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Note

A novel heterocyst glycolipid detected in a pelagic N_2-fixing cyanobacterium of the genus *Calothrix*

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ABSTRACT

Previous studies have shown that heterocyst glycolipids (HGs) are unique markers for N_2-fixing heterocystous cyanobacteria. In this study, the HGs of a marine pelagic *Calothrix* sp. CCY1611 isolated from the tropical western North Atlantic were analyzed by ultra-high pressure liquid chromatography–high resolution mass spectrometry and it was shown that this organism contains an unusual C_{28} triol HG with a methylated C_6 sugar (methyl-HG_{28} triol) head group. Gas chromatography–mass spectrometry analysis of the sugar released from the novel HG by acid methanolysis revealed that the sugar is likely 6-O-methyl-β-D-glucopyranose. We propose that this methyl-HG_{28} triol is a potential biomarker for pelagic members of the genus *Calothrix*.

1. Introduction

In all heterocystous cyanobacteria studied to date, the heterocyst cell wall contains heterocyst glycolipids (HGs) (Nichols and Wood, 1968; Abreu-Grobois et al., 1977; Gambacorta et al., 1995; Bauersachs et al., 2009a, 2014). These HGs almost universally comprise a hexose head group (hereafter C_6) glycosidically bound to long-chain diols, triols, or hydroxyketones (Bryce et al., 1972; Gambacorta et al., 1998; Bauersachs et al., 2009b, 2011), except for some marine endosymbiotic cyanobacteria which contain pentose head groups (Schouten et al., 2013; Bale et al., 2015, 2018). Previous studies reported that HGs show structural diversity depending on the family level within the cyanobacteria divisions (Bauersachs et al., 2009a, 2014, 2017). The heterocystous cyanobacteria of the genus *Calothrix* are characterized by the presence of the 1-(O-hexose)-3,25,27-octacosanetriol (C_6 HG_{28} triol) and 1-(O-hexose)-27-keto-3,25-octacosanediol (C_6 HG_{28} keto-diol) (Gambacorta et al., 1998; Bauersachs et al., 2009a; Wörmer et al., 2012). However, these studies on *Calothrix* focused predominantly on benthic strains, while this genus is also known from the pelagic where it occurs as a symbiont of marine diatoms, specifically of *Chaetoceros* (Foster et al., 2010, 2011). These symbioses are known as diatom-diazotroph associations. Previously, while analyzing the HG content of *Calothrix* sp. UTEX 2589, Schouten et al. (2013) found that alongside the C_6 HG_{28} triol and C_6 HG_{28} keto-diol there was also an unknown glycolipid eluting several minutes earlier than the known glycolipids. Based on mass spectral fragmentation patterns and molecular weight, this novel glycolipid was tentatively described as a HG_{28} triol containing a C_6 sugar moiety which contained either an additional keto group, e.g. glucuronic acid instead of glucose, or an additional methyl group, e.g. by methylation of one of the hydroxyl groups (Schouten et al., 2013). This novel glycolipid was not present in the majority of benthic *Calothrix* species examined to date, but was present in a *Calothrix* isolated from an intertidal microbial mat (CCY0202; Schouten et al., 2013). Here, we identified this novel HG in a pelagic *Calothrix* sp. CCY1611 that was isolated from the surface water of the tropical western North Atlantic using ultra-high pressure liquid chromatography–high resolution mass spectrometry (UHPLC–HRMS) and acid methanolysis.

