The distribution, biosynthetic origin and functional significance of Tyrian purple precursors in the Australian muricid *Dicathais orbita* (Neogastropoda: Muricidae)

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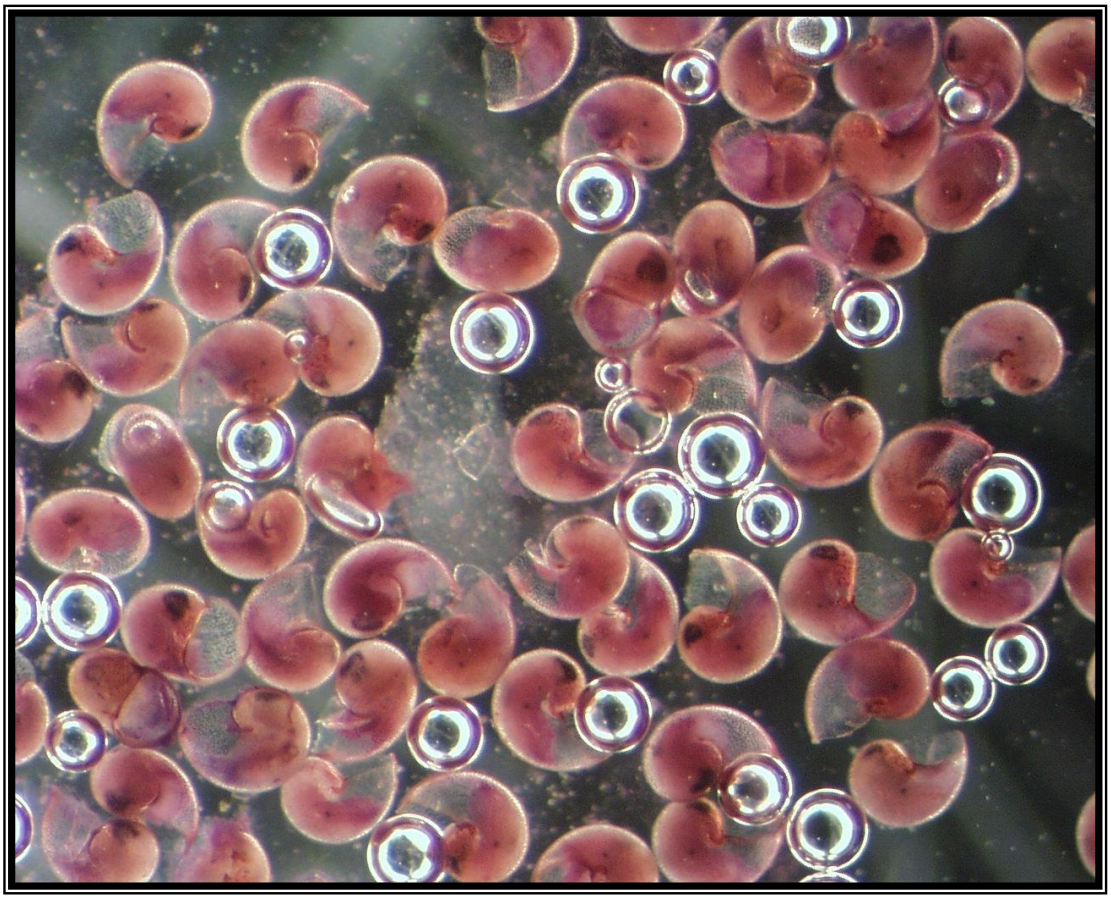

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Abstract

Information on the biosynthetic origin and functional advantage of marine mollusc natural products is not only essential to our understanding of chemical ecology, but to the development and responsible production of therapeutic agents. As demonstrating *in situ* activity is methodologically hindered, functions inferred by *in vitro* activity have been assumed for many secondary metabolites. The anatomical and ontogenetic distribution of natural products can not only provide information on the biosynthesis and storage of metabolites, but identify selective pressures likely to affect survivorship at a specific life stage. Thus, dissection and chemical analysis of distinct tissues, in combination with histochemistry may offer a valuable approach.

Marine gastropods of the Muricidae are renowned for the ancient dye Tyrian purple, which evolves from choline esters of bromoindoxyl sulphate in the hypobranchial gland through a series of enzymatic and photo-oxidative reactions. Prochromogen hydrolysis by arylsulphatase liberates neuromuscular active choline esters and cytotoxic bromoindole precursors, which also occur in muricid egg masses. Although visual accounts of dye pigments in the muricid gonoduct suggest precursors may be incorporated into egg masses from a maternal source, their biosynthetic origin and the evolutionary significance of the hypobranchial gland is unknown. Thus, the Muricidae, and in particular *Dicathais orbita* upon which most previous research has been focused, is an ideal model for this novel approach to natural product research.

