composition was co-injected with each sample. Long-term accuracy of the *n*-C_{38} standard was better than 0.35‰ and long-term precision was ≤0.30‰ ($n = 112$). CT standard and BHP $\delta^{13}C$ values were offset-corrected by subtracting the difference between known and measured *n*-C_{38} $\delta^{13}C$ values for each run. Finally, $\delta^{13}C$ values were corrected for acetate carbon by mass balance using the sample-specific $\delta^{13}C$ results for the CT standard. All results are reported in ‰ notation relative to Vienna Pee-Dee Belemnite (‰ VPDB) with uncertainty reported as ±1σ between replicate

Table 1
$\delta^{13}C$ values of compounds analyzed in this study. BHT, 2Me-BHT (*R. palustris*), and BHT-II (ODP site 964) values are corrected for acetate-derived carbon. Std. dev. is the ±1σ uncertainty between replicate injections and *n* is the number of injections for each sample.

	CT Standard			BHT			2Me-BHT or BHT-II		
	Mean (‰)	Std. dev. (‰)	*n*	Mean (‰)	Std. dev. (‰)	*n*	Mean (‰)	Std. dev. (‰)	*n*
R. palustris CGA009	−29.0	0.3	45	−33.4	0.6	94	−38.0	0.6	16
ODP site 964 E6H4 5–6 cm	−27.7	0.1	3	−24.8	0.2	3	−50.6	0.4	3

measurements after propagating uncertainty from acetate carbon correction.

3. Results and discussion

Given the relatively large on-column masses necessary for routine GC–IRMS measurements, baseline separation of BHP mixtures using HT-GC is prohibitively challenging (Sessions et al., 2013). Our method therefore first separates BHPs using the three-column UPLC method of Kusch et al. (2018), which baseline-separates BHT from 2Me-BHT in the *R. palustris* extract (Fig. 1a) and BHT from BHT-II in the Mediterranean sediment (Fig. 1b). This alleviates the issue of co-elution during HT-GC analysis and purifies the BHPs from most background contaminants. Fig. 1c–h shows that each UPLC fraction in this study contains a single compound of interest and that all BHPs, as well as the CT standard, are baseline-separated from background contaminants. Individual, intact BHP $\delta^{13}C$ values can then be determined by analyzing each UPLC fraction using HT-GC–IRMS. We note that the dominant 2Me-BHT and BHT-II isomers, if both present within the same TLE, would co-elute during semi-preparative UPLC and end up in the same fraction for HT-GC–IRMS analysis (Kusch et al., 2018). However, these compounds are baseline separated by ca. 0.5 min during HT-GC analysis, suggesting that our method would yield reliable $\delta^{13}C$ values for samples that contain both 2Me-BHT and BHT-II.

All resulting $\delta^{13}C$ values are shown in Table 1. The 4.7 ± 0.8‰ ^{13}C-enrichment in BHT relative to 2Me-BHT from the *R. palustris* culture is consistent with Summons et al. (1994), who observed similar differences between methylated and non-methylated BHPs in methanotrophic bacteria. The difference is unlikely to result from methylation alone, as it would require an unrealistically depleted ^{13}C composition of the methyl carbon. Rather, this has been interpreted as reflecting a separate, isotopically distinct pool of BHT that becomes methylated within the cell (Summons et al.,