2. Methods

2.1. Isolation and culturing

Calothrix sp. CCY1611 was isolated from surface water from the tropical North Atlantic Ocean collected during a research cruise

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onboard the R/V *Pelagia* in 2014 (Station 20, 64PE393, cf. Bale et al., 2018). A surface water sample (1.5 L) was filtered over a 47 mm GFF (Whatman, Maidstone, UK). The filter was placed in a disk filled with agarose (0.6%) solidified seawater from the same location and subsequently stored at −80 °C until transport to the laboratory at NIOZ. Isolation of diazotrophic cyanobacteria was performed by transferring the GFF filter to a Petri dish with a solidified artificial seawater T^0 medium (modified from Chen et al., 1996) with agarose (7 g L^{-1}) as the solidifying agent. The medium was supplemented with glucose (2 g L^{-1}) and the incubation was carried out in an incubator (model MLR-350, SANYO, Osaka, Japan) at 27 °C, with a 12–12 h light-dark cycle and a light intensity (photon density) of 20–30 μmol m^{-2} s^{-1}. Once colonies appeared on the filter, they were transferred to new agarose medium without glucose, and a pure culture was obtained after repeated transfers of single trichomes using standard microbiological techniques. The isolate was identified as a *Calothrix* sp. based on its morphology using a light microscopy and sequencing of the 16S rRNA gene (GenBank accession number MH364376). In order to characterize its HGs, the strain was grown for 40 days in T^0 liquid medium at 27 °C and harvested at stationary phase and stored at −20 °C until analysis.

2.2. Lipid extraction and analysis

The extraction of lipids from freeze dried biomass was carried out using a modified Bligh-Dyer extraction as described previously (Bale et al., 2013). UHPLC–HRMS was carried out as described by Bale et al. (2017) using an Agilent 1290 Infinity UHPLC was used, equipped with thermostatic auto-injector and column oven, coupled to a Q Exactive Orbitrap MS with Ion Max source with heated electrospray ionization (HESI) probe (Thermo Fisher Scientific, Waltham, MA, USA).