To confirm observations of dye pigments in muricid gonoducts and gain an understanding of their anatomical distribution, a liquid chromatography-mass spectrometry (LC-MS) method was developed to simultaneously quantify pigments,

precursors and the prochromogen, tyrindoxyl sulfate. The prochromogen was not only detected in albumen and capsule gland extracts, but bioactive intermediates and the dye 6,6'-dibromoindigo were also present in the latter. These findings provided preliminary evidence for the maternal provision of prochromogens in egg masses of *D. orbita* and identified regions within which to conduct histochemical investigations. Tyrindoxyl sulphate was also detected in male prostate gland extracts, along with the dibromoindigo isomer, 6,6'-dibromoindirubin and its oxidative precursor, 6-bromoisatin. This not only implies physiological differences exist between male and female gonoducts, but that these secondary metabolites are not solely intended for egg masses and may hold significance throughout the life cycle.

Histomorphological inspection of the pallial gonoduct-hypobranchial gland complex was conducted over the annual cycle to determine a mechanism for precursor transfer between these structures. Although an anatomical connection was not detected, the secretions of two hypobranchial cell types thought to be involved in Tyrian purple synthesis were of remarkable biochemical similarity to those of various capsule and albumen gland lobes. Together these findings implied the potential for natural product synthesis within the pallial gonoduct of *D. orbita*.

To establish the role of these glandular lobes in the incorporation of intracapsular fluid and capsule laminae, identical histochemical techniques were applied to transverse capsule wall sections. Biochemical correlations not only provided a simple method of deciphering the complex process of encapsulation in neogastropods, but effectively identified the destination of gonoduct secretions in egg capsules of *D. orbita*. Comparisons of capsule and gonoduct biochemistry revealed that the intracapsular fluid and inner capsule wall are secreted by the posterior

capsule gland lobe, the middle lamina by the lateral lobes and the outer layers by the dorsal lobe, albumen and pedal glands.

Investigation into the location of regulatory enzymes and precursors was conducted to establish the biosynthetic origin of Tyrian purple prochromogens and mechanisms governing bioactive precursor synthesis. Novel histochemical techniques for the localization of bromoperoxidase, the enzyme thought to facilitate prochromogen bromination, and tyrindoxyl sulphate were developed and applied to gonoduct, hypobranchial gland, and encapsulated larvae sections. Standard staining reactions for the indole precursor, tryptophan, and arylsulphatase were also applied. The histochemical approach adopted revealed that tyrindoxyl sulphate is *de novo* biosynthesized through the post-translational bromination of dietary derived tryptophan. Two biosynthetic sites were identified, one related to hypobranchial secondary metabolism and the second of significance to the presence of bioactive precursors in muricid egg masses.

Tryptophan is stored within secretory cells of the lateral hypobranchial epithelium and once exocytosed, is united with bromoperoxidase from supportive cells to form tyrindoxyl sulphate. Prochromogen synthesis also occurs in the subepithelial vascular sinus for storage and secretion by medial hypobranchial secretory cells. Bioactive precursor synthesis on the epithelial surface is regulated by the liberation of arylsulphatase from adjacent supportive cells. These findings not only provide evidence for *de novo* biosynthesis of Tyrian purple precursors, but are first account of natural product biosynthesis within the gastropod hypobranchial gland. Together these findings imply a naturally selected function for the synthesis of bioactive indoles in hypobranchial gland secretions of the Muricidae and Gastropoda.

Tyrindoxyl sulphate is also transported within the vascular sinus to lateral and dorsal capsule gland lobes where bromoperoxidase and arylsulphatase also occur. Arylsulphatase was also detected within the albumen gland, which along with the posterior capsule gland lobe, acts as a storage site for dietary tryptophan. Thus, tyrindoxyl sulphate and the constituents for prochromogen and precursor biosynthesis are introduced to intracapsular fluid and capsule laminae by the capsule gland. Histochemistry in combination with LC-MS revealed an identical biosynthetic profile within larval vitellus, which is elaborated during oogenesis and may also receive secretions from the albumen gland. Due to the absence of a hypobranchial gland in veligers, it appears that pelagic larvae rely on vitelline natural products until settlement and metamorphous. These findings together with the *in situ* antimicrobial activity of bromoindoles suggest Tyrian purple precursors are incorporated into muricid egg masses as a maternal investment in larval defence against pathogens.

The results of this investigation clearly highlight the benefits of adopting a histochemical approach to natural product research. This novel alternative to radioisotopes and *in situ* demonstration of bioactivity, can not only aid in the elucidation of secondary metabolic pathways and chemically mediated interactions, but identify mechanisms of metabolite regulation and differentiate between biosynthetic and storage tissues. Apart from providing insight into the ecological significance of muricid secondary metabolites, the biosynthetic information provided is valuable to our understanding of chemical phylogeny and biosynthetic enzyme sequencing for the environmentally sound development of natural products as biomedical agents.

Declaration

I certify that this thesis does not incorporate without acknowledgement, any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

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