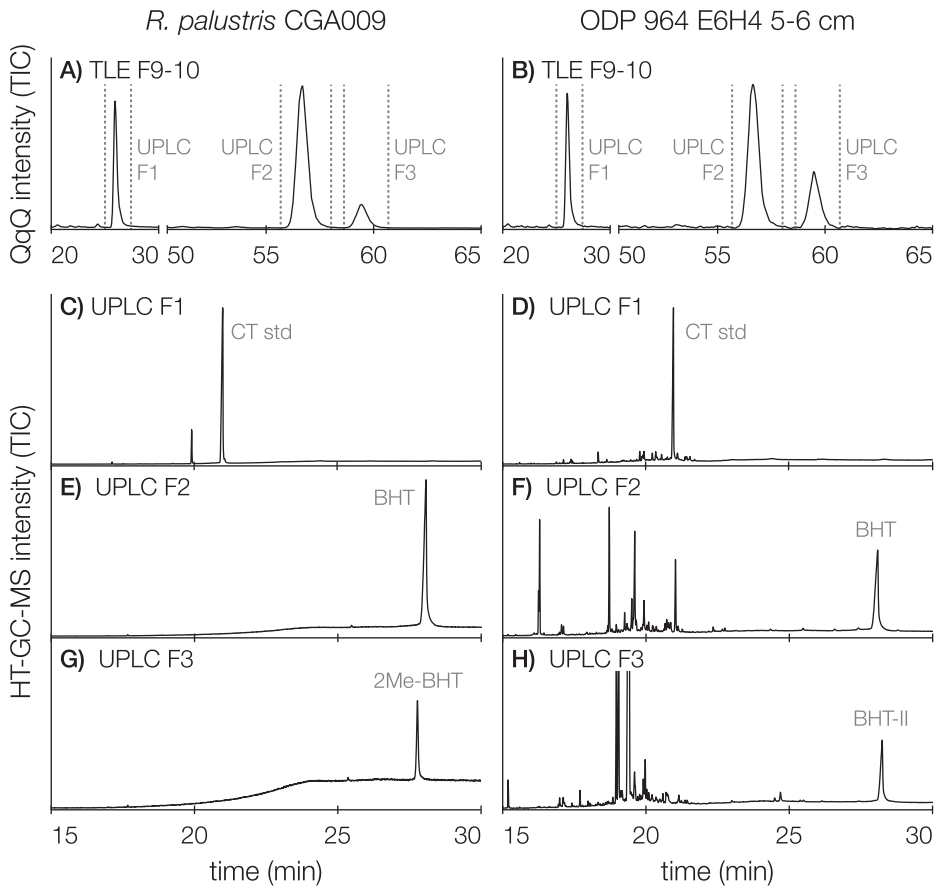

Fig. 1. UPLC–QQQ-MS chromatograms for the samples used in this study, showing the timing for each UPLC fraction (dashed lines) (A and B). HT-GC–MS chromatograms for each collected UPLC fraction: ULPC F1, which contains the CT standard (C and D); UPLC F2, which contains BHT (E and F); and UPLC F3, which contains 2Me-BHT (*R. palustris*) or BHT-II (ODP site 964) (G and H).

1994). Similarly, the observed 21‰ ^{13}C-depletion in Mediterranean Sea sediment BHT-II relative to BHT appears to validate the utility of BHT-II as a proxy for anammox bacteria (Rush et al., 2014).

Finally, by isolating milligram-scale masses of BHT and 2Me-BHT from *R. palustris* biomass, we tested the consistency of our δ^{13}C measurements across a range of quantities injected on-column for HT-GC–IRMS. Over a 20-fold dilution series, δ^{13}C values ranged from −34.7‰ to −31.8‰, averaging −33.4 ± 0.6‰ (n = 94). δ^{13}C values displayed a small yet statistically significant negative relationship with peak area (ordinary least squares; R^2 = 0.08; p = 0.006) and slightly larger uncertainty at smaller peak areas (1σ = 0.4‰ for area >3.0 Vs; 1σ = 0.8‰ for area $\leq$3.0 Vs). Although statistically significant, the area vs δ^{13}C trend is within measurement uncertainty and we did not correct for peak area bias.

We conclude that the method outlined here can produce stable, reproducible δ^{13}C values for intact, individual BHPs after derivatization to acetates (but without side-chain cleavage). Application of this method to environmental samples will likely provide novel insight due to its ability to separate a diversity of BHP structures and directly measure their individual δ^{13}C values.

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