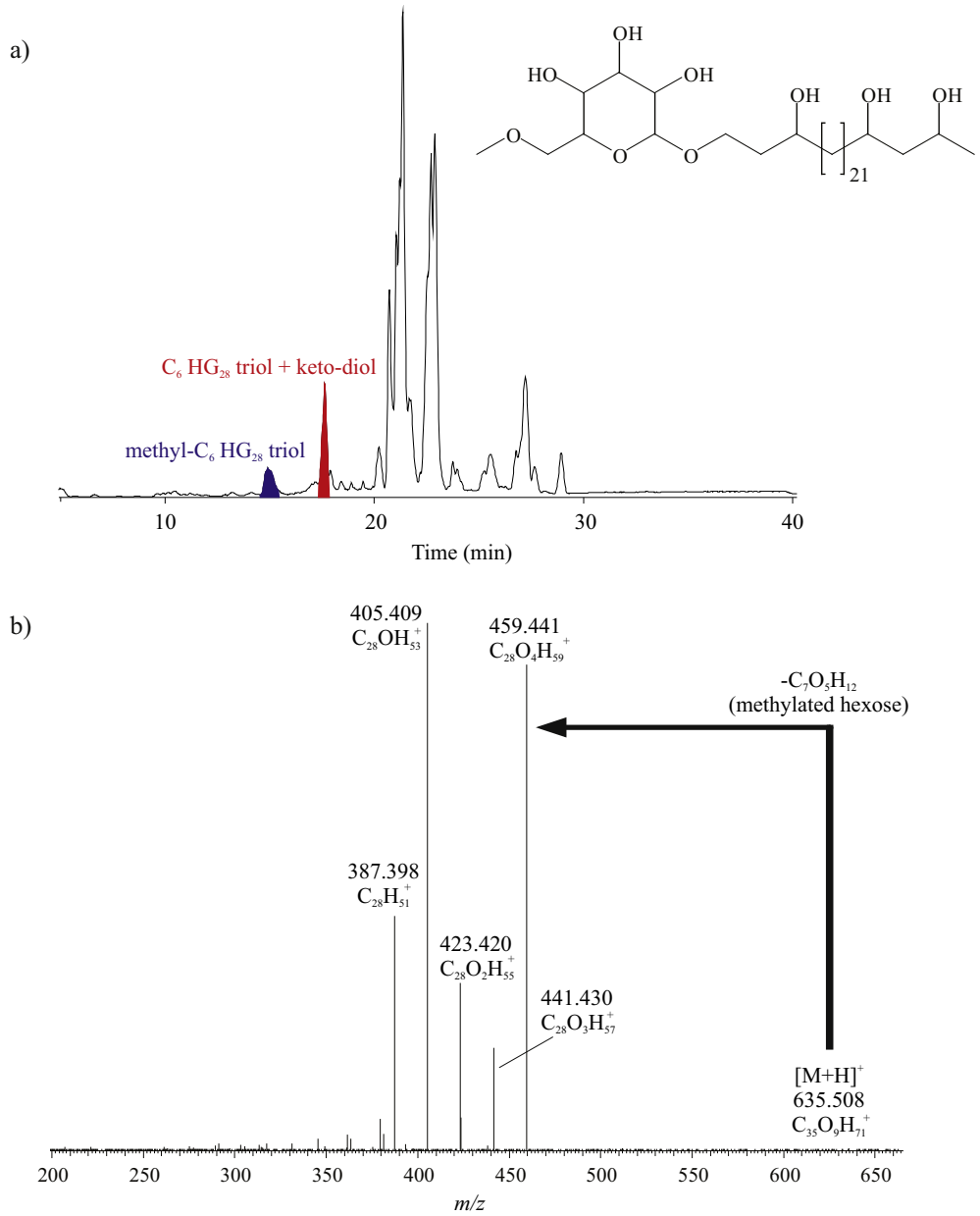

Fig. 1. (a) UHPLC-HRMS partial base peak chromatogram (Gaussian smoothed) showing the distribution of heterocyst glycolipids (filled peaks) in the Bligh and Dyer extract of *Calothrix* sp. CCY1611. Insert: proposed structure of the novel methyl-C_6 HG_{28} triol, 1-(O-6-O-methyl-β-D-glucopyranose)-3,25,27-octacosanetriol. (b) MS^2 spectrum of the novel methyl-C_6 HG_{28} triol with [M+H]+ 635.508. For interpretation of the acyl chain fragments see Bauersachs et al. (2009b).

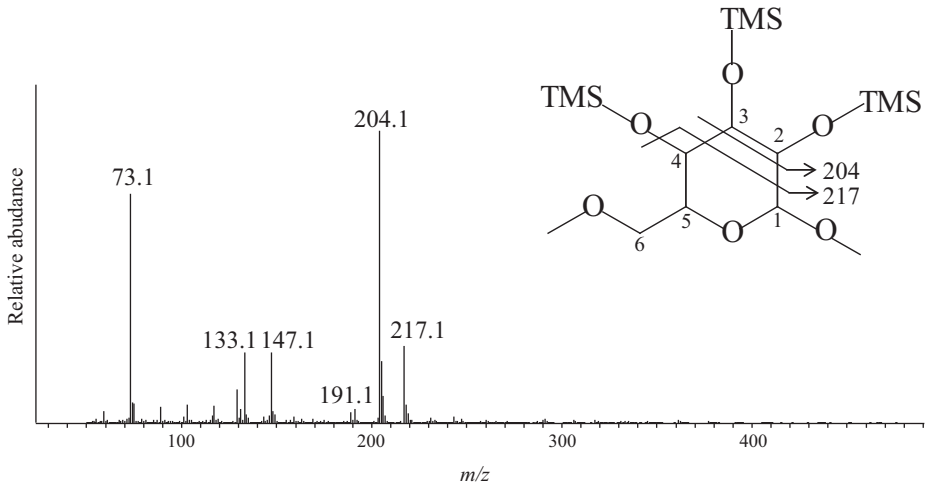

Fig. 2. Mass spectra of trimethylsilylated (TMS) sugar moiety of the novel heterocyst glycolipid with structure shown as insert.

