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Isolation of polyketides from *Prymnesium parvum* (Haptophyta) and their detection by liquid chromatography/mass spectrometry metabolic fingerprint analysis

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ABSTRACT

Prymnesium parvum is a microalga that forms blooms coupled with the presence of potent exotoxins; however, no chemical standards are currently available for the toxins. Streamlined methods are presented for the separation and enrichment of polyketide toxins, prymnesin-1 (prym1) and prymnesin-2 (prym2). Prymnesins were separated by reversed-phase chromatography and detected by positive-mode electrospray ionization MS to generate a unique metabolic fingerprint. More than 10 ions were detected and mass assignments were in agreement with predicted isotopic distributions for the intact compounds and related fragments; ions occurred as multiply protonated species and with common salt adducts. The most prevalent ion was observed at 919.88 *m*/*z*, which represents the aglycone $[prym_{agly} + 2H]^{2+}$ backbone structure common to both molecules. Expanded mass spectra for this and related ions were in excellent agreement (<0.5 ppm) with empirically derived spectra based on elemental composition and naturally occurring isotopes. These investigations have confirmed the isolation of polyketide prymnesins from whole cells, which heretofore has not been reproduced since their original characterization. Moreover, this study represents the first time these compounds have been verified in aqueous materials. These tools should allow the direct identification and analysis of polyketide prymnesins, which will greatly improve our understanding of these toxins in *P. parvum*.

More than 15 biologically active substances have been identified from chemical extracts of *Prymnesium parvum* and most have been documented as having lytic and/or other toxic effects [1–4]. These "prymnesins" have been described as glycolipids, galactolipids, polyketides, proteolipids, and, most recently, fatty acids [5–9]. Prymnesins are commonly known for their potent hemolytic and ichthyotoxic effects; however, published methods for the extraction of prymnesins have incorporated a variety of separation techniques, so it is often unclear which compounds are present under the various conditions [10]. Furthermore, toxic components have been found in the polar, intermediate, and nonpolar phases of extracts, but there are no standardized methods for the isolation of individual compounds. These complexities have made it extremely difficult to quantify toxicity associated with this organism with any consistency. The direct detection and quantitation of specific prymnesins using sensitive chemical and analytical methods (e.g., liquid chromatography/mass spectrometry (LC/MS)[1], GC/MS) would be preferable; however, many of these compounds have not been fully characterized and no standards are currently available.

Streamlined extraction methods are presented for the enrichment and detection of the two known polyketide metabolites from cells of *P. parvum*. Polyketide prymnesins prym1 ($C_{107}H_{154}Cl_3NO_{45}$) and prym2 ($C_{96}H_{136}Cl_3NO_{35}$) are renowned for their potent cytotoxicity and lethal effects on gill-breathing organisms, although these compounds have never been isolated outside of the original investigations [8,9] (Fig. 1). While the need for purified material was a prerequisite for structural elucidation by nuclear magnetic resonance (NMR), it was proposed that many of the chromatographic

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[1] *Abbreviations used:* ESI, electrospray ionization; EtOAc, ethyl acetate; FT-ICR, Fourier transform ion–cyclotron resonance; GC, gas chromatography; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; MeOH, methanol; NMR, nuclear magnetic resonance; PP, polypropylene; *n*-PrOH, 1-propanol; prym1, prymnesin-1; prym2, prymnesin-2; R_f, retention factor; RT, room temperature; SPE, solid-phase extraction; TLC, thin-layer chromatography; TIC, total-ion chromatogram; UV, ultraviolet; UTEX, Culture Collection of Algae at the University of Texas at Austin.

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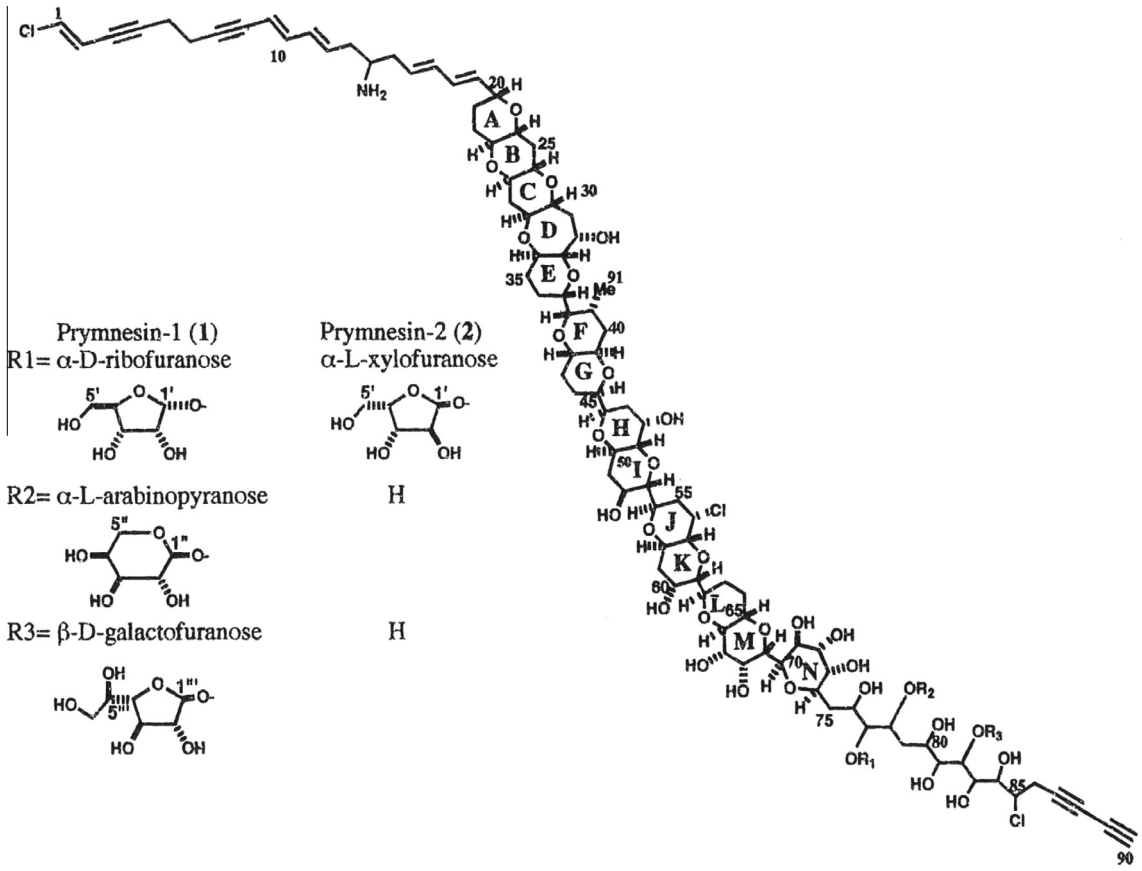

Fig.1. Structures of the toxic polyketides prymnesin-1 and prymnesin-2 are shown (from Igarashi et al. [9]).

steps described by Igarashi and colleagues [8,9] could be amended for the small-scale isolation of polyketide prymnesins from whole cells. Similarly, a solid-phase extraction (SPE) method was adapted for the concentration and fractionation of polyketide prymnesins in cell extracts, as well as aqueous samples. Last, LC/MS was used to create a "metabolic fingerprint" for the rapid identification of polyketide prymnesin-related compounds. Collectively, these simplified phytochemical isolations coupled with sensitive analytical instrumentation represent a significant contribution toward the detection of polyketide prymnesins with broader implications for monitoring this toxic bloom-forming alga.

Materials and methods

Culture maintenance

Unialgal cultures of *P. parvum* (UTEX 2797) were grown in 5 psu f/2 enrichment medium omitting silica (adapted from Guillard [11]). Erlenmeyer flasks containing 150 ml of culture each were maintained at 23 °C (room temperature, RT) on a gyratory shaking platform at approximately 150 rpm. The light source consisted of cool-white fluorescent bulbs with a 16/8 h (light/dark) photoperiod and a quantum flux of 40–45 μmol photons m^{-2} s^{-1} on the side of the flasks facing the light source (19–20 μmol photons m^{-2} s^{-1} on the opposing side). Cultures were monitored by light microscopy and spectrophotometry for A_{680nm} ($\varepsilon = 2.0 \times 10^{-7}$ cm^2 $cell^{-1}$).

Extraction of prymnesins from whole cells

The extraction procedure outlined was optimized for the extraction of polyketide prymnesins from 125–150 ml of dense cell culture (Fig. 2). All organic solvents were analytical grade (or better) and acquired from standard vendors. Extraction steps used dark amber polypropylene (PP) microcentrifuge tubes and/or borosilicate glass.

When culture densities approached 3–4.5 × 10^6 cells ml^{-1} (late-logarithmic phase), cells were harvested by centrifugation at 4500 rpm for 5 min at RT. The supernatant was decanted and the cells were extracted immediately or stored at −80 °C. Cell pellets were resuspended with 2 ml cold (−20 °C) acetone by gentle pipetting and vortex mixing and then split in half. Afterward, centrifugation was performed at 10,000 rpm for 5 min at 4 °C to sediment the cell debris. The (green to yellow–green) supernatant was discarded taking care not to carry over any particulates. This acetone wash was repeated two more times using 1 ml acetone per tube; additional rounds were performed until the supernatants were effectively clear of pigmentation. The remaining cell material was subsequently processed for the extraction of polyketide prymnesins.

Each acetone-extracted cell pellet was resuspended with 1 ml RT methanol (MeOH) by gentle pipetting and vortex mixing. This suspension was pelleted by centrifugation at 10,000 rpm for 5 min at RT and the (yellowish-green) supernatant was transferred to a borosilicate vial taking care not to carry over any cell debris. The extraction steps with MeOH were repeated three more times and the supernatants were pooled. Next, the cell pellet was resuspended with 1 ml RT 80% 1-propanol (*n*-PrOH) in HPLC-grade water (Omni-Solve, EMD Chemicals, Inc., Gibbstown, NJ, USA) and the mixture was homogenized for approximately 1 min. Afterward, the pestle was rinsed into the tube with 100 μl 80% *n*-PrOH and the cell homogenate was clarified by centrifugation at 4500 rpm for 5 min at RT. The resulting supernatant was transferred to the glass vial containing the MeOH fractions. The *n*-PrOH extraction, homogenization, and