To confirm the structure of the sugar in the novel HG, it was isolated using semi-preparative HPLC and the normal phase system as described by Bale et al. (2017). The column effluent was collected in 1 min fractions and the fractions containing the novel HG were pooled. Acid methanolysis was performed on the isolated compound and hydroxyl groups were converted into trimethylsilyl (TMS) ester derivatives using N,O-*bis*(trimethylsilyl)trifluoroaceta mide (BSTFA) and pyridine (1:1; 20 min at 60 °C) before analysis by GC–MS using a Thermo Trace DSQ as described in Schouten et al. (2013). The sugar was identified by comparison of its mass spectrum with library mass spectra (NIST Mass Spectral Library, Version 2.0, 2012) and three standards (methyl α-D-glucopyranoside, methyl-β-D-galactopyranoside and 3-O-methyl-D-glucopyranose, Sigma-Aldrich, St. Louis, USA).

3. Results and discussion

Analysis of *Calothrix* strain CCY1611 by UHPLC–HRMS (Fig. 1a) indicated the presence of a C_6 HG_{28} triol ($[M+H]^+$ *m/z* 621.493) and a C_6 HG_{28} keto-diol ($[M+H]^+$ *m/z* 619.478) and a novel HG ($[M+H]^+$ *m/z* 635.508), previously reported by Schouten et al. (2013) in two other *Calothrix* species. Initial structural identification was based on the $HRMS^2$ spectrum generated from the protonated molecule (Fig. 1b). The spectrum contained the same five ions as described in the MS^2 spectrum of the C_6 HG_{28} triol (Bauersachs et al., 2009b), at *m/z* 459.441, 441.430, 423.420, 405.409 and 387.398, suggesting that the alkyl chain is also a 3,25,27-octacosanetriol. The product ion at *m/z* 459.441 corresponded to a neutral loss of a head group of mass 176.067 Da ($C_7H_{12}O_5$). The accurate mass of the unknown HG $[M+H]^+$ ion (*m/z* 635.508) allowed us to distinguish between the two hypothesized structures for the head group by Schouten et al. (2013) since the accurate mass of the HG with an additional keto group on the C_6 sugar, e.g. glucuronic acid, ($C_{34}O_{10}H_{66}$) is 635.473, whereas accurate mass of the HG with an additional methyl group on the C_6 sugar ($C_{35}O_9H_{70}$) is 635.509. This demonstrates that the unknown HG compound is a methylated C_6 HG with a C_{28} triol core (methyl-HG_{28} triol). This was confirmed by GC–MS analysis of the sugar released by acid methanolysis of the isolated (by preparative HPLC) novel HG. The mass spectrum (Fig. 2) provided evidence that there was no methylation at the C-2, C-3 or C-4 position and that, due to the methanolysis of the alcohol chain, there was a methylation at the C-1 position (Petersson and Samuelson, 1968). The additional methylation was therefore determined to be at the C-6 position. Furthermore, the spectrum was similar to the reported mass spectrum to 6-O-methyl-β-D-glucopyranose (NIST Mass Spectral Library, Version 2.0, 2012), while its retention time and mass spectrum was different from our analysis of sugars with single methylations at the 1-O and 3-O position. Therefore, the novel HG was identified as 1-(O-6-O-methyl-β-D-glucopyranose)-3,25,27-octacosanetriol, a potential novel biomarker for cyanobacteria in the genus *Calothrix*.

To date, there have been no environmental reports of the methyl-HG_{28} triol, likely due to limitations of previous analytical methods such as selective reaction monitoring (SRM) (Bauersachs et al., 2009b; Bale et al., 2015), which did not include the transition of the methyl-HG_{28} triol. Further research examining more species of pelagic and benthic *Calothrix* should reveal if this novel HG is associated with pelagic strains of *Calothrix* or whether it has a wider distribution within the genus.

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